

Airway epithelial cell and macrophage regulation of pulmonary immunity

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Declaration of originality

I conducted or directly supervised all scientific work described in this thesis, with the following exceptions. Simone Walker managed surgical anaesthesia and operated Flexivent™ equipment during lung function measurements. Laura Denney performed all TGF- β bioactivity and influenza plaque assays described in Chapter 7, as well as the T cell, innate lymphoid cell and natural killer cell flow cytometry on influenza experiments described in that chapter. Dhiren Patel and Robert Snelgrove generated samples with which I performed the comparative analysis of mouse strains and characterisation of budesonide responses included in Chapter 3. Juho Vuononvirta generated samples with which I performed the kinetic analysis of the response to LPS inhalation described in Chapter 3. Franz Puttur and Juho Vuononvirta assisted with preparation of precision-cut lung slices for confocal microscopy. Quantitative PCR data in Figure 6.19d were generated by Su Yin Lim as part of a BSc project under my supervision. Wax embedding and sectioning of formalin-fixed lung tissue and haematoxylin and eosin staining were performed by Lorraine Lawrence. Flow cytometry, particularly cell sorting, was performed with assistance and guidance from Jane Srivastava, Jessica Rowley and Catherine Simpson of the Imperial College Flow Cytometry core facility. RNA sequencing and initial adapter trimming and FASTQC analysis of raw sequence data were performed by the Advanced Sequencing and Bioinformatics core facilities at the Francis Crick Institute.

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Abstract

Regulation of pulmonary immunity is reliant on the sentinel function of airway macrophages (AM) and conducting airway epithelial cells. The aim of this thesis was to examine regulatory mechanisms utilised by AM and airway epithelial cells, focussing on the cytokines IL-10 and transforming growth factor β 1 (TGF- β 1).

AM phenotype was examined in multiple models of pulmonary inflammation, establishing CD11b and MHC-II as robust AM activation markers. Transcriptomic analysis showed AM to increase expression of genes related to leukocyte recruitment, tissue repair, antigen presentation and metabolism by oxidative phosphorylation during allergic airway disease (AAD) driven by house dust mite (HDM) inhalation. However, AM also expressed genes encoding regulatory proteins such as TGF- β 1 and the retinoic acid-generating enzyme RALDH2 during AAD, suggesting that they retain immunoregulatory capacity even during established inflammation.

AM were shown to produce minimal IL-10 in response to pro-inflammatory stimuli and AM-derived IL-10 was redundant for regulation of immune responses to inhaled allergen or respiratory syncytial virus infection. Conversely, T cell-specific IL-10 deletion enhanced the severity of HDM-driven AAD, accompanied by pulmonary heightened type 1 immunity and dysregulated AM activation, indicating that T cells are the dominant IL-10 producing cells in the allergic lung and may regulate AM activation via IL-10.

Conditional knockout of TGF- β 1 in AM enhanced the type 2 immune response to HDM inhalation and increased production of the chemokine CCL2 by AM, suggesting a potential autocrine regulatory function of AM-derived TGF- β 1. Additionally, conducting airway epithelial club cells were shown to rapidly release TGF- β 1 into the airway lumen upon influenza infection. Conditional deletion of this TGF- β 1 source enhanced early interferon β production to limit viral replication, suggesting that club cell-derived TGF- β 1 acts as a pro-viral factor during influenza infection.

Collectively, these findings illustrate mechanisms by which immunoregulatory cytokines operate in context-specific niches during pulmonary inflammatory responses.

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List of abbreviations used

Abbreviation	Full term
AAD	Allergic airway disease
ALT	Alternaria alternata
AM	Airway macrophage(s)
APC	Antigen-presenting cell(s)
BAC	Bacterial artificial chromosome
BAL	Bronchoalveolar lavage
BATF3	B and T cell factor 3
BL	Baseline
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
BV	Brilliant Violet™
CCSP	Clara/club cell secretory protein
cDC(1/2)	Classical dendritic cell (1/2)
COPD	Chronic obstructive pulmonary disorder
cRPMI	Complete RPMI
DC	Dendritic cell(s)
dLck	Distal lymphocyte protein tyrosine kinase
DMEM	Dulbecco's Modified Eagle's Medium
dpi	Days post-infection
EC	Extracellular
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
eYFP	Enhanced yellow fluorescent protein
FBS	Foetal bovine serum
FDR	False discovery rate
FFU	Focus forming units
FMO	Fluorescence minus one
G-CSF	Granulocyte colony stimulating factor
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
gMFI	Geometric mean fluorescence intensity
GO	Gene Ontology
HBSS	Hank's Buffered Salt Saline
HDM	House dust mite
hpi	Hours post-infection
HRP	Horse radish peroxidase
i.n.	Intranasal
i.p.	Intraperitoneal
IC	Intracellular
IFN (α , β , γ , λ)	Interferon (α , β , γ , λ)
Ig (E,G1)	Immunoglobulin (E,G1)
IL	Interleukin
IL-10R (A, B)	IL-10 receptor (A, B)
ILC (1,2,3)	Type 1, 2, 3) innate lymphoid cells
IM	Interstitial macrophage(s)
IMM	Interstitial/inflammatory monocyte/macrophage
IN	Intranuclear
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity Pathway Analysis
IQR	Interquartile range
IRF (4, 5)	Interferon regulatory factor (4, 5)
I-Smad	Inhibitory Smad
Jak	Janus kinase
LAP	Latency-associated peptide
LEAF®	Low-endotoxin azide-free®
LPS	Lipopolysaccharide
LTBP	Latent TGF- β -binding proteins
M. tuberculosis	Mycobacterium tuberculosis
MAPK	Mitogen-activated protein kinase

Abbreviation	Full term
MCh	Methacholine
MDCK	Madin Darby canine kidney
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
mLN	Mediastinal lymph node
MMP	Matrix metalloproteinase
MMP	Matrix metalloproteinase
moDC	Monocyte-derived dendritic cell
mTOR	Mammalian target of rapamycin
NK cells	Natural killer cells
PAI-1	Plasminogen activator inhibitor-1
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS (-T)	Phosphate-buffered saline (+ 0.05 % Tween®-20)
PC(A)	Principal component (analysis)
PCLS	Precision-cut lung slice
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PM	Peritoneal macrophage
PMA	phorbol 12-myristate 13-acetate
PP1	Protein phosphatase 1
PPAR _γ	Peroxisome proliferator-activated receptor _γ
qPCR	Quantitative polymerase chain reaction
RALDH2	Retinaldehyde dehydrogenase 2
RCLB	Red cell lysis buffer
RGD	Arginine-glycine-aspartate
RNAseq	RNA sequencing
R-Smad	Receptor Smad
RSV	Respiratory syncytial virus
rtTA	Reverse tetracycline transactivator
SEA	Schistosoma egg antigen
SEAP	Secreted alkaline phosphatase
SEM	Standard error of the mean
Siglec	Sialic acid-binding lectin
SOCS (1, 3)	Suppressor of cytokine synthesis (1, 3)
SP-A/SP-D	Surfactant protein A/Surfactant protein D
STAT (1, 3, 6)	Signal transducer and activator of transcription (1, 3, 6)
TCID ₅₀	Tissue culture infectious dose ₅₀
TGF-β (1, 2, 3)	Transforming growth factor β (1, 2, 3)
TGF-βR (1, 2)	TGF-β receptor (1, 2)
Th (1, 2, 9, 17)	T helper (1, 2, 9, 17)
TLR	Toll-like receptor
TRE-Cre	Tetracycline response element-Cre
Treg	Regulatory T cell
VASP	Vasodilator-stimulated phosphoprotein
V-ATPase	Vacuolar type H ⁺ ATPase

1. Introduction

1.1. The pulmonary immune system: the importance of regulation

Maintenance of homeostasis in the mammalian lungs is essential for continuous effective gas exchange. Each breath exposes the airways to thousands of airborne particles, ranging from harmless environmental material to potentially lethal pathogens. Remarkably, the pulmonary immune system is able to discriminate between these particles, with inhalation of benign antigens generally resulting in immunological tolerance¹⁻³. In contrast, immune responses are effectively mounted to respiratory pathogens that present danger to the host. The airways are therefore a unique immune-environment interface at which balance between immunological tolerance and activation is crucial.

Human diseases in which there is failure to regulate the balance between airway tolerance and immunity illustrate the importance of pulmonary immune regulation. Allergic asthma affects millions of people worldwide and occurs due to aberrant immune responses to inhaled ubiquitous allergens such as the bodies and faecal pellets of house dust mites (HDMs), fungal spores and animal dander⁴. This inappropriate pulmonary immunity results in chronic peribronchial inflammation, generation of immunoglobulin E (IgE) class antibodies to allergen epitopes, hyperresponsiveness of the airways to irritants and bronchoconstrictors and structural changes to the conducting airway wall, termed airway remodelling. Together these pathobiological features result in impaired lung function, which is reversibly exacerbated in response to exposure to allergen, pollutants or respiratory infection^{4,5}. Regulation of immunity to harmless allergens such as HDM is therefore central to the prevention of asthma development and exacerbation.

Insufficient pulmonary immunity is also detrimental to health, as evidenced by the frequent occurrence of respiratory infections in patients with primary immunodeficiencies that limit innate immune responses to pathogens in the lung⁶. Conversely, much of the pathology occurring during respiratory infections such as influenza A virus results from bystander tissue damage caused by host immune cells⁷. Systems analysis of multiple mouse models of influenza infection showed a strong link between a gene expression pattern indicative of a heightened host immune response and lethality⁸. Moreover, assessment of the immune correlates of highly pathogenic human influenza strains showed rapid production of pro-inflammatory mediators and recruitment of innate leukocytes to be a key determinant of severity of these strains in mouse models^{9,10}. Balance between immune activation and regulation is therefore crucial for controlling the timing and magnitude of immune responses to allow clearance of infection without causing catastrophic tissue damage.

The work in this thesis focussed on understanding interactions between major cellular determinants of the balance between immune activation and regulation, which are introduced in the remainder of this section.

1.1.1. Innate barriers in the respiratory tract

Crucial to allowing discrimination between pathogenic and benign inhaled particles is the presence of physical barriers to separate the contents of the airway lumen from underlying blood vessels and submucosal leukocytes. In the lungs, this barrier is achieved by airway epithelial cells, which are tightly linked by interlocking junctional proteins that prevent intercellular passage of inhaled particles through the epithelium¹¹. Airway epithelium can be broadly divided into that of the conducting airways (bronchi and bronchioles) and alveoli, with the two differing in both composition and function.

Conducting airway epithelium is required to ensure that air is partially cleaned and delivered to the alveoli for gas exchange. Conducting airway epithelium contains goblet cells, a specialised epithelial cell subset that produce a protective mucus layer that blocks microbial access to underlying epithelium¹¹. Mucus is tethered to the airway epithelium by mucin proteins to form a spatially separated 'gel on brush' structure, which is escalated up the respiratory tract by the coordinated beating of cilia on the apical surface of a subset of conducting airway epithelial cells¹². The importance of this mucociliary clearance is highlighted by the failure of mice deficient in the mucus glycoprotein Mucin 5b (*Muc5b* gene) to clear bacterial lung infections¹³ and the frequent *Pseudomonas aeruginosa* colonisation of the defective mucus layers of cystic fibrosis patients¹⁴. Conducting airway epithelium also contains secretory club (formerly Clara¹⁵) cells, which produce a range of proteins with immunomodulatory and antimicrobial function, including Clara/club cell secretory protein (CCSP) and surfactant proteins (SP) -A and -D¹⁶ (see Section 1.2).

The primary function of alveolar epithelium, comprising type I and type II alveolar epithelial cells, is to permit gas exchange between the airways and underlying microvasculature. Type I cells are large and relatively flat, which provides a short distance for passage of gases, while type II cells are cuboidal and are required for production of protein and lipid components of surfactant¹⁷. While the principal function of surfactant is to reduce alveolar surface tension during ventilation to prevent collapse, it also contains proteins with antimicrobial functions such as SP-A and SP-D^{11,17}. Thus, both conducting airway and alveolar epithelium play important barrier roles in defence against inhaled pathogens. These cells also have critical non-barrier functions in control of immunity, which are discussed in section 1.2.

1.1.2. Lung-resident mononuclear phagocytes

Accompanying the epithelium and other stromal cells, healthy lungs contain a number of resident populations of macrophages and dendritic cells (DC) capable of phagocytosing inhaled particles and driving cell type- and context-dependent responses, ranging from tolerance to initiation of an adaptive immune response¹⁸.

Airway macrophages

Airway/alveolar macrophages are the most abundant of the resident mononuclear phagocytes in the healthy lung and make up the vast majority of leukocytes in the healthy human and murine airway lumen¹⁹. Airway-resident macrophages exist in close contact with airway epithelial cells, with roughly one macrophage present per every three alveoli in the steady-state mouse lung²⁰. These cells have historically been described as alveolar, however sampling from human or animal models by bronchoalveolar lavage (BAL) or whole tissue digestion does not discriminate between true alveolar macrophages and macrophages resident in the lumen of the conducting airways. In one published report, the authors attempted to distinguish between these compartments by dissecting apart the trachea and primary bronchi from the rest of the rat lung and lavaging each separately. This study suggested that macrophages obtained specifically from conducting airways displayed minor morphological differences and less effective opsonophagocytosis than true alveolar macrophages²¹. However, since this study used a relatively early cut-off along the length of the respiratory tract to designate macrophages as airway rather than alveolar and also pre-dates modern phenotypic and functional immunological methods, it is unclear to what extent the *in vivo* phenotype of macrophages in different portions of the airspace differ. Similarly, studies in healthy and asthmatic humans have compared resident macrophages obtained by sampling the conducting and distal airways by BAL with those obtained by sputum induction, which samples only the conducting airways^{22,23}. Airway macrophages were obtained in induced sputum, indicative of a population resident in the conducting airway lumen, which showed few differences in expression of macrophage lineage or activation markers compared with the equivalent cells from BAL, suggesting that similar cell populations exist in the conducting airways and alveoli. Considering this ambiguity regarding precise anatomical location, the umbrella term airway macrophage (AM) is used for airway lumen-resident macrophages throughout this thesis, in agreement with a recent literature review on these cells¹⁹.

AM are distinct from other lung mononuclear phagocyte populations based on their *in situ* differentiation, driven by the cytokine granulocyte macrophage colony stimulating factor (GM-CSF) and the transcription factor peroxisome proliferator-activated receptor (PPAR γ)^{24,25}. AM are also distinguished from other lung monocytes and macrophages by their high intrinsic autofluorescence²⁶ as well as surface expression of the integrin CD11c and, in mice, sialic acid-binding lectin (Siglec) F¹⁹ (**Fig. 1.1**). The mannose receptor CD206 is also ubiquitously expressed by AM in healthy human lung²⁷, while CD206 is expressed on a minority of murine AM at steady state²⁸ and is upregulated during allergic inflammation²⁸, pulmonary fibrosis²⁹ and in response to respiratory infection with the fungus *Aspergillus fumigatus*³⁰. The unique spatial niche and phenotype of AM has profound implications for their role in activation and regulation of pulmonary immunity, which are discussed at length in Section 1.3.

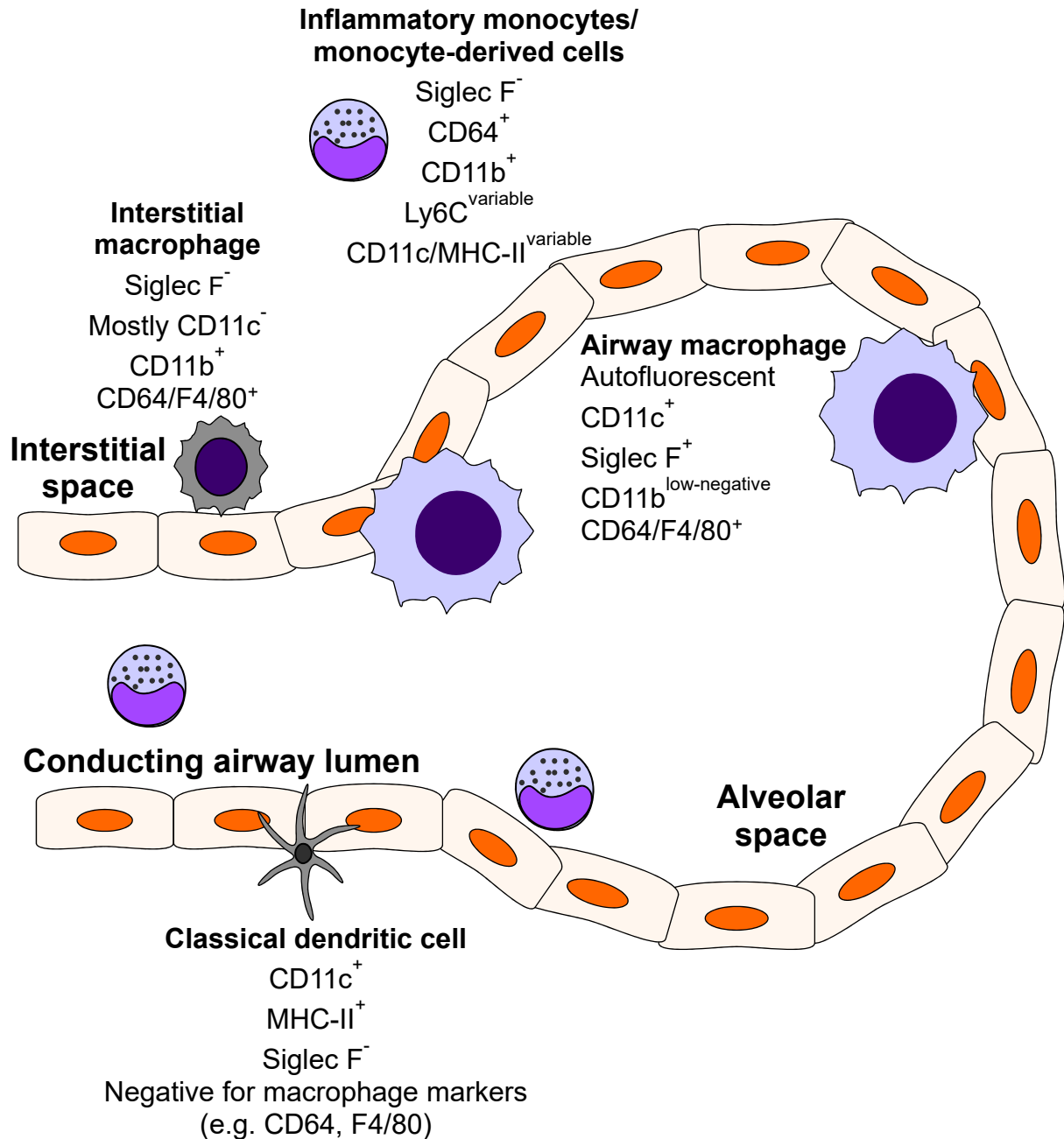


Figure 1.1. Anatomical and phenotypic distinctions between lung mononuclear phagocytes

Airway macrophages (AM) are present in the alveolar space and, to a lesser extent, the conducting airway lumen, where they form close associations with airway epithelial cells. They express macrophage markers such as CD64 and F4/80, but are distinguished by high intrinsic autofluorescence, expression of CD11c and Siglec F and, in the steady state, little to no expression of CD11b. Interstitial macrophages (IM) in the lung parenchyma are not autofluorescent, do not express Siglec F and most do not express CD11c. However IM express CD11b and macrophage markers (F4/80, CD64). Classical dendritic cell (cDC) are present in close association with the airway epithelium or in the submucosa. They do not express macrophage markers or Siglec F, but express both CD11c and MHC-II. Inflammatory monocytes can infiltrate the lung tissue and airway lumen and express variable surface markers depending on their state of activation or differentiation into monocyte-derived cells such as macrophages and DC. They do not express Siglec F, but express CD11b and CD64. Note that markers described here are for mouse cells. See text in section 1.1 for further details and references.

Interstitial macrophages

Interstitial macrophages (IM) are distinct from AM in both phenotype and anatomical location. As their name suggests, IM reside in the interstitial spaces of the lung parenchyma, in close contact with both DC and the basolateral surface of airway epithelial cells and are largely negative for CD11c^{31,32} (**Fig. 1.1.**). The functions of IM in pulmonary immunity are incompletely understood, but they are known to be highly phagocytic *in vitro* and have been suggested to restrict sensitisation to inhaled allergen *in vivo* by limiting the function of DC³¹. Three subsets of IM have been recently identified in mice, distinguishable by their surface marker and gene expression profiles and differing in their phagocytic capacity, including a subset that express CD11c³². It is likely that future work will reveal distinct functions for subsets of IM in pulmonary homeostasis and inflammation.

Classical and plasmacytoid dendritic cells

As in other tissues, lung-resident conventional DC consist of two subsets, classical DC1 (cDC1) and classical DC2 (cDC2)³³, both of which can be distinguished from pulmonary macrophages based on expression of CD11c, absence of the Fc γ receptor, CD64, and, in mice, absence of Siglec F²⁹ (**Fig. 1.1.**). cDC1 are dependent on the transcription factor B and T cell factor 3 (BATF3) for their development³⁴ and express the integrin CD103 in the lung, which allows their intimate association with airway epithelial cells that facilitates sampling of inhaled antigens^{35,36}. cDC1 are the most effective of DC subsets at cross-presentation of antigens to CD8⁺ T cells and so are crucial for antiviral immunity³⁴. cDC2 are dependent on the transcription factor interferon regulatory factor 4 (IRF4) and express CD11b in the lungs³⁷, where they reside in the submucosa³⁸. Lung cDC2 produce higher levels of a number of chemokines compared with cDC1 in response to stimulation by pathogen-associated molecular patterns (PAMPs) via Toll-like receptors (TLRs) *in vitro* and *in vivo*³⁹ and are essential for inception and maintenance of allergic airway disease (AAD)^{38,40,41}.

Both cDC1 and cDC2 reside in the lung in an immature state at homeostasis, with some constitutive migration to draining mediastinal lymph nodes (mLN)⁴⁰ thought to allow constant sampling of the lung environment and promote tolerance to self or harmless antigens⁴². Upon uptake of foreign antigen in the context of danger signals such as microbial PAMPs or pro-inflammatory cytokines, antigen-loaded cDC mature to increase their expression of co-stimulatory surface proteins such as CD40, CD80 and CD86 and display more substantial migration to mLN, where they present antigen to naïve T cells to initiate their expansion and polarisation^{43,44} (see Section 1.1.4).

Plasmacytoid DC (pDC), which in mice express intermediate levels of CD11c and are positive for the protein tyrosine phosphatase B220, are phenotypically and functionally distinct from cDC⁴⁵. A small number of pDC are present in the lungs at steady state, but at much lower frequencies

than cDCs⁴⁶. pDC are thought to be poor inducers of naïve T cell proliferation, but potent producers of the antiviral mediator interferon (IFN) α , suggesting that their function is likely to be in promotion of antiviral responses rather than direct instruction of adaptive immunity⁴⁷.

Other steady-state lung leukocytes

Importantly, macrophages and DC are not the only leukocytes present in the lung at steady-state. For example, a small number of innate lymphoid cells (ILCs) are maintained in the lung at steady state⁴⁸ and circulating classical monocytes constantly leave the blood and survey lung tissue, delivering antigen to draining lymph nodes⁴⁹. However, since these cells are much more abundant in the lung during immune responses they are discussed in later sections of this chapter.

Mast cells, like ILCs, are relatively rare tissue-resident leukocytes that are positioned close to blood vessels. This location positions mast cells optimally for rapid release of pre-formed and *de novo*-synthesised mediators involved in bronchoconstriction, vasodilation and cell recruitment, such as histamine, cysteinyl leukotrienes and chemokines, into the bloodstream. Mast cells are activated to release and synthesise these mediators by diverse stimuli, including cytokines, TLR agonists and cross-linking of surface-bound IgE by cognate antigen⁵⁰. The potential for mast cells to be activated in an IgE-dependent manner, along with their increased numbers in the lungs of asthmatics, means that they are key players in the pathogenesis of allergic airway inflammation⁵¹. However, mast cells are also implicated in immune responses to bacterial and eukaryotic pathogens⁵⁰.

1.1.3. Recruited innate leukocytes

Neutrophils

Exposure of airway epithelial cells and resident leukocytes to invading pathogens or allergenic material such as HDM results in rapid production of cytokines and chemokines that recruit innate effector leukocytes to the lungs^{5,7}. Neutrophils are among the first inflammatory cells rapidly recruited to the lung upon infection or injury, under control of mediators such as the chemokine CXCL1 and granulocyte colony-stimulating factor (G-CSF). Following extravasation into tissues, neutrophils can unleash a range of antimicrobial effector functions including phagocytosis and reactive oxygen species generation⁵². Neutrophils have also recently been shown to display crawling behaviour in pulmonary capillaries, which facilitates the detection and clearance of blood-borne bacteria, implicating them in the innate defence of the lung vasculature⁵³. The importance of neutrophils in pulmonary immunity is exemplified by the high rate of poorly controlled respiratory infections occurring in patients with genetic defects in neutrophil function⁶. However, the potent cytotoxic capacity of neutrophils means that they have great potential to cause immunopathology⁵² and excessive early neutrophil recruitment to the lung is associated with lethality during experimental influenza infection⁸. Neutrophilic inflammation is also typical of a subset of asthma patients, although the implications of neutrophilia on disease severity are currently unclear⁵, particularly since the presence of neutrophils in bronchial epithelium has been shown to correlate with improved lung function in severe paediatric asthmatics⁵⁴.

Eosinophils

Eosinophils, like neutrophils, are granulocytes recruited to sites of inflammation, including the lung, in response to chemokines such as CCL11 and CCL24. Eosinophils release a number of preformed cytotoxic granule proteins that can contribute to killing of large extracellular parasites, but are also capable of causing bystander pathology⁵⁵. The dependence of eosinophil recruitment on the cytokine IL-5 means that these cells are a major recruited granulocyte population in conditions where IL-5 is abundant, including helminth infection and AAD^{5,55}. Eosinophils are key anti-helminth effector cells⁵⁶ but can also contribute to the pathogenesis of AAD by producing molecules with bronchoconstriction capacity, such as cysteinyl leukotrienes⁵⁵, and driving airway remodelling⁵⁷.

Monocytes and monocyte-derived cells

Monocytes are recruited to the lung after infection, injury or allergen challenge with slightly delayed kinetics compared to neutrophils, in response to chemokines such as CCL2 and CCL7⁵⁸. The principal recruited monocyte subset are the classical or inflammatory monocytes, which express high levels of the surface molecule Ly6C in mice and are CD14^{hi} and CD16⁻ in humans⁵⁸. These cells contribute to both viral clearance and immunopathology during respiratory infection^{59,60} and can differentiate into inflammatory macrophages in the lung (**Fig. 1.1**) or adopt the phenotypic characteristics of resident AM^{61,62}. However, as discussed further in Section 1.3, the extent to which classical monocytes replace resident AM remains a matter of debate. In addition to infection, recruited classical monocytes are implicated in the pathogenesis of allergic airway disease⁶³, in part due to their differentiation into CD11c⁺ CD11b⁺ CD64⁺ antigen-presenting cells (APC), termed monocyte-derived DC (moDC; **Fig. 1.1**), which are the major DC-like cells in the lungs during HDM-driven AAD⁴⁰.

A separate population of monocytes, termed non-classical, express low levels of Ly6C in mice and are CD14^{low} and CD16⁺ in humans⁵⁸. These cells are thought to arise from classical monocytes in both mice⁶⁴ and humans⁶⁵, suggesting that they may be more terminally differentiated than classical monocytes. Non-classical monocytes are present in the lungs as well as other tissues at steady state⁶⁶ and are thought to be largely restricted to the vasculature, where they display patrolling behaviour and survey the luminal surface of endothelium⁶⁷. The function of non-classical monocytes in lung immunity is not well understood, but they have been shown to drive neutrophil recruitment upon intravascular PAMP sensing in other tissues⁶⁸, so may be required for sensing perturbations in the pulmonary vasculature.

Natural killer cells

Natural killer (NK) cells are another leukocyte population rapidly recruited to the lungs upon inflammatory insult and are particularly important in response to infection⁶⁹. NK cells recognise and kill infected host cells by detection of their altered expression of surface antigens, as well as

producing mediators that activate other leukocytes, principally IFN γ ⁶⁹. The crucial role of NK cells in clearance of pulmonary infection is demonstrated by the lethal influenza infection observed in mice lacking the activating NK cell receptor NKp46⁷⁰. In addition to infection, NK cell recognition of cell stress response molecules has been implicated in the pathogenesis of HDM-driven AAD⁷¹, highlighting the diverse protective and pathogenic roles of these cells in the lung.

1.1.4. T cells in adaptive pulmonary immunity

$\alpha\beta$ antigen receptor-bearing T cells are the major antigen-specific effectors in the adaptive phase of immunity to respiratory pathogens^{72,73} and airborne allergens⁵. CD8⁺ cytotoxic T cells are crucial to the clearance of respiratory viruses due to their ability to specifically kill infected host cells bearing viral antigens on surface class I major histocompatibility complex (MHC-I) molecules. However, excessive cytotoxic T cell responses can cause immunopathology and mortality during infection⁷⁴. CD4⁺ T helper cells are activated by APC, mainly DC, via recognition of antigenic peptides bound by class II MHC (MHC-II), and principally function by secreting cytokines that modulate the functions of other innate and adaptive leukocytes, as well as stromal cells⁷².

CD4⁺ T cells are classified on the basis of their cytokine production profiles and hallmark transcription factor expression. T helper type 1 (Th1) cells are generated in response to IL-12 and are characterised by expression of the hallmark transcription factor T-bet (*Tbx21/TBX21* gene in mouse/human) and production of IFN γ ⁷⁵. These cells are critical during many intracellular bacterial and viral infections, in part due to activation of the microbicidal functions of infected macrophages by IFN γ ⁷². Th1 cells and IFN γ are the major effectors of so-called type 1 immunity, which is implicated in autoimmune disease as well as bacterial and viral pathogen clearance⁷⁵.

Th2 cells are the antigen-specific effectors of type 2 immunity and are characterised by the hallmark transcription factor GATA3 and cytokines IL-4, IL-5 and IL-13⁷⁵. Differentiation of Th2 cells is driven by IL-4 and inhibited by IFN γ , providing potential for cross-regulation of these distinct T cell fates⁷⁶. IL-4 is a potent activator of antibody production from B cells, particularly IgE and the non-complement-fixing IgG isotype, IgG1^{77,78}, and induces an activation state in macrophages highly distinct from that of IFN γ ^{79,80} (see Section 1.3). In this way, IL-4 promotes generation of key effector cells and antibodies of type 2 immunity. IL-5 is required for eosinophil recruitment to lungs and their release from bone marrow to blood and is therefore central to the eosinophilic inflammation typical of type 2 immunity⁸¹. IL-13 shares a common receptor subunit with IL-4, IL-4R α 1, through which it induces a similar activation state to IL-4 in monocytes and macrophages^{80,82}. However, IL-13 also has non-overlapping functions with IL-4, such as promoting metaplasia of airway goblet cells and mucus hypersecretion and subepithelial fibrosis⁸³. IL-13 is also required for allergen-driven AHR in mice⁸⁴. Together the combined functions of these type 2 cytokines means that Th2 cells are widely implicated in the eosinophilic inflammation and IgE-dependent mast cell degranulation typical of asthma and other allergic disorders⁵. In addition, Th2 cells are crucial for clearance of helminth parasites⁸⁵, the

immunological mechanisms of which share several features with the pathogenesis of allergic asthma.

Th17 cells are the third major T helper cell subset, defined by their dependence on the transcription factor ROR γ t (*Rorc/RORC* gene in mouse/human). Th17 cells are the T helper cell component of so-called type 3 immunity, which centres on production of IL-17 family cytokines IL-17A, IL-17F and IL-22⁷⁵. IL-17A acts on stromal cells to drive production of antimicrobial peptides and neutrophil chemoattractants⁸⁶ and is implicated in clearance of pulmonary fungal infections⁷², autoimmunity and homeostatic immunity at barrier surfaces^{87,88}. Th17 cells are also implicated in the pathogenesis of neutrophilic sub-phenotypes of asthma⁵ and transfer of antigen-specific Th17 cells into mice drive a neutrophilic inflammation that persists after therapeutic administration of glucocorticoids⁸⁹.

Multiple CD4⁺ T cell subsets, including Th2 and Th17 cells, can produce the effector cytokine IL-9⁹⁰. However, IL-9 is also produced by a distinct subset of Th9 cells, which can arise from naïve CD4⁺ T cells⁹¹ or from plasticity of Th2 cells⁹². Th9 cells are relatively poorly studied compared to Th1, Th2 and Th17 cells, but production of IL-9 has been implicated in immunity to helminth parasites⁹³. Overexpression of IL-9 in the lungs of mice drives eosinophilic inflammation, mucus hypersecretion and mast cell hyperplasia⁹⁴, mediated in part by upregulation of type 2 cytokines IL-4, IL-5 and IL-13⁹⁵. Furthermore, IL-9 was shown to be a key factor in mast cell-dependent pathology in murine models of AAD^{96,97}. Th9 cells are therefore likely to contribute to type 2 immune responses in certain settings.

Effector T helper cell responses are balanced by the activity of regulatory T cells (Tregs). The best characterised Treg populations are those expressing the transcription factor FoxP3, which can arise either during thymic selection⁹⁸ or by peripheral induction and stabilisation of FoxP3 by expression by signals such as the cytokine transforming growth factor β (TGF- β ⁹⁹; see Section 1.4). Both thymic and peripheral FoxP3⁺ Tregs suppress effector T cell function by contact-dependent mechanisms and release of soluble mediators, as reviewed by Schmidt et al¹⁰⁰. FoxP3⁺ Tregs play crucial roles in regulation of T cell responses to respiratory infection, established allergic inflammation^{101,102} and induction of tolerance to inhaled inert antigens¹⁰³.

As discussed in detail in Section 1.4, production of the regulatory cytokine IL-10 is a key mechanism of Treg function at mucosal surfaces, including the lung^{102,104}. However, IL-10 production is not restricted to FoxP3⁺ Tregs¹⁰⁵ and can be produced to varying extents by Th1, Th2 and Th17 cells, as well as other FoxP3⁻ CD4⁺ regulatory T cell subsets¹⁰⁶. In addition, cell-intrinsic production of IL-10 by CD8⁺ T cells has been shown to regulate the immune response to respiratory viral infection and reduce immunopathology^{107,108}. Thus, multiple populations of T cells with regulatory capacity control inflammation in the lung.

1.1.5. Innate lymphoid cells

Research in recent years has yielded rapid advances in understanding of innate production of T helper cell-associated cytokines from ILCs. ILCs are located in tissues at homeostasis and lack

an antigen-specific receptor, instead expanding and producing effector molecules in response to local cytokine signals. Three major ILC subsets exist, with cytokine and transcription factor patterns analogous to CD4⁺ T cells. Type 1 ILCs (ILC1s) express T-bet and produce IFN γ , ILC2s express GATA3 and produce IL-5 and IL-13 and ILC3s express ROR γ t and produce IL-17A and IL-22¹⁰⁹. The role of ILCs as an innate cytokine source is well exemplified by ILC2s, which are sufficient to drive an allergic, type 2, immune response in the lungs of mice lacking T and B cells¹¹⁰ and are required for the initiation of AAD by protease inhalation¹¹¹. The importance of ILCs during established pulmonary immune responses is less clear, but ILC2s have been implicated in tissue repair following acute viral infection¹¹².

1.1.6. Sentinel cell model of the coordination of pulmonary immunity

The brief literature review in this section highlights some of the complexity of the immune interactions that occur in the lungs, with stromal cells, resident and recruited innate leukocytes and adaptive lymphocytes acting together in coordinated immune responses to pathogens and allergens (**Fig. 1.2.**). At the heart of this complexity is a requirement for sentinel cells that sense changes in the airway environment and instruct immune responses by providing both activating and regulatory signals. The focus of this thesis was on the sentinel role of airway epithelial cells and macrophages, which are discussed in depth in the following sections.

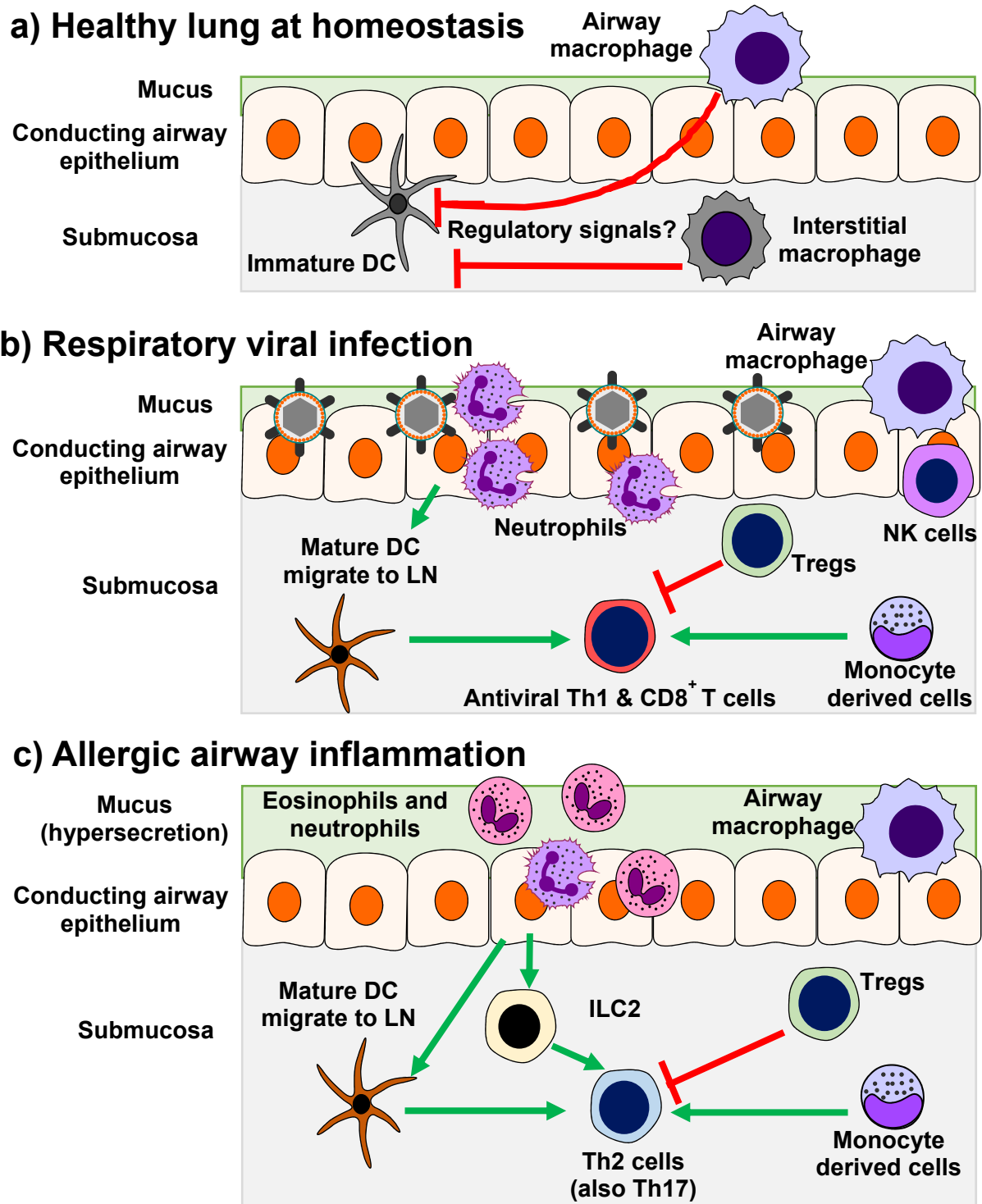


Figure 1.2. Immune cell interactions in the lung during homeostasis and inflammation

a) At steady state, AM are the principal leukocytes in the airway lumen, with a population of IM residing in the lung tissue. DC within and beneath the epithelial barrier scan for inhaled antigens, but are thought to be kept in an immature state by AM and IM. **b)** Respiratory infections such as influenza drive recruitment of neutrophils, monocytes and NK cells to the lung. DC maturation and migration to draining lymph nodes, driven by local cytokine signals results in a T cell response, dominated by Th1 and CD8⁺ T cells. **c)** Allergen exposure results in eosinophil-dominated inflammation, with neutrophils and monocytes also recruited. Epithelial cytokine release results in DC maturation and expansion and activation of ILC2, which together promote a Th2 cell response. Th17 cells can also be generated during allergic inflammation. Green arrows and red lines indicate positive and negative interactions, respectively.

1.2. Airway epithelial cells: an immunologically active barrier

In addition to their previously discussed barrier function, airway epithelial cells can produce an arsenal of mediators capable of initiating and regulating immune responses¹¹. The presence of airway epithelial cells at the interface with the external environment, in close proximity to DC, AM and other lung-resident leukocytes permits them to be key decision makers in the instruction of pulmonary immunity. Bone marrow chimera studies, in which TLR signalling is deficient in either haematopoietic or stromal cell compartments, have demonstrated a clear role for epithelial recognition of inhaled PAMPs for initiation of immune responses to allergens³⁶ and bacterial pathogens¹¹³. In addition to recognition of PAMP components of pathogens and environmental allergens, airway epithelial cells can be directly activated by intrinsic protease activity of certain allergens¹¹⁴, as well as responding indirectly to the cleavage products of proteinase allergens¹¹⁵. Airway epithelial cells also require signalling via the innate cytokine IL-1 α to mount an inflammatory response to HDM¹¹⁶, exemplifying how the epithelium integrates multiple signals to fine-tune the extent of immune reactions.

In response to activation of surface receptors by allergen inhalation, airway epithelial cells rapidly release mediators such as uric acid GM-CSF, CXCL1 and CCL2, which promote maturation of DC and recruitment of leukocytes to the airway^{36,116,117}. These leukocytes include monocytes that differentiate into moDC and contribute to T cell activation⁴⁰ (**Fig. 1.2**). Allergen exposure and other forms of epithelial disruption also cause release of IL-25 and IL-33^{36,114,116}, master regulators of ILC2 function that drive their expansion and production of type 2 cytokines^{109,110,118}, which in turn promotes a Th2 cell response^{111,119}. Epithelial cytokine and chemokine production is also highly relevant to respiratory infection. Airway epithelial cells are the initial site of infection for several common respiratory viruses¹²⁰⁻¹²² and conducting airway epithelial cells produce a range of cytokines and chemokines required for instruction of downstream immunity in response to influenza infection or treatment with the viral mimetic poly(IC) *in vivo*^{123,124}. The airway epithelium is also an important early source of the type I IFNs, IFN α and IFN β , and type III IFN, IFN λ , which act in an autocrine and paracrine manner to restrict viral replication^{125,126}. The epithelium is therefore a crucial site of immune recognition and initiation of inflammation in the lung.

In addition to instigating immune responses to pathogens or, in the case of allergens, inappropriately perceived threats, airway epithelial cells provide crucial regulatory signals implicated in maintenance of homeostasis. CD200 is constitutively expressed on the luminal surface of alveolar type II cells, which acts to limit activation of AM at steady-state and during inflammation¹²⁷. Epithelial-derived SP-A can dampen macrophage immune responses to certain lipopolysaccharide (LPS) serotypes *in vitro*¹²⁸ and is required for IL-4-dependent adoption of an AM phenotype associated with tissue repair¹²⁹. Both SP-A and SP-D can also inhibit pro-inflammatory cytokine production in macrophages *in vitro* by binding to the receptor SIRP α , in a manner that is prevented by the presence of necrotic cell debris¹³⁰, highlighting a potential

mechanism for injury sensing by these epithelial-derived mediators. Similarly, club cell-derived CCSP appears to have anti-inflammatory functions in the airways, since mice lacking CCSP expression display worsened immunopathology in response to several respiratory pathogens¹³¹⁻¹³³. Airway epithelial cells therefore provide both pro- and anti-inflammatory signals to drive measured immune responses while minimising immunopathology.

Inflammation is accompanied by tissue damage arising from both pathogen life cycles and the host immune response, necessitating repair processes to return to homeostasis. In the case of chronic lung inflammation, such as that in AAD, repeated cycles of injury and repair contribute to airway remodelling¹³⁴. Epithelial cells are an important source of mediators involved in tissue repair responses and, by extension, pathological mechanisms of airway remodelling, including endothelin-1, fibroblast growth factor, activin A and the pleiotropic cytokine TGF- β (discussed in detail in section 1.4)¹³⁵⁻¹³⁷. Thus, the airway epithelium is central throughout pulmonary immune responses, with roles in maintenance of homeostasis, initiation of inflammation and repair of injured tissue.

1.3. Airway macrophages: sentinels of pulmonary immunity

In addition to airway epithelial cells, AM are the most abundant cells in the healthy airway lumen and are therefore the first professional immune cells to encounter inhaled particles. Like the epithelium, AM have a key decision-making role in the initiation and maintenance of pulmonary immune responses¹⁹. AM display several differences from macrophages that differentiate from monocytes in tissues during inflammation and, by extension, from the frequently used *in vitro* model macrophages differentiated from human monocytes or murine bone marrow cells. These key features are reviewed in this section.

1.3.1. Development and origin of airway macrophages

AM, like the majority of tissue-resident macrophages, are now known to be derived from embryonic precursors and maintained largely independently of adult monocytes at homeostasis⁶¹. Work assessing the perinatal development of mice has shown population of the airways with AM to occur in the first days of life and to depend on the cytokine GM-CSF and the transcription factor PPAR γ , without which AM fail to develop^{18,24}. Importantly, when depleted by non-genotoxic ablation, AM were able to recover to baseline numbers within 9 days, in a manner dependent on local proliferation of residual AM under control of the cytokines macrophage colony-stimulating factor (M-CSF) and GM-CSF⁶¹, suggesting that homeostatic maintenance of AM occurs by proliferation of resident cells. Such *in situ* proliferation of tissue-resident macrophages is also a feature of the type 2 immune environment, where it is driven by IL-4¹³⁸ and, in the case of AM, concomitant binding of SP-A via the receptor myosin 18A¹²⁹.

Despite the tendency for AM to self-maintain by *in situ* proliferation, replacement by adult monocytes is possible and occurs following lethal irradiation, most likely due to reduction of the proliferative capacity of residual AM and changes to the lung environment caused by such severe

irradiation⁶¹. Long-term fate mapping studies using congenic bone marrow transplants in mice suggested around 40 % replacement of resident AM by monocyte-derived cells over a year, suggesting that homeostatic replacement of resident AM also occurs with age¹³⁹. This replacement was accelerated when acute lung injury was induced by administration of LPS to the airways, indicating that signals at the site of inflammation can promote replacement of resident AM by monocyte-derived cells¹³⁹. Using a more refined method of irradiation and bone marrow transfer, where lungs were shielded to protect AM from genotoxic depletion, monocyte differentiation into AM was demonstrated in a bleomycin mouse model of pulmonary fibrosis, suggesting that embryonic- and monocyte-derived AM can coexist in certain inflammatory settings¹⁴⁰.

The extent to which the origin of AM impacts function remains incompletely understood and is likely to differ depending on the context. A partially protected radiation chimaera model in which steady state AM were derived in equal proportions from embryonic precursors and adult monocytes showed remarkably few transcriptional differences between cells of different origin¹⁴¹. Similarly, adult monocytes, embryonic precursors or mature AM were all sufficient to populate the airway niche of AM-deficient mice lacking the receptor for GM-CSF, with few transcriptional differences apparent between the resultant AM⁶². Thus, at least at steady state, the airway microenvironment, rather than origin, appears to dominate control of AM gene expression and phenotype.

In the case of bleomycin-induced fibrosis however, monocyte- and embryonic-derived AM showed a number of differences in gene expression, with the monocyte-derived cells displaying increased expression of genes associated with the pathogenesis of fibrosis¹⁴⁰. Notably, few differences between monocyte- and embryonic-derived AM were observed after resolution of bleomycin-induced lung injury¹⁴⁰, suggesting that monocyte-derived AM adopt a gene expression profile close to that of true resident cells as homeostasis is restored. A situation in which AM of mixed origin but with similar overall phenotype coexist might be expected to occur in humans, who live longer and experience much higher microbial burdens than laboratory mice, which could conceivably result in greater contribution of circulating monocytes to the AM pool. However present technology does not allow precise tracking of cellular origins in humans as is possible in mice. Overall, existing data suggest that AM phenotype may depend upon combined effects of cellular developmental origin and microenvironmental signals of inflammation and tissue repair.

1.3.2. Airway macrophage phenotype and immune regulatory function

In addition to their embryonic origin and unique surface marker expression profile, the steady-state immunological phenotype of AM is also distinct from that of inflammatory monocyte-derived macrophages¹⁹. This phenotype is thought to be principally tolerogenic, actively restraining inflammatory responses to harmless environmental antigens¹⁹. Much of the evidence for this comes from *in vivo* depletion of AM in laboratory rodents using the cytotoxic drug clodronate, loaded into liposomes that are selectively phagocytosed by AM and cause cell death. *In vivo* AM

depletion results in heightened functional maturation of DC and increases the proliferative capacity of lung-resident T cells^{142,143}, suggesting that AM actively suppress the DC-T cell axis of initiation of adaptive immunity. Accordingly, liposomal depletion of AM enhances inflammation in multiple models of airway allergen exposure^{63,144-146}, suggesting that AM are required to limit pulmonary immune responses *in vivo*. A limitation of these studies is that clodronate depletion leaves remains of apoptotic AM in the airways that are ineffectively phagocytosed (Lloyd laboratory, unpublished observations), which could confound results by affecting inflammation. However, complementary studies have shown that transfer of naïve AM into AM-depleted rats that had been systemically sensitised to ovalbumin was sufficient to dampen the type 2 immune response to subsequent ovalbumin challenge¹⁴⁶, in a manner dependent on reduced DC antigen uptake and migration to mLN^{147 148}. Similar results were obtained with naïve AM transferred into mice sensitised to ovalbumin by inhalation using LPS as an adjuvant¹⁴⁵. Thus, steady-state AM possess an intrinsic capacity to restrict pulmonary immune responses.

The mechanisms by which AM prevent immune responses to innocuous antigens are not completely understood. Consistent with the heightened DC activation observed following AM depletion *in vivo*, AM prevent DC maturation when co-cultured *in vitro* across a semi-permeable membrane¹⁴³, suggesting that AM-derived soluble mediators restrain DC function at steady state. Co-culture experiments have also shown that naïve AM limit mitogen- or antigen-driven proliferation of T cells *in vitro*^{149,150}, are poor antigen-presenting cells^{150,151} and impair the responses of T cells to secondary stimulation¹⁵², suggesting potential direct suppression of T cell function by AM. AM exposed to inert protein antigen also induce FoxP3 expression and Treg function in naïve T cells when cultured *in vitro* and when co-transferred transferred into the airways, in a manner superior to that of antigen-pulsed DC¹⁵³. The extent to which these regulatory contacts between AM, DC and T cells occur locally in the lung remains to be determined, particularly since AM are non-motile in the steady state²⁰. However, AM have been shown by microscopy to exist in close proximity to DC processes interdigitating between alveolar epithelial cells in the steady state lung, which could feasibly allow homeostatic communication between these cell types¹⁴³. Additionally, multiple distal AM can signal in a coordinated manner to one another by sending synchronised waves of calcium ions via attached epithelial cells, acting to dampen neutrophil recruitment in response to LPS inhalation²⁰. Such long range signalling could theoretically allow AM to instruct other leukocytes *in vivo*, but this is not understood at this time.

Importantly, the tolerogenic state of AM can be broken in response to recognition of sufficient signals of danger, allowing initiation of immunity to threats such as infectious organisms. AM are a key early source of type I IFN during experimental RSV infection^{60,154}, which is responsible for rapid recruitment of inflammatory monocytes to the lung⁶⁰. AM are also required for the early production of pro-inflammatory mediators, such as TNF α and IL-6, and for recruitment of NK cells in this RSV model¹⁵⁴. Similarly, neutrophil recruitment to the airways, an early indicator of pulmonary inflammation, has recently been shown to drive inflammasome activation in AM,

resulting in release of active pro-inflammatory IL-1 β ¹⁵⁵. Production of inflammatory mediators, such as the chemokines CCL3 and CCL24, by AM is also apparent in human allergic asthma¹⁵⁶ and mouse models of AAD¹⁵⁷, indicating that activation of AM is a feature of both protective and dysregulated pulmonary immunity.

The effects of such 'activation' of AM *in vivo* on the proposed interactions between AM, DC and T cells in the lung are unclear, particularly since the latter cell types are concurrently engaged during immune responses *in vivo*, making isolation of the particular effects of AM difficult in these settings. Macrophages collected from BAL of rats during lung inflammation induced by bacillus Calmette-Guérin injection were no longer inhibitory to mitogen-induced T cell proliferation *in vitro*¹⁵⁸, suggesting that inflammatory signals may overcome the suppressive activity of AM. However, this study pre-dates distinction between resident AM and recruited monocyte-derived cells, so must be interpreted with caution. Others have shown that AM obtained from allergen-sensitised animals produce more pro-inflammatory cytokines and fail to dampen AAD when transferred into sensitised and challenged recipients, in contrast to transfer of naïve AM^{145,146}. Since transferred AM in these studies were obtained between sensitisation and onset of allergic inflammation^{145,146}, the observed effects are likely to reflect true reprogramming of resident AM to overcome their tolerogenic function.

Collectively, these data suggest that tolerogenic AM can be reprogrammed in response to pro-inflammatory stimuli and adopt a state that favours immune activation. However, this is now known to be an oversimplification, with AM likely to play a complex and multifaceted role throughout inflammation. Clodronate-mediated depletion of AM during established HDM-driven AAD results in exacerbated type 2 immunity and eosinophilia¹⁴⁴, indicating that AM regulate pulmonary immunity even when they themselves are in an activated pro-inflammatory state. AM were also shown to be crucial for resolution of inflammation after cessation of HDM exposure, which was delayed when AM were depleted¹⁴⁴. Further studies are therefore required to dissect the pro-inflammatory and tolerogenic mechanisms of AM employed in response to perturbations from homeostasis.

1.3.3. Macrophage polarisation and plasticity: implications for airway macrophages

The terms 'activation' and 'polarisation' are used largely interchangeably to describe perturbations in macrophage phenotype driven by exogenous stimuli such as cytokines and microbial PAMPs¹⁵⁹. The idea of macrophage polarisation, in particular, centres on the distinction between so-called 'classical' activation by LPS and the type 1 cytokine IFN γ ¹⁶⁰ and 'alternative' activation by the type 2 cytokines IL-4 and IL-13^{80,161}.

Classical macrophage activation induces expression of genes associated with microbicidal function such as the inducible nitric oxide synthase (iNOS; *Nos2/NOS2* gene in mouse/human) and increases expression of MHC-II and IL-12, which promote type 1 adaptive immune responses⁸⁰. The classically activated macrophage phenotype promotes killing of intracellular

bacteria such as *Mycobacterium (M.) tuberculosis*¹⁶² and mice lacking IFN γ expression are highly susceptible to *M. tuberculosis* infection¹⁶³. In contrast, alternative macrophage activation by IL-4 or IL-13 drives expression of genes related to immunity to extracellular parasites and tissue repair⁸⁰. IL-4 and IL-13 stimulation is inhibitory to control of intracellular *M. tuberculosis* infection by macrophages¹⁶⁴, but macrophages activated by these cytokines *in vivo* promote eosinophil recruitment during experimental helminth infection¹⁶⁵ and are necessary for worm clearance upon secondary infection⁸⁵, highlighting the context-dependent functions of different macrophage activation states.

The association of these distinct functional states of macrophages with responses to distinct types of pathogen and hallmark cytokines of adaptive immunity led to classification of binary classical and alternative activation states as 'M1' and 'M2', respectively, in line with the Th1 and Th2 dichotomy¹⁶⁶. However, multiple lines of evidence now show that this binary view of macrophage activation is an oversimplification. Macrophages display substantial plasticity and the capacity to integrate signals from multiple pro-inflammatory and regulatory mediators, resulting in expression of combinations of distinct modules of genes to adopt a spectrum of activation states¹⁶⁷. A nomenclature directly stating the name of macrophage stimulus (e.g. M[IFN γ], M[IL-4]) has since been proposed to reflect this complexity¹⁵⁹ and is used where relevant throughout this thesis. Additionally, macrophage polarisation is not fixed and can be modified further by subsequent stimulations. IL-4 co-stimulation is able to repress a subset of IFN γ -induced gene expression responses, leading to a distinct transcriptional profile from that driven by either stimulus alone¹⁶⁸. Similarly, impaired antibacterial function of macrophages elicited by nematode infection *in vivo* could be reversed by *ex vivo* stimulation with LPS and IFN γ ¹⁶⁹. The situation is further complicated for tissue-resident macrophages, the phenotype of which also depends on integration of tissue signals from the microenvironment that act via specific combinations of enhancer elements to drive distinct gene expression profiles^{170,171}.

As long-lived tissue-resident cells^{61,139}, AM are clearly distinct from the classical and alternative activation dichotomy¹⁹. Their activation state instead reflects integration of multiple signals, including lung-specific cues such as surfactant proteins^{19,129}, along with those of the prevailing cytokine milieu during inflammation¹⁹. However, markers of classical and alternative activation have shown some use in understanding the function of AM during pulmonary homeostasis and inflammation. AM upregulate iNOS, typical of classically activated M[IFN γ]⁸⁰, during tuberculosis infection¹⁷². In contrast, AM constitutively express the chitinase-like protein Ym1¹⁷³, a marker of alternatively activated M[IL-4/IL-13]⁸⁰, expression of which is increased during HDM-driven AAD¹⁷⁴. However, reliance on individual markers of AM activation states is potentially misleading, as a transcriptomic approach showed substantial gene expression changes in resident AM during bleomycin-induced fibrosis, including upregulation of genes associated with both M[IFN γ] and M[IL-4] extremes of the macrophage polarisation spectrum¹⁴⁰. The importance of the balance

between extremes of AM activation is clearly demonstrated by the enhanced type 2 immunity and airway remodelling observed in HDM-driven AAD where AM were deficient in the master regulator of M[IFN γ] polarisation, interferon regulatory factor 5 (IRF5)¹⁷⁵, and were therefore biased towards M[IL-4]-like activation *in vivo*¹⁷⁶.

Polarisation of AM during pulmonary inflammation can have durable detrimental effects on their functionality. For example, AM, but not blood monocyte-derived macrophages, from patients with chronic obstructive pulmonary disorder showed decreased responsiveness to stimulation antigens from the bacterial respiratory pathogen *Haemophilus influenzae*^{177,178}. Similarly, sustained exposure of mice to inhaled HDM reduced expression of TLR2 on AM, limiting their ability to recognise and respond to *Streptococcus pneumoniae* and thus impairing control of infection¹⁷⁹. AM dysfunction during pulmonary inflammation appears to be due to signals from the altered airway microenvironment, since loss of tolerogenic function of AM in ovalbumin-sensitised rats could be reversed by brief *ex vivo* culture¹⁸⁰. Dissecting the signals regulating AM activation and phenotype *in vivo* is therefore crucial to understanding their functions in health and disease. The focus of this thesis was the role of the regulatory cytokines IL-10 and TGF- β 1 in the airway environment, as introduced in the remainder of this chapter.

1.4. Control of pulmonary immunity by IL-10 and TGF- β 1

1.4.1. Immune regulation by IL-10

IL-10 is a potent anti-inflammatory cytokine capable of diverse functions via direct action on both myeloid cells and lymphocytes¹⁸¹. IL-10 signals by binding to a heterodimeric receptor (referred to collectively in this thesis as IL-10R), comprising the ligand-binding subunit IL-10RA¹⁸² and the signalling partner IL-10RB, which also forms part of the receptor for other cytokines such as IL-22^{183,184}. Together IL-10RA and IL-10RB trigger transphosphorylation of associated Janus kinases (Jaks) Jak1 and Tyk2, which allow docking and phosphorylation of signal transducer and activation of transcription (STAT) transcription factors, STAT1 and STAT3, which then homodimerise and activate gene expression in the nucleus¹⁸⁵ (**Fig. 1.3.**). Canonical IL-10 signalling results in suppression of the pro-inflammatory responses to PAMPs or cytokines such as IFN γ and IL-6¹⁸⁶, including reduced activation of the transcription factor NF- κ B¹⁸⁷. Although IL-10 itself can activate STAT1, suppression of pro-inflammatory cytokine signals depends in part upon overall reduction of STAT1 phosphorylation¹⁸⁶, along with induction of expression of suppressor of cytokine synthesis 1 and 3 (SOCS1/3) proteins¹⁸⁶, which non-competitively inhibit Jak kinase activity to negatively regulate cytokine signalling^{188,189}. The relative refractoriness of the IL-10R to feedback inhibition by SOCS3 compared with inflammatory cytokine receptors has been proposed to allow IL-10 to suppress pro-inflammatory cytokine signals while avoiding excessive feedback inhibition of its own signalling¹⁹⁰. Mechanisms of IL-10 signalling outside of this canonical pathway are possible, such as induction of SOCS3 expression in neutrophils independently of STAT phosphorylation¹⁹¹ and survival of germinal centre B cells by increased expression of the anti-apoptotic factor BCL2¹⁹².

Initial studies of IL-10 function in the immune system focussed on its role as a type 2 cytokine, owing to its production by Th2 cells and suppression of Th1 cell generation, thereby mediating cross-regulation between the two subsets¹⁹³. This effect was largely shown to be due to IL-10 suppressing the APC function of macrophages and DC, thus limiting Th1 cell polarisation¹⁹⁴⁻¹⁹⁶. IL-10 was a particularly potent inhibitor of macrophage APC function in these studies, reducing their production of polarising cytokines and their ability to induce proliferation and IFN γ production from CD4⁺ T cells¹⁹⁴⁻¹⁹⁶. Such limitation of pro-inflammatory type 1 responses by IL-10 most likely evolved to limit immunopathology during immunity to pathogens. However, this can conversely impact clearance of intracellular bacteria such as *M. tuberculosis*, infection with which is cleared more effectively in global IL-10 knockout mice and mice lacking IL-10 expression specifically in T cells¹⁹⁷. IL-10 is therefore crucial to the balance between protective immunity and immunopathology.

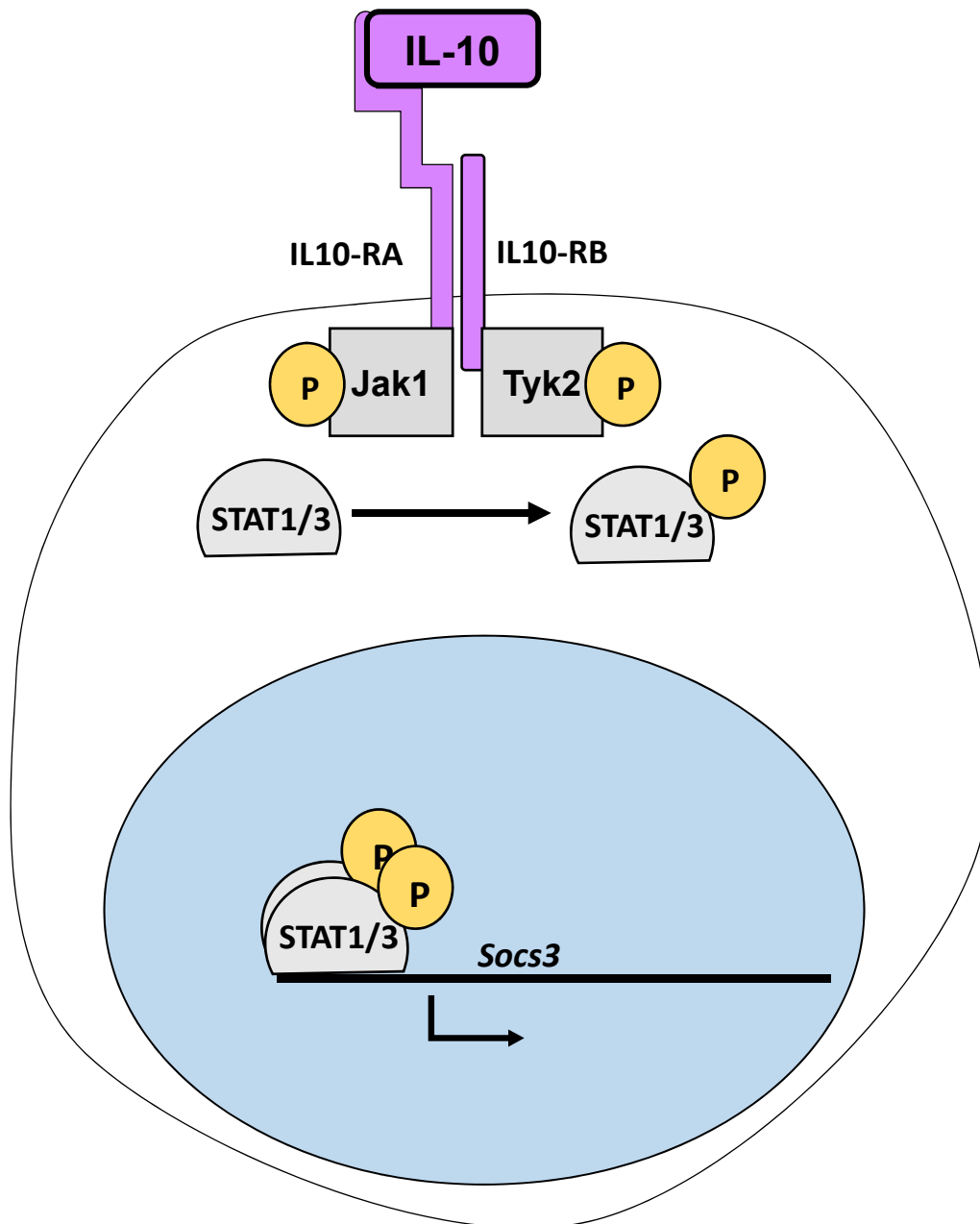


Figure 1.3. The canonical IL-10 signalling pathway

IL-10 binds to IL-10RA, allowing interaction with signalling partner IL-10RB and transphosphorylation of Janus kinases Jak1 and Tyk2. Phosphorylation by these kinases creates docking sites for STAT1 and STAT3, which become phosphorylated, dimerise and migrate to the nucleus where they control expression of genes such as *Socs3*/SOCS3.

IL-10 is now known to be more than a type 2 cytokine and can also be produced by a range of leukocyte populations, including FoxP3⁺ and FoxP3⁻ regulatory T cells, Th1 and Th17 effector T cells, CD8⁺ T cells, B cells, monocytes, macrophages and DC, as reviewed by Saraiva and O'Garra¹⁰⁶. The regulatory mechanisms upstream of IL-10 production show some differences between these cellular sources¹⁰⁶, which also have diverse functions *in vivo*, necessitating the context-specific study of IL-10 from individual cell types. Notably, many of the signals driving IL-10 expression are the same as those inducing pro-inflammatory cytokines, such as TLR4 signalling in monocytes, allowing autocrine regulation of pro-inflammatory cytokine responses via production of IL-10¹⁹⁸. However, these pathways are not entirely overlapping, as shown by the specific requirement of TNF receptor-associated factor 3 adaptor protein for induction of IL-10, but not pro-inflammatory cytokines, downstream of TLR activation¹⁹⁹. The balance between IL-10 and pro-inflammatory cytokine expression is therefore a key determinant of the magnitude of immune responses.

Global knockout of IL-10 in mice results in spontaneous colitis²⁰⁰, driven by an augmented Th1 response²⁰¹ and dependent on commensal enteric bacteria^{202,203}. Genetic defects in IL-10 or IL-10R are also associated with susceptibility to early onset inflammatory bowel disease in humans²⁰⁴. Subsequent studies have shown that T cell-derived IL-10 is crucial for prevention of spontaneous colitis in mice, at least in part by signalling to resident intestinal macrophages^{205,206}. Additionally, IL-10 derived from intestinal macrophages is required to control immunity to infection with the enteric pathogen *Citrobacter rodentium* and avoid excessive immunopathology²⁰⁷. The role of IL-10 as a regulator of tolerance to microbial colonisation in the intestine is therefore relatively well understood. However, as discussed in the following section, more questions remain about the regulatory functions of IL-10 in the respiratory tract.

1.4.2. IL-10 expression and function in the lung

The importance of IL-10 for regulation of pulmonary immune responses has been clearly demonstrated by work using global IL-10 knockout mice or systemic suppression of IL-10 signalling with a blocking antibody to IL-10RB. IL-10RB blockade enhances the severity of acute infection with either influenza or RSV, accompanied by augmented type 1 immune responses in the lungs^{107,108}. However, although these studies illustrate the importance of IL-10 for limiting immunopathology during acute infection, IL-10 is also necessary for viral persistence and limitation of the virus-specific T cell response during chronic infection with lymphocytic choriomeningitis virus^{208,209}. These extreme cases highlight the importance of IL-10 in the balance between pathogen clearance and immunopathology.

IL-10 knockout mice display worsened AAD in models of systemic sensitisation and inhalation challenge with ovalbumin²¹⁰ and repeated intranasal exposure to HDM²¹¹. Similarly, mice lacking IL-10RA expression in T cells show enhanced type 2 inflammation in a model of HDM-driven AAD, owing to decreased Th2 cell death²¹², supporting an important regulatory role for IL-10 in the allergic lung.

Despite the clear role for IL-10 in regulating pulmonary inflammation, the regulatory mechanisms governed by IL-10 signalling are incompletely understood, in part due to numerous cell types being proposed as major IL-10 sources in the lung. T cells have the clearest IL-10-dependent regulatory function in pulmonary immunity. IL-10-dependent regulation of immunopathology during acute influenza or RSV infection is due to its production by effector T cells^{107,108}, while transfer of IL-10-producing T cells into sensitised mice ameliorates allergic airway inflammation^{102,213}. Additionally, CD11c-negative pulmonary monocyte/macrophage cells, proposed to be IM, have been shown to produce IL-10 and to reduce allergic inflammation when transferred directly into the airways^{31,211,214}. IM transfer was sufficient to reverse the effects of global IL-10 knockout on HDM-driven inflammation²¹¹, suggesting that IM and monocyte-derived macrophages are potentially important IL-10 sources in the lung.

Interestingly, given their role as sentinels of pulmonary immunity, AM and bronchial epithelial cells have also been proposed as sources of IL-10; which could have implications for controlling thresholds of immune activation at the steady state, as well as regulating established inflammatory responses. However, results are inconsistent between reports. One study showed little to no IL-10 in AM of healthy human subjects by immunostaining, while bronchial epithelial cells from these individuals appeared to produce IL-10²¹⁵. Conversely, a separate study showed no immunostaining of IL-10 in epithelial cells in bronchial biopsies of healthy controls or asthmatics and no induction of *IL10* mRNA in primary bronchial epithelial cells or airway epithelial cell lines in response to pro-inflammatory cytokine stimulation²¹⁶. In contrast, the latter study showed positive IL-10 immunostaining on AM from BAL of healthy subjects²¹⁶. These inconsistencies mean that further elucidation is required of the mechanisms and cellular sources of IL-10 involved in regulation of pulmonary inflammation.

1.4.3. TGF- β signalling and activation

TGF- β 1 is a highly pleiotropic cytokine, with context-dependent and often contradictory functions in multiple aspects of cell and tissue biology²¹⁷, including in the immune system²¹⁸. TGF- β 1 is the prototypic cytokine of the TGF- β family, which also includes the TGF- β 2 and TGF- β 3 isoforms, and of the broader TGF- β superfamily, including activins, inhibins and bone morphogenic proteins²¹⁹. TGF- β 1, 2 and 3, collectively referred to as TGF- β or TGF- β isoforms throughout this thesis, share a common receptor complex and canonical signalling pathway²²⁰. Extracellular TGF- β binds first to a constitutively-active receptor serine/threonine kinase, TGF- β receptor type 2 (TGF- β R2), which can then recruit and phosphorylate a second receptor serine/threonine kinase, TGF- β receptor type 1 (TGF- β R1)²²¹. Phosphorylated TGF- β R1 can bind and phosphorylate cytoplasmic receptor Smad (R-Smad) proteins, Smad2 and Smad3, which accumulate in the nucleus and mediate TGF- β -driven gene expression changes, such as induction of the tissue repair mediator plasminogen-activator inhibitor-1 (PAI-1; *SERPINE2/Serpine2* gene in human/mouse)²²². Phosphorylated Smad2 and/or Smad3 form hetero-oligomers with the related protein Smad4, which does not directly bind to the TGF- β R complex but instead acts as a co-factor, or co-Smad, facilitating formation of oligomers capable of accumulation in the nucleus²²³

(Fig. 1.4.). Smad2 and Smad3 constantly shuttle between the nucleus and cytoplasm during active signalling, with repeated phosphorylation of R-Smads by active TGF- β R complexes allowing Smad2/3/4 oligomers to re-form and traffic to the nucleus²²⁴. In this way, the R-Smads dynamically sense TGF- β R activation state and Smad presence in the nucleus is therefore proportional to strength and duration of TGF- β signalling at the plasma membrane²²⁴. The Smad signalling pathway is also negatively regulated by the inhibitory Smad (I-Smad) Smad7. Smad 7 acts as an adaptor between TGF- β R and the E3 ubiquitin ligase Smurf2, which targets the receptor complex for proteasomal degradation²²⁵ and protein phosphatase 1 (PP1), which dephosphorylates TGF- β R1²²⁶ (Fig. 1.4.). Smad7 expression is rapidly induced by TGF- β signalling in an autoregulatory negative feedback mechanism²²⁷, but can also be induced by cytokines such as IFN γ , highlighting the potential for cross-regulation between signalling of TGF- β and other cytokines²²⁸.

In addition to canonical signalling through R-Smads, TGF- β activation of the TGF- β R complex can engage signalling pathways, including various mitogen-activated protein kinases (MAPKs), Rho-like GTPases and phosphatidyl-3-kinase lipid signalling, as demonstrated by activity of these pathways in model systems with defective Smad signalling²²⁹. These non-Smad pathways are less well studied than canonical TGF- β signalling but are implicated in key processes such as TGF- β -driven epithelial cell apoptosis, which is dependent on p38 MAPK but unaffected by expression of a dominant negative Smad3 protein that impairs canonical signalling²³⁰.

Another layer of complexity arises from the role of extracellular activation in regulation of TGF- β bioactivity. TGF- β isoforms are synthesised with a large amino-terminal pro-region, which is crucial for assembly of TGF- β homodimers inside the cell²³¹. The pro-region is cleaved during post-translational modification by furin and related proteases²³², but remains non-covalently associated with the carboxy-terminal cytokine region of TGF- β , preventing interactions with TGF- β R2 and thus maintaining TGF- β homodimers in an inactive latent state²³³. The former pro-region, or latency-associated peptide (LAP), mediates further contacts between TGF- β and the extracellular environment by covalent association with latent TGF- β -binding proteins (LTBPs), which anchors TGF- β to the extracellular matrix (ECM)²³⁴. TGF- β isoforms are therefore unique among cytokines in being exported from the cell in a form that is both spatially-restricted and incapable of signalling, allowing rapid temporal and spatial regulation of TGF- β bioactivity at the level of release from the latent complex.

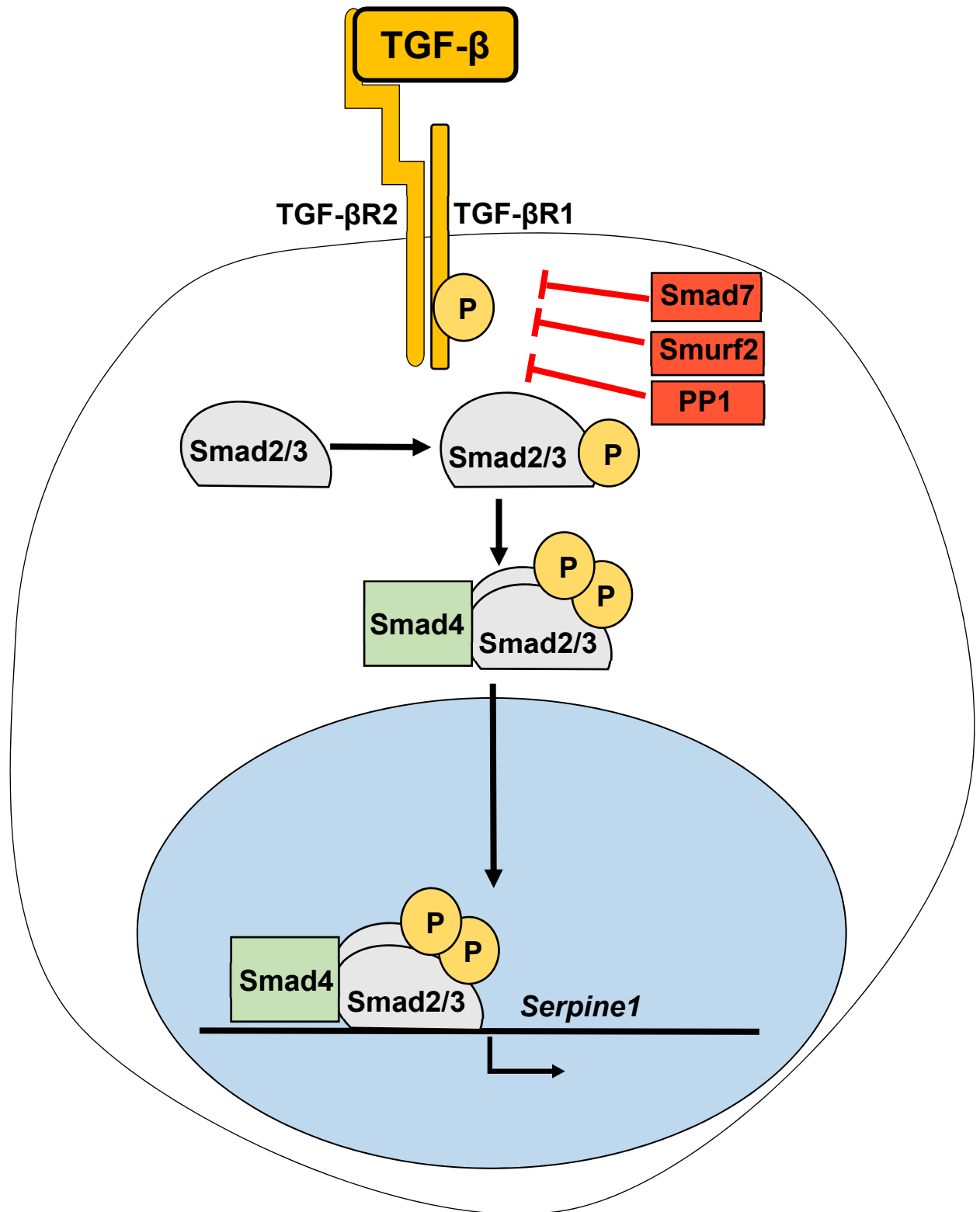


Figure 1.4. The canonical TGF- β signalling pathway

Active TGF- β binds TGF- β R2, which binds and phosphorylates TGF- β R1, creating docking sites for R-Smad (Smad2/3) binding and phosphorylation. Phosphorylated Smad2 and Smad3 form oligomers with co-Smad Smad4, which accumulate in the nucleus and control gene expression, such as induction of PAI-1 (*Serpine1*/*SERPINE1* gene). Negative regulators such as Smad7, Smurf2 and PP1 inhibit TGF- β signalling by promoting dephosphorylation, internalisation and degradation of the TGF- β R complex.

Latent TGF- β can be activated by proteases such as plasmin, thrombin and matrix metalloproteinases *in vitro*, although the *in vivo* relevance of these mechanisms is unclear²³⁵. Heat and acidic pH can also be used experimentally to activate latent TGF- β ²³⁶, but these are largely non-physiological conditions. Better understood *in vivo* is the importance of integrin interactions with LAP for activation of latent TGF- β 1 and TGF- β 3. LAP directly binds integrins via an arginine-glycine-aspartate (RGD) tripeptide sequence²³⁷, absence of which results in failure to activate latent TGF- β 1 *in vivo*²³⁸. Integrin-mediated activation is dependent on LTBP and is thought to require mechanical force exerted by the integrin against ECM-anchored TGF- β ²³⁹. Notably, LAP from TGF- β 2 lacks an RGD motif and so the mechanisms of its activation *in vivo* are less well understood than for the other isoforms. Integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ in particular have been shown to activate TGF- β 1^{240,241} and TGF- β 3^{242,243}. Activation of TGF- β by $\alpha_v\beta_6$ is thought to depend on its expression by epithelial cells^{244,245}, while $\alpha_v\beta_8$ is expressed by a number of immune cell populations and its expression on both DC and FoxP3⁺ Tregs has been shown to be important for TGF- β activation *in vivo*^{246,247}. Regulated integrin expression therefore presents a means of context-specific activation of TGF- β .

1.4.4. TGF- β 1 function in the immune system

Of the TGF- β isoforms, TGF- β 1 is by far the most widely implicated in the immune system²¹⁸. *Tgfb2* null mice die perinatally due to multiple developmental defects²⁴⁸, precluding meaningful immunological phenotyping, and *Tgfb3* null mice display defective palate fusion but no overt immunological disorder²⁴⁹. In contrast, global knockout of *Tgfb1* in mice does not cause gross developmental defects, but results in multi-organ inflammation that is fatal in the first weeks of life^{250,251}, highlighting the importance of TGF- β 1 in immune regulation.

Subsequent dissection of the mechanisms of TGF- β 1 immunoregulatory function *in vivo* demonstrated its importance in control of T cell responses. Conditional deletion of *Tgfb1* in T cells using CD4-Cre largely recapitulates the phenotype of the global TGF- β 1 knockout mouse line, with mice displaying lethal Th1-dominated immunopathology in multiple organs²⁵². Furthermore, mice with CD4-Cre-dependent knockout of TGF- β R2, that constitutively fail to receive TGF- β signals to T cells, also display fatal early-life autoimmunity, dominated by infiltration of Th1 and CD8⁺ T cells into organs^{253,254}. Together these studies indicated that a major immunological function of TGF- β 1 is control of T lymphocyte homeostasis.

However, subsequent experiments using a distal lymphocyte protein tyrosine kinase (dLck) Cre to delete TGF- β R2 did not recapitulate the lymphoproliferative disease observed with CD4-Cre-driven TGF- β R2 knockout²⁵⁵. This was proposed to result from lack of activation of the dLck promoter until late thymic development and release of T cells into the periphery²⁵⁵, indicating that the phenotype observed by Flavell, Rudensky and their colleagues^{253,254} was likely dependent on lack of TGF- β signalling to T cells throughout thymic development. However, the work of Zhang and Bevan²⁵⁵ confirmed an important role for TGF- β in regulating T cell homeostasis and preventing autoimmunity. dLck-Cre-driven TGF- β R2 knockout T cells caused lymphoproliferative

disease when transferred into *Rag1*^{-/-} B and T cell-deficient mice, in which exposure to self-antigens is increased, leading to the hypothesis that peripheral TGF- β signalling to T cells limits their responsiveness to low affinity T cell receptor ligands such as self-antigens²⁵⁵.

Regulation of T cell responses by TGF- β occurs in part through suppression of pro-inflammatory effector CD4⁺ T cell fates. TGF- β prevents differentiation of naïve CD4⁺ T cells into Th1 cells *in vitro* by reducing their responsiveness to the Th1-polarising cytokine IL-12²⁵⁶ and directly suppressing T-bet expression²⁵⁷. Accordingly, mice with limited TGF- β signalling to T cells mount an augmented protective Th1 response to the intracellular parasite *Leishmania*, clearing infection more rapidly than control mice²⁵⁷. TGF- β also prevents Th2 differentiation *in vitro* by suppression of GATA3 expression^{258,259}, demonstrating the potential of TGF- β to dampen both type 1 and type 2 immunity.

In contrast to its effects on Th1 and Th2 fates, TGF- β favours peripheral generation of inducible Tregs by inducing expression of FoxP3 in naïve CD4⁺ T cells^{99,153}. TGF- β may also promote stability of FoxP3 expression *in vitro* and *in vivo* by driving partial demethylation of conserved non-coding sequences in the *Foxp3* gene^{260,261}; although the latter mechanism is complex and incompletely understood²⁶². Mice lacking expression of both Smad2 and Smad3 in T cells display reduced peripheral Treg numbers²⁶³, consistent with generation and/or maintenance of these cells depending on canonical TGF- β signalling to T cells. In addition, TGF- β promotes IL-10 expression from both FoxP3⁺ and FoxP3⁻ CD4⁺ T cells in the periphery¹⁰⁵ and can cooperate with the myeloid cell-derived cytokine IL-27 to promote differentiation of IL-10-secreting regulatory T cells that are largely negative for FoxP3²⁶⁴. Thus, TGF- β has potential to direct multiple regulatory T cell phenotypes.

As well as promoting Treg generation, TGF- β production by Tregs is widely implicated in their anti-inflammatory functions. In T cell transfer models of colitis, antibody blockade of TGF- β ablates the protective effect of Treg transfer against pathogenic type 1 immunity²⁶⁵ and effector T cells expressing a dominant-negative TGF- β R2 were not suppressed by transferred Tregs *in vivo*²⁶⁶. TGF- β is therefore central to several aspects of regulatory T cell biology.

Although suppression of Th1 and Th2 differentiation and generation of peripheral Tregs supports a general anti-inflammatory function of TGF- β , TGF- β can also drive Th17 cell differentiation in cooperation with pro-inflammatory cytokines such as IL-6 and IL-1 β ²⁶⁷. Additionally, TGF- β promotes Th9 cell generation from naïve CD4⁺ T cells in combination with IL-4, which prevents TGF- β -dependent upregulation of FoxP3⁹¹ and can cause differentiated Th2 cells to adopt a Th9 like phenotype with reduced GATA3 expression that produce high levels of IL-9⁹². The range of effects of TGF- β on CD4⁺ T cell differentiation illustrates its diverse, potentially contradictory, functions in different settings (**Fig. 1.5**).

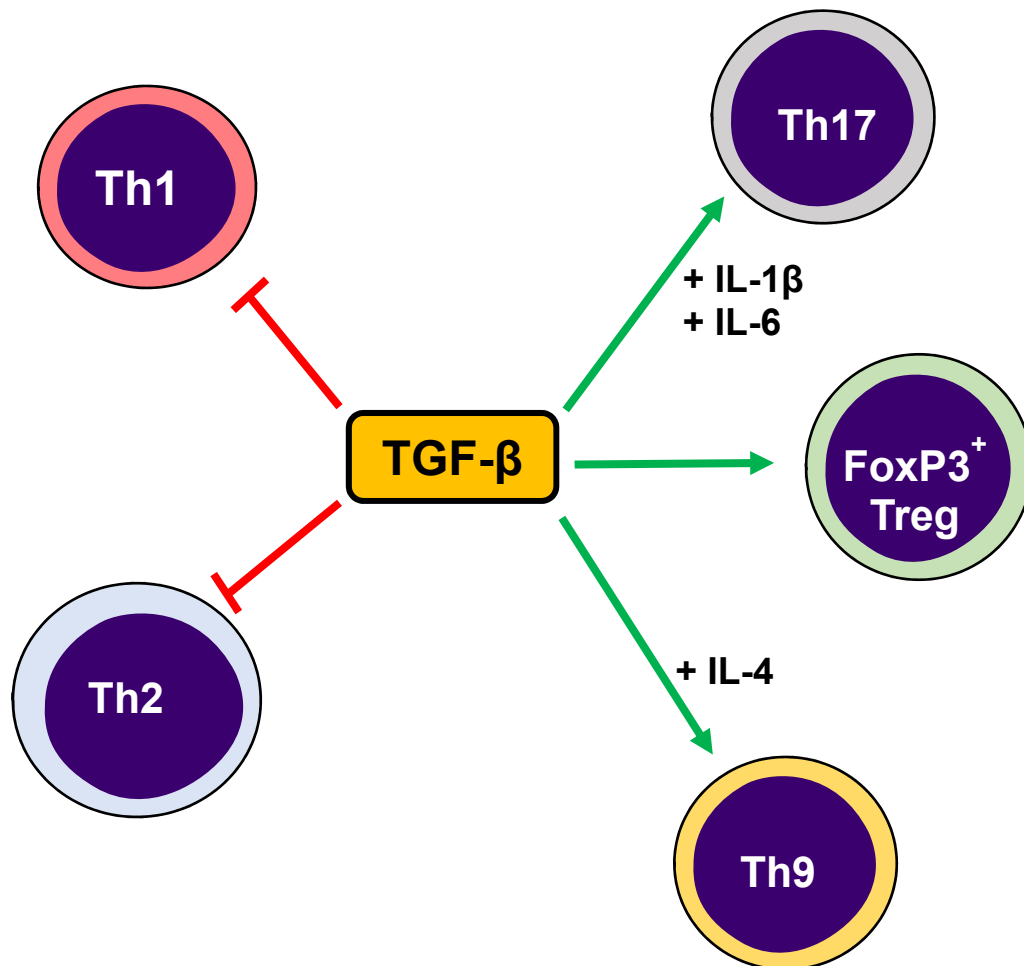


Figure 1.5. Context-specific control of CD4⁺ T cell phenotypes by TGF-β

Effects of TGF-β on generation of different T helper cell fates as described in Section 1.4.4. TGF-β directly suppresses Th1 and Th2 cell fates, in part by suppression of T-bet and GATA3 expression, respectively. TGF-β also promotes expression and maintenance of FoxP3 expression in peripheral Tregs and can drive Th17 and Th9 cell differentiation in combination with other cytokines. Green arrows and red lines indicate positive and negative interactions, respectively. See text in Section 1.4.4 for references.

The immune regulatory effects of TGF- β are not limited to T cells. For example, TGF- β suppresses proliferation and cytokine production by NK cells²⁶⁸ and deletion of TGF- β R2 on DC reduces their capacity to induce Tregs *in vitro* and *in vivo*, indicating that TGF- β promotes tolerogenic DC function²⁶⁹. TGF- β signalling is also required for development of epidermal Langerhans cells²⁷⁰ and a population of CD103⁺ CD11b⁺ intestinal DC that are necessary for local homeostatic generation of Tregs and Th17 cells in the gut²⁷¹. The pleiotropic functions of TGF- β in multiple different immune cell populations highlight the importance of studying TGF- β in specific organs, cell types and physiological contexts.

1.4.5. TGF- β 1 expression and function in the lung

TGF- β 1 is widely expressed in the healthy human and mouse lung, detectable in conducting airway and alveolar epithelial cells, AM, endothelial cells and peribronchial mesenchymal cells^{272,273}. TGF- β R2 is also expressed on multiple pulmonary stromal and immune cell subsets in the lung^{135,274}, allowing for a large number of potential TGF- β 1-mediated signalling events *in vivo*. In addition, expression of $\alpha_v\beta_6$ integrin on airway epithelial cells has been shown to be decisive in the control of pulmonary inflammation and fibrosis by local activation of TGF- β with anti-inflammatory and pro-fibrotic capacity^{240,244,245}, highlighting the importance of spatially restricted production and activation of TGF- β in the lung.

The potential of TGF- β to regulate pulmonary immunity has been widely demonstrated. For example, antigen-specific tolerance induced by repeated low-dose ovalbumin inhalation in mice is reliant on TGF- β expression on the surface of ovalbumin-specific regulatory T cells, which limit the proliferation of CD4⁺ effector T cells¹⁰³. In the case of experimental influenza infection, global antibody blockade of all three TGF- β isoforms resulted in increased immunopathology and mortality, likely due to impaired regulation of the T cell response²⁷⁵. However, the regulatory role of TGF- β during influenza infection appears to be context-specific, since lack of $\alpha_v\beta_6$ integrin expression on airway epithelial cells reduced disease severity, likely due to impaired TGF- β -dependent dampening of antiviral immunity in AM²⁴⁵.

Work using models of systemic sensitisation and airway challenge with ovalbumin have demonstrated a regulatory function for TGF- β 1 in the type 2 immune response to allergen. Heterozygous *Tgfb1*^{-/+} mice with reduced systemic TGF- β 1 expression displayed enhanced type 2 inflammation in the lungs²⁷⁶, while adoptive transfer of ovalbumin-specific CD4⁺ T cells engineered to overexpress TGF- β 1 reversed type 2 inflammation and AHR in an antigen-specific manner²⁷⁷. However, study of TGF- β in models of AAD is complicated by the central role of TGF- β in wound healing and fibrotic processes typical of the remodelling of the airway wall that occurs in asthma and experimental allergic inflammation²⁷⁸. TGF- β can promote a variety of features of airway remodelling, including epithelial cell apoptosis and ECM deposition²⁷⁸. Accordingly, targeted hyperactivation of TGF- β signalling in the airway epithelium using an adenoviral Smad2 overexpression vector enhanced peribronchial smooth muscle hyperplasia and collagen deposition following repeated HDM inhalation, without affecting inflammation¹³⁶. TGF- β therefore

has potential for both beneficial (immune regulation) and pathogenic (airway remodelling) functions in the allergic lung.

The magnitude of the conflicting effects of TGF- β appear to depend on the exact nature of the immune response to allergen. In a systemic sensitisation and inhalation challenge model of ovalbumin-driven AAD, a pan-TGF- β blocking antibody prevented airway remodelling when administered to mice with established disease, without enhancing pulmonary inflammation²⁷⁹. In contrast, the same antibody administered after establishment of allergic inflammation driven by HDM inhalation did not improve airway remodelling, but enhanced type 2 inflammation²⁸⁰. These conflicting results likely reflect differential involvement of TGF- β in regulation of immune responses generated by systemic sensitisation compared with mucosal sensitisation, as well as potential intrinsic features of HDM as an allergen compared with ovalbumin. The regulatory function of TGF- β in the lungs *in vivo* is therefore highly context-dependent and must be studied as such.

Context-specific study of TGF- β function in the lung is likely to centre on sentinel airway macrophages and epithelial cells, the major cell types expressing TGF- β 1 in the lung at steady state^{272,273}. Conditional deletion of TGF- β 1 in club cells of the conducting airway epithelium ablated TGF- β release into the airway lumen following intranasal challenge with HDM or IL-33, indicating that club cells release TGF- β 1 after allergen inhalation²⁷³. In contrast to global TGF- β 1 knockout mice^{250,251}, or mice lacking TGF- β 1 expression in T cells²⁵², club cell-specific TGF- β 1 knockout mice displayed no steady-state immunopathology and were instead protected from HDM- or IL-33-driven AAD, due to impaired ILC2 recruitment and activation²⁷³. Thus, this highly specific source of TGF- β 1 is redundant for regulation of the pulmonary T cell response, but an essential cofactor for ILC2 function in the allergic lung²⁷³.

Less is known about the specific role of AM-derived TGF- β in pulmonary immunity. TGF- β was shown to be required for optimal induction of FoxP3 in naïve T cells by AM in co-culture experiments¹⁵³, but the relevance of this mechanism *in vivo* has not been determined. Deletion of TGF- β 1 in myeloid cells using LysM-Cre ameliorated bleomycin-induced fibrosis in a genetically susceptible mouse model²⁸¹. However this mouse strain also featured spontaneous recruitment of monocytes to the lung²⁸¹, so it is unclear whether AM-derived TGF- β 1 was involved in fibrosis in this setting.

Also incompletely understood at this time is the role of TGF- β signalling to AM in regulation of their development and function. Mice lacking the epithelial integrin, $\alpha_v\beta_6$, display heightened spontaneous AM activation²⁴⁵ and develop emphysema due to overproduction of the AM-derived matrix metalloproteinase (MMP) 12²⁴⁴, suggesting that integrin-mediated activation of TGF- β in the vicinity of AM is important for their regulation. Furthermore, a study published during preparation of this thesis showed conditional deletion of TGF- β R2 in AM, using CD11c-Cre mice, to result in almost complete deficiency in AM, suggesting that, as well as regulating their activation, TGF- β is crucial for AM development and maintenance²⁸². In contrast, in an earlier

study, mice with LysM-Cre-driven deletion of TGF- β R2 in myeloid cells showed no decrease in AM numbers, but displayed enhanced emphysematous pathology following helminth-induced lung injury, along with evidence of AM dysfunction and elevated lung MMP activity²⁸³, consistent with TGF- β regulating AM activation *in vivo*. The contrasting effects of LysM-Cre- and CD11c-Cre-mediated TGF- β R2 deletion on AM development and maintenance can likely be explained by superior efficacy of CD11c-Cre at gene deletion in AM^{25,282}. However, these findings together highlight the need for better understanding of the role of different pulmonary TGF- β sources in regulating AM function *in vivo*.

1.5. Aims of thesis

From the brief review of existing literature in this chapter, it is clear that regulation of pulmonary immunity is complex and requires contributions from innate and adaptive leukocytes, as well as epithelial cells. The focus of this thesis was on better understanding the role of airway macrophages and epithelial cells as sentinels of pulmonary immune regulation, particularly the importance of IL-10 and TGF- β 1 in these processes.

Overall aim

Identify mechanisms by which IL-10 and TGF- β 1 mediate interactions between airway macrophages, epithelial cells, and pulmonary leukocytes to regulate immune responses in the lung.

Specific aims

- 1) Characterise changes in AM phenotype during allergic inflammation and pulmonary infection and describe how pulmonary inflammation affects IL-10 and TGF- β isoform expression in these cells
- 2) Determine the specific effects of AM- and epithelial cell-derived IL-10 and TGF- β 1 expression on pulmonary inflammation
- 3) Determine the effects of pulmonary IL-10 and TGF- β signalling on AM phenotype *in vivo* during inflammation

2. Materials and methods

2.1. Experimental animals

All work on live mice was conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and subsequent European Union directives. Mice were bred at Charles River Laboratories and housed in the Imperial College Central Biomedical Services facilities during experiments. In the case of genetically altered mice, littermates lacking either Cre recombinase or reporter allele expression from matched breeding were used as controls for all experiments. Mice were used for experiments at 6 to 9 weeks of age unless otherwise stated. Ageing of mice to 7 months, as described in Chapter 6, was carried out at Charles River Laboratories, with mice transferred to Imperial College a week prior to tissue harvest. Mice at both facilities were housed under specified pathogen-free conditions with free access to food and water.

Wild type C57BL/6J, BALB/c and 129 mice were purchased from Charles River Laboratories. All genetically altered mice were on a C57BL/6 genetic background, unless otherwise stated. 10BiT IL-10 reporter mice were originally generated by the Casey Weaver laboratory¹⁰⁵ and were obtained from the Anne O'Garra laboratory at the Francis Crick Institute, London with the kind permission of Professor Weaver. CD11c-Cre mice, original generated by the Boris Reizis laboratory²⁸⁴, were purchased from the Jackson Laboratory. *R26R^{eYFP}* stop-flox reporter mice were originally generated by the Frank Costantini laboratory²⁸⁵ and were a kind gift from Dr Brigitta Stockinger at the Francis Crick Institute, London. Mice with LoxP sites in the endogenous *Il10* locus (*Il10^{fl/fl}* mice), originally generated by Müller and colleagues²⁸⁶, were crossed to CD11c-Cre and CD4-cre²⁸⁷ mice in the Anne O'Garra laboratory (the Francis Crick Institute, London) to generate CD11c^{Il10} and CD4^{Il10} conditional knockout mice, respectively. Mice with LoxP sites in the endogenous *Tgfb1* locus (*Tgfb1^{fl/fl}*) on mixed genetic background (129/Black-Swiss/C57BL/6) were originally generated by Doetschman and colleagues²⁸⁸ and were purchased from the Jackson Laboratory. *Tgfb1^{fl/fl}* mice were crossed to CD11c-Cre mice to generate CD11c^{Tgf} mice, and to CCSP-reverse tetracycline activator (CCSP-rtTA) and tetracycline-inducible CMV-Cre mice²⁸⁹, originally obtained from the Max-Delbruck-Centrum für Molekulare Medizin (Berlin, Germany) with the kind permission of Professor Jeffrey Whitsett, to generate doxycycline-inducible bronchiolar epithelial club cell-specific *Tgfb1* knockout (epTGFβ1KO) mice²⁷³. Cre expression was induced in epTGFβ1KO mice by a single intraperitoneal (i.p.) injection of 2 mg doxycycline hyclate (Sigma-Aldrich).

2.2. Mouse models of allergic airway disease

2.2.1 Allergen exposure

All allergens were delivered by intranasal (i.n.) administration in a volume of 25 µl PBS. Briefly, mice were placed under light isoflurane anaesthesia and 25 µl of allergen solution dropped into nostrils to allow inhalation. Control mice received 25 µl PBS i.n. at the same frequency as allergen administration. For the *Alternaria alternata* (ALT)-driven AAD model, 10 µg ALT was administered 3 days per week for 1 to 3 weeks. A single batch of ALT (#227751) was obtained from Greer® and used for all studies described in this thesis

For the house dust mite (HDM)-driven model of AAD, 25 µg HDM was administered 5 days per week, except for when comparing multiple mouse strains, where mice received 25 µg HDM 3 days per week. Allergen challenge protocols are described in detail in the relevant results sections. 3 batches of HDM were purchased from either Greer® or Citeq Biologics and used for the work in this thesis, as detailed below.

- 1) Greer® batch #228282 was used for initial AM phenotyping experiments in Chapter 3 (sections 3.3-3.4) and for the RNAseq experiment described in Chapter 5.
- 2) Greer batch #290903 was used for studies comparing mouse strains in Chapter 3 (sections 3.6-3.7).
- 3) Citeq batch 15J02 was used for all experiments described in Chapter 6 and for 10BiT reporter mouse experiments described in Chapter 4.

2.2.2 Blockade of pulmonary IL-10 signalling with anti-IL10R antibody

Ultra-low endotoxin, azide-free (Ultra-LEAF™) preparations of rat anti-mouse IL10RB antibody (clone 1B1.3a) and rat IgG1 κ isotype control (clone RTK2071) were purchased from Biolegend. 50 µl of antibodies were delivered i.n. in a 50 µl volume of azide-free PBS 24h prior to the first HDM challenge and at twice weekly intervals throughout the allergen exposure protocol as detailed in Chapter 6.

2.2.3. Lung function assessment

Mice were placed under surgical anaesthesia with 50 mg/kg intraperitoneal (i.p.) pentobarbital (Sigma-Aldrich) and 100 mg/kg intramuscular ketamine (Ketaset, Zoetis). Tracheas were surgically exposed and cannulated. Mice were mechanically ventilated via cannulas using Flexivent™ apparatus (Scireq) as described previously²⁹⁰. Ventilation was performed using a tidal volume of 7 ml/kg body weight, a ventilation rate of 150 breaths/minute and a positive end-expiratory pressure of approximately 2 cm H₂O, comparable to normal breathing. Lung volume history was standardised by performing two deep inflations before making measurements of airway function. Measurements of resistance, elastance and compliance were determined from a user-defined protocol using the snapshot-150 perturbation, which is a single frequency sinusoidal waveform. Measurements were taken at baseline and in response to increasing concentrations of methacholine (Sigma-Aldrich), which was dissolved in PBS, nebulised and delivered via the inspiratory line of the ventilator. Airway resistance and pulmonary elastance and compliance were determined using flexiWare software (Scireq) by fitting data to a single compartment model using multiple linear regression as detailed below.

$$\text{Pressure} = (\text{resistance} \times \text{flow}) + (\text{elastance} \times \text{volume}) + \text{fitting constant}$$

Data points were excluded if the software-reported coefficient of determination, which indicates the quality of fit to the single compartment model and therefore the likely accuracy of measurements, was less than 0.9 of a maximum value of 1.

2.3. Mouse models of acute lung injury and pulmonary infection

2.3.1. Lipopolysaccharide (LPS)-induced acute lung injury

A single dose of 2.5 mg *in vivo*-grade LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) was delivered i.n. in a 50 µl volume of PBS and mice culled between 24-72 hours after LPS challenge. Control mice received 50 µl of PBS i.n.

2.3.2. Respiratory syncytial virus infection

RSV-A2 (American Type Culture Collection) in the form of HEP2 cell lysates was a kind gift from Dr James Harker (Imperial College London). $0.8-1 \times 10^6$ focus forming units (FFU) of RSV were administered i.n. in a volume of 100 µl, with control mice receiving 100 µl of uninfected HEP2 cell lysate. Mice were monitored once daily for weight loss and culled at time-points up to 7 days post-infection (dpi).

2.3.3. Influenza infection

X31 strain H3N2 influenza A virus was a kind gift from Dr Andreas Wack (Francis Crick Institute, London). 1.2×10^4 tissue-culture infectious dose₅₀ units (TCID₅₀) of X31 influenza were administered i.n. in a volume of 50 µl PBS. Control mice received 50 µl of PBS i.n. Mice were monitored once daily for weight loss and culled at time-points up to 6 dpi.

2.3.4. Antibody blockade of pulmonary interferon β signalling

To evaluate the effects of local IFN β blockade during influenza infection, mice were administered 100 µg of LEAF™ Armenian hamster anti-mouse IFN β (clone MIB-5E9.1, Biolegend) i.n. in a volume of 100 µl PBS, 1 day prior to influenza infection. Control mice received LEAF™ Armenian hamster IgG isotype control (clone HTK888, Biolegend) i.n.

2.4. Dissection and preparation of tissues

Mice were euthanised by intraperitoneal overdose of pentobarbital or cervical dislocation and death confirmed by severing a peripheral artery, as required for experiments and dictated under Schedule 1 of the Animals (Scientific Procedures) Act, 1986. In the case of lung function experiments or where blood was collected for flow cytometry, mice were terminally exsanguinated under terminal anaesthesia as described below.

2.4.1. Peripheral blood preparation for flow cytometry

Blood was obtained by cardiac puncture under terminal anaesthesia and added at a 1:4 ratio to red cell lysis buffer (RCLB; 155 mM NH₄CL, 13 mM KHCO₃ and 100 µM in demineralised water, pH 7.4), washed in a second change RCLB and suspended in complete RPMI (cRPMI; RPMI-1640 medium, containing 2mM L-glutamine, phenol red, 10 % foetal bovine serum [FBS] and 100 U/ml penicillin and streptomycin solution, all from Thermo-Fisher).

2.4.2. Bronchoalveolar lavage (BAL)

For standard experiments, tracheas were exposed and cannulated, before flushing airways with 3 changes of 400 µl PBS (Thermo-Fisher), recovering approximately 1 ml of BAL. BAL was centrifuged at 350 x g for 5 minutes to pellet cells. Supernatants were frozen at -80°C and cells suspended in cRPMI.

When requiring high yields of AM for cell sorting or *in vitro* cultures, as indicated in the relevant results sections, a high-volume protocol was employed. Mice were cannulated as above and airways flushed with 3 changes of 1 ml of 10 mM EDTA in PBS (Thermo-Fisher). Lungs were then inflated with a further 1 ml EDTA and mice left for 5 minutes at room temperature to allow disruption of interactions between airway leukocytes and epithelium. EDTA was removed and airways lavaged a further 3 times with EDTA. All 7 lavages were pooled and cells centrifuged and resuspended in cRPMI or buffer, as required for downstream experiments.

2.4.3. Mediastinal lymph nodes (mLN)

Individual mLN were separated with forceps and placed in cRPMI. Single cell suspensions were generated by passing through 70 µm cell strainers (BD) and resuspending in cRPMI.

2.4.4. Lung tissue

For standard experiments, lungs were removed *en bloc* after BAL and individual lobes dissected for use in different downstream applications. Right superior lobes were inflated with PBS via a major airway and fixed in 10 % buffered neutral formalin (Leica Biosystems) for 24 hours for histological use. Right middle and inferior lobes were frozen using liquid nitrogen or dry ice and stored at -80°C for subsequent quantification analysis. Right post-caval lobes were incubated in RNA/later™ solution for 24h at 4 °C then frozen at -80 °C until subsequent RNA extraction. To obtain single cell suspensions, whole left lungs were minced with scissors and incubated in cRPMI containing 150 µg/ml collagenase D and 25 µg/ml DNase I (both Roche diagnostics) in a shaking water bath at 37 °C for 1 hour. Tissue was then passed through 70 µm cell strainers and cell pellets resuspended in RCLB to lyse erythrocytes, before suspending cells in cRPMI.

For confocal imaging of lung tissue, BAL was not performed and lungs were instead inflated via a tracheal cannula with 2 % TopVision low melting point agarose (Thermo Fisher) in PBS and agarose allowed to solidify by placing the mouse on ice for 5 minutes. Lungs were then dissected and left lungs embedded in low melting point agarose and 200 µm precision-cut lung slices (PCLS) prepared by cutting in the horizontal plane with a vibratome. Slices were kept alive by transferring to cRPMI in black, clear-bottom 24-well µ-Plates (Ibidi) and maintaining at 37 °C and 5 % CO₂ until required.

2.4.5 Peritoneal lavage

Skin was removed from the mouse abdomen and peritoneal surface sterilised with 70 % ethanol. 4 ml of 10 mM EDTA in PBS was injected into the peritoneal cavity with a 27 G needle and the abdomen massaged to release resident cells. Peritoneal lavage fluid was recovered with a 23 G needle and resuspended in medium or buffer as required for downstream experiments.

2.4.6. Bone marrow extraction

Hind-legs were cut off at the hip and skin and muscle removed. Femurs and tibias were sterilised in 70 % ethanol and ends removed with a scalpel, before flushing bone marrow with sterile cRPMI via a 27 G needle. Large cell aggregates were separated by pipetting and cells resuspended in medium or buffer to obtain single cell suspensions.

2.4.7. Primary airway epithelial cells

Mice were tracheostomised and tracheas brushed between the larynx and primary bronchi with 4 mm interdental brushes (TePe®). Cells were immediately transferred to the required buffer or medium by submerging brushes.

2.5. Live, total and differential cell counts

When quantifying live cells as for cell cultures, cells were stained with 0.1 % Trypan Blue solution (Sigma-Aldrich) and Trypan Blue-negative cells counted by eye using Fastread 102 disposable counting chambers (Immune Systems). For all other purposes, total cell counts were obtained by staining cells with 0.02 % (w/v) crystal violet solution (Sigma-Aldrich), made up with 700 mM acetic acid in PBS.

For differential cell counts, 5×10^4 cells were adhered to Fisher Brand glass microscope slides (Thermo-Fisher) using a Cytospin® 3 cytocentrifuge (Thermo-Fisher) and fixed for 10 minutes in 100 % methanol. Cytocentrifuge preparations were rehydrated in cytospin buffer (4 mM NaH_2PO_4 and 5 mM KH_2PO_4 in deionised water, pH 7.4) for 30 minutes at room temperature, before immersing in Wright-Giemsa stain (Sigma-Aldrich) for 5 minutes, washing in cytospin buffer for 3 minutes and rinsing briefly with tap water. Macrophages and monocytes, eosinophils, neutrophils and lymphocytes were identified based on morphology, size and staining colour and cell numbers determined using the calculation below, counting at least 200 cells per slide.

$$\text{Total number of cell type x in tissue} = \frac{\text{\# cell type x counted}}{\text{\# total cells counted}} \times \text{Total crystal violet cell count per unit tissue}$$

2.6. Cell culture

2.6.1. Generation of bone marrow-derived macrophages and dendritic cells

To generate bone marrow-derived macrophages (BMDM), 3×10^6 bone marrow cells were cultured in 10 ml of cRPMI supplemented with 55 μM 2-mercaptoethanol (Thermo-Fisher) and 100 ng/ml recombinant human macrophage colony stimulating factor (M-CSF, Peprotech) in non-tissue culture-treated 10 cm petri dishes. After 3 days of culture, 5 ml of medium was removed and replaced with fresh supplemented cRPMI. After 7 days of culture, non-adherent cells were removed by washing with PBS and BMDM harvested by brief incubation with 0.05 % Trypsin-EDTA (Thermo-Fisher) and resuspending in cRPMI without M-CSF or 2-mercaptoethanol for use in experiments.

2.6.2. Stimulation of primary murine macrophages

1.5×10^5 live BAL cells, peritoneal lavage cells or BMDM were added to wells of 48-well plates (Corning) and macrophages allowed to adhere for 2 hours at 37 °C and 5 % CO_2 in a humidified incubator. In the case of peritoneal lavage, which contains a lower proportion of macrophages than BAL, only live cells of monocyte/macrophage morphology (large, round cells) were counted when preparing cell suspensions to normalise macrophage input between sample types. Medium was then removed after adhesion and replaced with 200 μl cRPMI containing stimulants at the

concentrations indicated in the relevant figures and legends. In experiments assessing TGF- β activity, macrophages were cultured in cRPMI lacking foetal bovine serum. Stimulants were as follows: LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich), Pam₃CSK₄ (Invivogen), poly(IC) (Invivogen), recombinant mouse IL-4 (Peprotech), recombinant mouse IL-10 (Peprotech) and recombinant mouse TGF- β 1 with carrier protein (R&D Systems). For *in vitro* RSV stimulations, 8 x 10⁵ FFU of RSV-A2 in 100 μ l of serum-free HEP2 cell lysate was added to cells for 1 hour to allow viral entry to cells, before addition of 100 μ l of cRPMI for the remainder of stimulation time.

2.6.3. Cell lines

Madin-Darby canine kidney epithelial (MDCK) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX™ and pyruvate supplements (Thermo-Fisher), further supplemented with 10 % FBS and 100 U/ml penicillin and streptomycin solution. MFB-F11 TGF- β signalling reporter cells, which produce secreted alkaline phosphatase (SEAP) in response to TGF- β -driven Smad signalling (Tesseur et al., 2006), were a kind gift from Dr Brigitta Stockinger (Francis Crick Institute, London) and were cultured in DMEM supplemented with 10 % FBS and 15 μ g/ml Hygromycin B solution from *Streptomyces hygroscopicus* (Sigma-Aldrich). Cell lines were maintained as adherent monolayers at 37 °C and 5 % CO₂ in a humidified incubator and routinely passaged by washing in sterile PBS and releasing from flasks by brief incubation with 0.05 % Trypsin-EDTA.

2.7. MFB-F11 Smad reporter cell assay for TGF- β bioactivity

10⁴ MFB-F11 cells were seeded per well of flat bottom 96-well plates in DMEM supplemented with FBS and Hygromycin B and incubated for approximately 15 hours at 37 °C and 5 % CO₂ in a humidified incubator. Cells were then washed by replacing medium with two changes of serum-free DMEM without Hygromycin B and incubated for 6 hours in a third change of serum-free DMEM at 37 °C and 5 % CO₂. Medium was then replaced with serum-free DMEM containing a standard concentration range of recombinant mouse TGF- β 1 or BAL supernatant diluted 1:2 with serum-free DMEM, all in duplicate. Cells were incubated at 37 °C and 5 % CO₂ for 24 hours, at which point supernatants were harvested and SEAP activity quantified using the Great EscAPE SEAP Chemiluminescence Kit 2.0 (Clontech), as per manufacturer's instructions. Luminescence was quantified using a Topcount NXT luminometer (Perkin Elmer). Bioactive TGF- β concentrations in BAL determined by interpolation of unknown values into standard curves generated in Prism v7 (Graphpad).

2.8. Flow cytometry

Up to 2 x 10⁶ cells in single cell suspension were transferred to 96-well round bottom plates (Corning) for staining. When subsequently assessing cytokine production by intracellular staining, suspensions were first stimulated *ex vivo* by incubating in cRPMI containing 20 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1.5 μ g/ml ionomycin free acid from *Streptomyces conglobatus* (Merck) and 5 μ g/ml Brefeldin A (Sigma-Aldrich) for 3.5 hours at 37 °C and 5 % CO₂ in a humidified incubator.

2.8.1. Staining of extracellular antigens

Cells were washed in flow cytometry grade PBS, before staining with either LIVE/DEAD™ Fixable Blue or Near-IR amine-reactive viability dye (Thermo-Fisher) for 20 minutes at room temperature in the dark. Excess viability dye was removed by two washes in PBS, before staining cells with fluorochrome-conjugated monoclonal antibodies (**Table 2.1**) in the presence of Fc Block (**Table 2.1**) for 30 minutes at 4 ° C in the dark. Antibody staining was performed in flow staining buffer, consisting of 1 % bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.1 % sodium azide (G-Biosciences). When analysing RSV-specific CD8⁺ T cells, cells were stained for one hour at room temperature with tetramers of biotinylated Db RSV M2187–195 at 1:50 dilution in flow staining buffer. Biotinylated Db RSV M2187–195 monomers were obtained from the NIH Tetramer Core Facility and tetramerised according to the manufacturer's guidelines using PE-conjugated streptavidin (Thermo-Fisher). After staining, cells were washed 3 times in flow staining buffer, before fixing for 15 minutes at room temperature. Fixation was performed using IC Fixation Buffer (Thermo-Fisher), unless subsequently staining for intranuclear FoxP3, where the Fixation/Permeabilisation Buffer kit (Thermo-Fisher) was used as per manufacturer's instructions.

2.8.2. Staining of intracellular and intranuclear antigens

Fixed cells were permeabilised by incubation in Permeabilisation Buffer (eBioscience) for 5 minutes at room temperature, before incubating with fluorochrome-conjugated monoclonal antibodies diluted in Permeabilisation Buffer for 30 minutes at 4 °C in the dark. Cells were then washed twice in Permeabilisation Buffer, before suspending in flow staining buffer for analysis.

2.8.3. Analysis of flow cytometry data

All flow cytometry data were acquired using an LSR Fortessa and FACSDiva software (both BD). Data were analysed using FlowJo software (TreeStar). For all analyses, non-cellular debris, dead cells and doublets were excluded prior to analysis and CD45 used to identify leukocytes (**Fig. 2.1a**). Hierarchical gating strategies for identifying myeloid cell populations are described in detail in Chapter 3. T cells, natural killer cells and ILCs were identified using gating strategies shown in **Fig. 2.1b**. Absolute numbers of cell populations were determined using the calculation below. Where relevant, geometric mean fluorescent intensity (gMFI) values for fluorescent parameters were determined in FlowJo software, normalising to mean values of negative control groups where necessary for pooling data from independent repeat experiments. Intrinsic autofluorescence was assessed by determining gMFI in a 530/30 nm filter after 488 nm excitation, in samples not stained with FITC- or Alexa-Fluor 488-conjugated antibodies.

$$\text{Total number of population } x \text{ in tissue} = \frac{\text{\# events in gate } x}{\text{\# non-debris cell events}} \times \text{Total crystal violet cell count per unit tissue}$$

Antigen	Clone	Fluorochrome(s)	Manufacturer	Dilution factor	Method
CD103	2E7	FITC	Biologend	100	EC
CD11b	M1/70	APC, PE-Cy7, BV605	Biologend	100	EC
CD11c	N418	APC-Cy7, BV421	Biologend	100	EC
CD19	6D5	BV605	Biologend	20	EC
CD206	C068C2	BV605	Biologend	50	EC
CD3	17A2	eFluor 450, PECy7	Thermo-Fisher	100	EC
CD4	RM4-5	FITC, Percp-Cy5.5, APC-Cy7	Biologend	100	EC
CD44	IM7	PE-610	Biologend	200	EC
CD45	30-F11	FITC, PERCP-Cy5.5, BV711	Biologend	200	EC
CD62L	MEL-14	BV605	Biologend	100	EC
CD64	X54-5/7.1	BV421	Biologend	50	EC
CD69	H1.2F3	APC	Biologend	100	EC
CD71	RI7217	PE-Cy7	Biologend	50	EC
CD8	53-6.7	BV605	Biologend	200	EC
CD90.1	OX-7	Percp-Cy5.5, PE/Dazzle™ 594	Biologend	100-200	EC
CD90.2	53-2.1	BV605	Biologend	100	EC
F4/80	BM8	PE-Cy7	Biologend	50	EC
FoxP3	NRRF-30	PE	Thermo-Fisher	100	IN
Haematopoietic lineage cocktail (supplemented with anti-CD5)	CD3 (17A2) B220 (RA3-6B2) CD11b (M1/70) TER-119 GR-1 (RB6-8C5) CD5 (53-7.3)	eFluor 450	Thermo-Fisher	50	EC
IA-IE (MHC-II)	M5/114.15.2	BV421, PERCP-Cy5.5	Biologend	100	EC
ICOS	C398.4A	PE-Cy7	eBioscience	100	EC
IFN- γ	XMG1.2	FITC, PE-Cy7	Thermo-Fisher	50	IC
IL-10	JES5-16E3	FITC	Thermo-Fisher	50	IC
IL-13	eBio13A	PE, APC	Thermo-Fisher	50	IC
IL-17A	eBio17B7	APC, PE-Cy7	Thermo-Fisher	50	IC
Ly6C	HK1.4	Alexa-Fluor 700	Biologend	100	EC
Ly6G	1A8	APC, BV510	Biologend	200	EC
NKp46	29A1.4	BV605, PE-Cy7	Biologend	100	EC
Siglec F	E50-2440	PE, BV421	BD	100	EC
SIRP α	P84	PE/Dazzle™ 594	Biologend	200	EC
CD16/32 Mouse Fc Block™	2.4G2	Unconjugated	BD	100	EC

Table 2.1. List of flow cytometry and confocal microscopy antibodies

EC, extracellular; IC, intracellular; IN, intranuclear; BV, Brilliant Violet™; BD, Becton Dickinson. All EC flow cytometry stains and confocal microscopy staining were performed in the presence of anti-CD16/CD32 to block Fc receptors (in bold).

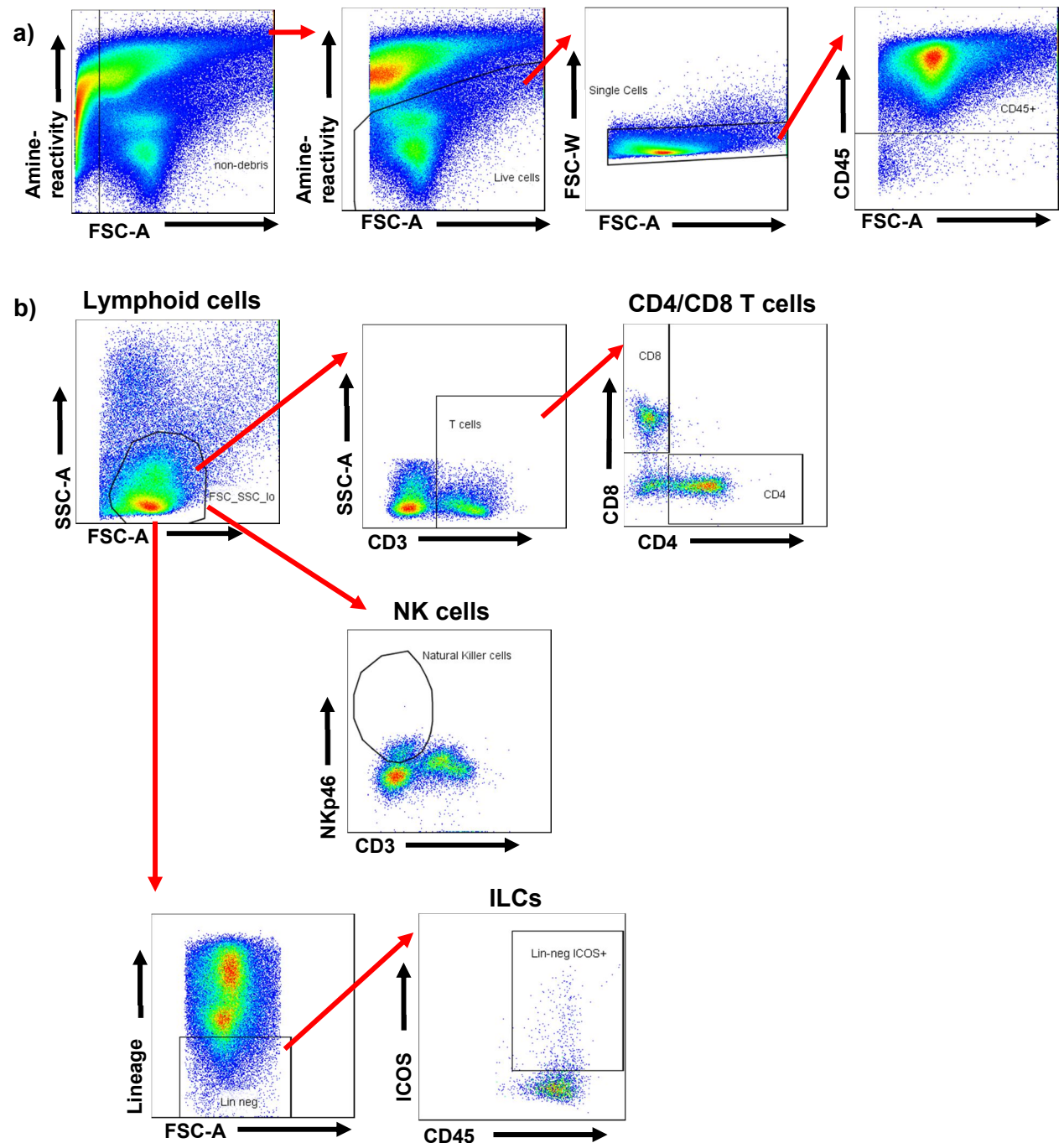


Figure 2.1. General flow cytometry gating strategies

Representative flow cytometry plots obtained by staining of single cell suspensions from lungs of a mouse exposed to HDM for 3 weeks to induce allergic inflammation. Red arrows indicate direction of hierarchical gating strategies. **a)** For all gating strategies, non-cellular debris (FSC^{low}), dead cells staining positive for amine-reactive viability dye and doublet events were excluded and CD45 staining used to identify leukocytes. **b)** Lymphoid cells were identified as $FSC-A/SSC-A^{low}$ and further subdivided. T cells were identified as $CD3^{+}$ and further discriminated on expression of CD4 or CD8. Natural killer (NK) cells were identified as $NKp46^{+} CD3^{-}$. Innate lymphoid cells (ILCs) were identified as lineage (CD3, B220, CD11b, TER-119, GR1, CD5)-negative cells positive for ICOS.

2.8.4. Flow cytometry sorting of airway macrophages from bronchoalveolar lavage

Single cell suspensions were stained with fluorochrome-conjugated antibodies in low-protein sorting buffer (0.1 % BSA, 1 mM EDTA, 25 mM HEPES, in PBS) in the presence of Fc Block (Table 2.1) for 30 minutes at 4 °C. Cells were then washed twice in sorting buffer and 50 µl/ml of 1 µM ToPro3 viability dye (Thermo-Fisher) added to suspensions immediately prior to sorting. Samples were sorted on a BD FACSAria using a 100 µm nozzle and purity settings, where the preceding and subsequent drops for each sorted cell were divided into 32 segments and the cell discarded if contaminating events were detected in the 16 segments nearest to the cell of interest. Cells were collected into sorting buffer and lysed for RNA extraction within 30 minutes of sorting.

2.9. Confocal microscopy on live precision cut lung slices

PCLS were incubated in cRPMI for a minimum of 1 hour after slicing to promote dissociation of agarose from tissue, before replacing with fresh cRPMI. PCLS were stained by addition of fluorochrome-conjugated monoclonal antibodies to cRPMI in µ-Plates in the presence of Fc Block (Table 2.1). PCLS were incubated for two hours at 37°C and 5 % CO₂ in a humidified incubator, before washing in one change of cRPMI. Medium was then removed and PCLS imaged using a Leica SP5 inverted microscope equipped with 405 nm, 488 nm, 543 nm and 633 nm lasers using a 10x objective lens. Leica Application Suite X (LAS X) software was used to acquire approximately 10 µm z-stacks of representative fields of view. Stacked images were processed using Imaris v8.0 software (Bitplane) to visualise and merge individual fluorescent channels.

2.10. Immunohistochemistry

For all fixed lung section staining, formalin-fixed right superior lung lobes were embedded in paraffin and 4 µm sections cut and attached to Superfrost™ Plus microscope slides (Sigma-Aldrich). For all staining, sections were dewaxed with Histo-Clear (National Diagnostics USA) and rehydrated through increasing concentrations of ethanol. Slide preparation, as well as haematoxylin and eosin (H&E) staining was performed by the Imperial College Histology Core Facility. All antibody staining and blocking steps were performed in a humidified chamber (made in-house). All antibodies were diluted in 1 % normal mouse serum (Sigma-Aldrich) in PBS. All imaging of formalin-fixed, paraffin-embedded lung sections was performed using a Leica DM2500 microscope and Leica Application Suite V4.7 (LAS V4.7) software.

2.10.1. TGF-β1 immunofluorescent staining

Sections were subjected to heat-mediated antigen retrieval by immersing slides in boiling 11.4 mM sodium citrate + 0.05 % Tween at pH 6 for 30 minutes. Sections were then blocked for 30 minutes at room temperature with 10 % normal donkey serum (Sigma-Aldrich) in PBS. Sections were stained with rabbit polyclonal anti-TGF-β1 antibody (Abcam) or rabbit IgG control (Vector Laboratories) overnight at 4 °C, before washing 3 times for 5 minutes in PBS + 0.05 % Tween-20 (Sigma-Aldrich; PBS-T). Sections were stained for one hour at room temperature with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch), washed 3 times in PBS-T and mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher), with DAPI for visualisation of nuclei.

2.10.2. Influenza NP protein immunohistochemistry

Endogenous peroxidases were blocked after rehydration of sections by immersing slides for 10 minutes in hydrogen peroxide (Sigma-Aldrich) in 100 % methanol (1 ml of 30 % hydrogen peroxide per 100 ml methanol, final concentration of 0.3 %). Heat-mediated antigen retrieval was then performed as described above. Sections were sequentially blocked using the Avidin/Biotin Blocking Kit (Vector Laboratories) and 10 % normal donkey serum in PBS, at room temperature. Sections were then incubated overnight at 4 °C with goat anti-influenza NP antibody (Abcam) or goat IgG control (Vector Laboratories), before washing 3 times with PBS-T. Sections were then incubated for one hour with biotinylated donkey anti-goat IgG (Jackson ImmunoResearch), followed by 30 minutes with VECTASTAIN® Elite® ABC reagent (Vector Laboratories), washing 3 times in PBS-T after each incubation. Sections were then incubated with 3,3'-diaminobenzidine horse radish peroxidase (HRP) substrate (Vector Laboratories) until desired staining intensity was obtained. Sections were counterstained by brief (around 30 seconds) immersion in Harris's haematoxylin, before dehydrating in increasing concentrations of ethanol, clearing in Histo-Clear and mounting with DPX mountant (Sigma-Aldrich).

The extent of bronchiolar NP staining was semi-quantitatively scored by a blinded investigator, using the following system (**Fig. 2.2**).

0: No NP staining

1: 1-25 % of epithelial cells in airway stained

2: 26-50 % of epithelial cells in airway stained

3: 51-75 % of epithelial cells in airway stained

4: 51-75 % of epithelial cells in airway stained.

An additional +1 score was added where one or more patches of dense staining was observed in an airway, giving a maximum score of 5 per bronchiole (representative images for each score in **Fig. 2.2**). The mean of all airways in one section (≥ 10) was reported for each mouse.

Score	% of cells with positive staining
0	0
1	1-25
2	26-50
3	51-75
4	76-100

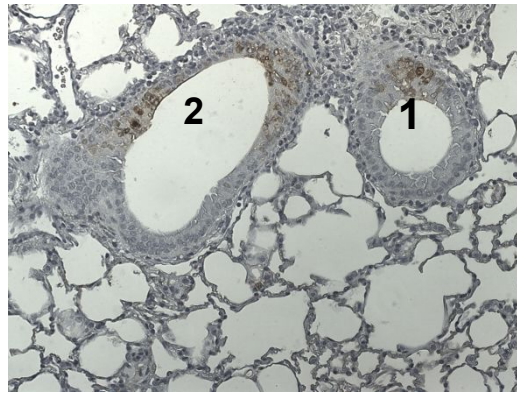
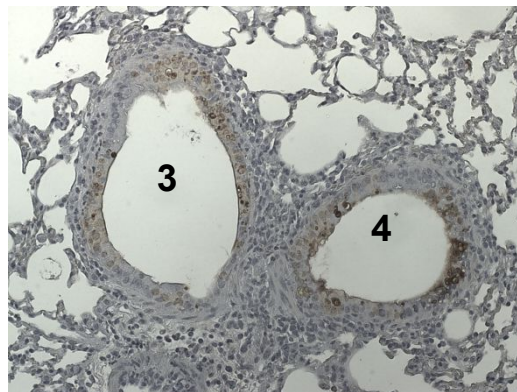
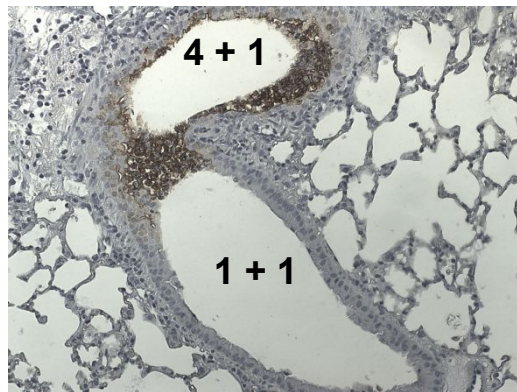


Figure 2.2. Scoring system for airway influenza staining

Representative 200x images of NP-stained lung sections exemplifying the semi-quantitative scoring system used to assess the extent of staining. Numbers within airways indicate scores for the proportion of cells stained. Arrowheads point to patches of dense staining, for which a score of +1 was added to the airway, resulting in a maximum score of 5 per airway.

2.11. Protein analyte quantification

2.11.1. Enzyme-linked immunosorbent assay (ELISA)

Middle right lung lobes were thawed on ice and mechanically homogenised at 50 mg tissue/ml in HBSS (Thermo-Fisher) supplemented with one cOmplete™ Mini, EDTA-free protease inhibitor tablet per 50 ml. Homogenisation was performed using the FastPrep®-24 homogeniser (MP Biomedicals) and homogenates centrifuged at 500 x g for 20 minutes at 4 °C to remove debris.

IL-4 was quantified using paired capture (clone 1B11, diluted in 0.1 M NaHPO₄) and biotinylated detection (clone BVD6-248, diluted in 1 % BSA 0.05 % Tween) antibodies and standardised to recombinant murine IL-4 (all BD), before incubating with Streptavidin-HRP Enzyme Conjugate (Vector Laboratories). All other murine cytokines were quantified using standardised sandwich ELISA kits from either eBioscience (Ready-Set-Go kits: IL-13, IL17A, IFN γ , GM-CSF, TNF α , IL-6 and IL-1 β ; Platinum ELISA: IFN- α) or R&D Systems (Duoset: CXCL1, CCL24, CCL2, IFN α 2/3, IL-33 and IL-10; Verikine™: IFN- β), according to the respective manufacturer's instructions.

All ELISAs were washed with PBS + 0.05 % Tween-20 and were developed after streptavidin-HRP incubation with TMB substrate (eBioscience) and reactions stopped by addition of an equal volume of 0.18 M sulphuric acid. Results were determined using a SpectraMax® i3x plate reader and SoftMax Pro 7 software (both Molecular Devices) to fit standard curves and interpolate unknown values.

2.11.2. LEGENDplex™ cytometric bead array assay

For more sensitive detection of soluble IL-10, a standardised LEGENDplex™ mouse IL-10 assay kit (Biolegend, limit of detection: 2 pg/ml) was used according to manufacturer's instructions. Briefly, anti-IL10 assay beads were bound to IL-10 in samples and recombinant IL-10 standards, before incubating with biotinylated anti-IL-10 detection beads and PE-conjugated streptavidin. Beads were then analysed on an LSR Fortessa flow cytometer and IL-10 concentrations determined using LEGENDplex™ software (Biolegend).

2.12. Influenza plaque assay

Plaque-forming influenza units were quantified using a modification of a published protocol²⁹¹, adapted to use a solid overlay medium. MDCK cells were grown in 12 well plates overnight to achieve ~90% confluent monolayers. Inferior right lung lobes were weighed and homogenised in 1 ml serum-free DMEM and 10-fold serial dilutions of lung homogenate added in duplicate to cell monolayers in 100 μ l volume. Cells were then incubated at 37 °C and 5 % CO₂ for one hour to allow infection, before washing away inoculum with PBS and overlaying cells with warm 1 x Minimum Essential Medium (MEM, Thermo Fisher), supplemented with 1 % L-glutamine, 100 U/ml penicillin and streptomycin solution, 1 mM HEPES (Thermo-Fisher), 0.14 % NaHCO₃ (Thermo-Fisher), 0.2 % BSA fraction V (Thermo-Fisher), 0.003 % diethylaminoethyl-dextran hydrochloride (Sigma-Aldrich) 0.6 % Oxoid agar (Thermo Fisher) and 50 μ g of L-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemical). Overlay was allowed to set at room temperature, before incubating plates upside down in a 37 °C 5 % CO₂

incubator for 3 days to enable plaque formation. After incubation, overlay medium was removed and cells stained with 0.1 % crystal violet in 20 % ethanol. Plaques were counted manually at the dilution factor yielding the greatest number of distinct plaques and plaque forming unit values per mg of lung tissue calculated as below.

$$\text{PFU/mg lung} = \frac{\text{Number of plaques} \times 10 \times \text{dilution factor}}{\text{Lung weight (mg)}}$$

2.13. Extraction and quality control of RNA

350 µl of RLT buffer (QIAGEN) containing 1 % β-mercaptoethanol (Sigma-Aldrich) was added to post-caval lung lobes, pelleted cell suspensions or scraped cell culture monolayers and mechanically disrupted with either a FastPrep®-24 homogeniser (MP Biomedicals; for lung lobes), or QIAshredder columns (QIAGEN; for cell suspensions and monolayers), as per manufacturer's instructions. Epithelial cells from tracheal brushings were lysed for RNA extraction by immersing brushes in 350 µl RLT + β-mercaptoethanol and vortexing tubes vigorously for 30 seconds. For lung homogenates and lysates of > 5 x 10⁵ cells, the RNeasy Mini Plus kit (QIAGEN) was used to extract RNA, as per manufacturer's instructions. For lysates of < 5 x 10⁵ cells, the RNeasy Micro Plus kit (QIAGEN) was used for RNA extraction, as per manufacturer's instructions. RNA concentration and integrity (RIN_e) value were obtained using the 2200 TapeStation instrument and reagents (Agilent), according to the manufacturer's standard protocol for RNA analysis.

2.14. Preparation of cDNA

For whole lung RNA samples, 1 µg of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher) with random primers and without RNase inhibition, as per manufacturer's instructions. For sorted or cultured cell RNA, the GoScript® Reverse Transcriptase System with random primers (Promega) was used for cDNA conversion according to manufacturer's instructions, using 3 mM MgCl₂. Input RNA concentrations for GoScript® reactions were normalised within individual experiments and were ≥ 100 ng where RNA yields allowed. All cDNA was diluted 1:4 in nuclease-free water prior to downstream applications.

2.15. Quantitative PCR

Quantitative PCR (qPCR) was performed using Taqman Fast Advanced Master Mix (Thermo Fisher) and FAM-conjugated Taqman primer/probe sets (Thermo-Fisher; **Table 2.2**). Reactions were performed in duplicate in 6 µl volumes, containing 2.4 µl cDNA, 0.3 µl nuclease-free water, 0.3 µl Taqman primer/probe and 3 µl Taqman master mix. Reactions were run in 384 well plates for 45 cycles on a ViiA7 instrument (Thermo-Fisher) using the recommended settings for Taqman Fast Advanced Master Mix. Threshold cycle (C_T) values were determined using ViiA7 software (Thermo-Fisher). Relative expression values were determined by normalisation of C_T values of target genes to mean C_T values of 2 housekeeping genes, as shown below.

$$\text{Relative expression value} = \frac{1}{2^{(C_T \text{ target gene} - C_T \text{ housekeeping genes})}}$$

Gene symbol	Taqman primer ID
<i>Actb</i>	Mm00607939_s1
<i>Arg1</i>	Mm00475988_m1
<i>Ccl2</i>	Mm00441242_m1
<i>Ccl24</i>	Mm00444701_m1
<i>Chil3/Chil3l3</i>	Mm_00657889_mH
<i>Gapdh</i>	Mm99999915_g1
<i>Hprt</i>	Mm03024075_m1
<i>Ifnb1</i>	Mm00439552_m1
<i>Ifng</i>	Mm01168134_m1
<i>Il10</i>	Mm01288386_m1
<i>Il10ra</i>	Mm00434151_m1
<i>Il10rb</i>	Mm00434157_m1
<i>Il13</i>	Mm00434204_m1
<i>Il1b</i>	Mm00434228_m1
<i>Il27</i>	Mm00461162_m1
<i>Il4</i>	Mm00445259_m1
<i>Il5</i>	Mm00439646_m1
<i>Inhba</i>	Mm00434339_m1
<i>Oas2</i>	Mm00460961_m1
<i>Smad2</i>	Mm00487530_m1
<i>Smad3</i>	Mm01170760_m1
<i>Smad4</i>	Mm03023996_m1
<i>Smad7</i>	Mm00484742_m1
<i>Tgfb1</i> (exon 5-6 for general use)	Mm01178820_m1
<i>Tgfb1</i> (exon 6-7 for knockout analysis)	Mm00441729_m1
<i>Tgfb2</i>	Mm00436955_m1
<i>Tgfb3</i>	Mm00436960_m1
<i>Tgfb2</i>	Mm03024091_m1
<i>Tnf</i>	Mm00443258_m1

Table 2.2. List of Taqman qPCR primer/probe assay sets

All primer/probe sets were FAM-labelled and purchased from Thermo Fisher. Assay sets were selected on the basis of having the least predicted chance of cross reactivity with off-target sequences and including primers targeting exon-exon junctions, to prevent amplification of genomic DNA.

2.16. Preparation of cDNA libraries for RNAseq analysis

200 ng of RNA was converted into a cDNA sequencing library with the TruSeq Stranded mRNA HT kit (Illumina), using the manufacturer's provided HT protocol. Briefly, mRNA was obtained by poly(A) enrichment, fragmented and used as a template for synthesis of short cDNA fragments. Uniquely barcoded adapters were then ligated to fragments of each sample and libraries amplified through 15 PCR cycles. This kit included strand-specific adapters, allowing the DNA strand from which transcripts derived to be determined during analysis.

Resultant libraries were quantified using the QuantiFluor® double-stranded DNA system and GloMax® plate reader (both Promega) to determine cDNA concentration (weight/volume) and the Agilent 2100 Bioanalyzer to determine DNA fragment sizes, all according to manufacturer's instructions. Molarity of sequencing libraries were determined based on concentration and fragment size data and used to normalise concentrations to 10 nM.

2.17. Sequencing of RNAseq libraries

Groups of 6 samples were pooled and run on single lanes of an Illumina HiSeq 4000, obtaining 75 base pair paired-end reads. Sequencing was performed by the Advanced Sequencing Facility at the Francis Crick Institute, who subsequently demultiplexed data and removed adapter sequences, providing FASTQ files for analysis.

2.18. RNAseq data analysis

2.18.1. Quality control of raw RNAseq data

Quality control parameters were obtained for each raw file using FASTQC software (Babraham Institute) and results aggregated and visualised using the MultiQC Python tool²⁹². Sequences with a mean phred quality score of 20 or below were removed prior to alignment.

2.18.2. Alignment of RNAseq reads

The COBWeb aligner within StrandNGS software (Strand LS) was used as per developer's recommendations to align sequence data to the Mm10 reference transcriptome and genome (University of California Santa Cruz). Briefly, the Ferragina-Manzini algorithm²⁹³ was used to index and compress the Mm10 reference, before matching seed regions within read sequences to the index. The Smith-Waterman algorithm²⁹⁴ was then used to determine the most similar mapping locations for each read around these seed regions, requiring a minimum match length of 25 base pairs, minimum sequence identity of 90 % and maximum gaps in overlap of 5 %. Only the best matched genomic location was reported for each read pair and reads were excluded if matching to 5 or more locations. Reads were also filtered post-alignment if either pair mapped to mouse ribosomal RNA or UniVec sequences, or if both pairs did not map to the same location.

2.18.3. Quantification and differential expression analysis

Aligned reads were attributed to Refseq (2013) genes and transcripts using annotations in StrandNGS software, as per developer's recommendations. Each read contributed counts of up to 1 to an exon, proportional to the length of the read mapping to that exon. Reads contributed a count of 1 if mapping entirely within an exon, with the score reducing proportionately with the

length of the read mapping to introns or other exons. Counting of reads towards distinct transcript isoforms of genes was achieved using a maximum likelihood estimation modified from that of Li et al²⁹⁵, based on the relative representation of all exons for a gene in the dataset. The DESeq algorithm²⁹⁶ was used to obtain normalised signal values for each transcript in each sample by dividing raw read counts by normalisation factor 'N', as summarised below, before log2 transforming resultant values.

'N' = median of 'Y' for all genes in sample

'Y' = raw reads of gene X in sample/geometric mean raw reads of gene X in all samples

StrandNGS software was used to calculate fold changes and compare normalised expression values between lists of genes in two experimental groups by moderated unpaired *t*-test, selected to account for relatively high levels of variance in the data. The Benjamini-Hochberg correction²⁹⁷ was applied to adjust *p* values for the false discovery rate due to multiple testing. Normalised expression values, fold changes and Benjamini-Hochberg-corrected *p* values were used for downstream analysis of data and are described in detail in Chapter 5.

2.18.4. Principal component analysis

Four principal components were determined from individual biological datasets using StrandNGS software and the two largest principal components (PC1 and PC2) visualised on two-dimensional axes. Biological groups were colour-coded to allow visualisation of separation between groups and intragroup variation.

2.18.5. Unsupervised Hierarchical clustering

Genes and individual samples in datasets were hierarchically clustered based on DESeq-normalised expression values using Ward's minimum variance method²⁹⁸ within StrandNGS software, dependent on the Euclidean distance between individual data points. No constraints were placed on clustering of samples from different experimental groups, so that clustering was unbiased. Gene clusters for further analysis were determined based on manual inspection of dendrograms for major sub-trees showing distinct patterns of gene expression between experimental groups.

2.18.6. Gene Ontology annotation of datasets

Selected gene sets of interest were assessed for overrepresentation of Gene Ontology (GO) terms by comparison to lists of genes annotated with GO terms in the publically available GO database²⁹⁹. Enrichment analysis was performed using Fisher's exact test within StrandNGS software to test the null hypothesis that the number of genes annotated with particular GO terms in datasets had occurred by chance. To focus the list of terms to highly-enriched biological processes, cellular component and molecular function terms were filtered and terms with a *p* value of ≤ 0.01 were carried forward for analysis. Resultant terms were further filtered for redundancy, to more clearly visualise the range of affected processes, using the REVIGO online tool³⁰⁰. Redundancy was determined using the SimRel score for similar GO terms, using a cut-off of 0.5, at which $< 1\%$ of randomly selected GO terms are marked as redundant³⁰¹. Scatter

plots of GO terms were generated using the *ggplot2* package in R (Cran) based on output code from online REViGO analysis. GO terms were clustered together in semantic space based on similarity and colour coded to denote *p* value of enrichment in the query dataset. Sizes of symbols on scatter plot were proportional to the number of total known genes associated with the GO term (i.e. its generality). Labels were included for highly enriched GO terms representative of distinct clusters.

2.18.7. Ingenuity pathway analysis (IPA)

Selected lists of Entrez IDs and associated expression values were uploaded to IPA (QIAGEN) and core analyses performed based on curated literature in the Ingenuity Knowledge Base. Analyses were restricted to only utilise experimentally observed relationships between gene products in *Mus musculus*. Enriched canonical pathways were determined based on a right-tailed Fisher's exact test, considering pathways with $p \leq 0.01$ significantly enriched. Enriched canonical pathways were manually curated based on percentage coverage in the query dataset, directionality (Z-score) of regulation (i.e. whether predicted increase or decrease of pathway agrees with pattern of gene expression) and redundancy (i.e. filtering highly similar pathways enriched based on near-identical genes), in order to report representative lists of pathways enriched within gene sets. Cytokine activity upstream of observed differential gene expression was performed with the 'Upstream Regulators' tool in IPA, comparing observed gene expression changes to known downstream effects of cytokines using Fisher's exact test and ranking results based on *p* value and Z-score. Colour-coded diagrams of genes in canonical pathways were generated using the 'My Pathway' function in IPA, overlaying fold change or normalised expression values from relevant datasets.

2.18.8. Visualisation of differential gene expression

Heat maps, box and whisker plots and profile plots were generated using StrandNGS. Volcano plots and Venn diagrams were generated in R using the *Calibrate* and *VennDiagram* packages, respectively. Word cloud visualisations of representative gene sets were generated using the 'REPT' function in Excel 2013 (Microsoft) to generate text files in which gene names are repeated a number of times proportional to either fold change or normalised signal values in the RNAseq dataset. Text files were then converted to word clouds with word sizes proportional to gene name abundance using the Wordle™ online tool (www.wordle.net).

2.19. Statistical analysis (non-transcriptomic data)

Chi-square (χ^2) analysis of mouse genotype occurrence rates was performed in Excel 2013. All other statistical analyses on non-transcriptomic data were performed in Prism v7 (Graphpad). Triplicate cell culture experiments, which showed low standard deviations but lacked sufficient repeats for non-parametric testing, were assumed to follow normal distributions to allow parametric statistical analysis. These data were expressed as means $\pm$ standard error of the mean (SEM) and groups compared by one-way ANOVA. If significant differences were determined between groups by ANOVA, individual groups were then compared by student's *t*-tests. Corrections for multiple comparisons were made by Bonferroni adjustment (corrected *p* value = raw *p* value x [number of comparisons made]). All other data were analysed by non-parametric Mann-Whitney U tests, when comparing only two groups, or Kruskal-Wallis tests when comparing multiple groups. If significant differences were determined between groups in Kruskal-Wallis tests, individual groups were then compared by Mann-Whitney U tests, with Bonferroni corrections for multiple comparisons as above. Correlation analysis of non-parametric data was performed by the Spearman method. For all non-transcriptomic analyses, a corrected *p* value of ≤ 0.05 was taken as sufficient to reject null hypotheses. Details of statistical tests performed for each dataset are provided in the respective figure legends.

3. Characterisation of airway macrophage activation during pulmonary inflammation

3.1. Introduction

Airway macrophages (AM) are key decision-making cells in the initiation, propagation and resolution of pulmonary immune responses¹⁹. AM are critical for initiation of early inflammatory responses to experimental RSV infection, including production of pro-inflammatory cytokines, such as TNF α , and recruitment of natural killer cells to the lung¹⁵⁴. Similarly, AM from mice or humans with allergic airway inflammation produce increased levels of pro-inflammatory cytokines and chemokines^{156,157} and can also adopt activation states associated with tissue repair and remodelling^{174,302}. AM can therefore play active roles in promoting inflammation, tissue repair and, possibly, airway remodelling in different inflammatory contexts. Conversely, depletion of AM using clodronate liposomes enhances type 2 inflammation in murine models of allergic airway disease (AAD)^{63,144,145} and worsens pulmonary inflammation following influenza infection³⁰³, endotoxaemia¹⁰ or unilateral lung contusion^{304,305}. Taken together these data suggest that AM are critical regulators of pulmonary immunity, even when they themselves are activated and contributing to inflammation.

Further research is clearly required to understand how pro-inflammatory and tolerogenic functions of AM are balanced during inflammation. AM activation is not a simple on/off process, nor a binary switch between tolerogenic and pro-inflammatory states, as the remarkable plasticity of macrophages can result in a range of context-dependent activation states^{80,167}. At the extremes of these states are classical activation, driven by stimuli such as lipopolysaccharide (LPS) and IFN γ (also known as M[IFN γ]¹⁵⁹), and IL-4/13-driven alternative activation⁸⁰ (also known as M[IL-4/13]¹⁵⁹). However, AM do not fit into either of these extremes at homeostasis, expressing molecules typical of both classical and alternative activation^{19,140}. Shifts in AM phenotype have been reported in human disease and experimental inflammation models, but these are generally incompletely explained by either M[IFN γ] or M[IL-4] extremes. For example, AM from tobacco smokers and COPD patients showed reduced expression of a subset of classical, M[IFN γ]-like, activation genes³⁰⁶, while AM from asthmatics expressed high levels of both the classical activation marker MHC-II and alternative activation marker, mannose receptor CD206³⁰⁷. In mouse models of house dust mite (HDM) allergen-driven AAD, increased expression of both IRF5, a key transcription factor in classical macrophage activation¹⁷⁵, and the alternative activation marker, chitinase-like protein Ym1, have been observed in AM to varying extents depending on the dose, number and frequency of HDM exposures¹⁷⁴. Similarly, bleomycin-induced fibrosis was accompanied by upregulation of genes associated with both classical and alternative activation by AM¹⁴⁰. AM activation during pulmonary inflammation is therefore non-binary and context-dependent.

Analysis of AM activation and phenotype is reliant on their discrimination from other myeloid cell populations, while assaying for parameters of activation. Multi-parameter flow cytometry is a powerful tool for such analysis, allowing parallel detection of multiple membrane-bound, cytoplasmic or nuclear proteins at single cell resolution, combinations of which can be used to

define cell populations of interest. Variably expressed antigens on cell populations can also be assessed as indication of cell activation or change in phenotype, as is well characterised for molecules such as the C-type lectin domain family receptor CD69 on T cells³⁰⁸.

Flow cytometric analysis of myeloid cells is limited by a lack of unique lineage-defining surface markers for many cell types with dramatically different lineages and functions. For example, the murine pan-macrophage marker F4/80 is expressed to varying extents on macrophages, depending on tissue location and origin³⁰⁹, but is also expressed by murine eosinophils³¹⁰, precluding its use for definitive identification of macrophages. The situation is further complicated for AM, which are defined in mouse models based on expression of surface markers common to dendritic cells (DC; CD11c) and eosinophils (Siglec F), in addition to canonical macrophage markers, potentially obscuring their identification during inflammatory states. Similarly, while low to negative expression of the integrin CD11b has proven useful for discrimination of AM from pulmonary interstitial macrophages (IM) and monocytes during homeostasis, CD11b is upregulated on AM during inflammation^{311,312}, meaning that this is not a fixed parameter for AM identification.

With these factors in mind, experiments were conducted to establish robust flow cytometry methods for discrimination of AM from other pulmonary myeloid cells and to assess AM activation in multiple models of lung inflammation.

3.2. Aims

1. Establish a flow cytometry strategy for identification and immunophenotyping of murine pulmonary myeloid cells, with a focus on AM, at steady state and during inflammation
2. Define AM activation markers and compare their expression in models of allergic and non-allergic pulmonary inflammation

3.3. A flow cytometry strategy for analysis of murine pulmonary myeloid cells

A central aim of this thesis was to determine changes occurring to AM during pulmonary inflammatory disease and how these changes impact the nature and outcomes of inflammation. To allow study of AM phenotype during both homeostasis and inflammation, it was necessary to establish a flow cytometry strategy that fit the following criteria:

1. Distinguish AM from possible confounding populations (e.g. interstitial macrophages, inflammatory monocytes, DC and eosinophils), at homeostasis and in diverse models of pulmonary inflammation
2. Allow parallel quantification of inflammatory cells (neutrophils, monocytes, eosinophils, DC) in the same sample
3. Include proteins expressed variably on AM depending on inflammation, for use as markers of AM activation
4. Utilise only cell surface antigens, removing the need for fixation and permeabilisation, allowing direct application of the method to sorting of live cells

To this end, a minimum panel of 10 parameters was determined for pulmonary myeloid cell analysis, by modification of a published strategy²⁹ (**Table 3.1**). C57BL/6 mice were exposed to intranasal HDM extract for 3 weeks to induce AAD, thus expanding and recruiting diverse myeloid populations in the lung, before harvesting lungs for flow cytometry.

After exclusion of debris, dead cells and doublets (see general gating strategy in Chapter 2), CD45⁺ cells were selected to exclude any contaminating stromal cells and larger debris. AM are highly autofluorescent²⁶, resulting in potential false-positive signal in gates used to exclude lymphoid cells from analysis. For this reason, AM were analysed separately from other myeloid cells by firstly gating on cells with high forward and side scatter properties, indicative of large cells with complex cytoplasm, to effectively exclude lymphocytes and smaller mononuclear cells independently of autofluorescence (**Fig. 3.1a**). Cells co-expressing Siglec F and CD11c were then selected, excluding Siglec F single positive (eosinophils) and CD11c single positive (e.g. DC). Of the CD11c⁺ Siglec F⁺ cells, those expressing the type 1 Fc γ receptor (Fc γ R1/CD64) were classed as AM (**Fig. 3.1a**).

Marker	Cell types	Optional?
Amine reactive dye	All dead cells	No
CD45	All leukocytes	No
CD103	cDC1	No
CD11b	Granulocytes, IMM, cDC2, moDC	No
CD11c	AM and DC, some IMM	No
Siglec F	AM and eosinophils	No
CD64	AM, IMM, moDC	No
Lineage (NKp46, CD19, CD90.2)	Lymphoid cells for exclusion	No
Ly6G	Neutrophils	No
MHC-II	DC, subset of AM and IMM	No
F4/80	AM, IMM, eosinophils	Yes
Ly6C	Classical monocytes and some monocyte-derived macrophages	Yes

Table 3.1. Flow cytometry panel for murine pulmonary myeloid cells

List of minimum surface antigens for discrimination of myeloid cell types in murine lung and BAL, plus additional markers (green rows) that provide optional extra information on monocyte and macrophage subsets. IMM, interstitial/inflammatory monocytes/macrophages.

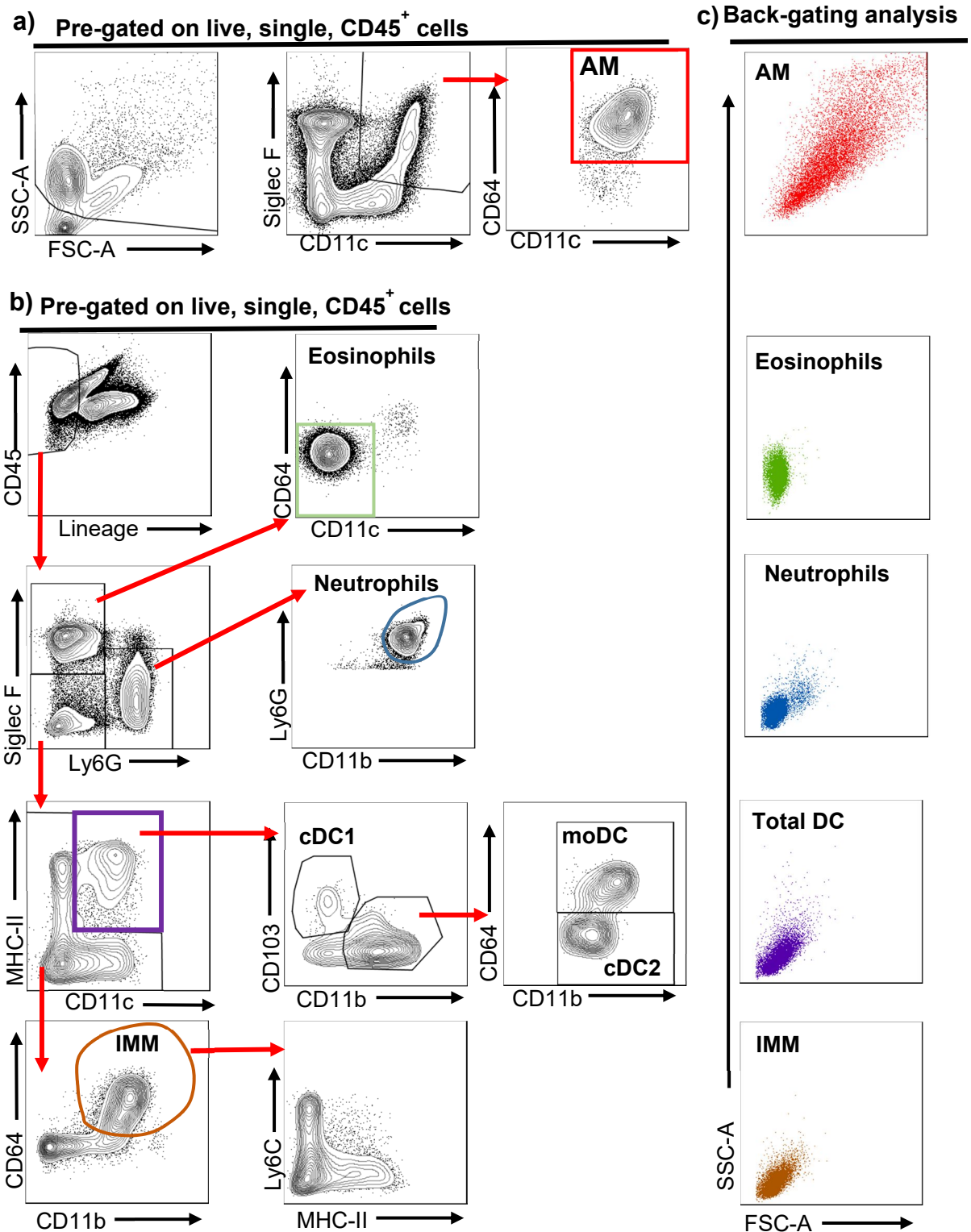


Figure 3.1. Gating strategy for murine pulmonary myeloid cells

Representative flow cytometry plots of lung cell suspensions from a C57BL/6 mouse exposed to intranasal HDM for 3 weeks. **a-b)** Hierarchical gating strategies for identification of AM (**a**), eosinophils, neutrophils, dendritic cells, interstitial/inflammatory monocytes and macrophages (IMM) (**b**). Red arrows indicate the direction of gating. **c)** Forward and side scatter distributions of the populations identified in (**a**) and (**b**) to confirm the expected size and internal complexity. Lineage, lymphocyte exclusion cocktail comprising CD19, CD90.2 and NKp46.

Other myeloid cells were identified in a hierarchical strategy (**Fig. 3.1b**), beginning by exclusion of NK cells (NKp46⁺), B cells (CD19⁺) and T cells and innate lymphoid cells (CD90.2⁺). Lineage-negative CD45⁺ cells were segregated based on expression of Siglec F and Ly6G. Siglec F⁺ cells were taken to be eosinophils if negative for AM markers CD11c and CD64⁺ (**Fig. 3.1b**). Cells co-expressing high levels of CD11b and the neutrophil marker Ly6G⁺ cells were classed as neutrophils (**Fig. 3.1b**). Siglec F/Ly6G double-negative cells were further examined for expression of CD11c and MHC-II. CD11c⁺ MHC-II⁺ cells were predominantly DC, with CD11b⁺ and CD103⁺ subsets detectable in lung (**Fig. 3.1b**). CD103⁺ CD11b⁻ DCs were consistent with pulmonary classical type 1 DC (cDC1)³³. CD11b⁺ DC could further be segregated based on expression of CD64, with CD64⁺ CD11b⁺ DC likely representing monocyte-derived cells with a DC-like phenotype (moDC), as described in depth by Lambrecht and colleagues^{40,313}, and CD64⁻ CD11b⁺ DC being classical type 2 DC (cDC2)³³.

Of the cells outside the total DC gate (**Fig. 3.1b**), cells co-expressing CD11b and CD64 comprised a mixed population of resident IM and inflammatory monocytes and macrophages (together abbreviated to IMM). IM are a relatively poorly understood subset of lung-resident mononuclear phagocytes compared to AM. IM do not express Siglec F and the majority are negative for CD11c, but they express the canonical macrophage markers F4/80, CD64 and CD11b^{31,32,214}. Therefore, unlike AM, IM share many surface markers with inflammatory monocytes and macrophages at homeostasis. Furthermore, it is highly likely that multiple subsets of IM exist, based on both function and anatomical location within the lung³². At steady state, the IMM gate likely consists of multiple IM populations along with circulating monocytes and tissue surveying monocytes^{49,314}, while during inflammation this population would also encompass infiltrating monocytes and macrophages at different stages of differentiation. Consistent with this, the IMM population from lungs of HDM-treated mice consisted of cells varying in expression of MHC-II and the inflammatory monocyte marker Ly6C (**Fig. 3.1b**), analogous to the 'monocyte-macrophage waterfall' pattern of macrophage maturation from monocytes in the intestinal mucosa at steady state and during inflammation³¹⁵. Because of the complex fate-mapping strategies required to truly discriminate between expanding resident macrophages and recruited monocyte-derived cells (see references^{140,214} for examples), the precise origin and nature of IMM was not inferred from flow cytometry data and these cells were generally analysed as a single population in subsequent experiments unless otherwise stated.

To confirm that the above gating strategies identified appropriate populations of cells, forward and side scatter properties of resultant populations were examined. The AM gate consisted of by far the largest cells (**Fig. 3.1c**), as expected given the known large size and complex cytoplasm of these cells³¹⁶. Eosinophil and neutrophil gates consisted of cells with lower FSC-A than AM, but high SSC-A (**Fig. 3.1c**), indicative of granulocytes. Finally, both total DC and IMM gates showed relatively high FSC-A, but lower SSC-A than granulocytes (**Fig. 3.1c**), consistent with a mononuclear phagocyte morphology. Overall, this analysis confirmed that a combination of antibodies to 9 surface antigens, plus a viability dye, could be used to distinguish major myeloid

cell populations in the allergic lung, while allowing capacity for parallel study of further markers (e.g. F4/80, Ly6C) on capable flow cytometers (**Table 3.1**). These definitions were applied to all subsequent studies described in this thesis (**Table 3.2**), unless otherwise specified.

Since the gating strategy described above was established in HDM-driven AAD, the robustness of this method of AM identification across pulmonary inflammation models remained to be determined. To assess this, C57BL/6 mice were subjected to models of repeated exposure to either HDM, which causes a pulmonary type 2 immune response with a Th17 and neutrophilic component³¹⁷, or the fungal allergen *Alternaria alternata* (ALT), which drives more severe type 2 inflammation than HDM in mice³¹⁸, with less neutrophilia. Allergens were administered for either one week (early allergic inflammation protocol; **Fig. 3.2a**) or 3 weeks (established allergic airway disease protocol; **Fig. 3.2b**). In parallel, separate mice were infected with X31 H3N2 influenza virus and tissues analysed at 3 days post-infection (dpi; **Fig. 3.2c**), the peak timepoint for airway neutrophilic inflammation in this model³¹⁹. Experimental protocols were staggered such that all mice were analysed at the same time (**Fig. 3.2**), to avoid any batch effects during analysis. An extensive high volume bronchoalveolar lavage (BAL) protocol (see Chapter 2.4.2.) was employed to maximise sampling of AM from throughout the bronchoalveolar spaces.

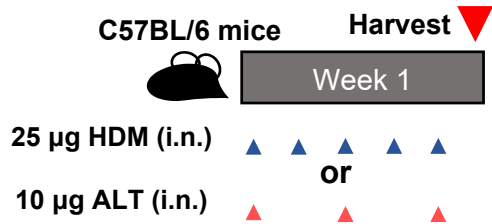
Both 3 week allergen exposure and acute influenza infection caused significant increases in airway inflammation, as measured by total leukocyte counts in BAL (**Fig. 3.3a**). Modest increases in BAL cell counts above PBS control levels were apparent in 1 week allergen-exposed mice, which did not reach statistical significance (**Fig. 3.3a**), consistent with these models reflecting the early stages in inception of AAD. As expected, 3 week allergen exposure protocols resulted in significantly increased airway eosinophil counts, which were not observed with influenza infection (**Fig. 3.3b**). Influenza infection instead drove substantial airway neutrophilia (**Fig. 3.3b**). A small increase in airway neutrophil numbers, which did not reach statistical significance, was observed in 3 week HDM-treated mice, but not in the 3 week ALT model (**Fig. 3.3a**), indicating that these models reflect mixed eosinophilic/neutrophilic and highly eosinophilic allergic inflammation, respectively. Despite the major changes in airway granulocyte numbers, AM counts in BAL did not differ substantially from those in PBS controls, with the exception of a trend towards a decrease (Mann-Whitney $p = 0.1$, **Fig. 3.3b**) in 3 week ALT-treated mice, suggesting that sustained exposure to this allergen leads to partial loss of AM.

Cell type	Flow cytometry profile
AM	FSC-A/SSC-A ^{hi} , CD11c ⁺ , Siglec F ⁺ CD64 ⁺
Eosinophil	Lineage ⁻ , CD11c ⁻ , CD64 ⁺ , Ly6G ⁻ , Siglec F ⁺ ,
Neutrophil	Lineage ⁻ , Siglec F ^{low-neg} , CD11b ⁺ , Ly6G ⁺
Interstitial/inflammatory Monocyte/macrophage (IMM)	Lineage ⁻ , [exclude CD11c/MHC-II double positive cells], CD11b ⁺ , CD64 ⁺
cDC1	Lineage ⁻ , CD11c ⁺ , MHC-II ⁺ , CD103 ⁺ , CD11b ⁻
cDC2	Lineage ⁻ , CD11c ⁺ , MHC-II ⁺ , CD103 ⁻ , CD11b ⁺ , CD64 ⁻
moDC	Lineage ⁻ , CD11c ⁺ , MHC-II ⁺ , CD103 ⁻ , CD11b ⁺ , CD64 ⁺

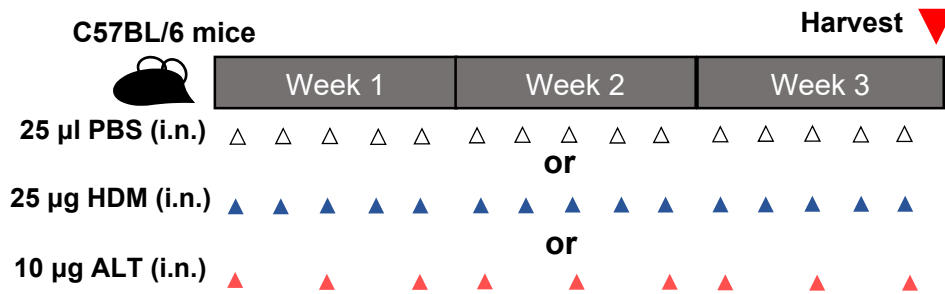
Table 3.2. Flow cytometry definitions for myeloid cell identification

Summary of the hierarchical approach used to define subsets of myeloid cells as outlined in Fig. 3.1., after pre-gating on live, single, CD45⁺ cells. These definitions are used for all flow cytometry described hereafter, unless otherwise specified. Lineage, lymphocyte exclusion cocktail comprising CD19, CD90.2 and NKp46.

a) Early allergic inflammation protocol



b) Established allergic airway disease protocol



c) Acute influenza infection protocol

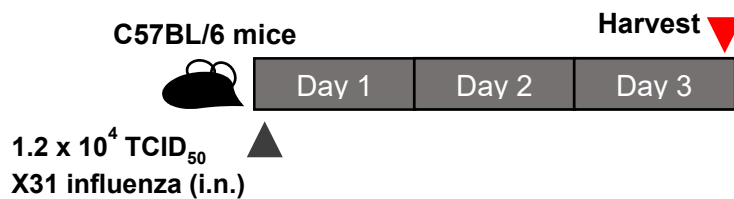


Figure 3.2. Experimental models for assessment of airway macrophage phenotype

Schematics of experimental inflammation models used for analysis of AM phenotype by flow cytometry. Protocols were run in parallel in adult female C57BL/6 mice and BAL harvested for analysis on the same day for all mice, 24h after final allergen challenge and 72h after influenza infection. **a-b)** 25 µg house dust mite (HDM) or 10 µg *Alternaria alternata* (ALT) were administered intranasally 5 or 3 times per week, respectively, for 1 (**a**) or 3 (**b**) weeks. Control mice received 25 µl PBS intranasal PBS 5 times a week for 3 weeks. **c)** Mice were infected intranasally with 1.2 TCID₅₀ X31 (H3N2) influenza 3 days prior to harvest.

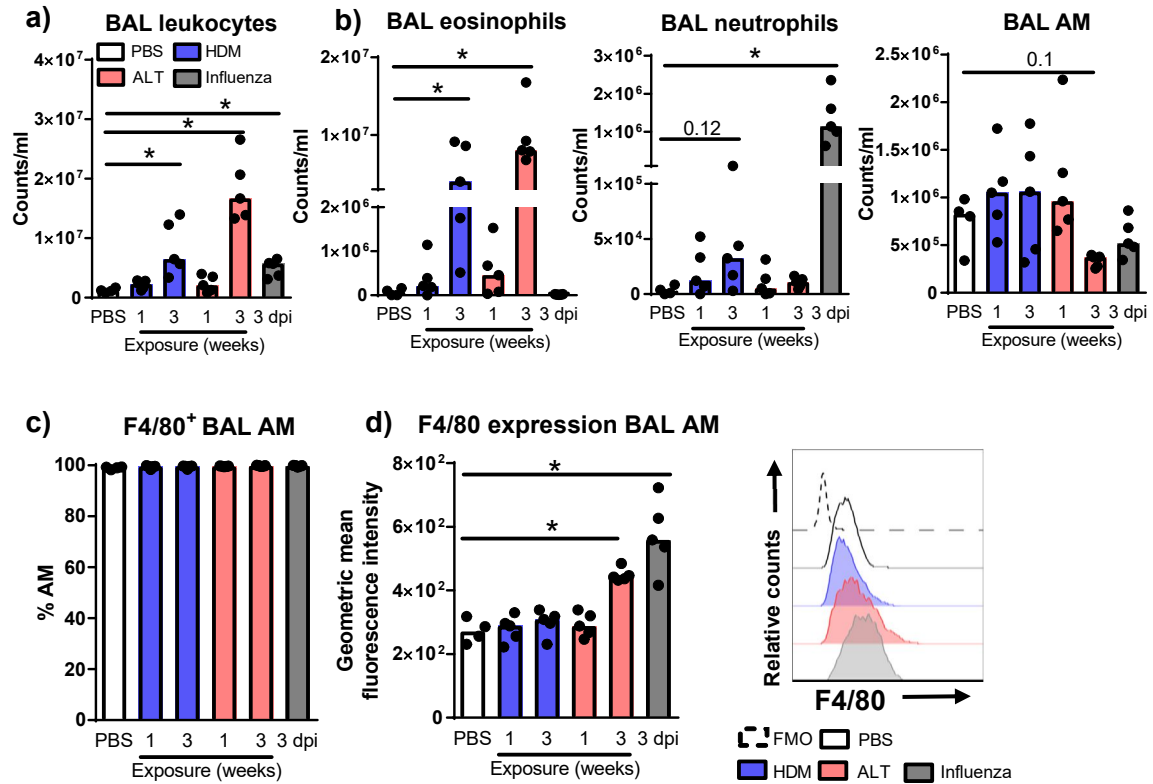


Figure 3.3. Validation of AM flow cytometry method across inflammation models

BAL cell number and phenotype were assessed by flow cytometry. **a)** Total crystal violet cell counts in BAL. **b)** Numbers of eosinophils, neutrophils and AM in BAL. **c)** Percentage of CD11c⁺ CD64⁺ Siglec F⁺ AM staining positively for F4/80. **d)** Geometric mean fluorescence intensity of F4/80 staining on AM (left), with representative histograms from 3 week allergen-, PBS- and influenza-treated mice (right). FMO, fluorescence-minus-one control. Scatter/bar plots show medians of 4-5 replicate mice per group. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$. Where relevant, corrected p values are stated between groups.

The surface antigen F4/80 and/or the cytoplasmic granular protein CD68 are frequently used to mark murine macrophages by flow cytometry, but were not included in the core gating strategy described above, in line with a recently published approach²⁹. As a validation step to determine whether the Siglec F⁺ CD11c⁺ CD64⁺ AM gate reliably marked *bona fide* macrophages, F4/80 expression on AM was assessed in all models. F4/80 expression was detected on 100 % of AM in all models tested (**Fig. 3.3c**) and its expression on AM, measured as geometric mean fluorescent intensity (gMFI) of flow cytometry staining, was increased in the 3 week ALT and influenza groups (**Fig. 3.3d**). Notably, the ALT and influenza models that led to increased F4/80 expression caused the most severe eosinophilic and neutrophilic airway inflammation, respectively (**Fig. 3.3b,c**), suggesting that F4/80 expression may be increased on AM in more severe inflammatory settings.

To further confirm that CD11c⁺ Siglec F⁺ cells in the allergic murine lung were AM, live precision-cut lung slices (PCLS) from mice exposed to either intranasal HDM or PBS control for 3 weeks were co-stained with CD11c, Siglec F and the epithelial cell marker EpCAM, for analysis by confocal microscopy. In PBS-treated mice, CD11c staining was localised to cells of mononuclear phagocyte morphology (arrowheads in **Fig. 3.4a**). These CD11c⁺ cells showed bright Siglec F staining and were intimately associated with airway epithelium (white pseudocolour in **Fig. 3.4a**), consistent with marking resident steady state AM²⁰.

In HDM treated mice, Siglec F single staining marked eosinophils clustered in inflammatory foci around airways, while CD11c⁺ Siglec F⁺ cells were round with large cytoplasm and resided in close contact with airway epithelial cells, strongly suggestive of AM (arrowheads and white pseudocolour in **Fig. 3.4b**). While the majority of large, CD11c⁺ cells showed Siglec F staining, this was generally dimmer than that seen for eosinophils, and a subset of these cells appeared to be Siglec F-negative (white box in **Fig. 3.4b**). While these CD11c single-positive cells may represent DC or CD11c⁺ infiltrating mononuclear phagocytes, their large size and location were suggestive of AM that with reduced Siglec F expression, as has been described previously in bleomycin-induced fibrosis¹⁴⁰. Thus, Siglec F and CD11c co-staining appears to be selective for AM in a PCLS system, but variability in the amount Siglec F expression means that further optimisation of this method will be required for reliably marking 100 % of AM in the allergic lung for visualisation by microscopy.

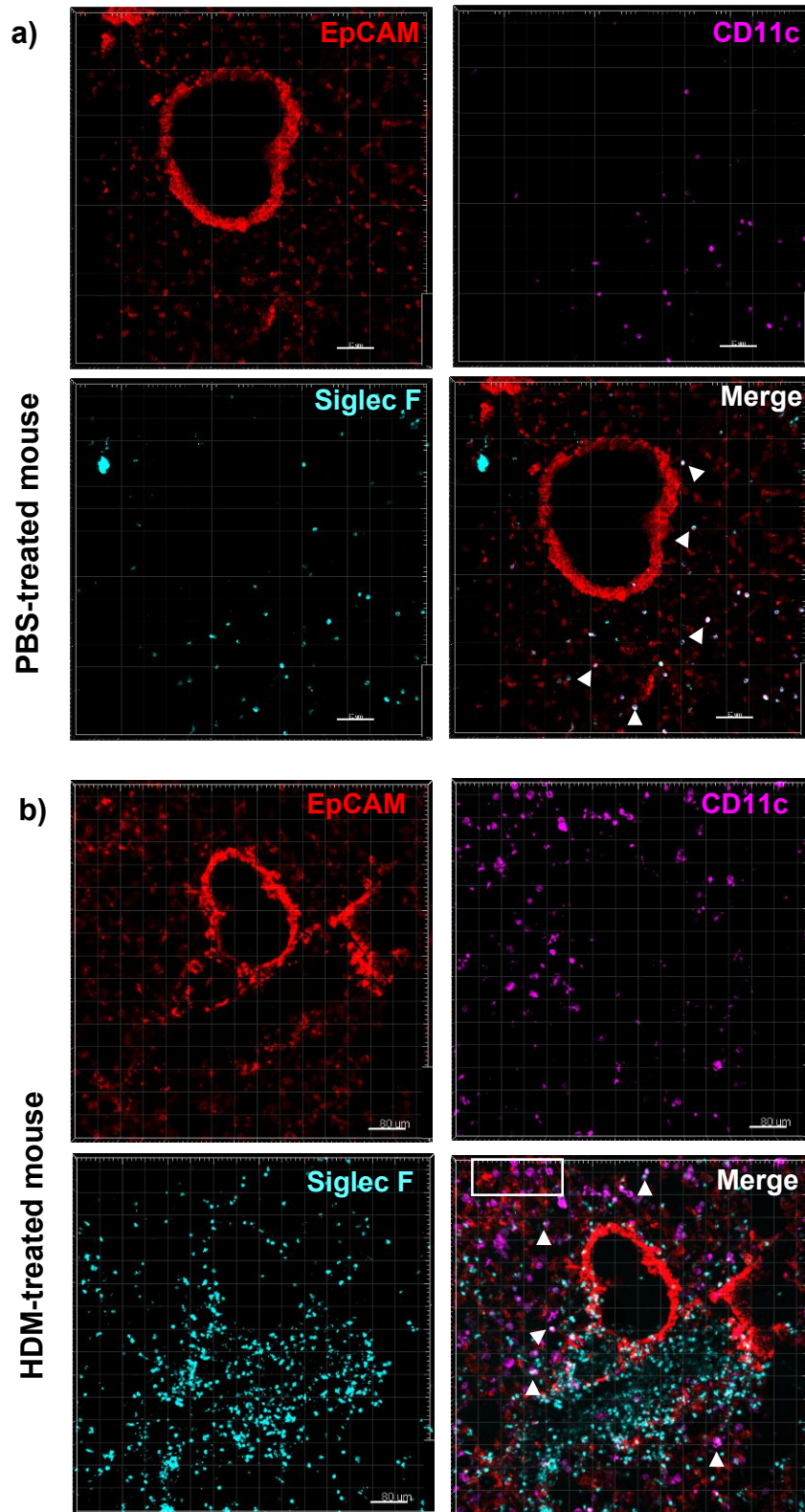


Figure 3.4. Co-expression of Siglec F and CD11c in AM *in situ*

Confocal images of live lung slices from mice treated for 3 weeks with either intranasal PBS (**a**) or HDM (**b**). Slices were stained with antibodies to CD11c and Siglec F, along with EpCAM to mark airway epithelium. White arrowheads in merged images indicate examples of Siglec F⁺ CD11c⁺ AM, which appear white due to overlay of cyan and magenta pseudocolours. White box in the merged image on (**b**) indicates CD11c-single positive cells with mononuclear phagocyte morphology. Images are representative of 2-3 fields captured from 2 PBS and 3 HDM-treated mice.

3.4. Characterisation of AM phenotype in models of pulmonary inflammation

3.4.1. AM increase in size and vacuolar morphology during inflammation

To begin to understand changes occurring to AM during pulmonary inflammation and identify parameters by which to quantify their activation *in vivo*, AM phenotype was assessed by flow cytometry. Using forward and side scatter of laser light as surrogate measurements of cell size and internal complexity, respectively, CD11c⁺ Siglec F⁺ CD64⁺ AM were confirmed to be large and heterogeneous in size and complexity (**Fig. 3.5a**). AM FSC-A and SSC-A properties were increased in mice treated with allergens for 3 weeks and, to a lesser extent, during acute influenza infection (**Fig. 3.5a**), which could also be seen by calculating geometric means of these values (**Fig. 3.5b**). No changes were observed in AM from mice exposed to HDM or ALT for 1 week (**Fig. 3.5b**), indicating that the mild inflammation occurring at these timepoints was insufficient for morphological changes to AM. Autofluorescence, an inherent property of AM arising from their fluorescent cytoplasmic contents²⁶, was increased in parallel with FSC-A and SSC-A (**Fig. 3.5b**), further supporting increased internal complexity of these cells. No significant change in geometric means of these parameters was observed for AM from influenza-infected mice (**Fig. 3.5b**), as these AM were heterogeneous in size and complexity, with only a minority showing light scatter properties distinct from PBS controls (**Fig. 3.5a**), which would have been missed by median or geometric mean calculations.

The changes to AM size and morphology suggested by light scatter properties were verified by visualisation of BAL AM on cytocentrifuge slides preparations of BAL cells. While AM from PBS-treated mice were fairly uniform in size and morphology, those from lung inflammation models were more heterogeneous, with larger cells apparent in HDM and influenza groups and the largest AM present in the ALT-treated mice (**Fig. 3.5c**). AM from the allergen-treated mice in particular showed additional morphological changes compared with PBS controls, including increased cytoplasmic vacuolation and more ruffled plasma membrane (**Fig. 3.5c,d**, arrowheads highlight examples), suggestive of increased uptake of material into internal compartments. Thus, AM activation during pulmonary inflammation is accompanied by distinctive changes in size and morphology.

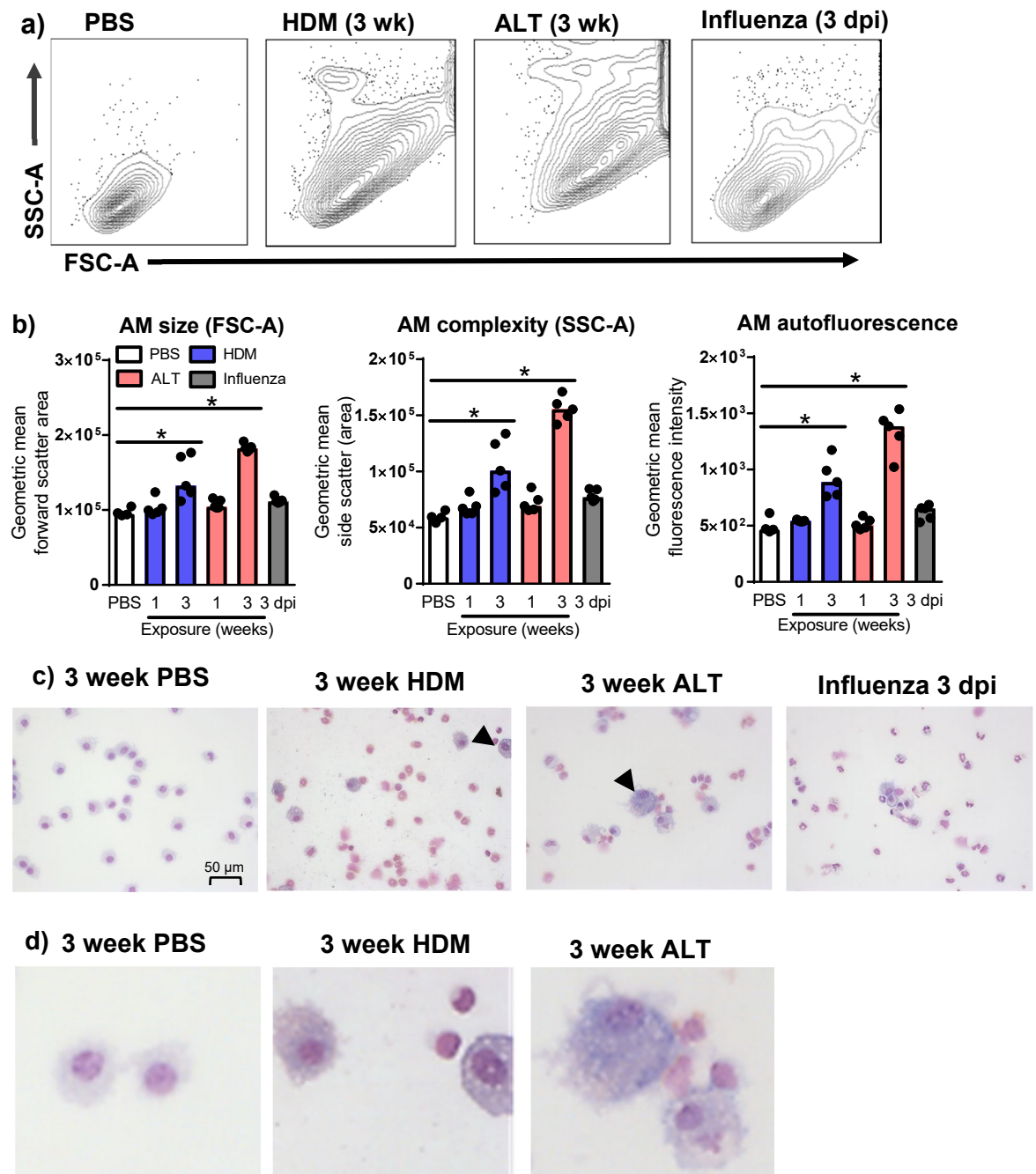


Figure 3.5. Changes in AM size and morphology during inflammation

AM from BAL were analysed by flow cytometry. **a)** Representative plots of forward scatter and side scatter areas as an approximation of AM size and internal complexity. **b)** Geometric means of AM forward scatter area, side scatter area and autofluorescence (in a 530/30 nm filter after 488 nm excitation), as determined by flow cytometry. **c)** Representative images of AM in Wright-Giemsa stained cytocentrifuge preparations of whole BAL at 400x magnification. Arrowheads point to AM exemplifying altered morphology. **d)** Larger images of the cells marked in (c) compared to PBS controls. Scatter/bar plots show medians of 4-5 replicate mice. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$.

3.4.2. Context-dependent expression of AM activation markers during inflammation

Flow cytometric analysis showed only a small proportion of AM from PBS control mice to express surface proteins associated with inflammatory monocytes and activated macrophages, such as CD11b, MHC-II and the receptor for iron-bound transferrin, CD71³²⁰ (**Fig. 3.6a**), consistent with published data¹⁹. The mannose receptor CD206, a marker of alternative activation^{80,321,322}, was also only expressed on a minority of steady state AM (**Fig. 3.6a**), consistent with a previous report²⁸. Upregulation of these proteins was therefore compared between inflammation models.

The proportion of CD11b⁺ AM significantly increased in influenza-infected mice and mice with established, but not early stage, AAD (**Fig. 3.6a**), consistent with reports of CD11b upregulation being a common feature of AM activation in mice^{311,323}. AM from 3 week ALT-treated mice, which had the most severe overall airway inflammation (**Fig. 3.3a**), showed the highest proportion of CD11b⁺ AM at around 80 % (**Fig. 3.6a**), suggesting a relationship between the extent of airway inflammation and expression of CD11b on AM. Influenza and 3 week allergen treated mice also displayed significant increases in the proportion of AM expressing MHC-II, typically expressed during classical macrophage activation³²² (**Fig. 3.6a**). Expression of CD71 and CD206 by AM was significantly increased following 3 week HDM and ALT exposure, with the greatest proportions of positive cells for these markers on AM from ALT-treated mice (**Fig. 3.6a**). In contrast to CD11b and MHC-II, expression of CD71 and CD206 was not significantly elevated on AM during acute influenza infection (**Fig. 3.6a**), suggesting that expression of these markers by AM is context-specific and favours allergic inflammation over the acute neutrophilic immune response to virus.

In addition to upregulation of proteins with little to no expression on naïve AM, the effects of inflammatory challenges on expression of constitutively expressed AM surface proteins were also examined by quantifying gMFI. CD11c was highly expressed on AM under all conditions, but intensity of staining was significantly increased in 3 week HDM and ALT groups (**Fig. 3.6b**), indicating that upregulation of CD11c on AM is a feature of sustained allergic inflammation. Upregulation of CD64 and signal regulatory protein α (SIRP α), a potential negative regulator of macrophage phagocytosis³²⁴, was apparent on AM of influenza-infected and 3 week ALT-treated mice, but not those from HDM-treated mice (**Fig. 3.6b**). This pattern of results suggests that increased expression of CD64 and SIRP α not typical of all allergic inflammation and instead rely on factors present during ALT and influenza-driven inflammation. Finally, reduced expression of Siglec F was observed on AM from 3 week allergen-treated mice, but not those from the influenza group (**Fig. 3.6b**). Notably, this downregulation appeared to be largely due to emergence of a Siglec F^{low} population, which encompassed a minority of AM from HDM-treated mice and the majority of those from the ALT group (**Fig. 3.6b**). These findings are consistent with the confocal microscopy described earlier in this chapter, which revealed a range of Siglec F staining intensities, from bright to undetectable, on CD11c⁺ AM in lungs of HDM-treated mice (**Fig. 3.4b**). Thus, allergic inflammation seems to drive either downregulation of Siglec F on a subset of AM, or generation of new AM with relatively low expression of Siglec F.

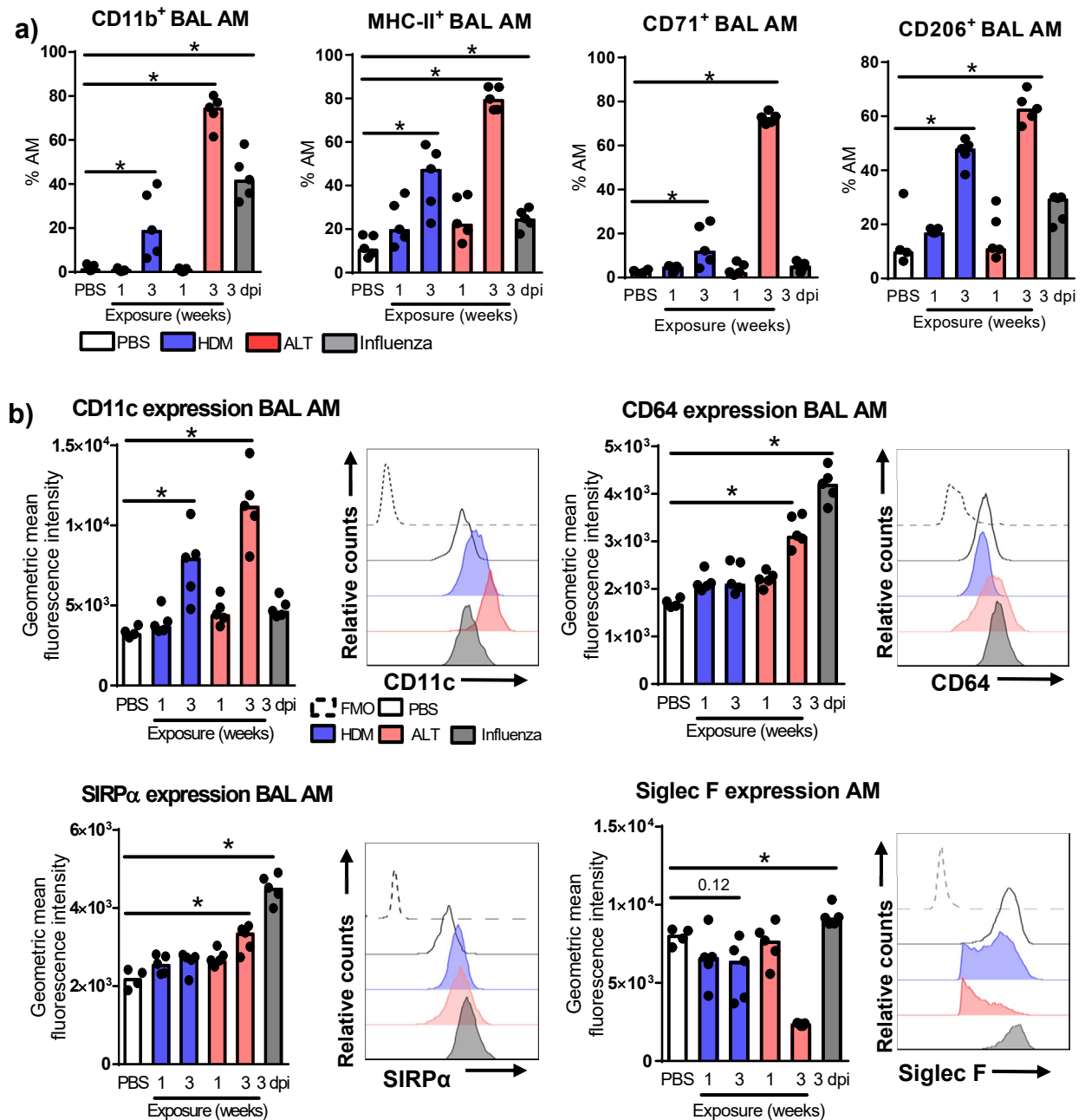


Figure 3.6. Changes in AM surface marker expression during inflammation

AM from BAL were analysed by flow cytometry **a)** Percentages of AM with positive surface staining for CD11b, MHC-II, CD71 and CD206. **b)** Geometric mean fluorescence intensities of staining for the indicated markers, with representative histograms (right). FMO, fluorescence minus one control. Scatter/bar plots show medians of 4-5 replicate mice per group. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann-Whitney tests between pairs of groups. *, $p \leq 0.05$. Where relevant, corrected p values are shown between groups.

These experiments confirm that, while AM show relatively uniform morphology and surface molecule expression at homeostasis, perturbation of the pulmonary microenvironment during inflammation results in substantial changes in the phenotype of AM. These changes consisted of increased expression of a number of surface molecules associated with pro-inflammatory monocyte and macrophage functions and, in the case of allergic inflammation, downregulation of the constitutively expressed protein Siglec F. These changes did not appear to follow a classical or alternative activation paradigm, with AM from mice with AAD increasing expression of both MHC-II and CD206 (**Fig. 3.6a**). Importantly, the changes seen during inflammation were not identical across all models, illustrating the context-specificity of AM activation (**Table 3.3**).

Marker	Baseline expression	HDM (3 week)	ALT (3 week)	Acute influenza
CD11c	++	+++	+++	++
CD64	+	+	++	++
F4/80	+	+	++	++
Siglec F	+++	++	+	+++
CD11b	-	++	+++	++
MHC-II	+	++	++	++
CD71	-	+	+++	-
CD206	+	+++	+++	+

Table 3.3. Surface markers of AM activation *in vivo*

Relative surface expression of markers described in figures 3.3 and 3.6. Baseline reflects expression on AM from PBS-treated C57BL/6 mice. Minus signs indicate minimal expression of the marker by AM. Plus signs indicate the relative amount of positive expression of markers. Markers in red were increased from naïve AM, while markers in green were decreased.

3.5. Kinetic analysis of AM activation during acute lung inflammation

The experiments described in Section 3.4 identified increased expression CD11b and MHC-II as markers of AM activation common to both allergic inflammation and acute infection, while reduced Siglec F expression appeared to be restricted to sustained allergic inflammation (**Table 3.3**). To better understand the kinetics with which these protein expression changes occur in AM, a reductive inflammation model was employed in which AM phenotype was assessed throughout progression of acute lung injury following a single challenge with the TLR4 agonist LPS. Adult female C57BL/6 mice received 2.5 µg of intranasal LPS, a dose that had previously been determined to induce substantial, resolving, neutrophilic inflammation (Vuononvirta and Carlin, unpublished observations). LPS was administered in a large (50 µl) volume to promote penetrance along the respiratory tract and increase direct contact with AM, with control (0h) mice receiving the same volume of PBS. BAL cells were harvested at 24, 48 and 72 hours following challenge (**Fig. 3.7a**).

The observed peak in neutrophilic inflammation occurred at 24h post-challenge with LPS, but airway neutrophilia remained elevated at 72h (**Fig. 3.7b**). A population of CD64⁺ CD11b⁺ IMM, most likely inflammatory monocytes, were detectable in BAL at 24h post-challenge and were further increased at 72h (**Fig. 3.7b**), consistent with delayed recruitment of monocytes to the airway compared with neutrophils. AM numbers were also increased around two-fold above baseline levels by 72h post-challenge (**Fig. 3.7c**), congruent with the increase in airway IMM (**Fig. 3.7b**), suggestive of possible differentiation of infiltrating monocytes into mature Siglec F⁺ CD11c⁺ AM, although further experiments would be required to confirm this.

To determine the stage at which AM phenotype changed during this inflammatory process, expression of AM activation markers were assessed along the time-course. An increased proportion of MHC-II⁺ AM was apparent from 24h post-challenge and had increased further by 48h (**Fig. 3.7d**), consistent with activation of AM occurring rapidly following LPS challenge. As observed for allergen and influenza challenges, the proportion of CD11b⁺ AM was increased in LPS-challenged mice (**Fig. 3.7d**). However, this proportion exceeded that seen with allergen and influenza at close to 100 % and reached this maximum by 24h post-challenge (**Fig. 3.7d**). Despite this plateau, CD11b expression, as assessed by gMFI, continued to increase from 24 to 72h post-challenge (**Fig. 3.7e**), in a pattern similar to that seen for MHC-II⁺ AM (**Fig. 3.7d**). Expression of AM activation markers CD11b and MHC-II therefore occurs rapidly following LPS challenge *in vivo* and increases further as neutrophilic inflammation wanes and monocyte recruitment progresses.

Finally, Siglec F expression was examined by gMFI and showed a small decrease from baseline levels at 24h post-challenge, although this was transient and gMFI had returned to initial levels by 48h (**Fig. 3.7e**). This shows that reduction in Siglec F surface expression on AM is not limited to sustained inflammation as observed in AAD models (**Fig. 3.6b**) and in bleomycin-induced fibrosis^{29,140}, but may require more than a single challenge with LPS to persist.

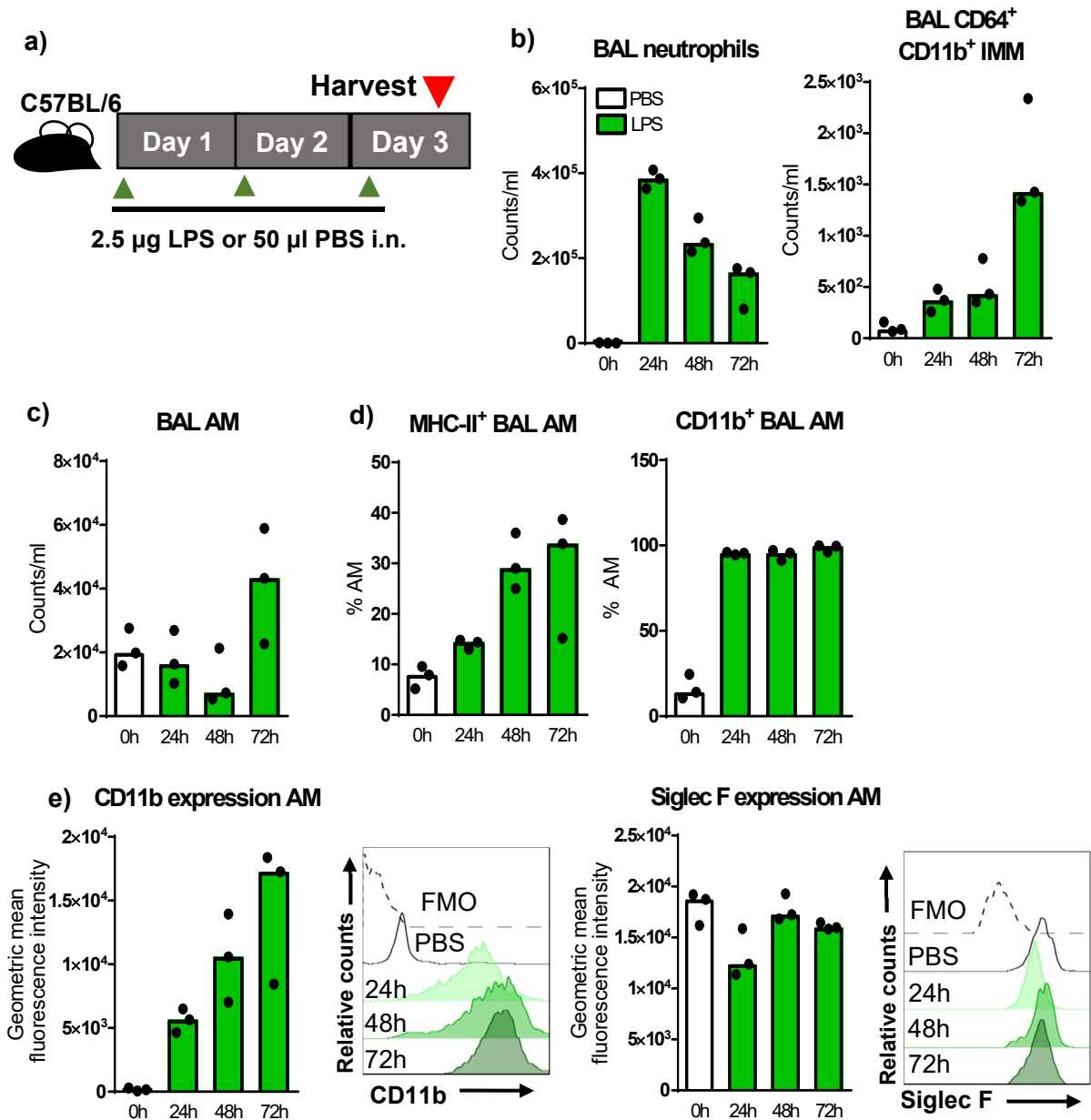


Figure 3.7. Changes in AM phenotype in LPS-driven acute lung injury model

a) Schematic of experimental model. C57BL/6 mice were administered 2.5 µg of intranasal LPS, with doses staggered to achieve 24h, 48h and 72h timepoints. Control mice received intranasal PBS and were harvested after 72h, to achieve the 0h timepoint. Cells in BAL were analysed by flow cytometry. **b-c)** Absolute numbers of neutrophils, CD64⁺ CD11b⁺ Siglec F⁻ IMM (**b**) and AM (**c**) in BAL. **d)** Percentages of AM with positive surface staining for MHC-II or CD11b. **e)** Geometric mean fluorescence intensity of CD11b and Siglec F in AM, with representative histograms. FMO, fluorescence minus one control. Scatter/bar plots show medians of 3 replicate mice.

3.6. Phenotypic changes to AM are partially affected by genetic background in murine models of distinct allergic airway disease phenotypes

The above studies highlighted different AM activation profiles in models of eosinophilic and neutrophilic airway inflammation. In C57BL/6 mice, the 3 week intranasal HDM exposure model of AAD results in a mixed granulocytic airway inflammation, dominated by eosinophils but also featuring a substantial number of airway neutrophils (**Fig. 3.3b**). Work in our department has shown that exposure of BALB/c and 129 mouse strains to identical HDM regimens results in airway inflammation with greater neutrophilia and eosinophilia, respectively, than C57BL/6 mice (Patel, Snelgrove, Lloyd, unpublished observations), presenting an opportunity to study AM activation and phenotype in different inflammatory settings arising from the same initial challenge. To assess changes to AM surface marker expression in the eosinophil^{hi} 129 and neutrophil^{hi} BALB/c HDM models, female mice of each strain were challenged for 3 weeks with HDM or PBS control, using C57BL/6 mice as comparators (**Fig. 3.8a**). HDM was administered 3 times per week in this experiment, rather than 5 times (**Fig. 3.8a**), due to the known increased susceptibility of BALB/c and 129 strains to AAD compared to the relatively resistant C57BL/6 strain.

Flow cytometry of BAL cells confirmed the expected distinct airway inflammation phenotypes between mouse strains. Eosinophil counts in HDM-treated 129 mice were significantly greater than those in C57BL/6 and BALB/c, while HDM-treated BALB/c mice displayed the highest BAL neutrophil counts (**Fig. 3.8b**). Steady state AM numbers were comparable in BAL of all three strains, however 129 mice displayed significantly higher numbers of AM than the other strains following HDM treatment (**Fig. 3.8c**), suggesting that AM maintenance, differentiation or proliferation may differ in this eosinophil^{hi} AAD model.

HDM treatment increased the percentage of AM expressing MHC-II in all three strains, with no significant differences observed between steady state or HDM-induced proportions of MHC-II⁺ AM between strains (**Fig. 3.8d**). HDM also resulted in upregulation of CD11b on AM in all strains; however, while around 40-50 % of AM were CD11b⁺ in HDM-treated C57BL/6 and 129 mice, a significantly lower proportion of CD11b⁺ AM were present in BALB/c mice (**Fig. 3.8d**). Notably, 129 mice showed the highest proportion of CD11b⁺ AM at steady state (**Fig. 3.8d**; median 12.9 %), suggesting that AM in these mice express different levels of macrophage functional receptors to other strains, even in the absence of inflammatory insult. The 129 strain was also outstanding in upregulation of CD71 during HDM-driven AAD. CD71 expression was increased on the surface of AM from all strains following HDM treatment, but was significantly higher in HDM-treated 129 mice than C57BL/6 or BALB/c (**Fig. 3.8e**). In contrast, the proportion of CD206⁺ AM induced by HDM treatment did not significantly differ between strains (**Fig. 3.8e**). Siglec F expression on AM was decreased following 3 week HDM exposure in all three strains, but the decrease was greatest in C57BL/6 mice, possibly in part due to these mice having the highest Siglec F gMFI in the absence of allergen challenge (**Fig. 3.8f**).

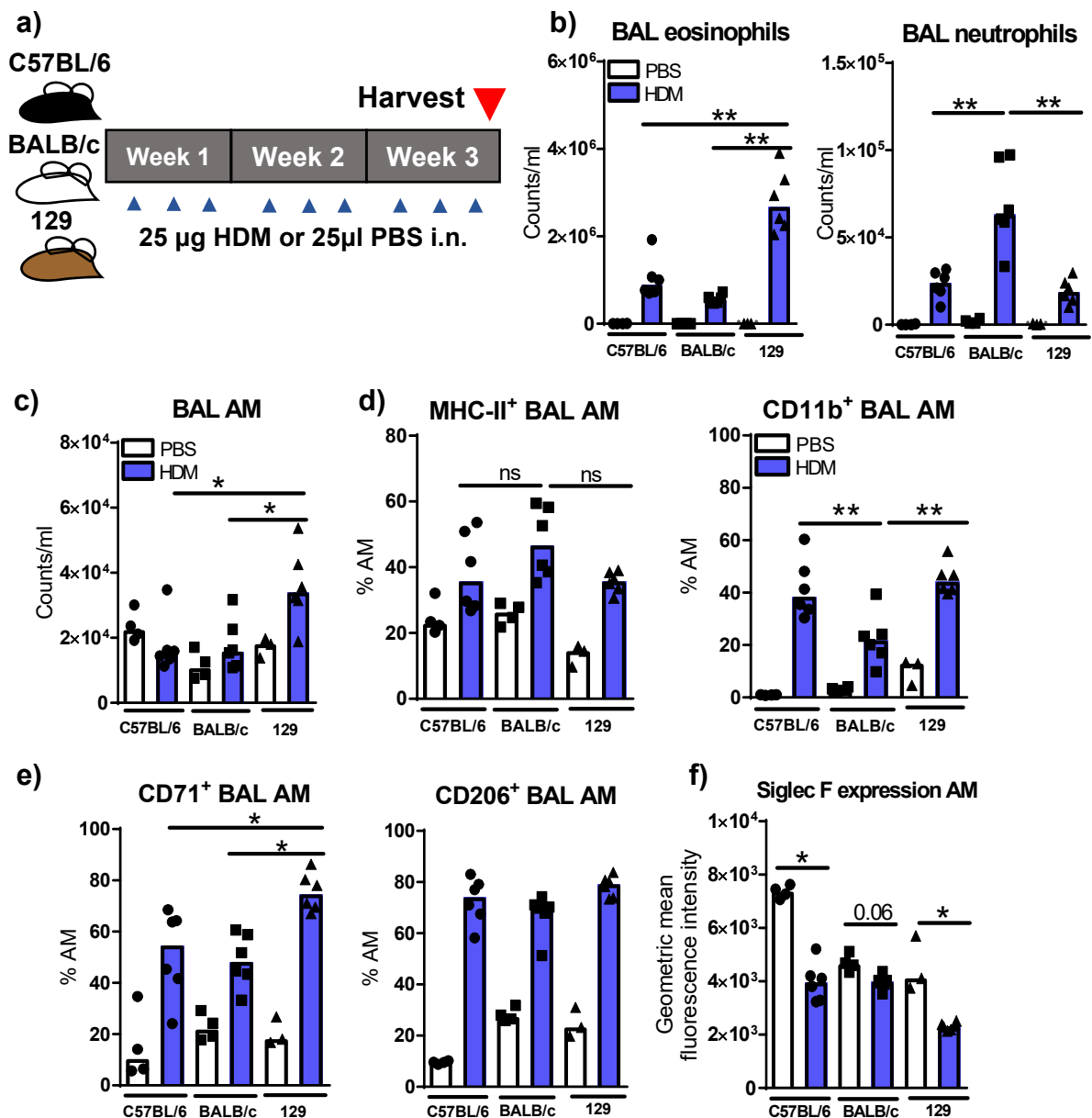


Figure 3.8. Expression of AM activation markers in neutrophilic and eosinophilic models of allergic airway disease

a) Experimental model. 3 laboratory mouse strains received intranasal HDM or PBS vehicle control 3 times per week for 3 weeks, before analysing BAL cells by flow cytometry 24h after final challenge. **b-c)** Absolute numbers of eosinophils and neutrophils (**b**) and AM (**c**) in BAL. **d-e)** Percentage of AM with positive surface staining for MHC-II or CD11b (**d**) and CD71 or CD206. (**e**). **f)** Geometric mean fluorescence intensity of Siglec F staining on AM. Scatter/bar plots show medians of 3-6 replicate mice per group. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant. Where relevant, corrected p values are shown between groups.

Overall, this comparative analysis confirmed that the parameters of AM activation during HDM-driven AAD defined in C57BL/6 mice held true for 129 and BALB/c strains, despite their distinct airway granulocyte profiles. However, the magnitude of changes to some of these parameters, such as CD71 expression and downregulation of Siglec F, varied between the three models. Further study of these differences may provide insights into the molecular and cellular determinants of AM activation during allergic inflammation.

3.7. AM activation during HDM-driven allergic airway disease is partially prevented by inhaled corticosteroid

Given the consistent AM activation observed during HDM-driven AAD, a pertinent question was whether these changes were sensitive to inhaled corticosteroids, the major maintenance therapy for asthma patients (see www.brit-thoracic.org.uk/standards-of-care/guidelines/). To address this, mice were treated with the clinically relevant glucocorticoid budesonide (BUD) intranasally one day prior to initial HDM challenge and five days per week throughout the HDM protocol, before collecting BAL cells for analysis 24h after final HDM challenge (**Fig. 3.9a**). BALB/c and 129 mice were used in this study, to represent the extremes of neutrophil^{hi} and eosinophil^{hi} AAD, respectively (**Fig. 3.9a**).

As expected, HDM treatment induced airway eosinophilia in both BALB/c and 129 mice, which was limited by budesonide treatment, although a substantial number of BAL eosinophils remained in 129 mice treated with budesonide and HDM (**Fig. 3.9b**), consistent with incomplete suppression of the eosinophil^{hi} AAD phenotype by inhaled corticosteroids. Airway neutrophils were also increased by HDM and significantly reduced with budesonide treatment in both strains (**Fig. 3.9b**). BAL AM numbers were not significantly altered in either strain by HDM or budesonide, although, as observed in the previous study, total AM numbers were generally higher in 129 than BALB/c mice (**Fig. 3.9b**).

Suppression of eosinophilic and neutrophilic inflammation in BALB/c mice by inhaled budesonide was accompanied by complete prevention of HDM-driven increases in proportions of AM expressing MHC-II, CD11b and CD71 and a partial reduction in CD206 expression (**Fig. 3.10a**). Budesonide also reduced baseline MHC-II expression and increased CD206 expression on AM in PBS-treated BALB/c mice, although these differences did not reach statistical significance after adjustment for multiple comparisons (**Fig. 3.10a**, $p = 0.06$), suggesting that inhaled corticosteroids modulate the steady state phenotype of AM. In contrast to these markers, the reduction in Siglec F gMFI observed on HDM-treated BALB/c AM was not prevented by budesonide treatment. Indeed, budesonide alone significantly reduced the gMFI of Siglec F staining on AM (**Fig. 3.10b**). Thus, most, but not all HDM-driven changes in AM activation marker expression were sensitive to prophylactic inhaled corticosteroid treatment in the BALB/c model of neutrophil^{hi} AAD.

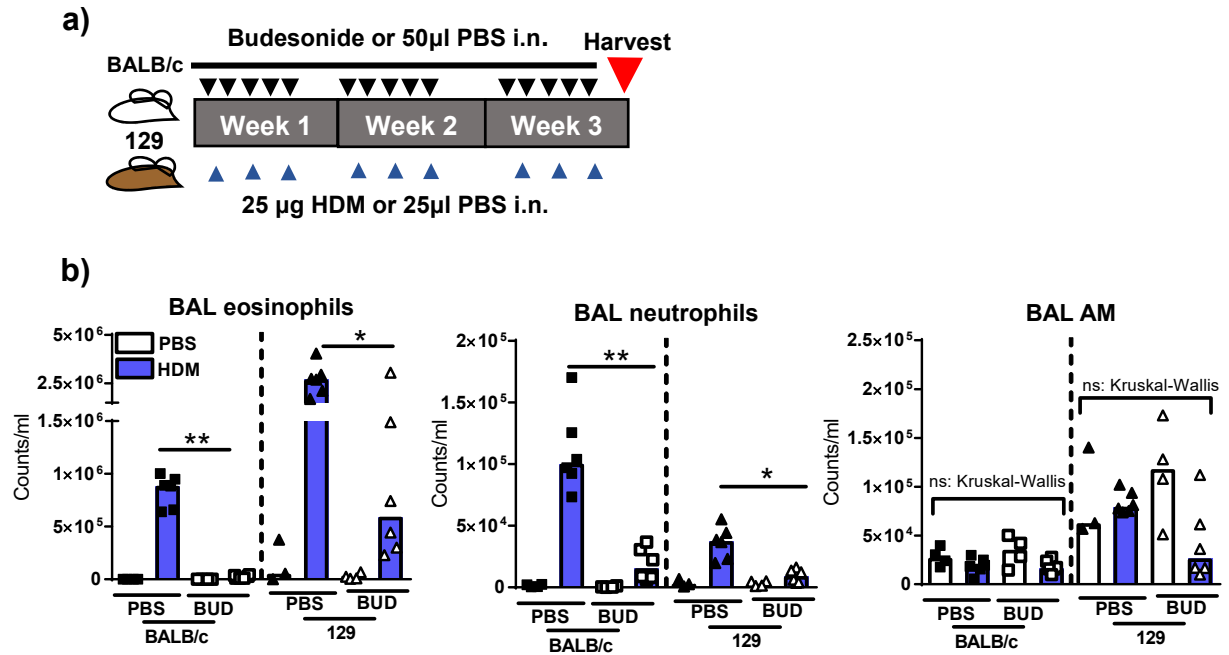


Figure 3.9. Inhaled corticosteroid suppression of HDM-driven allergic airway disease

a) Schematic of budesonide treatment in neutrophil^{hi} (BALB/c) and eosinophil^{hi} (129) models of allergic airway disease. Intranasal budesonide was administered 24h prior to initial HDM challenge and then 5 days per week throughout the HDM exposure protocol. BAL was analysed by flow cytometry 24h after final HDM challenge. **b)** Numbers of eosinophils, neutrophils and AM in BAL. Scatter/bar graphs show are medians of 4-6 replicate mice per group. Statistics: Kruskal-Wallis test, followed by Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant.

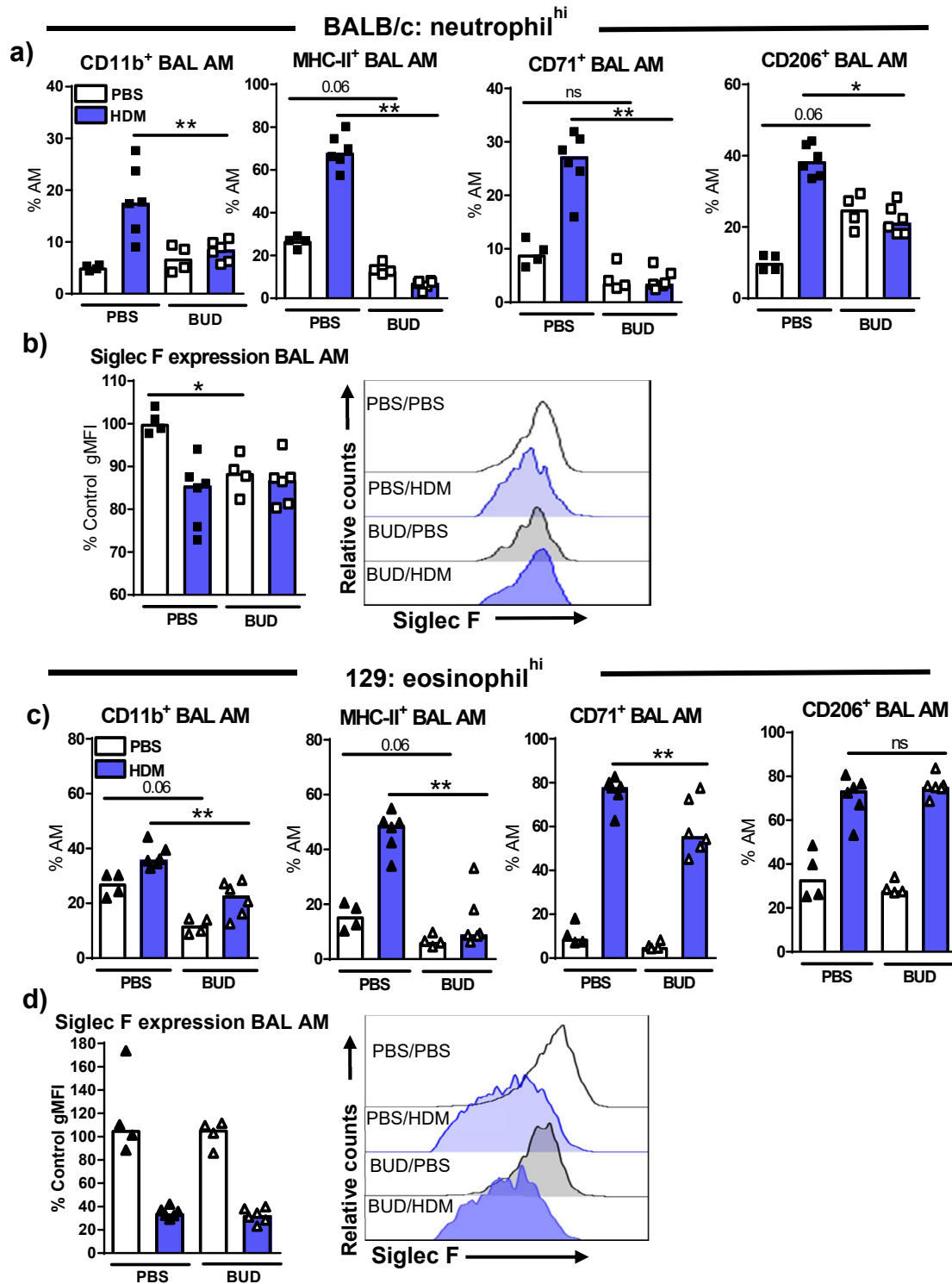


Figure 3.10. Partial steroid sensitivity of HDM-driven AM activation marker expression

Flow cytometric analysis of AM from experiments outlined in **Fig. 3.9**. **a)** Percentage of AM expressing MHC-II, CD11b, CD71 and CD206 in BAL from BALB/c mice. **b)** Geometric mean intensity of Siglec F staining on AM from BALB/c mice, normalised to median value obtained in PBS/PBS controls, with representative histograms. **c-d)** Matched data to **a** and **b**, respectively, obtained from 129 mice. Scatter/bar plots show medians 4-6 replicate mice per group. Statistics: Kruskal-Wallis test, followed by Bonferroni-corrected Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant. Where relevant, corrected p values are shown between groups.

Dampening of HDM-driven eosinophil^{hi} inflammation by budesonide in 129 mice was also accompanied by limitation of MHC-II and CD11b expression on AM to levels seen in PBS control mice (**Fig. 3.10c**), indicating complete suppression of upregulation of these surface molecules by the inhaled corticosteroids. Decreases were also apparent ($p=0.06$) in baseline expression of these markers by AM from PBS control mice when treated with budesonide alone (**Fig. 3.10c**), further demonstrating that inhaled corticosteroid treatment affects the steady state phenotype of AM. Although HDM-driven CD71 expression on AM was significantly reduced by budesonide in 129 mice, this reduction was only by around 25 % and the proportion of CD71⁺ AM remained above that seen in PBS control mice (**Fig. 3.10c**). This partial reduction was in contrast to the complete reduction seen in BALB/c mice (**Fig. 3.10a**), possibly reflecting the high level of expression of this receptor on AM in HDM-treated 129 mice compared with other strains (**Fig. 3.8e**). No limitation of the HDM-driven increase in CD206 expression by budesonide was observed in 129 mice (**Fig. 3.10c**), in contrast to the partial reversal in BALB/c mice (**3.10a**). Similarly, reduced Siglec F gMFI on AM of HDM-treated 129 mice was not prevented by budesonide, although there was no effect of budesonide alone on Siglec F expression on AM from PBS-treated mice (**Fig. 3.10c**), in contrast to that seen in BALB/c (**Fig. 3.10b**).

Together, these steroid sensitivity experiments indicate that expression of MHC-II and CD11b by AM, both at baseline and during allergic inflammation, occurs in a manner inhibited by corticosteroid inhalation, while reduced Siglec F expression on AM during AAD is steroid-refractory. Moreover, the different extents of reduction of CD71 and CD206 expression by budesonide observed in different mouse strains suggest that AM can remain partially activated even in the presence of steroid and that suppression of AM activation by corticosteroids is likely to vary depending on the inflammatory context. These data suggest that AM activation is a complex process, involving a combination of steroid-sensitive and –insensitive pathways.

3.8. Discussion

The overall aim of the work described in this chapter was to better understand the activation state of AM in different pulmonary inflammatory contexts. These findings were intended to inform the design and methodology of subsequent experiments in which IL-10 and TGF- β signalling were manipulated *in vivo*.

Through these preliminary experiments, a minimal flow cytometry panel for analysis of murine pulmonary myeloid cells was developed and shown to robustly distinguish AM from other macrophage and DC populations at homeostasis and during inflammation. Identification of murine AM, eosinophils, neutrophils, cDC1, cDC2, moDC and a mixed population of inflammatory monocytes and interstitial macrophages (IMM) could be achieved without using the typical mouse macrophage antigens CD68 and F4/80, as suggested in a recent publication²⁹. However, F4/80 remains useful as an additional confirmatory marker of monocytes and macrophages, so was included along with Ly6C in subsequent studies in this thesis, to allow more detailed analysis of these cells where necessary.

Comparison of AM surface molecule expression across multiple inflammation models revealed activation markers common to multiple inflammation states and those restricted to only a subset of models. Increased expression of CD11b and MHC-II on AM occurred consistently in allergen models in all mouse strains tested and in a manner prevented by inhaled corticosteroids. Expression of these proteins was also elevated following influenza infection and after LPS inhalation, suggesting that CD11b and MHC-II are robust activation markers for AM in both allergic and non-allergic inflammation. These findings are in accordance with published data in which CD11b expression on murine AM was increased in multiple models of airway inflammation, independently confirming that this is a useful parameter for studying activation of murine AM^{311,323}. However, the distinction in human AM is less clear, since low levels of CD11b have been reported on these cells in the absence of overt disease, which was increased in individuals with BAL cytology indicative of airway inflammation³¹¹. This report therefore suggests that, in humans, a continuum of CD11b expression on AM exists and that the binary expression observed on mouse AM may be due to a failure of laboratory mice to adequately reflect the low level of CD11b expression on AM of humans and other animals living in a less controlled environment. However, the work of Duan *et al*³¹¹ was based on a relatively small number of donors, only three of which were healthy non-smokers and the majority of whom were over 60 years of age. Further research is therefore required, in young healthy humans and matched cases of inflammatory disease, to better evaluate CD11b expression as an activation marker for human AM.

Increased expression of MHC-II on AM has previously been reported in animal models of airway inflammation²⁹ and in human diseases, including asthma^{307,325}. Although IL-4 can induce MHC-II expression in mononuclear phagocytes³²⁶, MHC-II has traditionally been viewed as a marker of classical activation of macrophages, as it is potently induced by IFN γ ^{80,322}. However, AM are now appreciated to exist outside of the extremes of classical and alternative activation¹⁹, expressing

both MHC-II and the alternative activation marker CD206, to varying extents, in both healthy individuals and asthmatics^{307,327}. Indeed, large proportions of AM in both the HDM and ALT models of AAD expressed MHC-II and CD206 in the present studies. The increased expression of MHC-II observed on AM in the allergic airway therefore likely reflects integration of one or more of the many signals occurring in the airway microenvironment following repeated allergen inhalation⁴. Supporting a need for sustained mucosal allergen exposure to increase MHC-II expression on AM in a type 2 immune setting, experiments in this chapter showed no increase in MHC-II expression on AM of mice receiving intranasal HDM or ALT for only one week. Moreover, rat AM did not upregulate MHC-II in a systemic sensitisation and airway challenge model of AAD³²⁸, which is known to differ markedly in mechanisms of inflammation from mucosal sensitisation models^{4,329}.

Increased expression of CD71 and CD206 on AM was observed in AAD models in multiple mouse strains, but not during acute influenza infection. This suggests that the mechanisms of induction of these proteins in AM were distinct from those driving CD11b and MHC-II expression, likely requiring signals present in the allergic airway but absent early in acute influenza infection. Upregulation of CD71 and CD206 on AM has previously been demonstrated in bleomycin-induced pulmonary fibrosis²⁹, the pathobiology of which has a strong tissue repair component³³⁰, suggesting that potential common drivers of AM CD71 and CD206 expression may exist between fibrosis and AAD models. Interestingly, the highest proportions of CD71⁺ AM in the present work were observed in the most eosinophilic conditions tested: the 3 week ALT model in C57BL/6 mice and the 3 week HDM model in 129 mice, suggesting a possible link between the degree of airway eosinophilia and the extent of CD71 expression on AM. It will therefore be interesting to explore the functional importance of CD71 expression on AM and to examine CD71 expression in asthma patients, particularly since increased expression of this molecule was only partially sensitive to inhaled corticosteroid treatment in highly eosinophilic 129 mice.

Increased expression of the alternative macrophage activation marker CD206 and classical activation marker MHC-II on the same cell populations during HDM- and ALT-driven AAD further reinforces the idea that AM do not fit into the classical/alternative activation dichotomy¹⁹, even during type 2 immunity. CD206 is expressed by all lung macrophages in healthy adults²⁷ and in all AM obtained in paediatric BAL samples (Cook, Saglani, Lloyd, unpublished observations). The increased CD206 expression observed during AAD in mice in the present work is consistent with results from human asthma, where increased CD206 expression on AM has been reported³⁰⁷. The incomplete suppression of HDM-driven CD206 upregulation on AM by prophylactic budesonide treatment fits with published *in vitro* studies where corticosteroids increased expression of CD206 on macrophages³³¹ and may have implications for AM phenotype in asthma patients, for whom inhaled corticosteroids are the major maintenance therapy. The precise functions of CD206⁺ AM in the allergic airway are not clear, but CD206 has been implicated in protective macrophage functions such as phagocytosis and apoptotic cell clearance³³².

Another major change observed in AM during inflammation was in their intensity of Siglec F expression, which was decreased in AAD models, but not during acute influenza infection. Siglec F expression was bimodal in chronic HDM or ALT-treated mice, indicating the emergence of a Siglec F^{low} AM population. Low Siglec F expression has been reported to mark AM derived from recruited monocytes, rather than embryonic precursors^{29,140,141}, suggesting that Siglec F^{low} AM may represent cells that have recently differentiated from inflammatory monocytes. However, transient reduction in Siglec F expression was also observed on AM 24h after LPS inhalation, a timepoint prior to peak recruitment of inflammatory monocytes to the lung, suggesting that Siglec F expression can also be reduced on resident AM in a cell-intrinsic manner. Notably, reduced Siglec F expression was still observed in AM from chronic HDM-treated mice in the presence of budesonide, despite marked suppression of airway inflammation. Thus, reduced Siglec F expression on AM in the allergic lung may result from both intrinsic downregulation by resident AM and incomplete upregulation of Siglec F by recruited monocyte-derived cells that have differentiated into AM.

Distinguishing between resident and recruited macrophages is likely to be important in understanding the contribution of AM to allergic inflammation, since macrophages derived from inflammatory monocytes have been demonstrated to promote type 2 pulmonary inflammation, whereas resident AM are more tolerogenic^{63,333}. However, the extent to which AM have that have fully differentiated from inflammatory monocytes truly differ from resident AM that have been activated during inflammation remains an open question. Confident tracing of macrophage origin requires complex experimental approaches using lineage tracing mutant mice, which were outside the scope of this thesis, particularly given the requirement for additional complex transgenic mice in later experiments (see Chapter 6). However, future studies may benefit from distinguishing resident and recruited AM during AAD to better understand how the allergic microenvironment shapes macrophage phenotype.

Overall, these comparative phenotyping studies identified increased CD11b and MHC-II and decreased Siglec F as robust markers of AM activation, which could be assessed in future studies. Although sufficient for the purposes of establishing methodology, these experiments were not without caveats. Firstly, comparisons between AAD models and influenza infection are not definitive, since only one influenza timepoint was used, while the immune environment differs dramatically over the course of influenza infection⁷, with likely consequences for AM phenotype. However, the influenza-treated mice in this study were principally intended as a model of acute, neutrophilic inflammation to compare with chronic allergen exposure, meaning that a full time-course was outside the scope of the chapter. Secondly, while prophylactic budesonide treatment experiments clearly demonstrated suppression of a subset of HDM-driven AM activation parameters, these changes could conceivably depend upon limitation of inflammation throughout allergen exposure, rather than revealing true corticosteroid reversibility of these phenotypic changes. It would therefore be necessary to administer budesonide therapeutically, after establishment of AAD, to better understand the sensitivity of AM activation to inhaled

corticosteroids. Finally, while providing markers to assay for AM activation *in vivo*, these experiments did not explore the functional importance of increased expression of surface proteins by AM. For example, CD11b can function in phagocytosis and modulate responses to complement and TLR4 agonists in myeloid cells in a cell type-dependent manner³³⁴, suggesting that expression of CD11b by AM may promote pro-inflammatory features of AM during inflammation. However, further experiments would be required to test these functions.

3.9. Conclusions

Together, the results describe here demonstrate that AM show profound changes in phenotype in response to pro-inflammatory stimuli, increasing expression of molecules associated with inflammatory monocytes and macrophages. These changes are context-specific, with distinct combinations of markers expressed and altered during allergic disease and acute neutrophilic inflammation, including expression of proteins associated with both classical and alternative macrophage activation. Importantly, markers of AM activation during HDM-driven AAD vary in their sensitivity to suppression by inhaled corticosteroids, which may have implications for therapeutic reversal of AM dysfunction during allergic inflammation.

4. Characterisation of airway macrophage production of Interleukin 10 and Transforming Growth Factor β 1

4.1. Introduction

In Chapter 3, the surface protein expression of AM was examined in mouse models of pulmonary inflammation and shown to shift towards that of more pro-inflammatory macrophages; displaying increased CD11b, MHC-II and CD71 expression and reduced expression of Siglec F to varying extents depending on the model of inflammation used. Such inflammatory responses are typically accompanied by intrinsic regulatory mechanisms to limit potentially pathogenic inflammation. This is likely to be the case with AM during pulmonary inflammation, since depletion of AM during established house dust mite- (HDM-) driven allergic airway disease (AAD) enhances allergic airway inflammation¹⁴⁴, indicating that even after exposure to pro-inflammatory stimuli, AM retain a regulatory function. The mechanisms by which AM regulate pulmonary immunity at homeostasis and during inflammation are not well characterised. Early studies using co-culture systems and *in vivo* depletion suggested that AM achieve this by being poor antigen-presenting cells (APC)¹⁵¹, suppressing T cell activation^{149,152,335} and reducing the antigen presenting cell function of dendritic cells (DC)^{143,147}, therefore limiting the activation of pulmonary T cells to inhaled antigens³³⁶. Others have shown that antigen-loaded CD11c⁺ Siglec F⁺ pulmonary macrophages (i.e. AM) preferentially induce FoxP3 expression by CD4⁺ T cells *in vitro* and when transferred into the airway lumen¹⁵³. However, the extent to which these interactions with T cells and DC occur physiologically *in vivo*, without experimental transfer of cells, is unclear.

Production of the cytokines IL-10 and TGF- β 1 is a potential mechanism of limiting immune responses, which may be employed by AM. Loss of IL-10 function increases the severity of allergen-^{210,213} and respiratory virus-driven inflammation^{107,108} in experimental models, indicating the importance of this cytokine in regulation of pulmonary immunity. However, although some studies have suggested that AM express IL-10 and that this expression is altered in asthmatics^{156,216,337}, a role for AM-derived IL-10 in regulation of allergic inflammation is yet to be demonstrated. AM have previously been shown to be a minor source of IL-10 during mouse models of infection with respiratory syncytial virus (RSV)¹⁰⁸ and *Mycobacterium tuberculosis*¹⁹⁷, but similar studies have not been described using models of AAD. Further analysis of AM IL-10 expression in response to inflammatory stimuli, *in vitro* and *in vivo*, is therefore required to better understand the potential for AM to regulate immune responses via IL-10 production.

The importance of TGF- β 1 in regulation of pulmonary immunity is well accepted, as evidenced by the potential for systemic pan-TGF- β blockade to enhance airway inflammation in models of AAD²⁸⁰ and respiratory infection²⁷⁵. However, although AM are known to be major pulmonary producers of TGF- β 1 at homeostasis and during inflammation^{273,338}, the contribution of AM to TGF- β -dependent mechanisms of immune regulation in the lung is not understood. *In vitro* studies have shown TGF- β 1 to be necessary for AM induction of FoxP3 expression in CD4⁺ T cells, in concert with retinoic acid¹⁵³, but the relevance of this mechanism *in vivo* is not known. The context-dependent, pleiotropic functions of TGF- β signalling in T cell polarisation²¹⁸, airway remodelling^{136,137,339} and cell recruitment to the airways²⁷³, mean that characterising TGF- β 1

expression in AM is essential for understanding how these cells fit into the complex immunoregulatory networks that function in the lung.

Equally important to studying production of TGF- β 1 and IL-10 by AM is understanding the responsiveness of AM to these cytokines, which are proposed to maintain their tolerogenic state *in vivo*¹⁹. Mice unable to effectively activate TGF- β in the airway due to absence of integrin $\alpha_v\beta_6$ develop emphysema, likely due to failure to restrict production of MMP12 by AM²⁴⁴, while TGF- β signalling is required for perinatal development and maintenance of AM *in vivo*²⁸². Additionally, both IL-10 and TGF- β drive expression of the regulatory receptor CD200R on AM¹²⁷. However, the effects of TGF- β and IL-10 on AM are likely to be context-dependent and vary with the prevailing cytokine milieu, necessitating the study of these interactions both at steady state and during inflammation.

A major overall aim of this thesis was to understand how TGF- β 1 and IL-10 function to regulate immunity in the lung and how AM are involved in these processes. Initial studies of AM production of, and responsiveness to, TGF- β 1 and IL-10 were therefore conducted to guide subsequent experiments in which these cytokines were to be modulated *in vivo*.

4.2. Aims

1. Assess AM IL-10 and TGF- β isoform production and responsiveness *in vitro*
2. Assess expression of TGF- β 1 and IL-10 by AM *in vivo* at steady state and during inflammation and compare their expression to other cellular sources

4.3. Comparing IL-10 production by AM to other murine macrophage subsets

To assess cytokine production by AM, an *in vitro* system was established in which responses of primary murine AM to inflammatory stimuli could be assessed and compared to macrophages of different origins. Peritoneal macrophages (PM) were used as a comparator tissue resident macrophage that develop in a distinct anatomical location to AM, but are also predominantly derived from embryonic precursors in young adult mice³⁴⁰. In addition, M-CSF-differentiated bone marrow-derived macrophages (BMDM) were used a control macrophage derived from adult haematopoietic precursors and frequently used to study macrophage function *in vitro* (**Fig. 4.1a**). Cells were obtained by bronchoalveolar lavage (BAL; AM), peritoneal lavage (PM) or 7 day culture with M-CSF (BMDM) and subsequently adhered to tissue culture plates to enrich for macrophages (**Fig. 4.1a**). BAL and BMDM preparations contained a high proportion (> 95 %) of CD45⁺ CD64⁺ F4/80⁺ macrophages prior to adhesion enrichment, while macrophages made up around 50 % of peritoneal lavage (**Fig. 4.1b**). PM and BMDM were predominantly CD11b⁺ CD11c⁻, in contrast to the CD11b⁻ CD11c⁺ AM (**Fig. 4.1b**). Adhesion enrichment of all three populations removed contaminant cells, leaving behind only large mononuclear cells with morphology characteristic of macrophages (**Fig. 4.1c**).

BMDM are known producers of IL-10³⁴¹ and so were used as a positive control for IL-10-induction. To compare IL-10 production in macrophage subsets, cells were initially activated with LPS, which drives IL-10 production in BMDM via the Myd88 and TRIF pathways downstream of toll-like receptor 4 (TLR4) and type I interferon (IFN) production^{341,342}. To maximise potential for IL-10 production in this system, LPS treatment was also combined with IL-4, which was shown previously to enhance LPS-driven IL-10 fluorescent reporter signal from BMDM³⁴³.

Il10 mRNA was present at similar relative expression levels in resting PM and BMDM cultured in medium alone, but was undetectable in AM under these conditions (**Fig. 4.2a**). LPS increased *Il10* mRNA expression in all three macrophage subsets, but levels remained very low in AM (raw qPCR threshold cycle values > 35). IL-4 did not affect *Il10* mRNA levels in any of the cell types, alone or in combination with LPS (**Fig. 4.2a**). Consistent with their low levels of *Il10* mRNA, IL-10 protein was undetectable by ELISA in supernatants of resting and LPS-treated AM (lower detection limit 31.25 pg/ml), but was significantly increased in PM and BMDM after LPS stimulation (**Fig. 4.2b**). Absence of assayable levels of IL-10 in AM supernatants was confirmed by Legendplex™ cytometric bead array, in which supernatants from resting or LPS-treated AM did not reach the 2 pg/ml lower limit of detection (not shown). Lack of induction of IL-10 in AM did not result from failure of LPS stimulation, since LPS significantly increased *Tnf* mRNA (**Fig. 4.2c**) and TNFα protein secretion (**Fig. 4.2d**) in all three macrophage subsets. IL-4 had no effects on resting or LPS-driven *Tnf* gene expression (**Fig. 4.2c**).

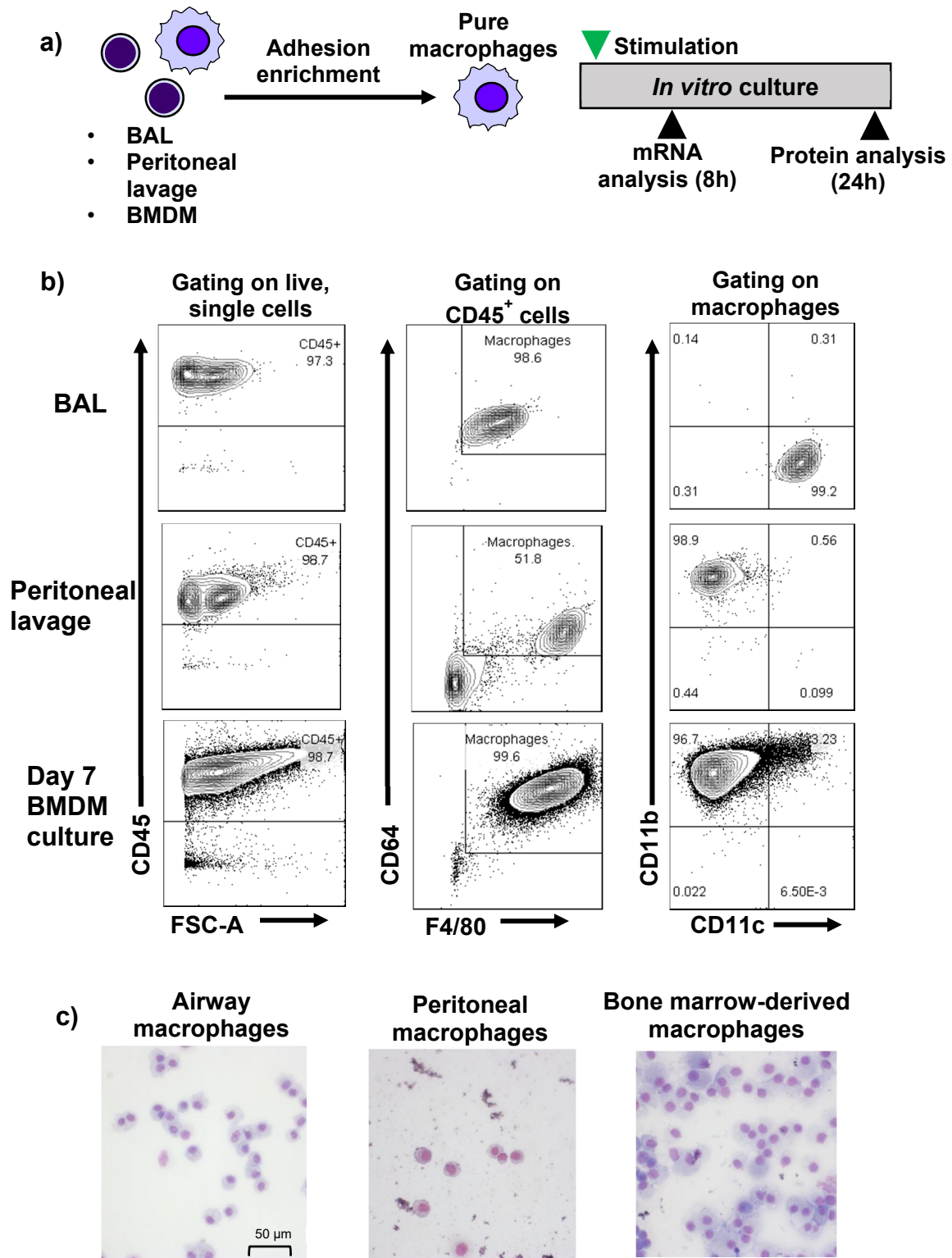


Figure 4.1. *In vitro* system for stimulation of mouse macrophage subsets

a) Bronchoalveolar lavage (BAL), peritoneal lavage, or M-CSF-differentiated bone marrow-derived macrophages (BMDM) were enriched by adhesion before stimulation. RNA in cell lysates and protein in supernatants were quantified after 8h and 24h of stimulation, respectively, unless otherwise stated.

b) Representative flow cytometry plots showing macrophage ($CD64^+ F4/80^+$) composition and macrophage phenotype ($CD11c$ and $CD11b$ expression) of BAL, peritoneal lavage and BMDM cultures, prior to adhesion enrichment. **c)** Representative images of cytocentrifuge preparations of AM, PM and BMDM, following adhesion enrichment, taken under 400x magnification.

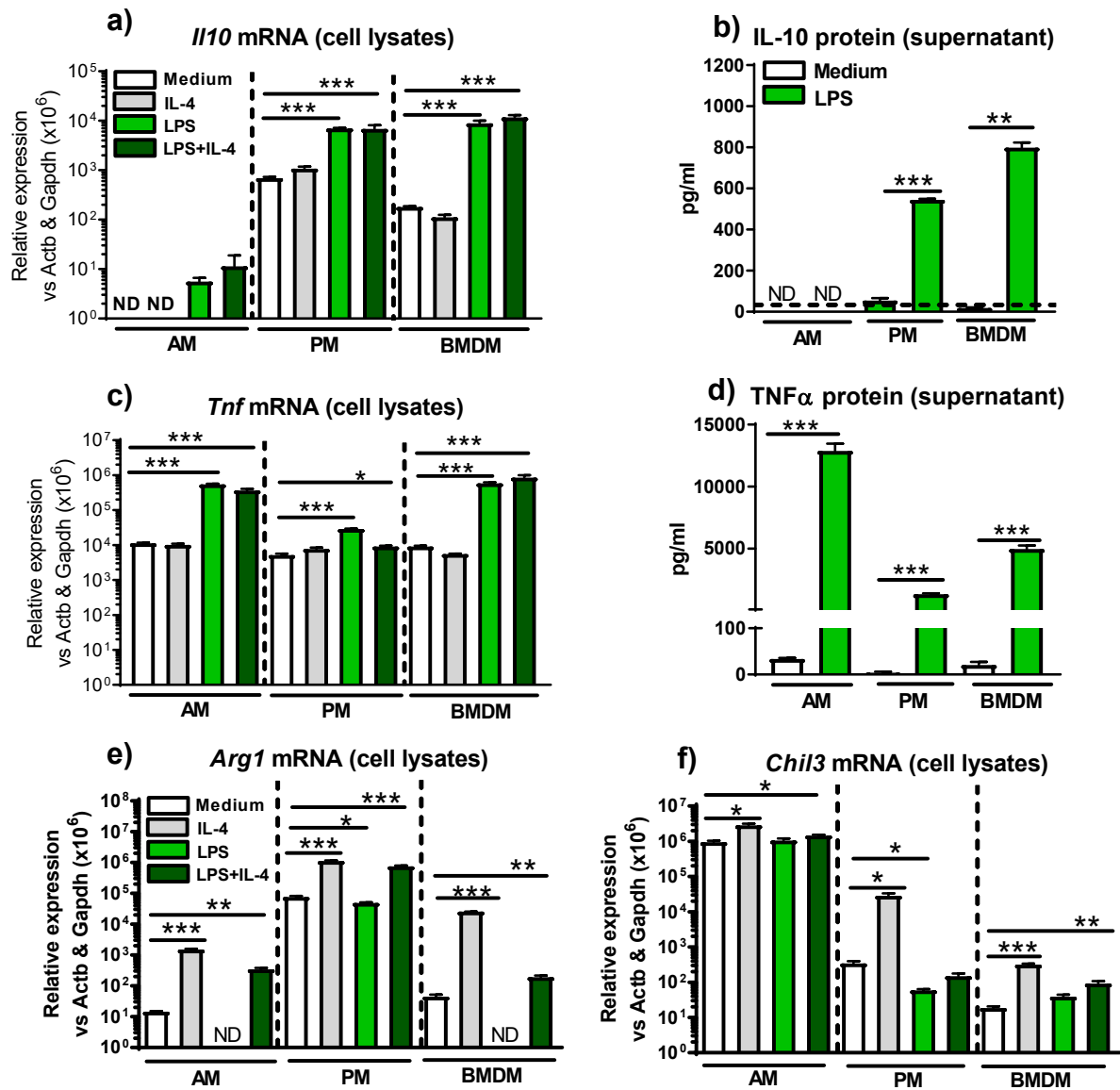


Figure 4.2. IL-10 production by stimulated macrophages

Macrophages were prepared from C57BL/6 mice as described in Fig. 4.1 and stimulated with 500 ng/ml LPS and/or 200 ng/ml IL-4. Control cells were incubated in medium alone. **a)** qPCR data showing *Il10* mRNA levels in macrophage lysates, relative to housekeeping genes. **b)** ELISA data showing IL-10 concentration in macrophage culture supernatants. Dashed line indicates limit of detection. **c)** qPCR data showing *Tnf* mRNA levels in macrophage lysates relative to housekeeping genes. **d)** ELISA data showing TNFα concentration in macrophage culture supernatants. **e-f)** qPCR data showing *Arg1* (**e**) and *Chil3* (**f**) mRNA levels in macrophage lysates relative to housekeeping genes. Data are means of triplicate cultures $\pm$ SEM and are representative of three (**a-d**) or one (**e-f**) independent experiment(s). Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ND, not-detectable.

The failure of IL-4 to enhance LPS-induced IL-10 is in contrast to the published report from Kamanaka et al., which showed increased IL-10 reporter signal from BMDM where IL-4 and LPS were combined³⁴³. To confirm that the IL-4 concentration used in this experiment, approximately equivalent to the 2500 bioactive units per ml reported by Kamanaka and colleagues³⁴³, was effective in this system, expression of genes typical of IL-4-dependent macrophage activation was assessed. IL-4 alone significantly increased expression of mRNA for the arginine metabolising enzyme arginase (*Arg1*; **Fig. 4.2e**) and mammalian chitinase like protein Ym1 (*Chil3*; **Fig. 4.2f**) in all three macrophage subsets, indicating productive transcriptional regulation by IL-4 in this system. LPS, a classical macrophage activation stimulus⁸⁰, decreased expression of *Arg1* in all three subsets and *Chil3* in PM (**Fig. 4.2e,f**). Reduction of *Arg1* gene expression by LPS was overcome by co-stimulation with IL-4 in all macrophage subsets (**Fig. 4.2e**), indicating that macrophages were capable of integrating LPS and IL-4 signals. Thus, the results seen were not due to inactivity of the recombinant IL-4, but instead reflect a failure of IL-4 to further enhance LPS-driven IL-10 production at the concentration and timepoints used in these experiments. These experiments therefore showed AM to be poor producers of IL-10 *in vitro* compared to PM and BMDM, in response to TLR4 stimulation with LPS.

IL-10 expression in macrophages can be driven by pattern recognition through multiple receptors, many of which operate through distinct but overlapping pathways¹⁰⁶. It therefore remained possible that IL-10 induction in AM required stimulation via one or more alternative TLRs to TLR4. To address this, IL-10 induction in AM was assessed in response to agonists of TLR2 and TLR3, alone and in combination with LPS, and compared to BMDM. TLR2 was selected as it is known to mediate IL-10 induction in BMDM in response to certain bacterial cell wall components³⁴⁴ and was found to be the most highly expressed TLR gene in naïve AM by microarrays performed as part of the Immunological Genome Project³⁴⁵, suggesting that AM are primed to receive signals via TLR2. TLR3 was selected because it is expressed by resting AM (Immunological Genome Project, not shown), mediates IL-10 production in response to double stranded DNA in BMDM and operates solely via the TRIF-IRF3 pathway, as opposed to TLR2 and TLR4, which rely partially on Myd88³⁴¹.

Neither Pam3Csk4, nor poly(IC) increased *Il10* mRNA levels in AM to the same extent as LPS, with all three stimulations resulting in very low *Il10* qPCR signals (**Fig. 4.3a**, raw Ct > 36). Additionally, neither Pam3Csk4, nor poly(IC) enhanced LPS-induced *Il10* mRNA levels in AM (**Fig. 4.3a**), suggesting that there was no additive effect of different TLR agonists on IL-10 production in AM. In line with low levels of *Il10* mRNA, IL-10 protein was undetectable in AM supernatants from all stimulation conditions (**Fig. 4.3b**). Despite failing to induce substantial levels of IL-10, all three TLR agonists significantly increased *Tnf* mRNA expression (**Fig. 4.3c**) and Pam3Csk4 increased levels of TNF α protein in AM supernatants (**Fig. 4.3d**). Analysis of TNF α production also confirmed that combinations of TLR agonists could have additive effects on cytokine production in AM, since addition of either Pam3Csk4 or poly(IC) resulted in significantly increased levels of TNF α mRNA (**Fig. 4.3c**) and protein (**Fig. 4.3d**) compared with LPS

stimulation alone. Thus, AM responded effectively to combinations of TLR2, TLR3 and TLR4 ligands *in vitro*, but this did not result in production of substantial levels of IL-10.

In contrast, but in line with published literature^{341,344}, Pam3Csk4 and poly(IC) significantly enhanced IL-10 production from the BMDM positive control (**Fig. 4.3a,b**), although the small increase in *Il10* mRNA induced by Pam3Csk4 did not reach statistical significance after adjusting for multiple comparisons (Bonferroni-adjusted *p* value = 0.1; **Fig. 4.3a**). Addition of poly(IC) in combination with LPS also significantly increased *Il10* mRNA levels in BMDM compared to LPS stimulation alone (**Fig. 4.3a**), while both Pam3Csk4 and poly(IC) increased IL-10 protein release in combination with LPS in these cells (**Fig. 4.3b**). These results indicate that the concentrations and combinations of TLR agonists used were effective at inducing IL-10 expression in murine macrophages. Pam3Csk4 and poly(IC) also significantly increased LPS-induced TNF α production in BMDM compared to LPS alone (**Fig. 4.3d**). Notably, levels of TNF α released in response to these combined TLR stimulations were higher in AM than BMDM cultures (**Fig. 4.3d**), indicating that the lack of IL-10 detection in AM supernatants under these conditions did not result from a relatively poor cytokine response to these stimuli compared to BMDM and instead reflects a selective lack of induction of IL-10 expression.

Finally, the possibility remained that TLR stimulation alone was insufficient to drive IL-10 production in AM and that a more complex stimulus was required. To assess IL-10 production by AM in response to a live pathogen, cells were infected with the A2 subtype of respiratory syncytial virus (RSV) *in vitro* or stimulated with LPS as a comparator. BMDM were included as a positive control for IL-10 production. RSV infection increased *Il10* mRNA in AM, with relative expression significantly greater than that obtained with LPS. However, RSV-induced *Il10* expression in AM remained relatively low and was comparable to the level seen in resting BMDM (**Fig. 4.4a**). RSV infection increased *Il10* by more than 20-fold in BMDM, although to a lesser extent than LPS stimulation (**Fig. 4.4a**). Consistent with little to no induction of IL-10 by *in vitro* RSV infection of AM, IL-10 was undetectable in supernatants of RSV-infected AM when assayed by ELISA (limit of detection 31.25 pg/ml, **Fig. 4.4b**) or cytometric bead array (limit of detection 2 pg/ml, not shown). This was in contrast to BMDM, in which RSV significantly increased IL-10 concentrations in supernatants, although as observed at the mRNA level, the amount of IL-10 protein in supernatants was lower than that observed with LPS stimulation (**Fig. 4.4b**). Lack of IL-10 induction in RSV-infected AM was not due to failure of the virus to drive a cytokine response, since *Tnf* mRNA was induced by RSV infection in both AM and BMDM, with significantly higher levels in RSV-treated than LPS-stimulated AM (**Fig. 4.4c**). IL-10 production from AM therefore remained negligible even after culture with live virus.

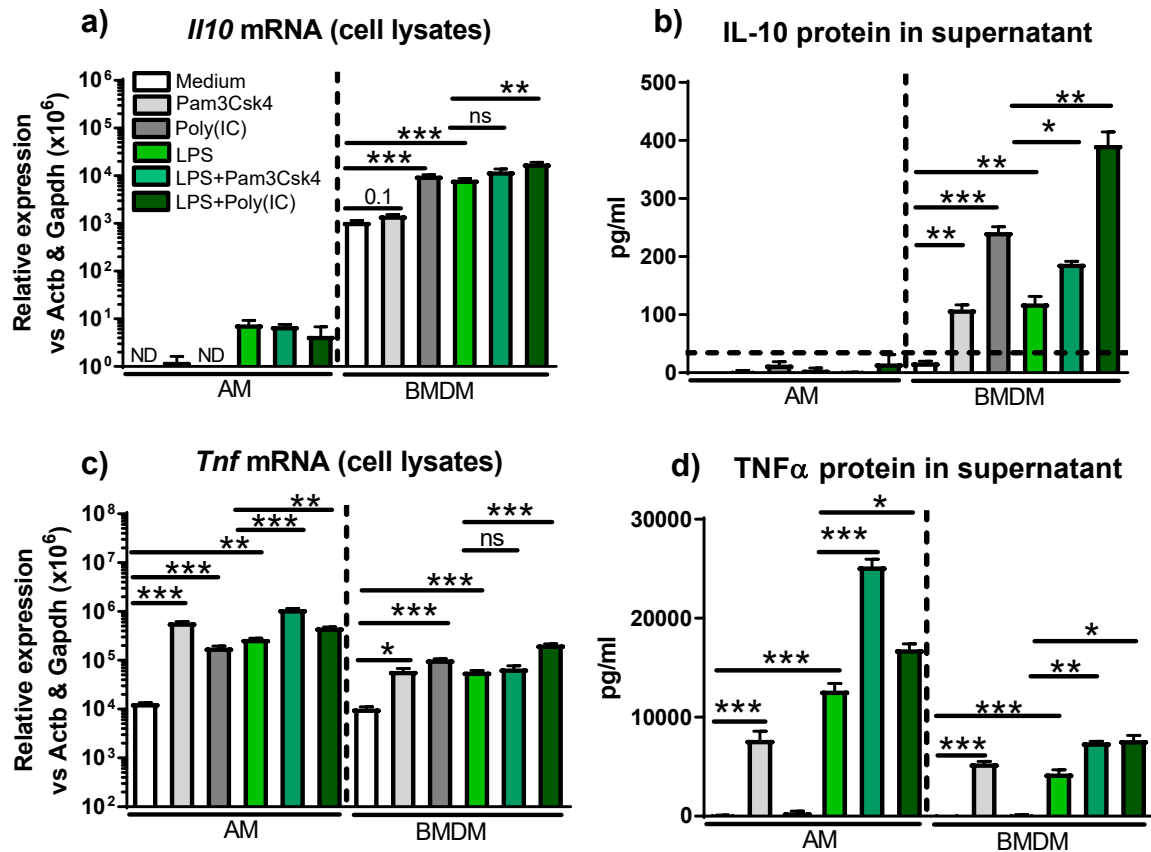


Figure 4.3. IL-10 production by macrophages stimulated with TLR2, TLR3 and TLR4 agonists

Macrophages were prepared from C57BL/6 mice as described in **Fig. 4.1** and either stimulated with 500 ng/ml LPS and/or 500 ng/ml Pam3CSK4 or 10 μ g/ml poly(IC). **a)** qPCR data showing *Il10* mRNA expression in macrophage lysates relative to housekeeping genes. **b)** ELISA data showing concentrations of IL-10 in macrophage culture supernatants. Dashed line shows the limit of detection. **c)** qPCR data showing *Tnf* mRNA expression in macrophage lysates relative to housekeeping genes. **d)** ELISA data showing concentrations of TNF α in macrophage culture supernatants. Data shown are the means of triplicate culture wells $\pm$ SEM and are representative of two independent experiments. Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ND, not-detectable.

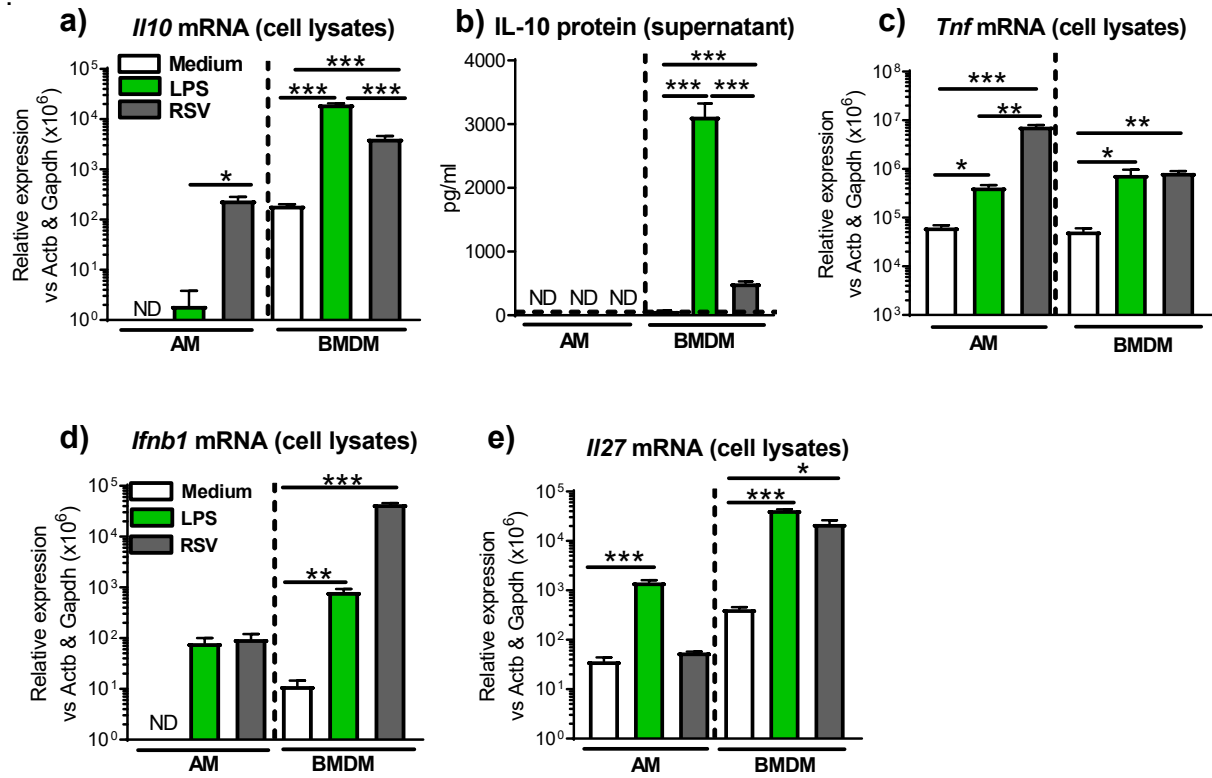


Figure 4.4. Macrophage IL-10 production following *in vitro* viral infection

Macrophages were prepared from C57BL/6 mice as described in **Fig. 4.1** and either stimulated with 500 ng/ml LPS or infected with 5 MOI RSV-A2. Control cells were cultured in medium alone. **a)** qPCR data showing relative *Il10* expression in macrophage lysates relative to housekeeping genes. **b)** ELISA data showing levels of IL-10 in culture supernatants. Dashed line indicates limit of detection. **c-e)** qPCR data showing *Tnf* (**c**), *Ifnb1* (**d**) and *Il27* (**e**) mRNA expression relative to housekeeping genes. Data are means of triplicate cultures $\pm$ SEM and are representative of two independent experiments, except for **d-e**, which represent a single experiment. Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ND, not-detectable.

Optimal transcription of IL-10 by BMDM stimulated with TLR agonists is reliant on autocrine production of type I IFNs³⁴² and IL-27³⁴⁶. The minimal *Il10* gene expression observed from stimulated AM *in vitro* could therefore feasibly arise from failure to produce type I IFN and/or IL-27 upon stimulation, resulting in absence of these mediators in the culture system. To address this, *Ifnb1* (IFN β) and *Il27* gene expression was quantified in lysates of LPS- and RSV-treated macrophages. Both LPS and RSV induced *Ifnb1* expression in AM and BMDM, although relative expression of *Ifnb1* was more than 10-fold higher in stimulated BMDM than AM (**Fig. 4.4d**). *Il27* mRNA was detectable in AM under all conditions tested and LPS, but not RSV, increased *Il27* expression in AM (**Fig. 4.4e**). As observed for *Ifnb1*, higher *Il27* mRNA levels were observed in stimulated BMDM than AM (**Fig. 4.4e**). Lack of substantial IL-10 expression by LPS- and RSV-stimulated AM could therefore not be attributed to absence of *Ifnb1* and *Il27* expression; however further experiments will be required to determine whether increased amounts of these mediators would enhance IL-10 production by AM.

Overall, naïve AM showed a markedly reduced capacity to produce IL-10 in response to pro-inflammatory stimuli, compared to peritoneal and bone marrow-derived macrophages.

4.4 Defining the *in vivo* sources of IL-10 during pulmonary inflammation

In vitro stimulations of primary AM were useful for understanding their gene expression at steady state and the response to individual stimuli when in isolation. However, since the phenotype and function of AM is dependent on their microenvironment and interactions with other cell types¹⁹, it was necessary to assess IL-10 production by AM *in vivo*. While intracellular staining for cytokines in *ex vivo* stimulated cells is a well-established means of assessing cytokine production by flow cytometry, it is biased by the choice of stimulus and is best suited for T cells, which can be driven to produce cytokines by either specific or non-specific activation of T cell receptor signalling³⁴⁷. In the case of IL-10, several reporter mouse strains have been developed, which express fluorescent proteins in the *Il10* locus to mark IL-10-producing cells without the need for *ex vivo* stimulation³⁴⁸. However, sensitivity of fluorescent IL-10 reporter mice is limited such that they predominantly detect cells expressing relatively high amounts of IL-10, mainly T cells, especially at baseline³⁴⁸. Lower level production of IL-10, as might be expected from AM, is therefore likely to be missed using fluorescent reporter systems.

To circumvent these limitations and compare IL-10 production by AM and other lung cells *in vivo*, the 10BiT IL-10 reporter mouse was employed. The 10BiT mouse features several copies of a bacterial artificial chromosome (BAC), containing an interrupted *Il10* gene encoding CD90.1 (*Thy1.1*), integrated randomly into the C57BL/6 mouse genome, resulting in CD90.1 expression on the surface of cells capable of producing IL-10 *in vivo*: so called 'IL-10-competent' cells¹⁰⁵ (**Fig. 4.5a**). As well as marking IL-10-competent T cells¹⁰⁵, 10BiT mice have previously been successfully used to detect IL-10 production capacity in myeloid cells³⁴⁹⁻³⁵¹, suggesting that this reporter would provide the best means of detecting IL-10-producing AM *in vivo*.

a) 10BiT bacterial artificial chromosome: ~12 copies integrated in genome

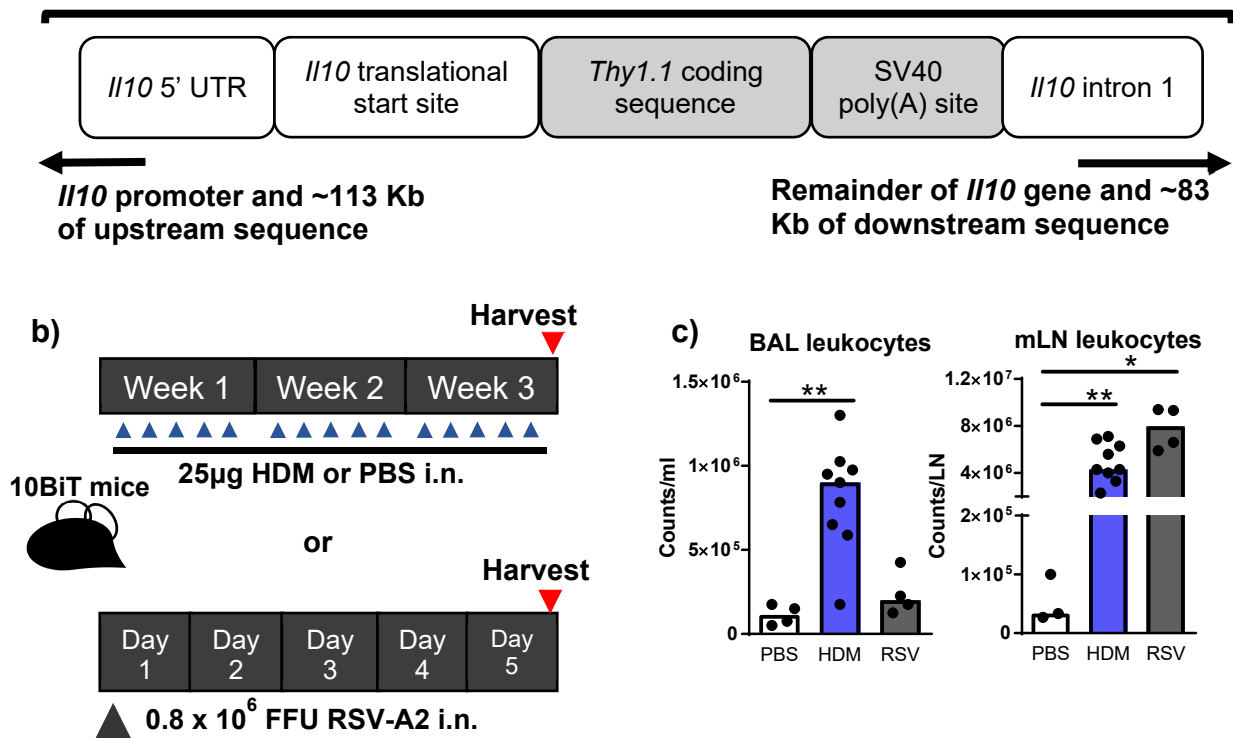


Figure 4.5. Reporter mouse model for identifying cellular sources of IL-10

a) Outline of composition of the bacterial artificial chromosome (BAC) present in multiple copies in 10BiT mice, containing a *Thy1.1* coding sequence within the *Il10* locus, followed by an SV40 early poly(A) site to promote polyadenylation and translation of *Thy1.1* mRNA. **b)** Experimental protocols. 10BiT mice received intranasal HDM for 3 weeks or were infected with RSV-A2 5 days prior to harvest. Control mice received intranasal PBS for 3 weeks. **c)** Total crystal violet cell counts of BAL and mLN cell preparations. **d)** Total numbers of CD90.1/*Thy1.1*/10BiT⁺ CD45⁺ cells in lung digests. **e)** Representative flow cytometry plots showing 10BiT reporter signal and CD45 expression, pre-gated on live, single cells. Data shown are medians of 4-9 replicate mice per group. HDM data are pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-adjusted Mann Whitney tests between the indicated groups. *, $p \leq 0.05$, **, $p \leq 0.01$.

To allow examination of IL-10-competent cells during allergic airway inflammation, 10BiT mice were exposed to intranasal HDM daily for 3 weeks and compared to non-inflamed control mice that received intranasal PBS (**Fig. 4.5b**). In parallel, another group of 10BiT mice were infected with RSV to allow comparison to non-allergic pulmonary inflammation (**Fig. 4.5b**). RSV was selected as an *in vivo* stimulus, as this virus had induced the strongest *Il10* mRNA signal in AM *in vitro* of all stimuli tested (**Fig. 4.4**), suggesting that RSV infection may drive AM to produce IL-10 *in vivo*. RSV-infected mice were analysed at 5 days post infection (**Fig. 4.5b**), a timepoint at which both innate leukocytes and IL-10⁺ T cells are detectable in the lungs^{108,352}, with the aim of parallel analysis of AM, infiltrating innate leukocytes and T cells.

HDM-treated mice showed a substantial pulmonary inflammatory response, as evidenced by increased leukocyte numbers in BAL and mediastinal LN (mLN; **Fig. 4.5c**). RSV-infected mice showed no elevation in total airway leukocyte numbers compared to PBS controls, consistent with this timepoint being after the initial innate inflammatory response to virus³⁵², but mLN leukocyte numbers were significantly elevated (**Fig. 4.5c**) due to the substantial activation of the T cell response by this timepoint³⁵². 10BiT reporter⁺ cells were quantified by flow cytometry using a fluorochrome-conjugated anti-CD90.1 antibody. 10BiT⁺ cells were detectable in lung digests at baseline in PBS-treated mice and their numbers were significantly increased in mice treated with HDM or RSV (**Fig. 4.5d**). 10BiT signal was restricted to the CD45⁺ leukocyte fraction of cell suspensions, rather than CD45⁻ stromal cells, few of which are obtained with the lung digest protocol used in these experiments (**Fig. 4.5e**).

Further analysis of 10BiT expression by flow cytometry revealed variable reporter expression across different cell populations in the lung. However, few (median < 4 %) AM obtained in lung digests showed positive reporter signal under all conditions tested (**Fig. 4.6a-b**). These data support the conclusion that AM are not a homeostatic IL-10 source *in vivo* and do not become a major IL-10-producing population during HDM-driven AAD or in the early stages of RSV infection.

Despite minimal detection in AM, several other myeloid cell subsets showed substantial IL-10 reporter signal. CD11b⁺ CD64⁻ CD103⁻ lung cDC2 were present at low levels lungs of PBS-treated control mice and 10BiT⁺ cDC2 were essentially undetectable in these samples (**Fig. 4.6a**). However, both cDC2 and CD64^{hi} CD11b⁺ moDC showed substantial 10BiT signal in the lungs of HDM and RSV-treated mice, with 10BiT reporter expression being particularly high on cDC2 in the RSV group (median 39 %, **Fig. 4.6a-b**). The strongest 10BiT⁺ pulmonary myeloid cell signal was associated with an F4/80⁺ CD64⁺ CD11b⁺ Siglec F⁻ non-DC population, a surface phenotype indicative of interstitial and inflammatory monocytes and macrophages (IMM). Around 15 % of IMM showed positive IL-10 reporter signal in PBS-treated mice and this proportion increased by more than two-fold in both HDM and RSV models (**Fig. 4.6a-b**), supporting the notion of a resident homeostatic population of IL-10-producing myeloid cells which expands during inflammation^{31,214}.

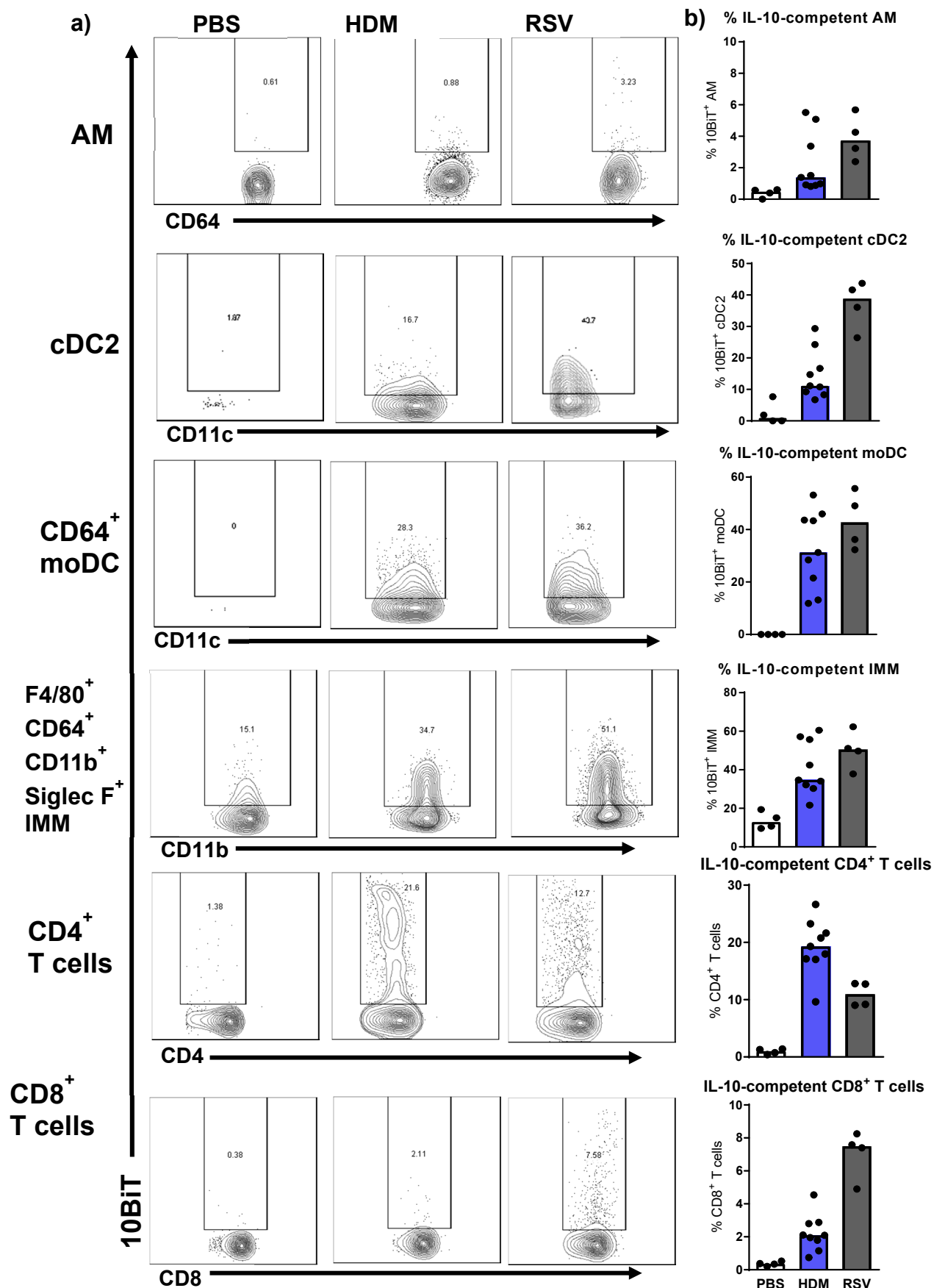


Figure 4.6. Expression of 10BiT IL-10 reporter by pulmonary leukocytes

Representative flow cytometry plots (**a**) and quantitative data (**b**) showing proportions of major leukocyte subsets staining positively for 10BiT (CD90.1) in lung digests from mice treated with PBS, HDM or RSV as detailed in Fig. 4.5. Scatter/bar plots show medians of 4-9 replicate mice per group. HDM data are pooled from two independent experiments.

A small proportion (< 2 %) of CD4⁺ T cells showed IL-10 reporter staining the lungs of PBS control mice, suggesting that CD4⁺ T cells are not a major pulmonary IL-10 source prior to initiation of an immune response. However, a substantial proportion of lung CD4⁺ T cells were 10BiT⁺ in HDM-treated and RSV-infected mice (**Fig. 4.6a-b**). IL-10-competent CD8⁺ T cells were essentially absent from the lungs of PBS control mice and only a minor proportion of CD8⁺ T cells were 10BiT⁺ in HDM-treated mice (< 4 %, **Fig. 4.6a-b**), as expected given the dominance of CD4⁺ T cells in the T cell response to inhaled HDM⁵. In contrast, RSV-infected mice showed a substantial proportion of 10BiT⁺ CD8⁺ T cells in lung tissue, consistent with published literature describing these cells as an IL-10 source during RSV infection in mice¹⁰⁸ (**Fig. 4.6a-b**). Thus, while neither CD4⁺ nor CD8⁺ T cells are a major source of IL-10 at homeostasis in the murine lung, IL-10-competent T cells populate the lung during inflammation.

To better estimate the relative contributions of leukocyte populations to pulmonary IL-10 production, absolute numbers of 10BiT⁺ cells were compared (**Fig. 4.7**). Consistent with the low proportions of 10BiT⁺ AM observed under all conditions, numbers of IL-10-competent AM were low compared to other pulmonary leukocyte populations (**Fig. 4.7**), consistent with AM being minor IL-10 producers *in vivo* during HDM- or RSV-driven inflammation. Despite a substantial proportion of cDC2 expressing the 10BiT reporter in HDM and RSV-treated mice, these cells were relatively low in number in lung digests compared to other IL-10-competent populations (**Fig. 4.7**), suggesting that cDC2 represent a minor proportion of IL-10-competent leukocytes in the lung during inflammation. IL-10-competent moDCs were present at a relatively high level in a subset of HDM-treated mice, but their numbers were highly variable (**Fig. 4.7**). By far the most abundant IL-10-competent cell types common to both HDM- and RSV-driven lung inflammation were IMM and CD4⁺ T cells, suggesting that these are the major pulmonary innate and adaptive sources of IL-10, respectively (**Fig. 4.7**), prompting further analysis of the phenotypes of these cells.

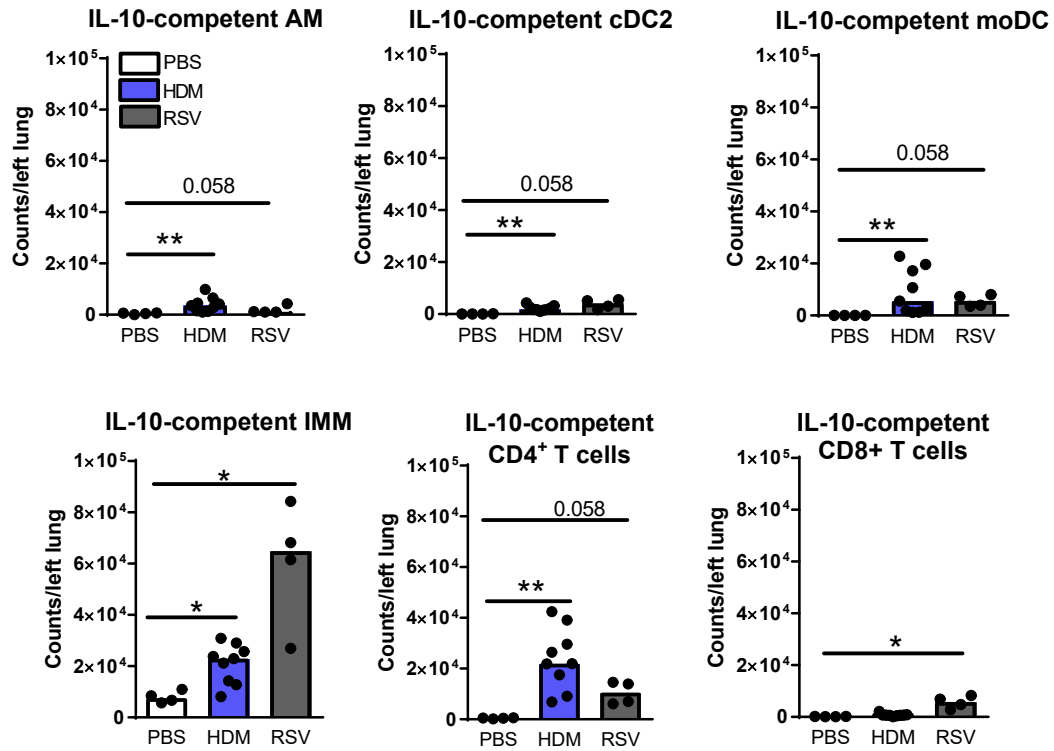


Figure 4.7. Abundance of IL-10-competent lung leukocyte populations

Total numbers of $10\text{B}\text{iT}^+$ leukocytes from the populations shown in Fig. 4.6 were calculated and plotted on the same scale to aid comparisons of relative abundance. Scatter/bar plots show medians of 4-9 replicate mice per group. HDM data are pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$. Where relevant, corrected p values are shown between groups.

More detailed analysis of IL-10-competent IMM revealed these cells to be a mixed population, varying in expression of CD64 and Ly6C (**Fig. 4.8a**), suggestive of different subsets and differentiation states of monocytes and monocyte-derived macrophages. To determine how IL-10 competency varied between these subsets, IMM were sub-classified into three major populations: P1 (CD64^{low-mid}, Ly6C⁺), P2 (CD64^{low-mid}, Ly6C⁻) and P3 (CD64^{hi}, Ly6C⁻) and proportions compared between 10BiT⁺ and 10BiT⁻ IMM (**Fig. 4.8b**). In lungs of PBS-treated control mice, both 10BiT⁺ and 10BiT⁻ IMM predominantly fit into the P1 or P2 gates (**Fig. 4.8b**), generally expressing low to intermediate levels of CD64, suggestive of resident macrophage, or circulating or patrolling monocyte populations, as opposed to mature inflammatory macrophages. CD64^{hi} Ly6C⁻ P3 cells were evident in lungs of both HDM- and RSV-treated mice; consistent with these cells being an inflammatory population. However, these cells were predominantly represented among 10BiT⁻ IMM (**Fig. 4.8b**), suggesting that relatively few of these inflammatory cells produce IL-10 in these models. In contrast, P1 cells were the dominant population of 10BiT⁺ cells in lungs of HDM- and RSV-treated mice, representing more than 50 % of IL-10 competent IMM in both models of inflammation (**Fig. 4.8a-b**).

To better understand the nature of these populations, forward and side scatter properties of P1-3 gates were examined as proxy measurements of cell size and morphology. P1 cells had relatively low forward and side scatter properties (**Fig. 4.8c**), suggestive of small monocyte-like phenotype. Along with their low to intermediate expression of CD64, which was increased during inflammation, and high expression of Ly6C (**Fig. 4.8a**), these cells were likely to be classical monocytes⁶⁰. P2 cells showed similar light scatter properties to P1 (**Fig. 4.8c**), consistent with monocyte or small macrophage morphology. Given their lack of Ly6C expression and low to intermediate CD64 expression, these cells were likely to comprise Ly6C⁻ patrolling monocytes⁶⁷ and resident IM²¹⁴. P3 cells showed higher forward and side scatter properties than other populations (**Fig. 4.8c**), suggestive of a large size as expected of macrophages. Together with the high CD64 expression of these cells, their lack of the inflammatory monocyte marker Ly6C (**Fig. 4.8a**) and absence in lung digests of PBS control mice, cells in P3 are likely to represent mature inflammatory macrophages^{214,315}. Overall, these results confirm that a large proportion of non-AM mononuclear phagocytes are competent IL-10 producers during pulmonary inflammation and that Ly6C⁺ CD64^{low-mid} classical monocyte-like cells are the major IL-10-competent IMM population in the HDM and RSV models.

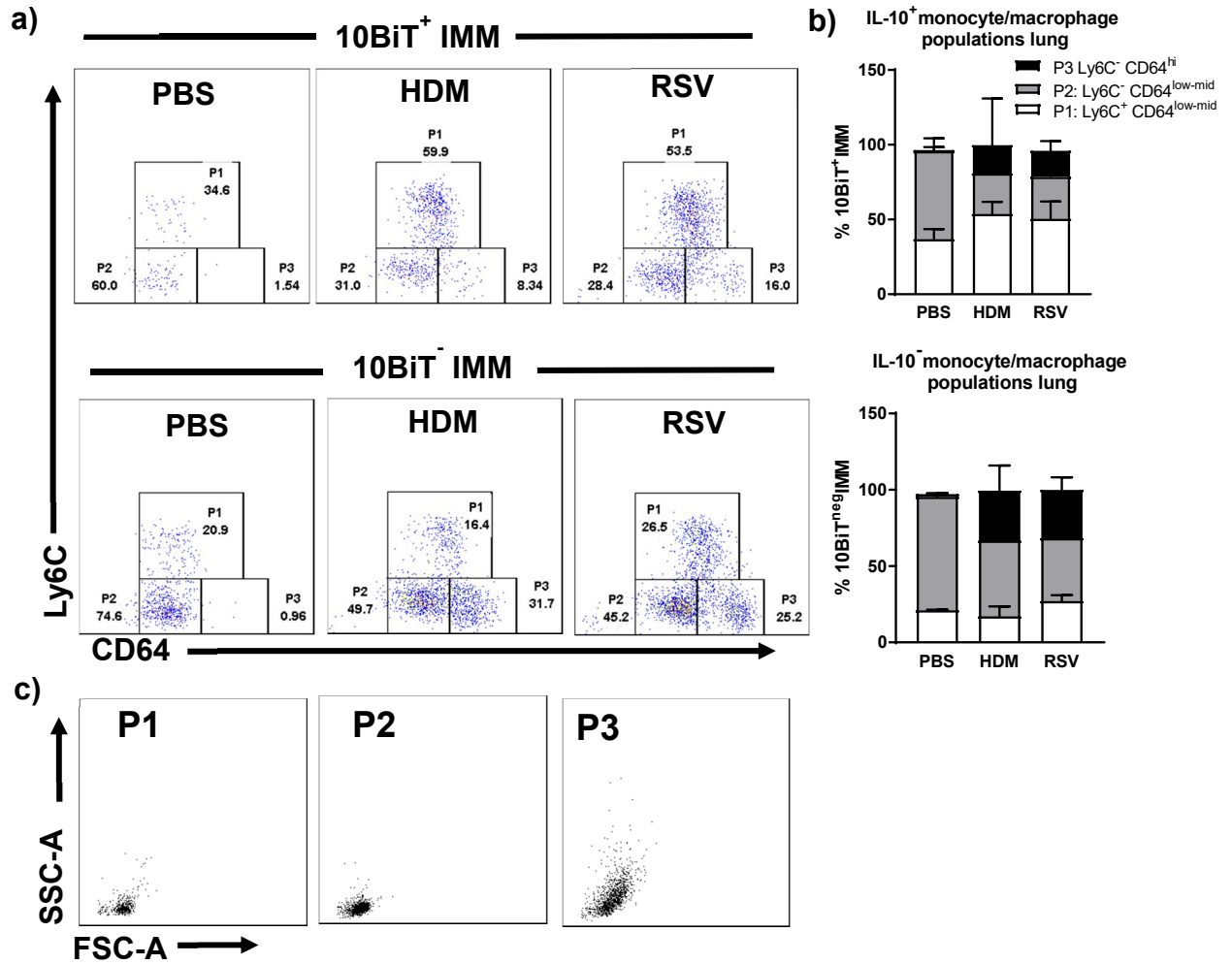


Fig. 4.8. Distinct pulmonary IL-10-competent monocyte and macrophage populations

a) Representative flow cytometry plots showing distributions of CD64 and Ly6C expression by F4/80⁺ CD11b⁺ IMM, either positive or negative for the 10BiT reporter, from lungs of mice treated with PBS, HDM or RSV as detailed in **Fig. 4.5**. 3 major populations were identified: P1-P3 **b)** Stacked bar plots showing proportions of IMM falling into P1-P3 gates. Data shown are medians $\pm$ IQR of 4-9 mice per group. HDM data are from two independent experiments. **c)** Representative flow cytometry plots showing forward and side light scatter properties of events in P1-3 gates. Plots shown are from a HDM-treated mouse. Numbers on flow cytometry plots indicate the percentages of events in gates.

IL-10 can be derived from multiple CD4⁺ T cell subsets, including both FoxP3⁺ and FoxP3⁻ regulatory T cells^{105,106} and effector T helper subsets¹⁰⁶, with the *in vivo* function of IL-10 likely to differ depending on the cellular source. Pulmonary 10BiT⁺ CD4⁺ T cells displayed a CD44^{hi} CD62L⁻ memory cell phenotype (**Fig. 4.9a,b**), indicative of previous antigen-experience, suggesting that IL-10 production from pulmonary CD4⁺ T cells was driven in a manner dependent on antigen stimulation of the T cell receptor in both HDM- and RSV-driven inflammation. Consistent with an activated phenotype of IL-10-competent CD4⁺ T cells, the 10BiT⁺ population contained a higher proportion of cells positive for the inducible T cell activation markers CD69 and ICOS than their 10BiT⁻ counterparts (**Fig. 4.9c**).

Given the known contribution of FoxP3⁺ T cells to IL-10 production during inflammation^{104,105} it was important to determine whether the IL-10-competent CD4⁺ T cells arising during pulmonary inflammation expressed FoxP3. Although FoxP3⁺ cells were significantly more highly represented among HDM-elicited 10BiT⁺ than 10BiT⁻ CD4⁺ T cells (median 10 % vs 6 %; **Fig. 4.9d**), the majority of IL-10-competent CD4⁺ T cells in the allergic lung did not express FoxP3 (**Fig. 4.9d**). Together these data suggest that antigen-experienced FoxP3⁻ CD4⁺ T cells are the major IL-10-competent lymphocyte population in the lungs during HDM-driven AAD.

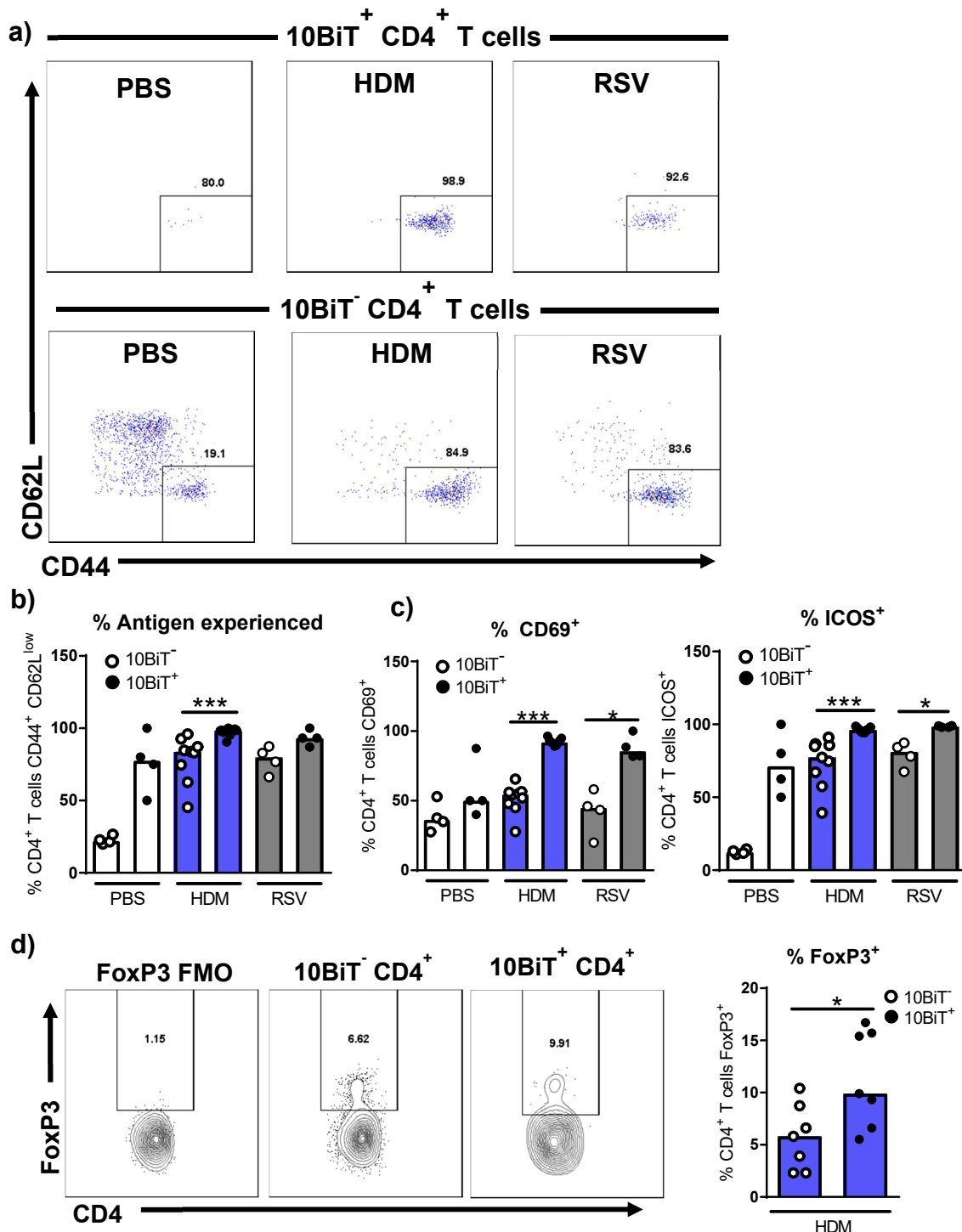


Figure 4.9. Activated phenotype of IL-10-competent CD4⁺ T cells in the inflamed lung

a) Representative flow cytometry plots showing proportions of 10BiT⁺ or 10BiT⁻ CD4⁺ T cells with antigen-experienced (CD44⁺ CD62L^{low}) phenotype. **b)** Quantification of data represented in (a) for multiple mice. **c)** Proportions of 10BiT⁺ and 10BiT⁻ CD4⁺ T cells with positive surface staining for CD69 and ICOS activation markers. **d)** Representative flow cytometry plots (left) and quantitative data (right) showing proportions of 10BiT⁺ and 10BiT⁻ CD4⁺ T cells with positive intranuclear staining for FoxP3. FoxP3 FMO control plot shows 10BiT⁺ CD4⁺ T cells stained with all antibodies except for FoxP3. Scatter/bar plots show medians 4-9 replicate mice per group. HDM data are pooled from two independent experiments. Numbers on flow cytometry plots indicate the percentages of events in gates. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; ***, $p \leq 0.001$.

4.5. Assessing the IL-10 responsiveness of macrophage subsets *in vitro*

Having established that AM are poor producers of IL-10 compared to other macrophage subsets and pulmonary leukocytes, it remained necessary to determine whether AM could respond effectively to exogenous IL-10, to determine whether AM are a likely target for IL-10 from other cellular sources *in vivo*. IL-10 responsiveness of BMDM is well established^{195,196}, however given the clear differences in cytokine production between AM and macrophages generated *in vitro* (see **Figs. 4.2-4.4**), it was critical to test the IL-10 response of cells obtained directly from the airways. Firstly, expression of IL-10 receptor subunits by AM was compared to that in PM and BMDM, both at baseline and following activation with LPS. All three macrophage subsets showed high relative expression of mRNA for both IL-10R subunits, *Il10ra* and *Il10rb*, at baseline (**Fig. 4.10**). LPS significantly upregulated both *Il10ra* and *Il10rb* gene expression in AM by 2.8- and 2.5-fold respectively (**Fig. 4.10**), suggesting that IL-10 responsiveness may increase in activated AM. *Il10ra* levels were also increased by LPS in BMDM, although by a lesser extent (1.5-fold; **Fig. 4.10**). In contrast to AM, LPS treatment caused modest decreases in *Il10rb* expression by both PM and BMDM. These data support all three macrophage subsets being primed to receive IL-10 signals, with potential for modulation of IL-10 responsiveness upon activation via TLR4.

Detection of high levels of mRNA for a cytokine receptor does not necessarily indicate expression of functional protein on the cell surface. Several attempts were made to stain airway cells for cell surface and intracellular IL-10RB using the major commercially available clone (1B1.3a), but staining was variable and inconclusive regardless of the buffer and staining protocol used (not shown). Instead, to directly confirm that macrophages expressed functional IL-10 receptors, cells were stimulated with LPS *in vitro* in the presence or absence of increasing concentrations of recombinant IL-10.

As expected from their high *Il10ra* and *Il10rb* gene expression, AM, PM and BMDM were all sensitive to inhibition of LPS-driven TNF α release by recombinant IL-10, in a concentration-dependent manner (**Fig. 4.11a**). Normalisation of these data to observe relative inhibition showed 50 % inhibition of IL-10 release by the maximum concentration of IL-10 tested in AM, compared to around 85 % in PM and 70 % in BMDM (**Fig. 4.11b**). The superior suppression of TNF α production by IL-10 in PM likely reflects the relatively low levels of TNF α release observed with LPS alone (**Fig. 4.11a**). Suppression of TNF α production by IL-10 was at least partially at the transcriptional level, consistent with the known mechanism of action of IL-10 on macrophages¹⁹⁶, since significant reductions in *Tnf* mRNA were observed at 10 ng/ml LPS in all macrophage subsets (**Fig. 4.11c**). Suppression of the pro-inflammatory LPS response in AM was not limited to TNF α , with significant reductions in LPS-driven *Il1b* and *Il27* mRNA observed at the highest concentrations of IL-10 (**Fig. 4.11d**). Thus, AM from naïve, wild-type mice showed a functional response to IL-10 comparable to that of macrophages from other origins. These data suggest that AM are primed to receive IL-10 signals at steady state.

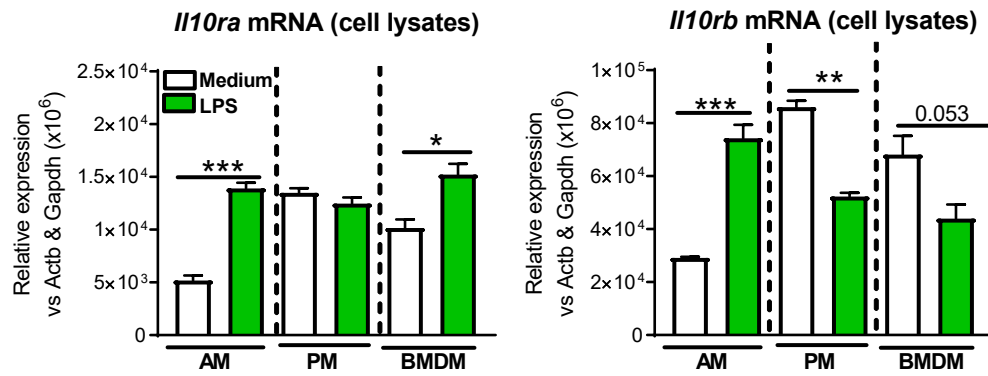


Figure 4.10. Expression of IL-10 receptor subunits by murine macrophage subsets

Macrophages were prepared and stimulated as described in Fig. 4.1. Data show *Il10ra* and *Il10rb* mRNA levels in macrophage lysates relative to housekeeping genes, as determined by qPCR. Data shown are the means of triplicate cultures $\pm$ SEM and are representative of three independent experiments. Statistics: t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Where relevant, p values are shown between groups.

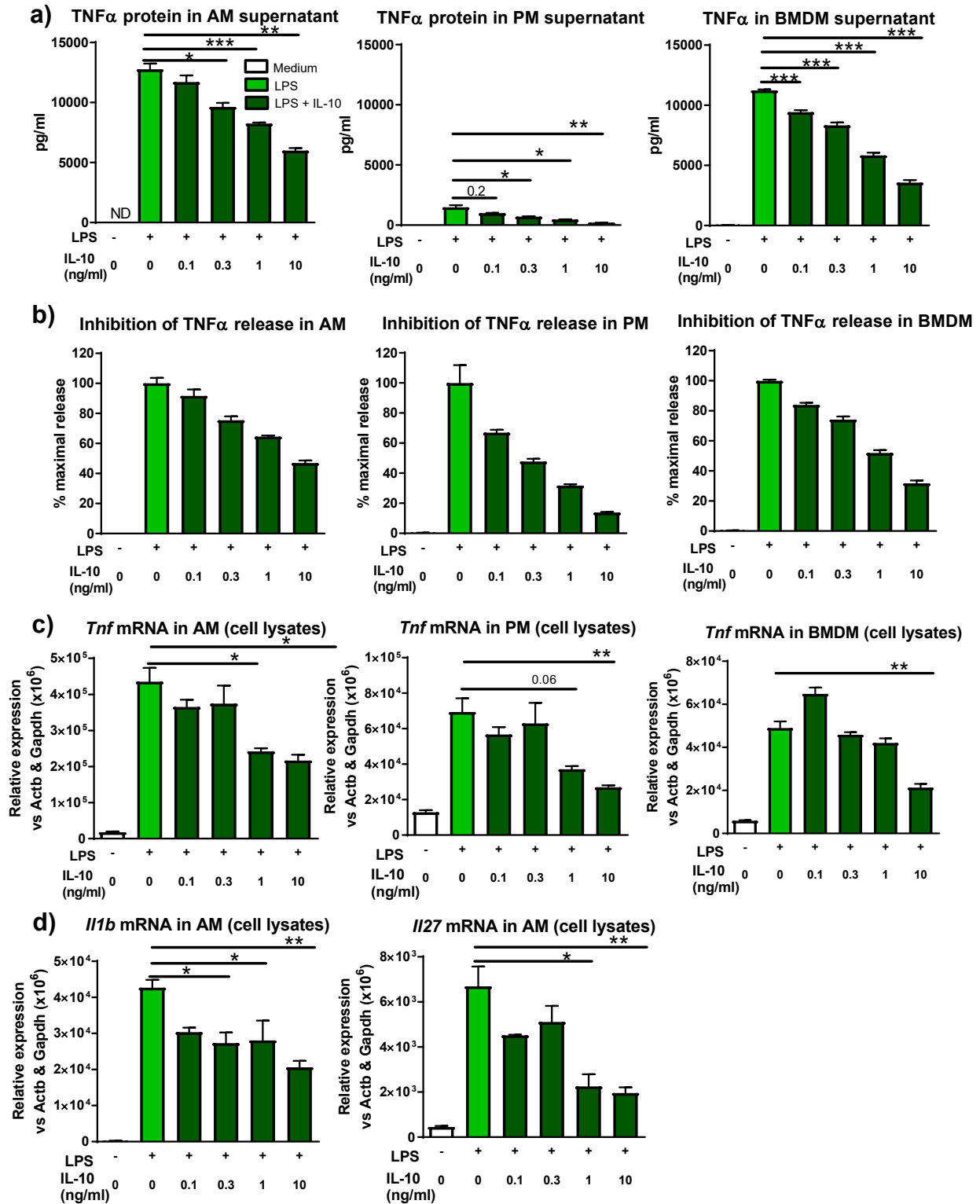


Figure 4.11. Responsiveness of macrophage subsets to IL-10

Macrophages were prepared as described in **Fig. 4.1** and stimulated with 500 ng/ml LPS for 8h in the presence of increasing concentrations of recombinant murine IL-10. **a)** ELISA data showing TNFα concentrations in culture supernatants. **b)** Values in **(a)** normalised to the maximum obtained with LPS alone, to demonstrate relative inhibition of TNFα release by IL-10. **c-d)** *Tnf* (**c**), *Il1b* and *Il27* (**d**) mRNA expression in cell lysates relative to housekeeping genes, as determined by qPCR. Data shown are the means of triplicate cultures, ± SEM, from a single experiment. Statistics: one-way ANOVA followed by Bonferroni-corrected t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Where relevant, corrected p values are shown between groups.

4.6. TGF- β 1 is the most abundant TGF- β isoform in AM

The previous experiments established that murine AM are unlikely to be major contributors to pulmonary IL-10 during allergic inflammation and RSV infection, but are sensitive to IL-10 suppression of pro-inflammatory cytokine production. The other key regulatory cytokine of interest in this thesis, TGF- β 1, is known to be produced, to varying extents, by many haematopoietic and stromal cell lineages²¹⁸, including AM^{273,338}. However, it remained necessary to determine whether AM-derived TGF- β 1 levels varied in response to pro-inflammatory stimuli in the experimental systems required for this thesis. In addition, although TGF- β 1 is the most abundant and active TGF- β isoform in the immune system²¹⁸, TGF- β 2 and TGF- β 3 act on the same receptor and signalling pathway, potentially allowing for redundancy. Determining expression of these alternative isoforms was therefore important in understanding potential for AM to signal via the TGF- β pathway.

To compare expression of TGF- β isoforms in resting and activated AM, cells were obtained by BAL from naïve mice and treated with LPS or medium-only control. PM and BMDM were used as comparators, as in previous experiments. As expected, *Tgfb1* was the most abundant TGF- β isoform transcript in all macrophage subsets tested, with at least 10-fold greater relative expression than *Tgfb2* and *Tgfb3* in stimulated or resting cells (**Fig. 4.12a**). LPS treatment caused a small 1.5-fold increase in *Tgfb1* gene expression in AM, had no effect on PM and resulted in a significant 2-fold decrease in mRNA levels in BMDM (**Fig. 4.12a**). Importantly, neither resting nor LPS-induced expression of *Tgfb1* differed substantially between AM, PM or BMDM, suggesting that none of these subsets produced exceptionally high levels of *Tgfb1* mRNA among macrophages.

While expressed at very low levels at baseline, LPS stimulation increased *Tgfb2* expression in AM by 30-fold (**Fig. 4.12a**), suggesting AM can upregulate minor TGF- β isoforms upon activation. In contrast, LPS treatment resulted in a 20-fold decrease in *Tgfb2* mRNA levels in PM, while this isoform was undetectable in BMDM under either condition (**Fig. 4.12a**). *Tgfb3* was by far the least abundant isoform in all of the macrophage subsets tested, consistent with its primarily stromal cell-associated functions in tissue development³⁵³, and was undetectable in AM by qPCR (**Fig. 4.12a**). BMDM showed the highest relative mRNA expression of *Tgfb3* among the three macrophage subsets, however this was decreased 50-fold by LPS stimulation (**Fig. 4.12a**).

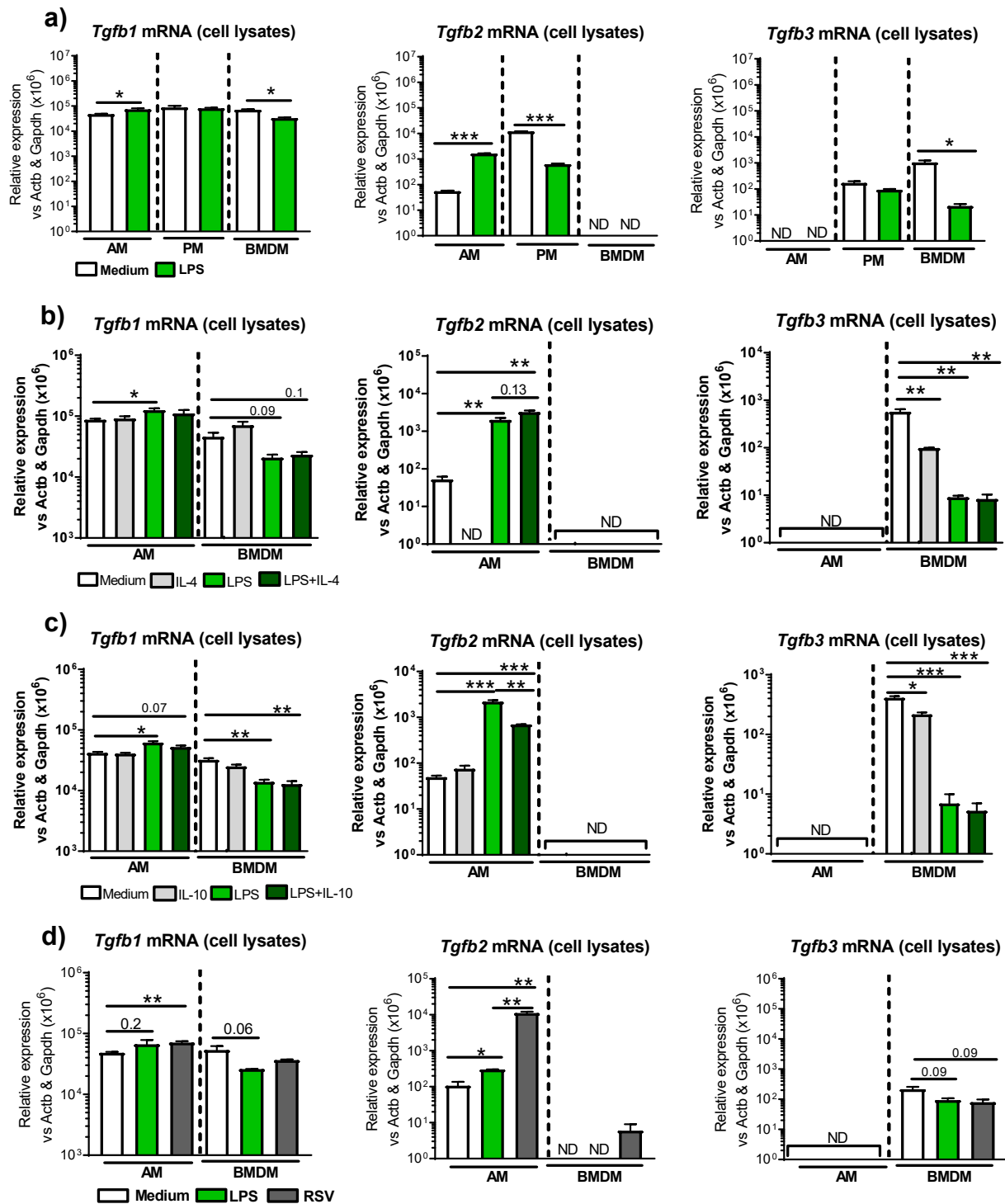


Figure 4.12. Expression of TGF- β isoforms by murine macrophages

Macrophages were obtained and stimulated as described in **Fig. 4.1**. Graphs depict qPCR data showing expression of *Tgfb1*, *Tgfb2* and *Tgfb3* mRNA relative to housekeeping genes, in lysates of macrophages stimulated with LPS alone (**a**), LPS $\pm$ 200 ng/ml IL-4 (**b**), LPS $\pm$ 10 ng/ml IL-10 (**c**) or 5 MOI RSV compared to LPS (**d**). Data shown are the means of triplicate cultures $\pm$ SEM and are representative of one (**c**), two (**d**) or three (**a-b**) independent experiments. Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ND, not-detected. Where relevant, corrected p values are shown between groups.

TGF- β expression in macrophages can be increased as part of the regulatory and tissue repair-related, 'alternative' activation programmes induced by cytokines such as IL-4 and IL-10³⁵⁴. The effects of these cytokines on TGF- β isoform expression in AM were therefore examined, alone and in combination with LPS, using BMDM as a comparator. IL-4 did not affect resting or LPS-induced *Tgfb1* expression in either macrophage type, nor did it significantly modify the LPS-induced increase in *Tgfb2* gene expression in AM (**Fig. 4.12b**). However, the low levels of *Tgfb2* mRNA detected in resting AM were no longer detectable after IL-4 treatment (**Fig. 4.12b**), suggesting that IL-4 suppresses *Tgfb2* expression in these cells. Similarly, the low levels of *Tgfb3* in BMDM were significantly reduced by IL-4 (**Fig. 4.12b**), further demonstrating modulation of TGF- β isoform expression by this cytokine.

As observed for IL-4, IL-10 did not alter resting or LPS-induced levels of *Tgfb1* in AM or BMDM (**Fig. 4.12c**). In contrast, IL-10 significantly reduced LPS-induced *Tgfb2* mRNA levels in AM (**Fig. 4.12c**), suggesting that the pathway mediating LPS induction of *Tgfb2* in these cells is negatively regulated by IL-10. Similarly to IL-4, IL-10 alone significantly reduced *Tgfb3* mRNA levels in BMDM (**Fig. 4.12c**), indicating that the low level of *Tgfb3* expression displayed at baseline by these cells is sensitive to suppression by multiple cytokines.

To determine whether TGF- β isoform expression in AM could be modulated to a greater extent in response to a complex stimulus, rather than a cytokine or TLR ligand, AM were infected with RSV and isoform expression examined by qPCR. BMDM were included as a comparator and LPS stimulation was used as a positive control for modulation of TGF- β isoform expression in both cell types. RSV infection caused a small but significant increase of around 1.5-fold in *Tgfb1* mRNA levels in AM, but did not affect expression of this isoform in BMDM (**Fig. 4.12d**). In contrast, RSV increased *Tgfb2* mRNA expression in AM by more than 100-fold, to levels significantly greater than those obtained with LPS stimulation at the same timepoint (**Fig. 4.12d**). RSV-infected BMDM also showed detectable *Tgfb2* mRNA, which was not seen in either resting or LPS-stimulated cells (**Fig. 4.12d**). However, this was close to the detection limit of the PCR assay (raw threshold cycle values >35) and therefore likely represents very low levels of *Tgfb2* transcript. Finally, *Tgfb3* mRNA remained undetectable in AM following RSV infection, while RSV caused an around 2-fold decrease in levels of this isoform in BMDM, comparable to that observed with LPS (**Fig. 4.12d**).

Together these results show that although *Tgfb1* is the most abundant TGF- β isoform in multiple macrophage subsets, expression of the lesser isoforms *Tgfb2* and *Tgfb3* differs markedly between different subsets of macrophages and can be altered by inflammatory stimuli and cytokine signals. Of particular note, *Tgfb2* was upregulated by pro-inflammatory stimuli in AM, in a manner inhibited by IL-10.

Detection of TGF- β mRNA is insufficient to determine the amount of active protein present in a system. Not only must TGF- β mRNA be translated to protein, but all three TGF- β isoforms require release from their extracellular latent form for activity^{218,236}. In an attempt to assess the production

of active TGF- β by AM in comparison to other macrophage subsets, LPS stimulations were repeated in a serum-free system and TGF- β bioactivity quantified in supernatants using a Smad reporter bioassay. However, bioactive TGF- β levels were undetectable in all conditions tested (detection limit 31.25 pg/ml, not shown), meaning that the experiment was inconclusive. This result may be due to insufficient TGF- β mRNA being translated to protein, or a lack of cell intrinsic capacity to activate latent TGF- β in artificial conditions of the culture system. For this reason, mRNA was used as a readout of TGF- β production in subsequent experiments using isolated cells.

4.7. Localisation of pulmonary TGF- β 1 expression *in vivo* at homeostasis and during inflammation

Having compared *Tgfb1* expression between distinct subsets of macrophages *in vitro*, it was necessary to assess the relative contribution of AM to TGF- β 1 levels *in vivo*. Previous work from our group showed AM to be a major population displaying positive immunostaining for TGF- β 1 in the normal murine lung and to remain positive for TGF- β 1 during HDM-driven AAD, accompanied by large numbers of recruited TGF- β 1⁺ leukocytes (²⁷³ and Denney, Lloyd *et al.*, unpublished observations). To independently affirm these findings in a different allergen-driven AAD model, C57BL/6 mice were challenged intranasally with the fungal allergen *Alternaria alternata* (ALT) for 3 weeks and TGF- β 1 expression assessed. To allow further dissection of pulmonary TGF- β 1 expression following allergen challenge, mice were assessed at 24, 48 and 72 hours after the final ALT administration (**Fig. 4.13a**). Both pulmonary eosinophilia and the proportion of CD4⁺ T cells producing IL-13 had significantly declined by 72h after final ALT challenge (**Fig. 4.13b,c**), consistent with the type 2 immune response beginning to resolve in these mice over the time-course. Despite this, TGF- β bioactivity remained significantly elevated in the airways of ALT-treated mice 72h after final allergen challenge (**Fig. 4.13d**).

To identify the cellular sources of TGF- β 1 across this time-course, formalin-fixed paraffin embedded lung sections were examined by immunofluorescence staining for TGF- β 1. Identity of cell types staining positive for TGF- β 1 was inferred from morphology and anatomical location. Consistent with published data^{272,273}, in non-inflamed PBS-treated control mice, TGF- β 1 staining was localised predominantly to bronchiolar epithelial cells and large mononuclear cells intimately associated with epithelium: a morphology typical of AM (**Fig. 4.13e**). Some staining was also observed in elongated cells forming alveolar walls, determined to be alveolar epithelial cells, in agreement with published research²⁷².

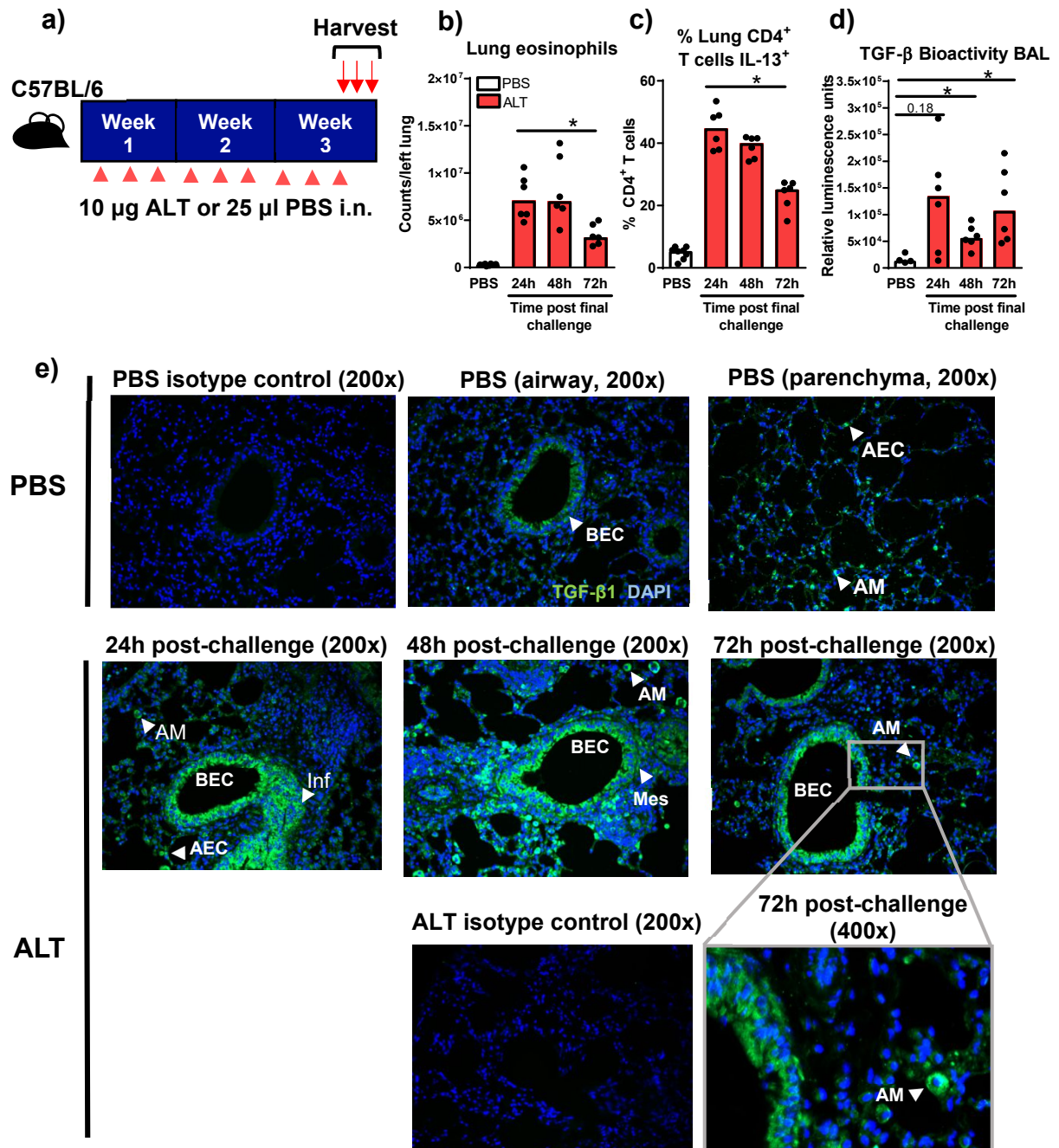


Figure 4.13. Lung TGF-β1 expression at homeostasis and during allergic airway disease

a) Adult female C56BL/6 mice received 10 µg intranasal ALT or PBS control 3 times per week for 3 weeks and were analysed at 24, 48 or 72 hours after final challenge. **b)** Eosinophil numbers in lung digests enumerated by flow cytometry. **c)** Percentage of CD4⁺ T cells in lung digests with positive intracellular IL-13 staining as determined by flow cytometry. **d)** Relative luminescence values obtained by TGF-β bioassay of BAL. **e)** Representative images TGF-β1 immunostaining in lung sections. White arrowheads and text indicate examples of TGF-β1⁺ cells: BEC, bronchiolar epithelial cell; AEC, alveolar epithelial cell; AM, airway macrophage; Inf, inflammatory infiltrate; Mes, mesenchymal cell. Scatter/bar plots show medians of 4-6 replicate mice per group. Statistics: Kruskal-Wallis followed by Bonferroni-adjusted Mann-Whitney tests between the indicated groups; *, $p \leq 0.05$. Where relevant, corrected p values are shown between groups.

In lungs of ALT-treated mice, AM and bronchiolar and alveolar epithelial cells remained positive for TGF- β 1, but additional staining was detected on peribronchial and perivascular inflammatory infiltrates, as well as mesenchymal cells surrounding the airways (**Fig. 4.13e**), likely to be smooth muscle cells or fibroblasts. Notably, despite all staining and imaging being performed together with identical parameters, TGF- β 1 staining was visibly more intense in ALT-treated than PBS-treated mice, including in AM (**Fig. 4.13e**). This suggests that AM and epithelial cells increase their expression of TGF- β 1 protein following allergen exposure *in vivo*, consistent with published data for the bronchiolar epithelium²⁷³. Increased fluorescent staining on AM from ALT-treated mice could conceivably arise from increased intrinsic autofluorescence of AM in this model; however, no fluorescent signal was observed from AM in any isotype control images from PBS or ALT treated mice (**Fig. 4.13e**), suggesting that increased staining intensity in AM reflected true increase in the quantity of TGF- β 1 protein.

These data support AM being major producers of TGF- β 1 at homeostasis and during allergic inflammation- the functional importance of which was be tested directly in subsequent experiments.

4.8 Expression of canonical TGF- β signalling molecules in AM

Further to characterising TGF- β isoform expression in AM relative to macrophages from other sites and origins, it was necessary to determine benchmarks of TGF- β responsiveness in AM which could be used for subsequent studies. Since many of the functions of TGF- β occur via the canonical Smad2/3-dependent signalling pathway²²⁰, gene expression of molecular components was assessed in naïve primary AM and compared to PM and BMDM. Cells were assessed in the resting state (medium only) and after stimulation with TGF- β 1, activation with LPS alone or treatment with LPS in the presence of increasing concentrations of TGF- β 1. Experiments were performed in serum-free conditions to exclude the influence of bovine TGF- β present in foetal calf serum.

The type 2 TGF- β receptor TGF- β R2 binds to TGF- β family members at the initiation of signalling and is crucial for cellular responses to TGF- β 1, 2 and 3²²¹. *Tgfb2* mRNA was detected at high relative levels in all three macrophage subsets in the absence of stimulation (**Fig. 4.14a**), consistent with these cells being primed to receive TGF- β signals at steady state. LPS treatment significantly decreased *Tgfb2* levels by around 1.6-fold in PM and 2.5-fold in AM and BMDM (**Fig. 4.14a**), suggesting that activation via TLR4 may reduce the ability of macrophages to receive TGF- β signals at the cell surface. TGF- β 1 did not affect *Tgfb2* expression in AM or BMDM, either alone or in LPS-stimulated cells (**Fig. 4.14a**). In contrast, TGF- β 1 alone significantly decreased *Tgfb2* expression in PM (**Fig. 4.14a**), suggesting that regulation of *Tgfb2* expression by its own ligand varies between macrophage subsets. TGF- β 1 also appeared to reverse the reduction of *Tgfb2* gene expression induced by LPS in PM at the highest concentration tested, although this did not reach statistical significance after correction for multiple comparisons (**Fig. 4.14a**).

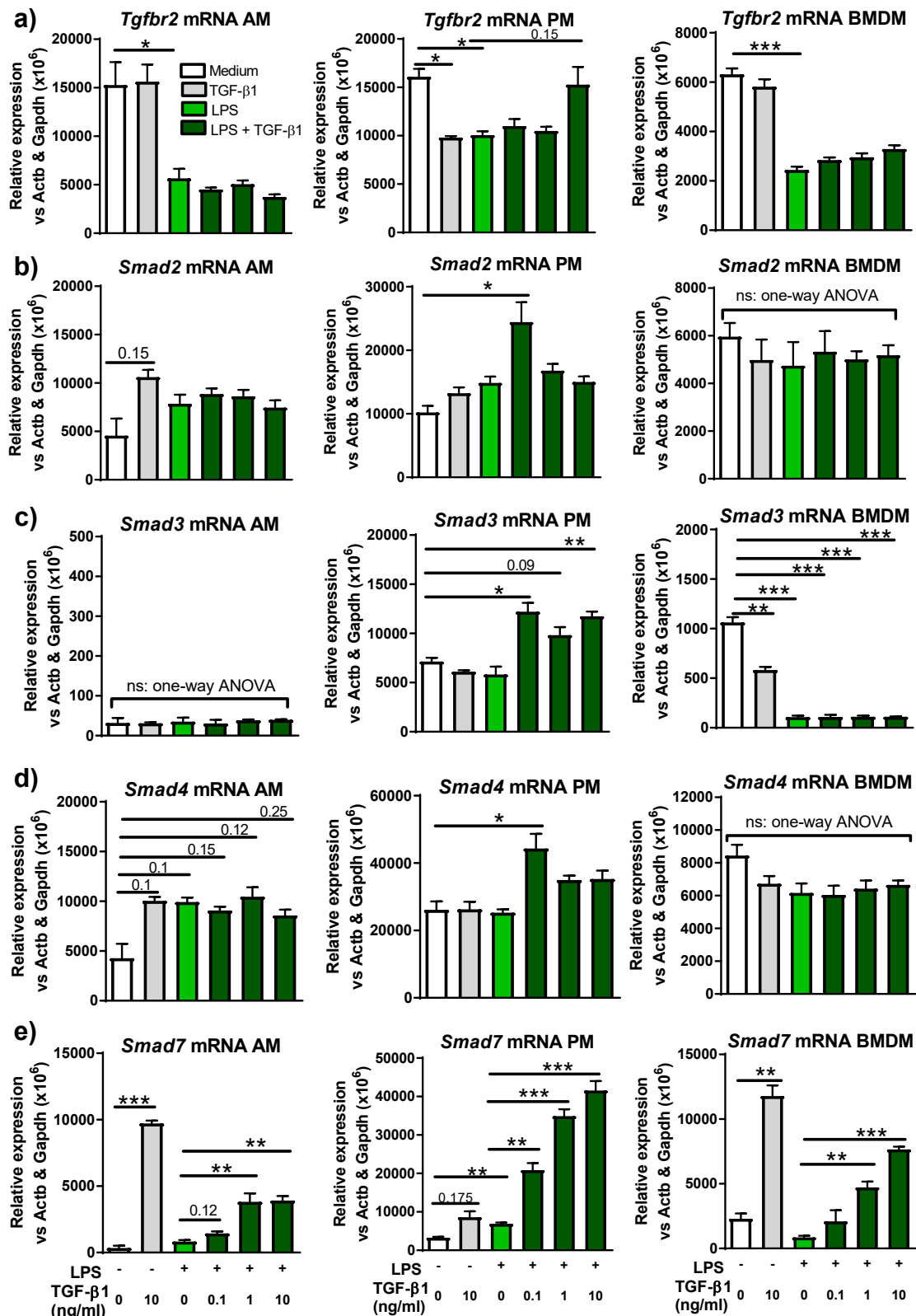


Figure 4.14. Expression of canonical TGF-β signalling genes by murine macrophages

a) Macrophages were serum starved for 2 hours prior to stimulation for 8h with LPS ± increasing concentrations of TGF-β1. Graphs display qPCR data showing expression of *Tgfb2* (a), *Smad2* (b), *Smad3* (c), *Smad4* (d) and *Smad7* (e) mRNA relative to housekeeping genes in macrophage lysates. Data shown are the means of triplicate cultures from a single experiment ± SEM. Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant; ND, not detected. Where relevant, corrected p values are shown between groups.

One or both of the receptor Smads; Smad2 and Smad3, are required for canonical gene expression responses to TGF- β downstream of TGF- β R2 ligation²²². As observed for *Tgfb2*, *Smad2* mRNA was detected at high relative levels in all three macrophage subsets tested (**Fig. 4.14b**), suggesting that these cells can transduce TGF- β signals via Smad2 at steady state. No significant changes in *Smad2* gene expression were induced by TGF- β 1 or LPS stimulation in AM or BMDM (**Fig. 4.14b**), suggesting that Smad2 expression is relatively stable at the mRNA level in these cells. A significant 2.4-fold increase in *Smad2* gene expression was observed in PM upon treatment with LPS and TGF- β 1, but only at the lowest concentration of TGF- β 1 tested (0.1 ng/ml; **Fig. 4.14b**), further supporting a distinct TGF- β response in PM compared to AM and BMDM and indicating that TGF- β -induced gene expression changes do not necessarily increase with ligand concentration.

Unlike *Smad2*, relative mRNA expression *Smad3* varied greatly between macrophage subsets at steady state. Relative expression was around 7-fold higher in PM than BMDM at baseline and was nearly undetectable under all conditions tested in AM (**Fig. 4.14c**). Similarly to *Smad2*, *Smad3* expression was elevated in PM treated with LPS in combination with TGF- β 1, but not either stimulus alone (**Fig. 4.14c**). Conversely, TGF- β 1 alone decreased *Smad3* mRNA levels by around 50 % in BMDM, while LPS stimulation decreased expression by approximately 10-fold- to levels close to the lower limit of detection by qPCR (**Fig. 4.14c**). Addition of TGF- β 1 did not further modulate decreased *Smad3* expression in LPS-treated BMDM (**Fig. 4.14c**).

Smad2/3 trafficking to the nucleus occurs in complex with the mediator protein Smad4²²². *Smad4* mRNA was readily detectable in AM, as well as PM and BMDM, further supporting intact TGF- β signalling in these cells (**Fig. 4.14d**). Expression patterns of *Smad4* in stimulated macrophages closely mirrored those of *Smad2*, with no significant changes observed in stimulated BMDM (**Fig. 4.14d**). Both TGF- β 1 and LPS appeared to increase *Smad4* mRNA expression in AM by around 2-fold, although these changes did not reach statistical significance after correcting for multiple comparisons (**Fig. 4.14d**). In a manner analogous to *Smad2* and *Smad3* expression, *Smad4* was significantly induced in PM by a combination of LPS and 0.1 ng/ml TGF- β 1 (**Fig. 4.14d**). Thus, both *Smad2* and its binding partner *Smad4* are expressed by resting AM, PM and BMDM and show varying sensitivity to TGF- β 1 and LPS-induced signals in these different cell types. Together these data suggest that AM have an intact TGF- β signalling pathway at steady state and are likely to signal via Smad2, rather than Smad3, based on low gene expression of the latter transcription factor.

Finally, expression of *Smad7*, a negative feedback regulator of TGF- β receptor signalling²²⁷, was examined in AM and compared to PM and BMDM. As expected from a known TGF- β -induced gene²²⁷, TGF- β 1 stimulation increased *Smad7* mRNA levels in all three macrophage subsets (**Fig. 4.14e**), although only a relatively subtle around 2-fold increase was observed with PM, which failed to reach statistical significance after correcting for multiple comparisons (**Fig. 4.14e**). Similarly, TGF- β 1 increased *Smad7* expression in LPS-stimulated macrophages in a

concentration-dependent manner (**Fig. 4.14e**), highlighting *Smad7* gene expression as a robust indicator of TGF- β signalling in macrophages. PM were distinct from other subsets in that *Smad7* levels achieved by TGF- β 1 and LPS together exceeded those with TGF- β 1 alone (**Fig. 4.14e**), suggesting that TGF- β 1 signalling can synergise with the response to LPS in different ways in different macrophage subsets. Notably, baseline *Smad7* mRNA levels were lowest in AM among the macrophage subsets tested and under all conditions examined (mean relative expression 345, compared with 3200 in PM and 2300 in BMDM; **Fig. 4.14e**). However further experiments would be required to determine whether this increases the capacity of AM for TGF- β signalling.

4.9 Assessing AM sensitivity to TGF- β 1 *in vitro*

Having confirmed that AM express molecules required for canonical TGF- β signalling, the effects of TGF- β 1 on cytokine production by these cells was assessed, with the intention of both confirming TGF- β responsiveness of AM and identifying robust indicators of TGF- β anti-inflammatory function for use in subsequent studies.

As expected, LPS stimulation increased *Il27* mRNA expression in serum-free cultures of AM, PM and BMDM. This LPS-induced *Il27* mRNA expression was significantly reduced in the presence of TGF- β 1 in all macrophage subsets, in a concentration-dependent manner (**Fig. 4.15a**). TGF- β 1 also decreased resting *Il27* expression in these cell types, although this did not reach statistical significance in AM after correction for multiple comparisons (**Fig. 4.15a**). These effects were not consistent for all LPS-induced cytokines, since LPS-induced *Tnf* expression was only significantly decreased by TGF- β in BMDM (**Fig. 4.15b**). There was a non-significant trend towards suppression at higher TGF- β concentrations in AM, while TGF- β 1 only decreased resting *Tnf* expression in PM (**Fig. 4.15b**). Differential TGF- β 1 responses were also apparent at the level of TNF α protein. TGF- β 1 did not significantly affect LPS-induced TNF α protein concentrations in AM supernatants (**Fig. 4.15c**), while significant suppression of TNF α release was apparent in BMDM from 1 ng/ml TGF- β 1 (**Fig. 4.15c**). Conversely, TGF- β 1 caused a concentration-dependent increase of LPS-induced TNF α protein levels in PM (**Fig. 4.15c**). The reasons for this contrasting response in PM were unclear from this experiment, but may in part result from LPS-induced TNF α production being substantially lower from PM compared with that from AM and BMDM (**Fig. 4.15c**).

These data were sufficient to confirm that all three types of macrophage tested were responsive to TGF- β 1, with suppression of LPS-induced *Il27* gene expression identified as a consistent TGF- β 1 response across AM, PM and BMDM. However, the different effects of TGF- β 1 observed on TNF α expression between these subsets illustrates the highly cell type-specific nature of TGF- β signals.

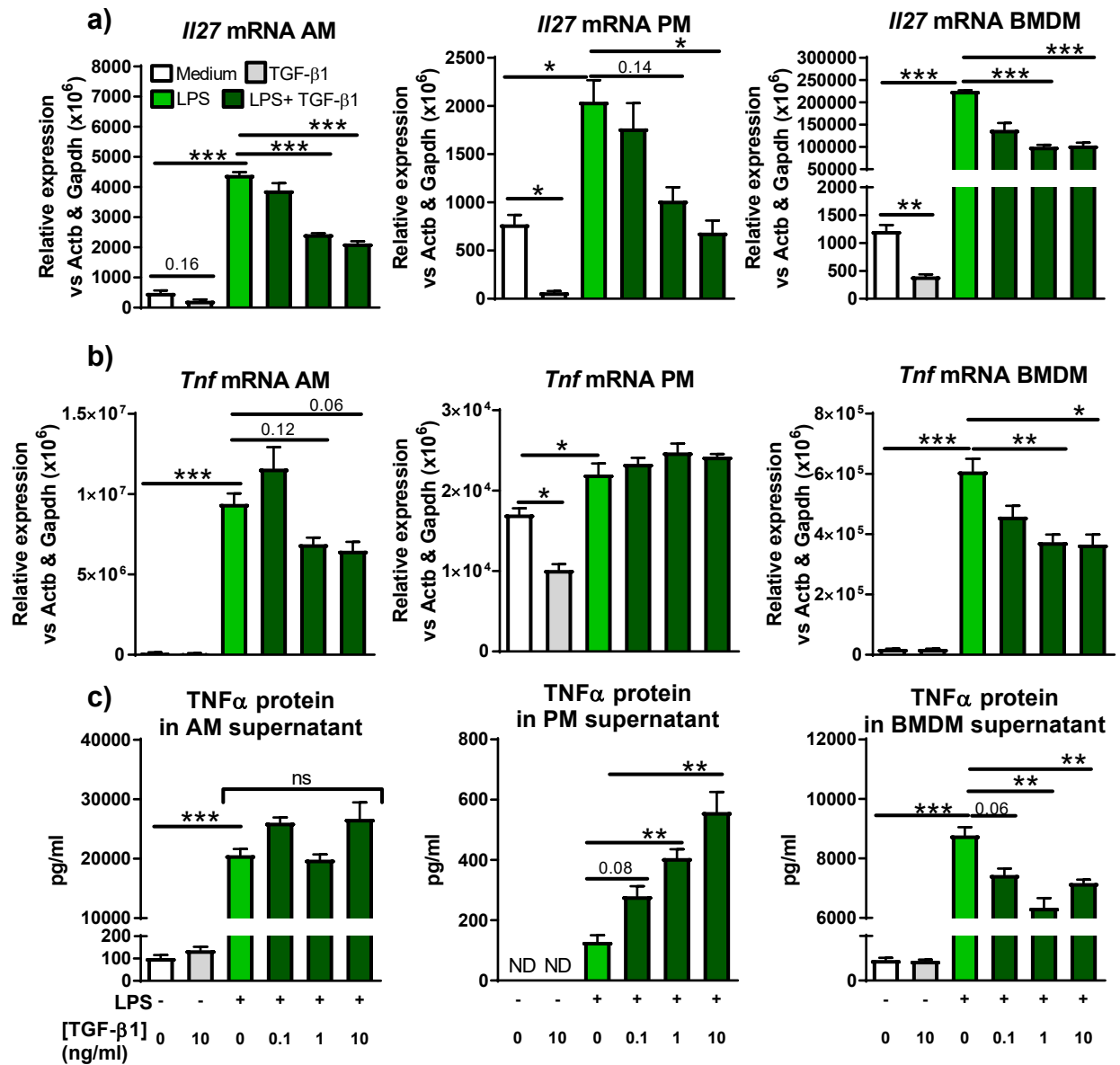


Figure 4.15. Concentration- and cell type-dependent effects of TGF-β1 on inflammatory cytokine production by macrophages

Macrophages were serum starved and stimulated as described in Figure 4.14. **a-b)** qPCR data showing expression of *I/27* (**a**) and *Tnf* (**b**) mRNA relative to housekeeping genes. **c)** ELISA data showing TNFα concentrations in macrophage supernatants. Data shown are the means of triplicate cultures ± SEM from a single experiment. Statistics: one-way ANOVA followed by Bonferroni-adjusted t-tests between the indicated groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ND, not-detectable.

4.10. Discussion

The aim of this chapter was to characterise production of the regulatory cytokines IL-10 and TGF- β 1 by AM in response to pro-inflammatory and polarising stimuli and to compare this to other pulmonary leukocytes and macrophages from different tissue sites and origins. These studies were intended to inform the design of subsequent experiments in which IL-10 and TGF- β signalling pathways were manipulated *in vivo*.

4.10.1. AM are poor producers of IL-10 in response to several inflammatory stimuli

IL-10 expression by AM was examined *in vitro* and *in vivo* and compared to other macrophages and pulmonary leukocytes. AM produced little to no detectable IL-10 during pulmonary inflammation *in vivo*, or after *in vitro* stimulation with a range of TLR agonists, in marked contrast to PM and BMDM. These findings suggest that murine AM have little capacity to produce IL-10 in response to several pro-inflammatory stimuli. Although lack of IL-10 production from murine AM has not been a focal conclusion in any published work to date, its absence has been demonstrated by other groups at both the protein secretion³¹ and transcriptional levels^{153,170}, independently affirming the findings in this chapter. The mechanistic basis of the failure of AM to produce IL-10 under conditions driving high levels of IL-10 secretion from PM and BMDM was not clear from the present work, but did not appear to be due to failure to produce the IL-10-inducing cytokines IFN β ³⁴² or IL-27³⁴⁶ in response to stimulation. However, exogenous administration of these cytokines to AM would be required to determine whether increased concentrations would allow IL-10 production. Understanding the molecular basis of the minimal IL-10 production from AM will require thorough investigation, for example at the level of chromatin accessibility, transcription factor recruitment or the magnitude of signals such as extracellular signal-regulated kinase (ERK) phosphorylation, the strength of which correlates with IL-10 production by myeloid cells³⁵⁵. However, these studies fell outside of the scope of this chapter, for which determining expression levels of IL-10 was sufficient.

A potential criticism of this *in vitro* analysis is the sole use of C57BL/6 mice, as IL-10 expression is known to differ substantially with genetic background, at least in BMDM³⁵⁶. However, when compared to BALB/c mice, BMDM from the C57BL/6 strain produced superior levels of IL-10 in a type I IFN-dependent manner, indicating that C57BL/6 mice should be suitable for detection of IL-10 from IL-10-competent macrophages. A more serious caveat of these experiments is their focus on mouse, rather than human, AM. Reports of IL-10 production by human AM in the literature generally suggest that these cells can produce IL-10, but results have been variable with regards to quantities of IL-10 and whether its production was increased in AM from asthmatics^{156,216,337}. Importantly, human AM were shown to produce much less IL-10 than peripheral blood monocytes in culture¹⁵⁶, suggesting that although human AM may be better producers of IL-10 than those from laboratory mice, the relative superiority of monocyte-derived cells over AM in IL-10 production described in this chapter may remain true in humans. Given the varied results in the literature, it will be necessary to compare matched airway and blood monocyte-derived macrophages from human subjects to determine the relative IL-10 production

in response to TLR agonists. Further, since corticosteroids were shown to increase IL-10 production from human AM *in vitro*¹⁵⁶, budesonide treatment of naïve murine AM will be included in future experiments, to determine whether corticosteroid signalling is sufficient to drive mouse AM to produce IL-10.

Importantly, AM showed little to no IL-10 reporter signal during RSV infection or HDM-induced AAD, suggesting that, even after chronic allergen exposure, AM were unlikely to produce substantial amounts of IL-10 *in vivo*. These data are in agreement with a recent publication, in which AM displayed minimal IL-10 reporter signal during AAD or after *in vivo* administration of a TLR9 agonist, which instead resulted in expansion of IL-10-producing interstitial and monocyte-derived macrophages²¹⁴. However, it remains possible that low, or even undetectable, levels of IL-10 could be relevant for AM immunoregulatory capacity or that the relevant timepoints for IL-10 production by AM were missed in these experiments. *In vivo* manipulation experiments were therefore required to exclude a role for AM-derived IL-10 in regulating pulmonary inflammation, which are described in Chapter 6.

Reporter mouse analysis revealed a population of F4/80⁺ CD64⁺ CD11b⁺ Siglec F⁻ IMM to be the major IL-10-competent pulmonary innate leukocytes. These findings support those of Bureau and colleagues, who have previously demonstrated IL-10 production from F4/80⁺ CD11b⁺ cells that they reported to be IM and recruited monocyte-derived macrophages^{31,214}. Indeed, the data in this chapter support these IL-10 competent mononuclear phagocytes being a mixed population, consisting of a combination of inflammatory monocytes and macrophages with varying expression of CD64 and the inflammatory monocyte marker Ly6C. In addition to these monocytes and macrophages, CD4⁺ T cells, mostly negative for FoxP3, were the other major IL-10-competent leukocytes in the allergic lung, which is pertinent given the known regulatory capacity of multiple subsets of IL-10-secreting T cells in AAD³⁵⁷. As expected for cytokine-producing CD4⁺ T cells, 10BiT⁺ T helper cells showed strong evidence of activation via the T cell receptor, suggesting that IL-10 production was induced in response to antigen, rather than in an innate-like manner. This apparent requirement for antigen, together with the relatively low numbers of 10BiT⁺ CD4⁺ T cells compared with IMM in the lungs of non-inflamed PBS control mice, suggests that CD4⁺ are not a major IL-10 source in lung homeostasis, but become relevant during inflammation. Experiments to directly test this hypothesis are described in Chapter 6.

A caveat of the IL-10 reporter experiments in this chapter is the use of a reporter in which several copies of a BAC are inserted randomly into the mouse genome, in contrast to most cytokine reporter mice, in which the endogenous locus is modified^{105,348}. Consequently, despite being under much of the same transcriptional and translational control¹⁰⁵, the 10BiT IL-10 reporter allele is more abundant in the genome than endogenous *Il10* and is not necessarily surrounded by the same chromatin landscape, such that the 10BiT reporter could feasibly overestimate IL-10 production. However, the increased sensitivity of this reporter compared with fluorescent reporter mice³⁴⁸ was required to maximise the capacity to detect potential IL-10 production from myeloid

cells and so was the best available option for global analysis of leukocytes. For better understanding of CD4⁺ T cells, which showed the strongest 10BiT signal, it would be more appropriate to use a mouse line directly reporting from the endogenous *Il10* locus, such as those described by Madan, Karp and colleagues³⁵⁸, to identify active IL-10 producers *in vivo*.

4.10.2. TGF- β 1 is the most abundant TGF- β isoform in AM

In contrast to IL-10 and in line with published data^{272,359}, AM expressed high levels of *Tgfb1* mRNA under all conditions tested and showed positive TGF- β 1 immunostaining *in vivo*. *Tgfb1* gene expression in AM was relatively stable, showing minimal changes in response to inflammatory stimuli and polarising cytokines. However it remains possible that these stimuli increased levels of TGF- β 1 protein in macrophages, although bioactive TGF- β was not detectable in the cell culture system used for these studies. *In vitro* AM culture fails to replicate the *in vivo* airway environment, where proteases and integrins from neighbouring cells can activate latent TGF- β ^{218,235,236,240,244}. Thus, despite minimal regulation at the transcriptional level, it remains likely that inflammatory stimuli can increase levels of AM-derived TGF- β 1 *in vivo* through concerted actions on AM and interacting cells.

A major finding from this chapter was the significant upregulation of *Tgfb2* in AM upon stimulation with either LPS or RSV, indicating the potential for simultaneous production of distinct TGF- β isoforms by activated AM. Both isoforms act on the same receptor complex and therefore have capacity for functional redundancy, although TGF- β 1 is far more widely implicated in the immune response²¹⁸. TGF- β 2 could therefore conceivably contribute to the pool of AM-derived TGF- β during inflammation.

4.10.3. AM are primed to receive IL-10 and TGF- β signals *in vivo*

Importantly, AM obtained directly from the BAL of mice expressed the canonical signal transduction molecules required to respond to IL-10 and TGF- β and displayed concentration-dependent suppression of LPS-induced gene expression by these cytokines *in vitro*, suggesting that AM are primed to receive signals from these cytokines *in vivo* at steady state. This is particularly relevant in the case of IL-10, which is known to perform many of its functions via suppression of macrophage activation^{195,196}, including in the intestine^{205,206}, a mucosal surface with some immunological parallels to the airway. Regarding TGF- β 1, high levels of production of and responsiveness to this mediator by AM supports the likelihood of autocrine signalling in the airway microenvironment, as has recently been proposed²⁸², particularly given the proximity of AM to epithelial cells bearing integrins capable of activating latent TGF- β ^{240,244}. If autocrine TGF- β 1 is indeed an essential signal for regulating AM, then deletion of AM-intrinsic TGF- β 1 would be expected to affect the phenotype of AM at baseline and following inflammatory challenge, a hypothesis addressed in Chapter 6 of this thesis.

4.11. Conclusions

The data in this chapter strongly suggest that the pro-inflammatory phenotypes of AM occurring during allergic airway disease and respiratory infection are not accompanied by increased expression of IL-10, suggesting that the regulatory capacity of AM in these settings is independent of IL-10 production. Instead, IMM and CD4⁺ T cells were the major pulmonary IL-10-competent leukocytes during inflammation. In contrast to IL-10, TGF- β 1 is expressed at high levels by AM at homeostasis. These data suggest that, *in vivo*, IL-10 from other cellular sources may signal to AM in a paracrine manner, while TGF- β 1 signalling to AM may be autocrine or paracrine. These models were tested in Chapters 6 and 7, guided by the results described here.

5. Transcriptomic analysis of airway macrophages during allergic airway disease

5.1. Introduction

In Chapter 3, flow cytometry was used to determine phenotypic changes occurring to airway macrophages (AM) in different models of pulmonary inflammation, from which changes to the function and behaviour of AM *in vivo* could be inferred and used to assess AM activation in subsequent experiments. Following this, in Chapter 4, the IL-10 and TGF β pathways were examined by qPCR and ELISA analysis of macrophage subsets subjected to different inflammatory stimuli. However, such analyses are limited by biased selection of parameters to measure and therefore may miss potentially important changes to known or novel pathways in AM.

The role of AM in the pathobiology of allergic airway disease (AAD) is complex and incompletely understood³⁶⁰, thus presenting an interesting model for studying their immunoregulatory function. AM are a major source of pro-inflammatory mediators during AAD^{156,157} and can promote type 2 immunity and airway remodelling when dysregulated in the absence of the transcription factor IRF5¹⁷⁶. Conversely, AM are required for effective regulation of the type 2 immune response to allergen and resolution of house dust mite- (HDM-) driven pulmonary inflammation^{144,145,361}. Moreover, there is evidence that the regulatory function of AM is at least partially lost following allergic sensitisation^{145,147}. This multifaceted role of AM in the allergic lung demonstrates the need for unbiased ‘-omics’ analysis of these cells in AAD models to better understand the molecular basis of their pro-inflammatory and immunoregulatory functions.

The transcriptome of tissue-resident macrophages has been the subject of a number of studies in recent years, which demonstrated the importance of the local microenvironment for dictating gene expression and phenotype^{141,170,171}. This is particularly true for AM, since the airway niche has been shown to be sufficient to differentiate diverse macrophage precursors into cells essentially identical to endogenous AM^{62,362}. It follows that the profound changes to the airway microenvironment during allergic inflammation, such as that induced by repeated HDM inhalation, are likely to integrate with the tissue-specific signals that control steady state phenotype of AM^{62,170}, resulting in an ‘AM[HDM]’ gene expression profile distinct from both homeostatic AM and inflammatory macrophages in other tissue sites. However, there are no published studies examining the effects of repeated airway HDM exposure on the transcriptome of AM.

In addition to the local environment, the ontogeny of AM may also influence their gene expression. AM are largely self-maintained by local proliferation, with replacement from circulating monocytes occurring after lethal irradiation⁶¹ and gradually with age^{139,340}. However, as discussed earlier in this thesis, the extent of monocyte replacement of resident AM during AAD is unclear, as are the effects of AM origin on gene expression in this setting. Two studies have shown little to no difference between transcriptomes of embryonic- and adult monocyte-derived AM at steady state^{62,141}. However, monocyte-derived AM showed greater induction of genes related to fibrosis than AM resident in lungs prior to disease onset¹⁴⁰, indicating that ontogeny may impact AM gene expression during disease states. In the context of AAD, two papers have suggested that

monocyte-derived macrophages recruited to the lung differ from resident AM by promoting pathogenesis of the disease^{63,363}. However, neither study determined whether these were monocytes that had differentiated into AM-like cells, or represented a distinct population. The phenotype of AM in the allergic lung is therefore likely to reflect integration of local tissue and inflammatory signals, potentially along with a transcriptional 'memory' of cellular origin.

The principal aim of this chapter was to examine gene expression changes in AM from a model of HDM-driven AAD, to highlight biological processes relevant to the pro-inflammatory and regulatory functions of AM in the allergic lung. To this end, RNA sequencing (RNAseq) was performed on flow cytometry-sorted AM from bronchoalveolar lavage (BAL) of HDM-treated mice (AM[HDM]) and compared to those from PBS-treated non-inflamed controls (AM[PBS]).

Although transcriptomic analysis of purified cell subsets is desirable for specific attribution of gene expression changes to a particular cell type, it is not always possible due to the requirement for specialist equipment, such as fluorescence-activated cell sorters. An attractive alternative is to identify gene expression signatures of a cell type of interest detectable in unrefined biological samples containing mixed populations of cells, which are more readily obtained and require less prior processing that may influence gene expression. Such approaches also provide a snapshot of gene expression derived from all cell types within a tissue compartment, which when compared to transcriptomes from individual cell types can allow inferences of the contribution of different cell populations to distinct molecular signatures to be made. Dominant neutrophil gene expression signatures have previously been detected in whole lung tissue and used to infer the contribution of neutrophils to the immunopathogenesis of lethal influenza⁸, supporting the utility of such an approach. Comparison of the transcriptomes of sorted AM and unrefined BAL cells from HDM-treated mice was therefore proposed as a means of assessing the relative contribution of AM to gene expression patterns suggestive of biological processes relevant to AAD.

Collectively, data from this chapter were intended to provide insights into changes occurring to AM during allergic inflammation, while comparing gene expression profiles of AM to other leukocytes in the allergic airway.

5.2. Hypothesis and aims

Hypothesis

AM from BAL of mice with established HDM-induced AAD (AM[HDM]) will show a transcriptional profile composed of distinctive gene signatures of type 2 immunity and immune regulation, which will be detectable in unenriched BAL cell RNA preparations.

Aims

1. Obtain RNA from sorted AM and total BAL cells of HDM-treated mice of sufficient quality for RNA-seq
2. Identify and functionally annotate differentially expressed genes in AM[HDM] versus AM from PBS-control mice (AM[PBS])
3. Compare gene expression patterns in AM[HDM] and whole BAL from HDM-treated mice (BAL[HDM])

5.3. Sample collection and sorting of airway macrophages

Pure AM are readily obtained from BAL of naïve mice by selective adhesion onto tissue culture plastic, due to their abundance among airway leukocytes and high expression of adhesion molecules³⁶⁴. However, this is no longer possible during allergic inflammation, where AM are a minority population in the BAL (see Chapter 3), precluding separation of AM from infiltrating leukocytes by adhesion-enrichment. It was therefore necessary to establish a flow cytometry sorting method for purification of AM from BAL of HDM-treated mice.

To induce AAD in which to study AM, C57BL/6 mice were exposed to intranasal HDM or PBS control for 3 weeks and BAL harvested 24h after final allergen challenge (**Fig. 5.1a**), a timepoint at which robust allergic inflammation and AHR are detectable in mice³¹⁷. BAL was performed using a high-volume protocol (see Section 2.4.2) to maximise cell yield for sorting. As expected, total airway cell counts were significantly elevated in HDM-treated mice (**Fig. 5.1b**), principally due to increased numbers of eosinophils and lymphocytes, which were almost completely absent from BAL of PBS control mice. (**Fig. 5.1c**). Total AM numbers in BAL were comparable in HDM-treated and PBS control mice (**Fig. 5.1c**).

AM were sorted using the minimum criteria defined in Chapter 3, where AM were defined as CD45⁺ cells expressing Siglec F, CD11c and CD64 (**Fig. 5.1d**). An average of 3×10^5 AM were obtained per PBS-treated mouse, while median recovery from HDM-treated mice was lower at 0.75×10^5 (**Fig. 5.1e**). Lower AM recovery from HDM-treated mice occurred despite total AM numbers in BAL being comparable between experimental groups (**Fig. 5.1c**), highlighting the difficulty of sorting cells that are at low frequency in the starting material. Sort purity checks obtained for a subset of samples showed AM from PBS mice to be > 96 % pure, while purity from HDM-treated mice was 89-93 % in two samples, with one AM[HDM] sample (AM[HDM]#1) displaying lower purity at 81 % (**Fig. 5.1f**). Reduced purity in sample AM[HDM]#1 was due to relatively high levels of contaminant Siglec F⁻ cells (**Fig. 5.1f**), highlighting this sample as a potential source of error.

To allow parallel analysis of total airway cell transcriptomes from the HDM-driven AAD model, a separate group of mice were exposed to HDM or PBS for 3 weeks and BAL cells directly lysed for RNA extraction. As for the mice used for sorting, total BAL cell numbers were significantly increased in HDM-treated mice (**Fig. 5.2a**), consistent with induction of allergic airway inflammation. As expected, Wright-Giemsa staining showed increased numbers of eosinophils and lymphocytes in BAL of HDM-treated mice (**Fig. 5.2b,c**) along with a decreased proportion of macrophages and monocytes, including AM, in BAL (**Fig. 5.2b,d**).

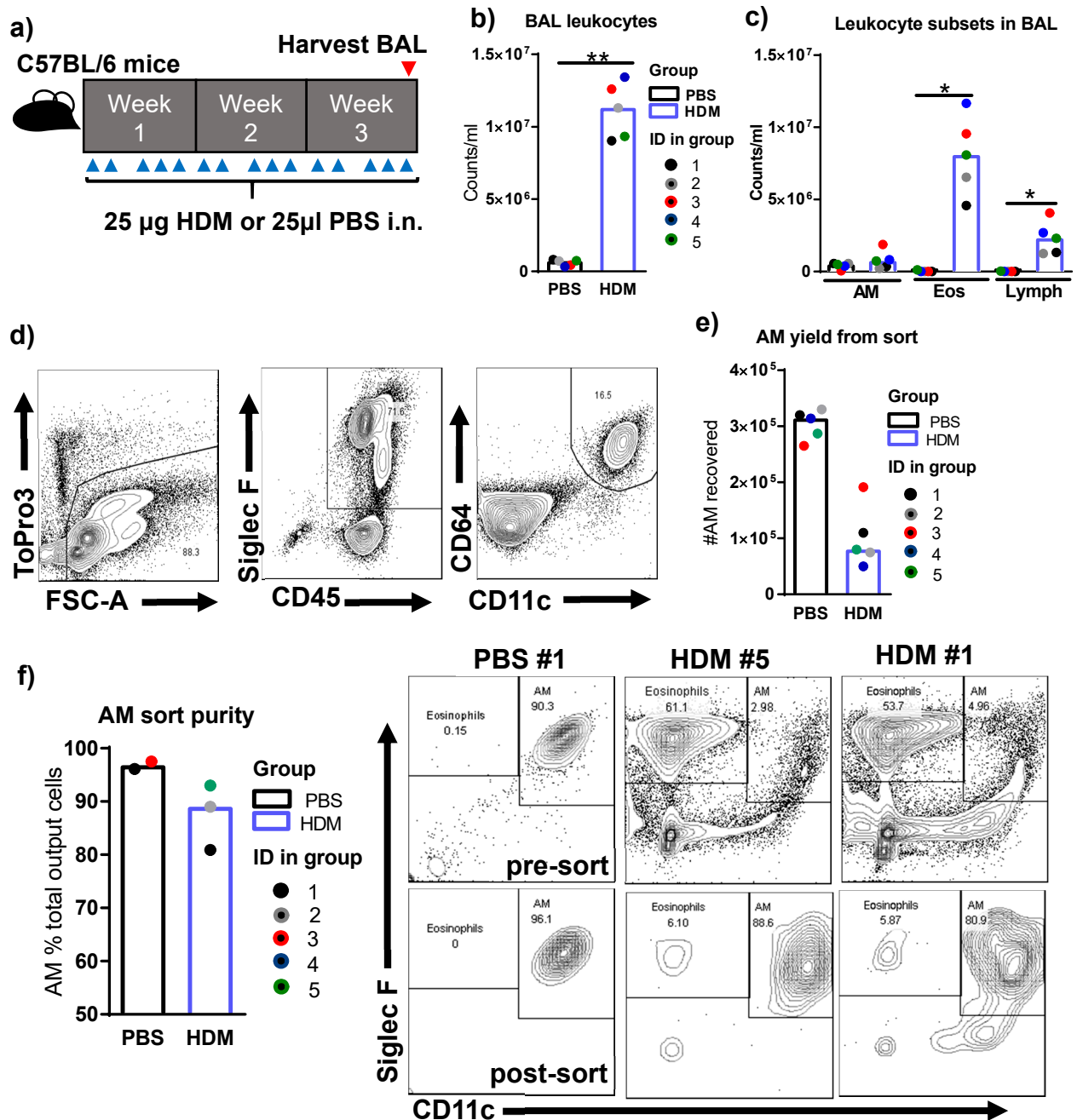


Figure 5.1. Flow cytometry sorting of AM from mouse HDM model

a) Adult female C57BL/6 mice received intranasal HDM or PBS control 5 days per week for 3 weeks. Mice were culled and BAL harvested 24h after the final allergen challenge **b)** Total crystal violet cell counts in BAL. **c)** Total AM, eosinophils (Eos) and FSC^{low}/SSC^{low} CD45⁺ lymphoid cells (Lymph) in BAL as determined by flow cytometry. **d)** AM sorting strategy: live cells were selected and CD45⁺ Siglec F⁺ CD11c⁺ CD64⁺ cells determined to be AM. **e)** Numbers of sorted AM as reported by the flow sorter. **f)** Representative purity values of sorted AM samples from PBS and HDM groups (left) and representative flow cytometry plots showing Siglec F⁺ CD11c⁺ AM among total cells before and after sorting (right). Plots in (f) are pre-gated to exclude FSC^{low} debris. Numbers show proportions of events in gates. Scatter/bar plots show medians of 5 mice per group, with samples colour-coded within each group. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$; **, $p \leq 0.01$.

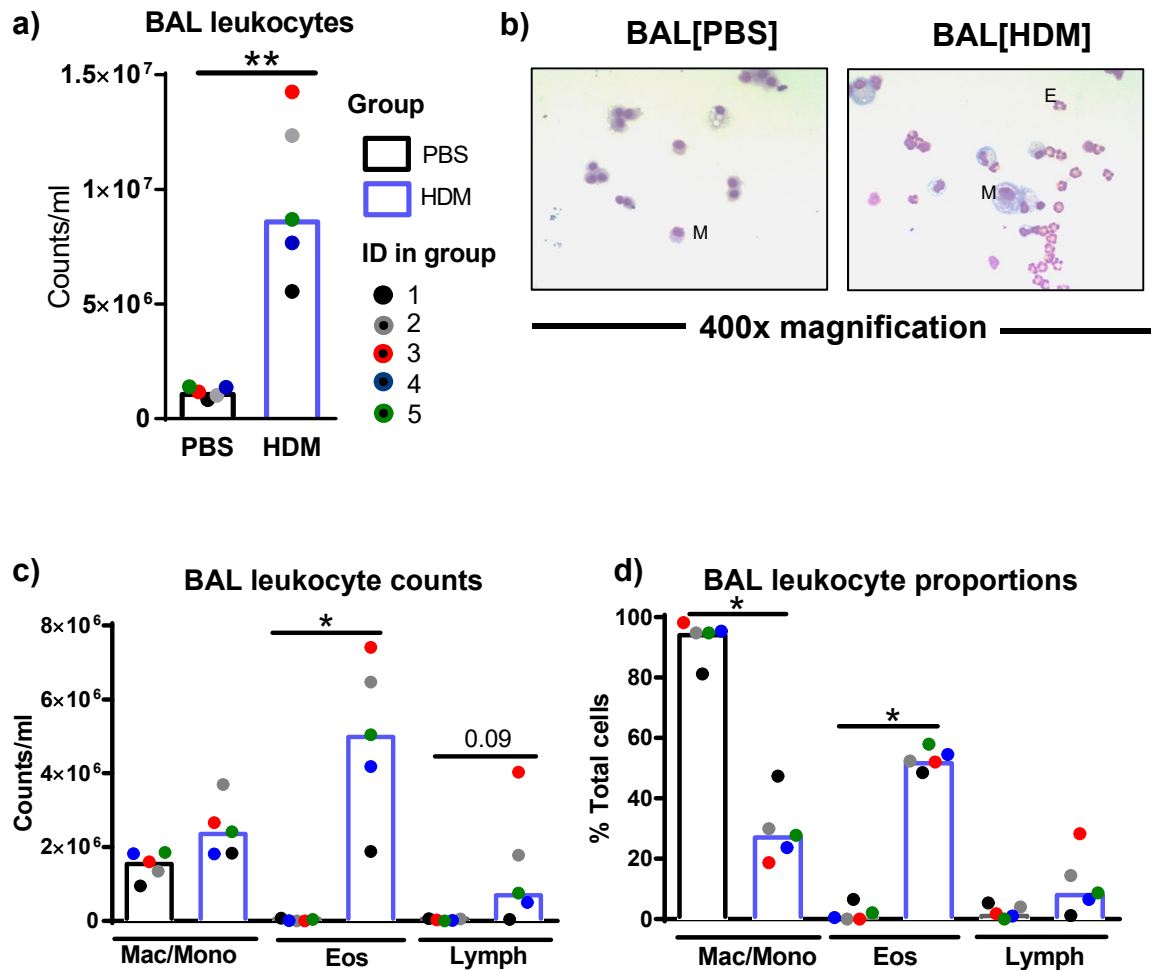


Figure 5.2. Unsorted BAL samples used for RNA-seq

Mice were exposed to intranasal PBS or HDM for 3 weeks before harvesting BAL, as described in **Fig. 5.1a**. **a)** Total crystal violet cell counts in BAL. **b)** Representative Wright-Giemsa-stained cytocentrifuge preparations of BAL of PBS and HDM-treated mice. M, macrophage; E, eosinophil. **c-d)** Absolute numbers (**c**) and proportions (**d**) of macrophages/monocytes, eosinophil (Eos) and lymphoid cells (Lymph) as determined by differential counting of Wright-Giemsa-stained cytocentrifuge preparations. Scatter/bar plots show medians of 5 mice per group, with samples colour-coded within each group. Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. *, $p \leq 0.05$; **, $p \leq 0.01$. Where relevant, corrected p values are shown between groups.

Notably, one mouse in each group in the whole BAL analysis experiment showed unusual airway cytology. One HDM-treated mouse (BAL[HDM]#1) displayed relatively low total airway cell counts and numbers of eosinophils and lymphocytes (**Fig. 5.2a, c**), while mouse BAL[PBS]#1 showed a slightly elevated proportion of eosinophils and consisted of only 80 % AM, in contrast to the other mice in the group (**Fig. 5.2d**). These outliers with intermediate airway cytology were excluded prior to analysis by RNAseq in the interest of making valid comparisons between resting and inflamed airway cell populations.

5.4. Quality control of RNA, cDNA libraries and raw sequence data

The quality and reliability of RNAseq data are dependent on both the quantity and integrity of RNA used to generate sequencing libraries. Low input levels of RNA require more PCR amplification of resultant cDNA libraries, which can reduce library diversity and impair signal-to-noise in detection of low abundance transcripts³⁶⁵. Degradation of input RNA can result in a failure to align resultant library sequences to reference genomes and can markedly affect gene expression results due to biased sequencing of parts of the transcriptome that escape degradation^{366,367}. To confirm that that RNA samples obtained were suitable for RNAseq, quantity and quality were assessed by electrophoresis. All samples yielded > 250 ng of RNA (**Fig. 5.3a**), 200 ng of which was used for library preparation. AM and BAL samples showed little degradation, with all RNA integrity (RIN^e) values meeting the recommended 8/10 for Illumina sequencing (**Fig. 5.3b**).

Sequence libraries were prepared from RNA samples by poly(A) enrichment of mRNA, randomly-primed cDNA synthesis, adapter ligation and limited-cycle PCR amplification, as described in Chapter 2. Resultant libraries were normalised and sequenced using the Illumina HiSeq 4000 platform. After removal of adapter sequences, raw sequence data were inspected for quality. Sequencing yielded 30-170 million paired end reads (60-340 million total sequences) per sample, with no differences in sequencing depth observed between PBS and HDM groups (**Fig. 5.4a**). MultiQC analysis²⁹² was used to examine phred scores of raw reads, which indicate the probability of individual base calls being correct. Phred scores were above 30 across the length of reads in all samples (**Fig. 5.4b**), indicating that quality was not substantially reduced at the extreme ends of sequences and so the full reads could be used for alignment. Plotting the distribution of mean phred scores for all sequences showed less than 0.1 % of reads to have relatively low quality (mean phred < 20, **Fig. 5.4c**), which were filtered prior to alignment.

Duplicate non-unique sequences in transcriptome libraries can occur from true biological overrepresentation of sequences, microbial contamination of samples or excessive PCR amplification and have potential to impact results of differential expression analysis^{368,369}. AM and BAL libraries showed 60-85 % duplication. Notably, duplication was significantly higher in AM[HDM] samples than those from PBS-treated mice (**Fig. 5.4d**), suggesting that duplication was at least partially due to overrepresented transcripts expressed by AM in the HDM model. Analysis was therefore continued on all AM and BAL samples.

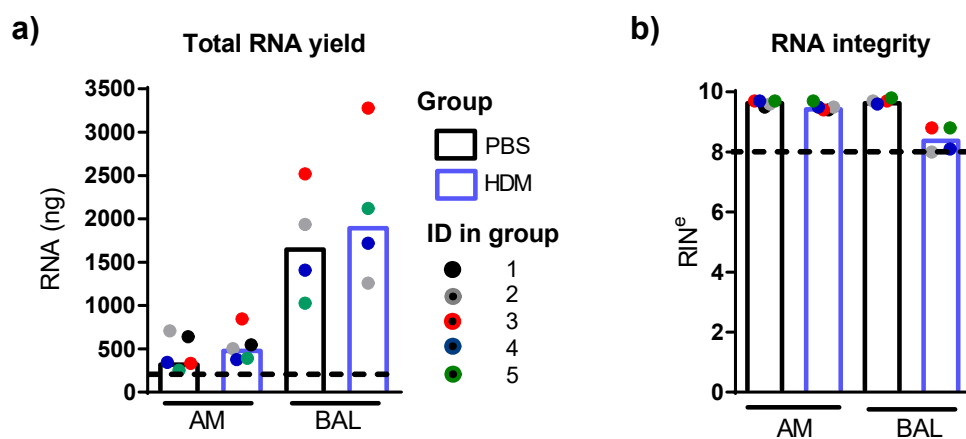


Figure 5.3. Quality control metrics of RNA for sequencing

RNA samples obtained from sorted AM and whole BAL and total blood were analysed using TapeStation electrophoresis and quantities (a) and integrity (b) determined. Scatter/bar plots show medians of the indicated number of replicate samples, with samples colour-coded within each group. Horizontal dashed lines indicate the minimum values required for the chosen library preparation protocol.

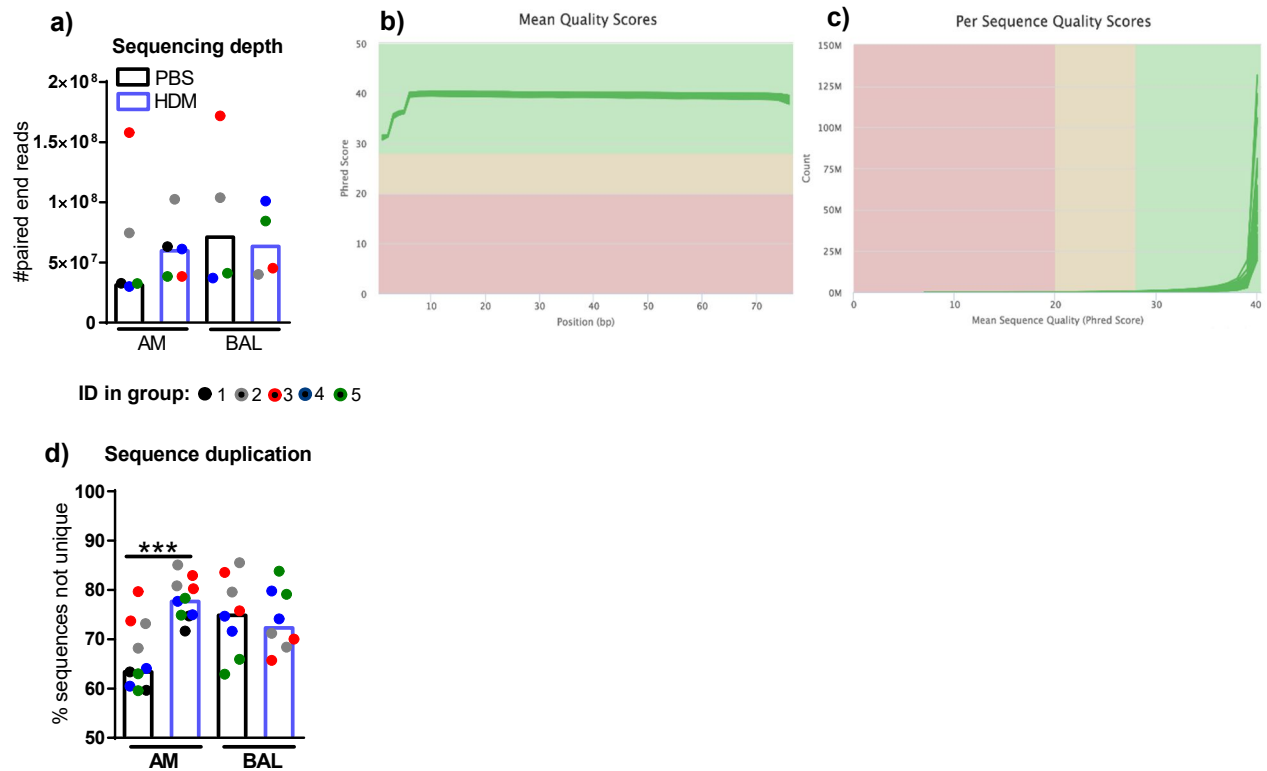


Figure 5.4. Quality control metrics of raw sequence libraries

Raw FASTQ files from Illumina sequencing were adapter-trimmed and subjected to FASTQC analysis.

a) Numbers of paired-end sequences obtained for each library. **b-c)** MultiQC analysis plots of all libraries showing mean phred quality scores at each read position (**b**) and numbers of reads with the indicated phred score across the entire sequence length (**c**). Red areas indicate lower thresholds for filtering, while green areas show optimal quality values for analysis. **d)** Percentages of sequences in each library identified as non-unique replicates. MultiQC plots show overlaid forward and reverse read averages from all sorted AM and whole BAL samples (total 36 sequence files). Scatter/bar plots show medians of 4-5 replicate samples, or 8-10 individual sequence files, with samples colour-coded within each group. Note that forward and reverse reads are shown as individual points of the same colour in (**d**). Statistics: Kruskal-Wallis test, followed by Bonferroni-adjusted Mann Whitney tests between indicated pairs of groups. ***, $p \leq 0.001$.

Filtered high-quality reads were aligned to the Mm10 reference genome and transcript quantities determined by maximum likelihood estimation and normalised by the DESeq method³⁷⁰. Samples were aligned and analysed in the first instance as two individual datasets: sorted AM and whole BAL. To minimise the likelihood of reads being mistakenly aligned to the incorrect genomic location, genes were filtered during alignment if they failed to map with ≥ 90 % identity to the reference genome, contained a continuous gap of ≥ 5 % in the matching sequence to reference sequence, or if paired reads mapped inconsistently from one another. Around 94 % of reads mapped successfully with the above criteria (**Fig. 5.5**). A small proportion of reads in libraries from both AM mapped to ribosomal RNA (rRNA; $< 10^{-6}$ %) or common contaminants arising from vectors, adapters and other molecular biology tools (UniVec database, < 0.1 %), which were also removed during the alignment process.

5.5. Determining analysis thresholds for the airway macrophage dataset

Having confirmed technical sequence quality, samples in the AM dataset were inspected for reproducibility between biological replicates. Preliminary principal component analysis (PCA) was performed on all genes with ≥ 20 raw reads assigned in ≥ 75 % (i.e. all but one) of samples in either experimental group, so as to ignore only the genes detected at lowest levels in AM for initial inspection of the data. AM[PBS] samples clustered tightly in two principal components accounting in total for 73 % of variation in the dataset (**Fig. 5.6a**), consistent with highly similar gene expression patterns in AM in the absence of inflammation. Variance between samples from HDM-treated mice was greater than that for PBS (**Fig. 5.6a**), as expected in the case of a deviation from immune homeostasis. However, one sample (AM[HDM]#1) was a clear outlier by PCA, clustering away from all other samples in PC2, which accounted for 10.7 % of variation in the dataset.

AM sample HDM#1 had previously been identified to have low sort purity (81 %, **Fig. 5.1f**), prompting further investigating of potential contamination in this sample. Reasoning that pure AM should not express molecules associated with T cell receptor signalling, regardless of activation state, raw read counts for a number of hallmark AM genes were normalised to sequencing depth for each sample and compared to genes typical of CD4⁺ T cells as a rough estimate of the degree of contamination. AM[PBS], which showed > 96 % purity and thus minimal contamination, displayed high relative read values (generally > 100) for AM genes and values of 0.1-1 for T cell 'contaminant' genes *Cd3e*, *Cd3g* and *Cd4* (**Fig. 5.6b**). Relative read values were similar to AM[PBS] for contaminant genes in the majority of AM[HDM] samples with the exception of AM[HDM]#1, which showed values between 5 and 25 (red dashed boxes in **Fig. 5.6b**), indicating that the relatively low purity of this sample had impacted gene expression data. Sample AM[HDM]#1 was therefore excluded and transcript quantification repeated in its absence, leaving a total of 4 biological replicates in the AM[HDM] group.

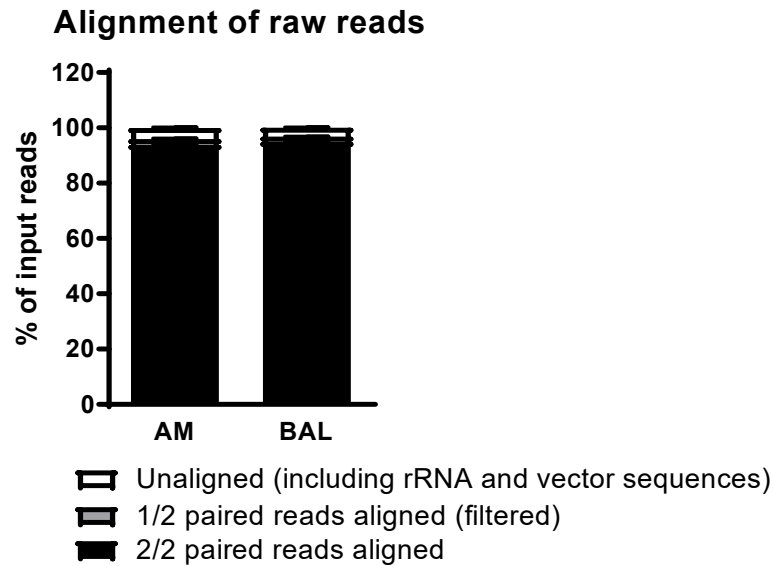


Figure 5.5. Alignment statistics of raw sequence reads

Raw paired-end read files were filtered to remove those with mean phred scores < 20, before aligning to the Mm10 genome. Sorted AM and whole BAL datasets were run in two separate alignments. Reads matching the mouse rRNA or UniVec databases or with < 90 % identity to the reference sequence were not aligned. Stacked bars show the proportions of reads aligning successfully (both paired ends map to same location), compared with unaligned reads and reads filtered due to mismatches between pairs. Data shown are means $\pm$ SEM of total reads subjected to alignment from combined PBS and HDM samples (8-10 per group).

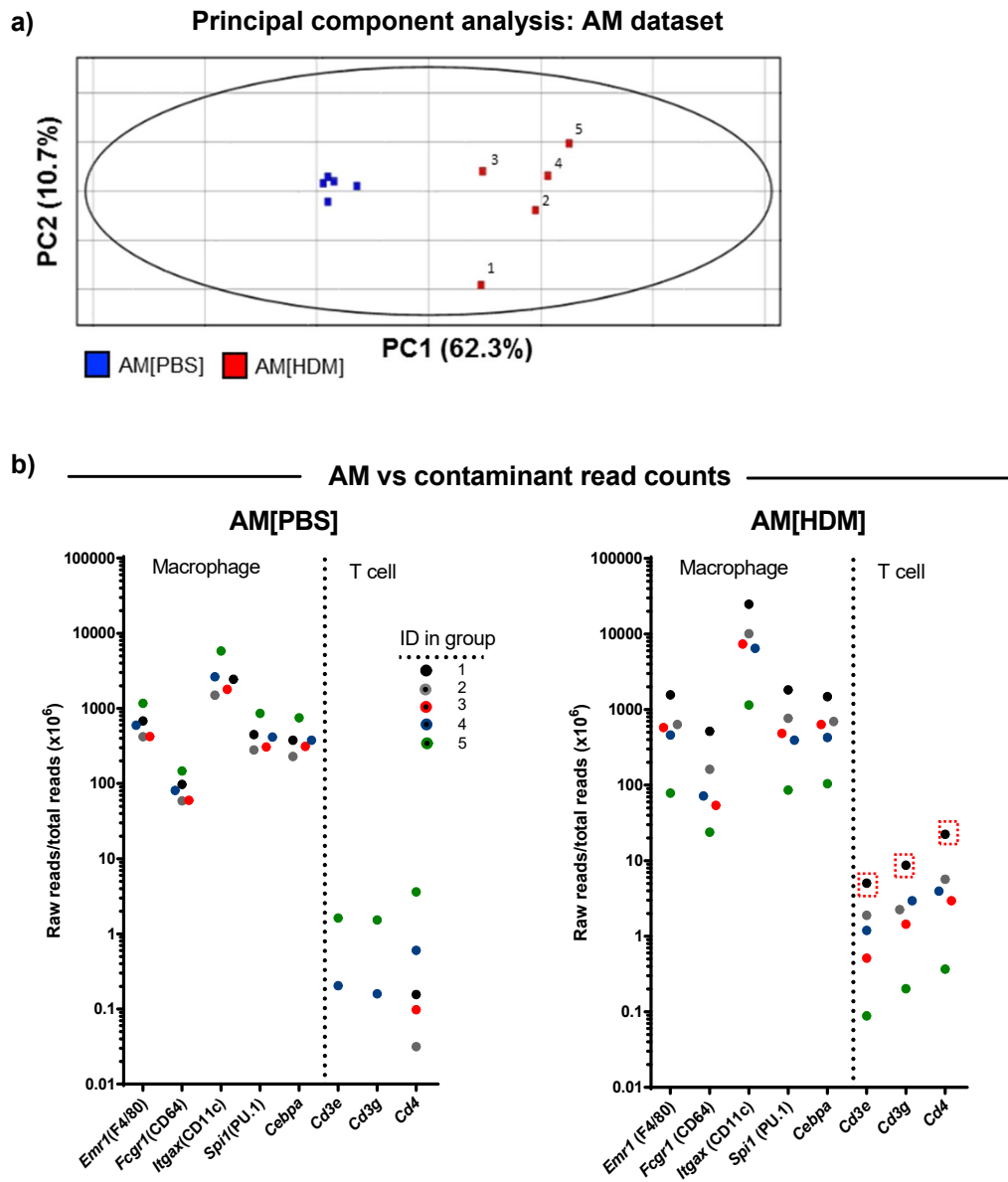


Figure 5.6. Outlier identification in the AM[HDM] dataset

DESeq-normalised expression data were filtered to remove genes with fewer than 20 raw reads in both experimental groups, before examining variation in data. **a)** Principal component analysis showing the two major principal components accounting for variation in all AM samples. ID numbers are shown for samples in the AM[HDM] group. **b)** Raw read counts for known AM genes and putative T cell contaminant genes were normalised to total aligned read counts and visualised for each sample in PBS (left) and HDM (right) groups, to identify samples with relatively high levels of non-AM contaminant reads. Red dashed boxes highlight likely evidence of contamination in sample AM[HDM]#1.

Examination of likely contaminant reads in the remaining AM[HDM] samples revealed low but consistent detection of lymphocyte-associated genes such as *Ii4*, *Cd3e* and *Ctla4*, generally with fewer than 100 raw reads, translating to normalised signal values ranging from 4.93 to 7.72. These genes have not consistently been reported to be expressed by AM and showed very low read counts when compared to genes known to be expressed in AM[HDM]: *Fcgr1*, *Il4ra*, *Itgam* and *Itgax*, suggesting they were highly likely to be low level contaminants. Importantly, since these proposed contaminant genes were not detected in AM[PBS] samples, even low level detection in AM[HDM] resulted in large fold changes from AM[PBS] (**Table 5.1**), which could erroneously suggest their upregulation by AM during HDM-driven AAD. To circumvent this issue, a lower threshold normalised signal value of 8 was imposed, filtering genes for which expression in ≥ 75 % of samples in both experimental groups was below this value.

5.6. Differential gene expression analysis in AM from HDM-treated mice

Following outlier removal and thresholding, 10 048 genes of the 17 121 initially assigned ≥ 1 read in the AM dataset remained for analysis (**Fig. 5.7a**). PCA of these genes showed AM[HDM] samples to cluster relatively closely in PC1, with no clear outlier, suggesting that variance could be attributed to true biological variation in gene expression within AM themselves, rather than different degrees of contamination (**Fig. 5.7b**). Box and whisker plots of gene expression value distributions confirmed this; AM[HDM] displayed more extreme gene expression values at the upper and lower ends of plots, but no clear outlier was observed (**Fig. 5.7c**). Thus, data were concluded to be successfully sanitised ahead of differential expression analysis.

PCA visualisations and box and whisker plot distributions indicated that the transcriptome of AM[HDM] differed markedly from PBS controls (**Fig. 5.7b,c**). To focus analysis on genes showing substantial and significant changes between groups, relatively stringent differential expression analysis was performed, considering genes significantly differentially expressed with a Benjamini-Hochberg false discovery rate (FDR)-corrected p value (moderated t-test) of ≤ 0.01 and an absolute fold change of ≥ 2 . Of 10 048 genes above threshold, 6264 passed the p value cut-off and 2352 had a fold change of ≥ 2 , with 2293 genes meeting both criteria (**Fig. 5.7d**). Roughly equal numbers of differentially expressed genes were increased (1143) and decreased (1150), although a proportion of the upregulated genes showed much greater fold changes than any downregulated genes in AM[HDM] (right-most cluster of genes on volcano plot in **Fig. 5.7e**), consistent with substantial induction of gene expression in AM during HDM-driven AAD.

Despite marked changes in gene expression in AM[HDM], around one third of genes (3725) did not meet either p value or fold change criteria for differential expression (**Fig. 5.7d**), indicating relatively consistent expression of these genes by AM at homeostasis and during AAD. Notably, these included several genes involved in innate immune responses, including the key pattern recognition molecules *Tlr3*, *Tlr4*, *Tlr7* and *Myd88* (**Fig. 5.7d**). AM activation during HDM-driven AAD therefore did not result in global dysregulation of gene expression related to innate recognition of pathogen-associated molecular patterns.

Gene	Raw reads AM[HDM] mean, n=4	Normalised signal AM[HDM] mean, n=4	Fold change AM[HDM] vs AM[PBS]
<i>Il4</i>	91	6.35	82
<i>Il13</i>	34	4.93	16
<i>Cd3e</i>	43	5.08	9.78
<i>Cd3g</i>	76	6.14	21.72
<i>Ctla4</i>	70	6.33	25.50
<i>Cd28</i>	63.5	5.75	18.74
<i>Cd19</i>	129	7.72	149.5
<i>Fcgr1</i>	3750	11.9	-1.23
<i>Il4ra</i>	33750	15.04	2.07
<i>Itgam</i>	7400	12.76	17.62
<i>Itgax</i>	333066	18.15	2.11

Proposed
contaminants

‘True’ signal

Table 5.1. Contaminant gene expression levels in AM[HDM] samples

All genes detected in the AM dataset, minus outlier sample AM[HDM]#1, were subjected to DESeq normalisation and fold change analysis, without prior filtering on raw or normalised expression values. A representative selection of genes are shown, to demonstrate the potential for low numbers of likely contaminant reads to result in large and misleading fold changes (shown in red).

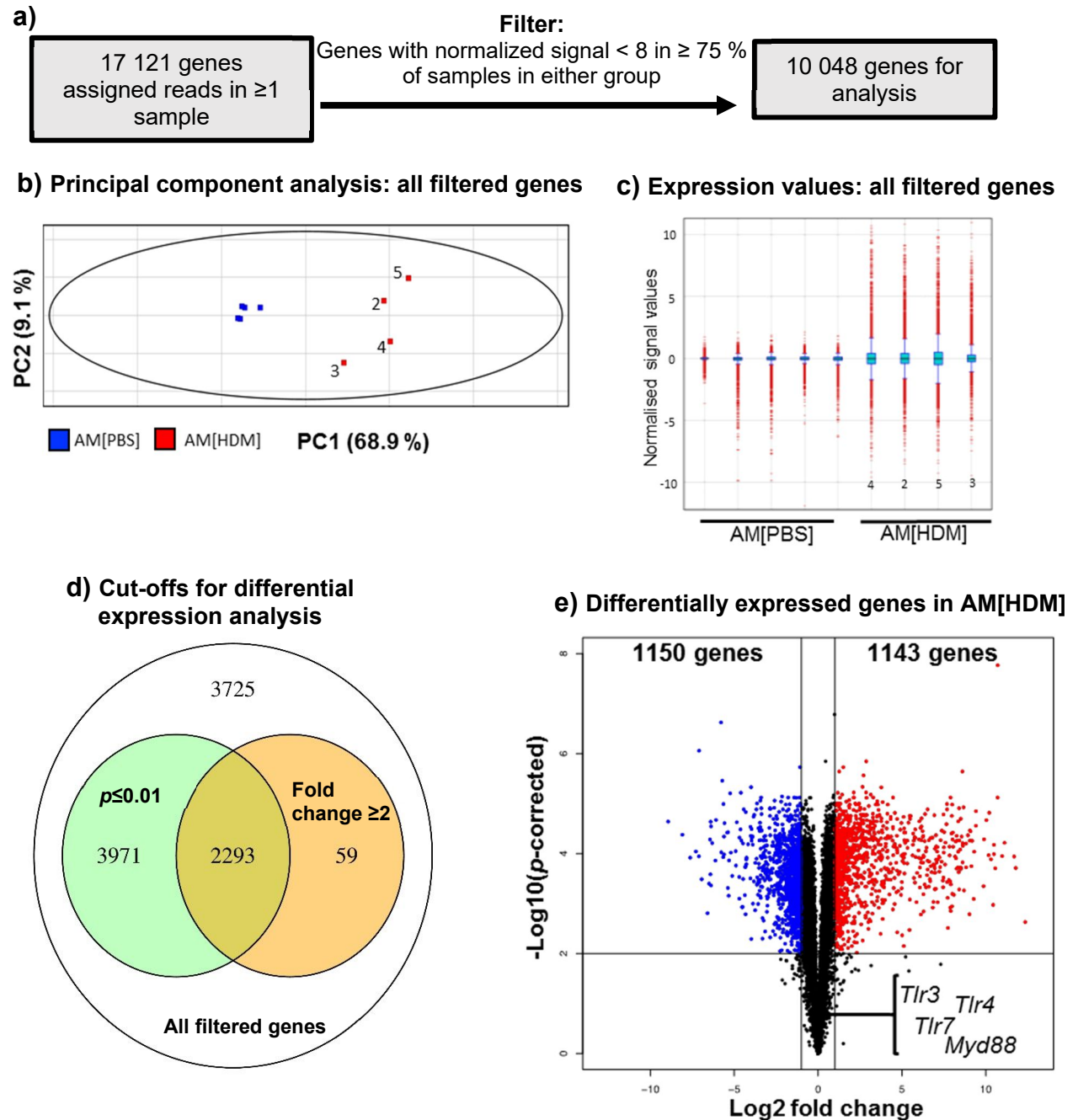


Figure 5.7. Differential gene expression analysis in AM[HDM]

a) Genes in the AM dataset were filtered to remove low level contaminant reads prior to analysis. **b)** PCA showing similarity of gene expression in sorted AM samples from PBS and HDM-treated mice. AM[HDM] group are annotated with sample numbers. **c)** Scatter plots of DESeq normalized expression values showing medians $\pm$ IQR (boxes) and ± 1.5 -times the IQR (whiskers), with more extreme outlying values shown in red. **d)** Venn diagram showing numbers of total genes (large circle) showing significantly different expression in AM[HDM] vs AM[PBS] ($p \leq 0.01$, moderated t -test with Benjamini-Hochberg correction) and/or a ≥ 2 -fold difference in mean normalized gene expression between AM[HDM] and AM[PBS]. **e)** Volcano plot showing transformed fold changes and corrected p values resulting from differential expression analysis described in (d). Up- and down-regulated genes are indicated in red and blue, respectively. Selected immune response genes showing no differential expression are marked on this plot.

5.6.1 AM express alternative activation genes at steady state and upregulate genes related to antigen presentation and phagolysosome function during HDM-driven AAD

To better understand the potential functional consequences of differential gene expression in AM[HDM], the 2293 differentially expressed genes were subjected to unsupervised hierarchical clustering based on DESeq-normalised expression values. As expected, AM[HDM] and AM[PBS] samples cleanly segregated by this method, with genes clustering based on their resting expression (in AM[PBS]) and expression in the HDM treatment group (**Fig. 5.8a**). Reasoning that genes sharing biological functions and regulatory pathways are likely to show similar expression patterns, 13 major clusters of genes were selected for functional annotation (**Fig. 5.8a**). Visualising differential expression of these clusters of genes in AM[HDM] as volcano plots showed a range of fold change patterns, including downregulated gene clusters (e.g. cluster 13) moderately increased gene clusters such (e.g. cluster 3) and highly upregulated gene clusters (e.g. cluster 7). Clusters were broadly categorised based on baseline expression (i.e. in AM[PBS]) and fold change in AM[HDM] to aid interpretation of functional annotations, as summarised in **Table 5.2**.

To assess the likely functional importance of the differential gene expression clusters apparent in AM[HDM], clusters were individually assessed for overrepresentation of genes in particular gene ontology (GO) terms and Ingenuity canonical pathways. Given that these analyses can yield long lists of highly similar annotations, Ingenuity pathway hits were manually curated to show the most highly enriched pathways, as well as, where relevant, less significantly enriched pathways representative of the full diversity of annotations. Similarly, a publically available tool, REVIGO³⁰⁰, was used to filter out highly similar GO terms, leaving highly enriched terms representative of those that were highly similar, in order to display a more complete picture of GO term enrichment in clusters. Notably, clusters 6, 10 and 11 were not significantly enriched for any GO terms or canonical pathways, suggesting that although they showed similar expression patterns to one another, genes in these clusters are not known to have related functions. These genes were therefore not analysed at this stage.

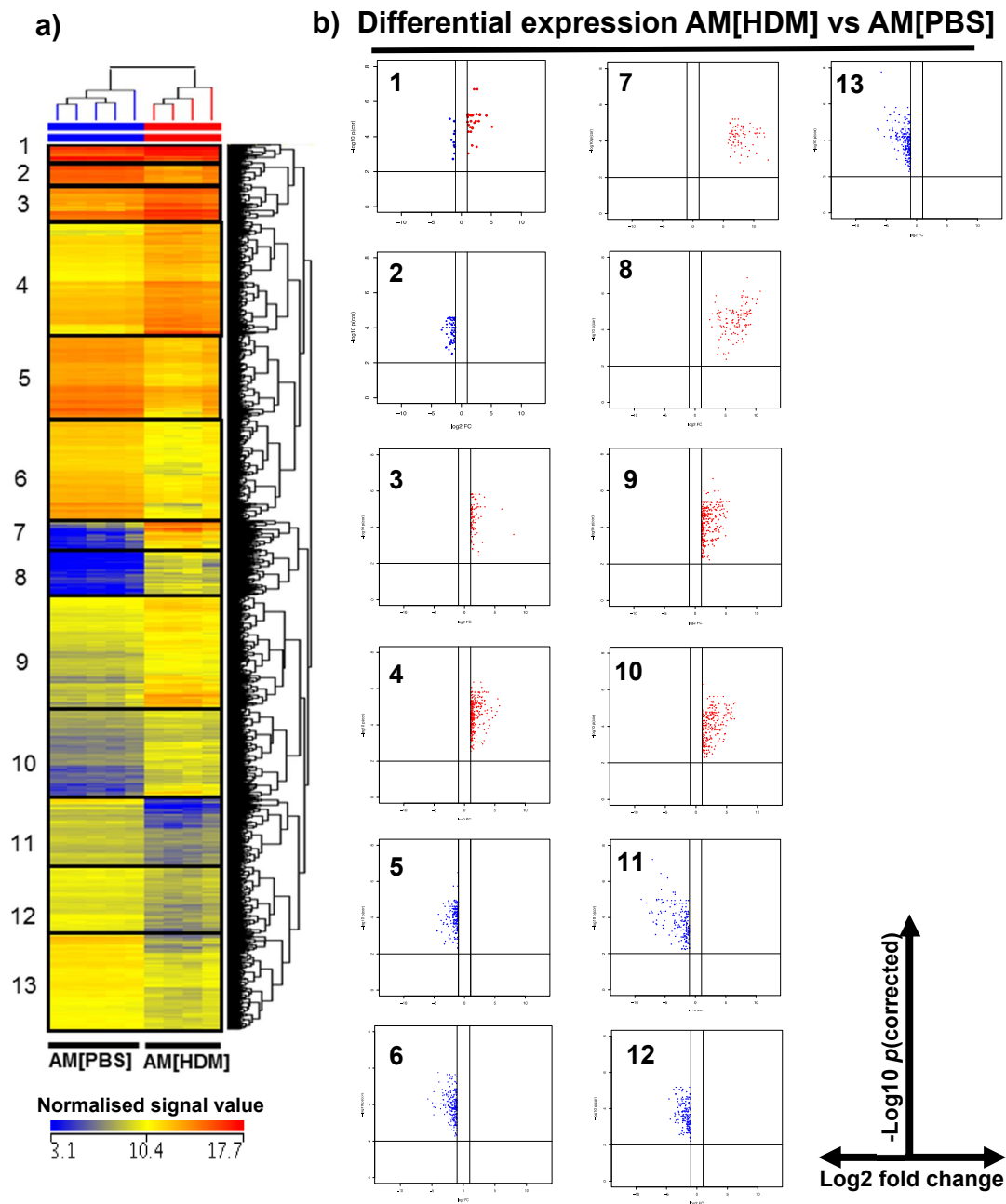


Figure 5.8. Distinct clusters of differential gene expression in AM[HDM]

a) All 2293 genes differentially expressed in AM[HDM] were subjected to unsupervised hierarchical clustering in the AM dataset. 13 major clusters were selected manually from the resultant dendrogram.

b) Volcano plots showing results of differential expression analysis (moderated t -test with Benjamini-Hochberg FDR correction) for each cluster on fixed axes, to allow comparison of the magnitude of differential gene expression between clusters.

Cluster	# genes	Expression in AM[PBS]	Change in AM[HDM]	
1	41	High	Increase	Decrease
2	60	High	Decrease	
3	97	High	Increase	
4	295	Moderate	Increase	
5	218	High	Decrease	
6	264	Moderate	Decrease	
7	73	Below threshold	Large increase	
8	121	Below threshold	Increase	
9	290	Low	Increase	
10	236	Low-below threshold	Increase	
11	173	Low	Decrease	
12	184	Low-moderate	Decrease	
13	241	Moderate	Decrease	

Table 5.2. Patterns of differential gene expression in AM[HDM]

Gene expression clusters depicted in **Fig. 5.8** were classified based on their initial expression (i.e. in AM[PBS]) and their change in AM[HDM], to guide functional interpretation of these clusters.

Cluster 1 was unique in containing both downregulated (10) and upregulated (31) genes, which clustered together based on very high expression in AM[PBS] (**Fig. 5.9a**). This pattern indicated that these genes were constitutively expressed at a high level by resting AM and were differentially expressed, but remained high, in AM[HDM], suggestive of genes related to core macrophage functions. Because expression of only 10 genes in this cluster was significantly decreased in AM[HDM] (**Fig. 5.9a**), functional annotation of downregulated genes was not performed. However, manual inspection of downregulated genes identified a 2.6-fold decrease in expression of apoptotic cell receptor *Axl*. (* in **Fig. 5.9a**). *Axl* is constitutively expressed by AM under control of GM-CSF and binds exposed phosphatidylserine on apoptotic cells via the adapter protein Gas6³⁷¹. AM from asthmatics show diminished expression of *AXL* mRNA³⁷², suggesting that loss of apoptotic cell recognition via *AXL* on AM may be a general feature of AAD, with possible implications for the function of these cells in clearing apoptotic cells arising during inflammation.

GO term and canonical pathway analysis of 31 upregulated genes in cluster 1 revealed enrichment for biological processes related to macrophage function, including tissue remodelling, antigen processing, cell adhesion, phagosome function and responses to stress ('unfolded protein response' and 'endoplasmic reticulum stress' canonical pathways; **Fig. 5.9b**), consistent with the high homeostatic expression of these genes in AM. Examination of the genes underlying these annotations highlighted upregulation of *Itgax* (CD11c; **Fig. 5.9c**), consistent with increased protein expression observed in this model by flow cytometry (see Chapter 3). Also upregulated were *Cd36*, encoding a scavenger receptor required for uptake of fatty acids during alternative macrophage activation³⁷³, and the chitinase-like protein *Chil3* (Ym1; **Fig. 5.9c**), another marker of alternative macrophage activation which has been shown previously to be expressed by naïve AM and upregulated during allergic inflammation^{173,174}. In accordance with the 'phagosome maturation' and 'antigen processing and presentation' annotations of this cluster, several members of the cathepsin family of lysosomal proteases (*Cts* genes) and antigen processing genes such as *B2m* (β 2-microglobulin) were upregulated in cluster 1.

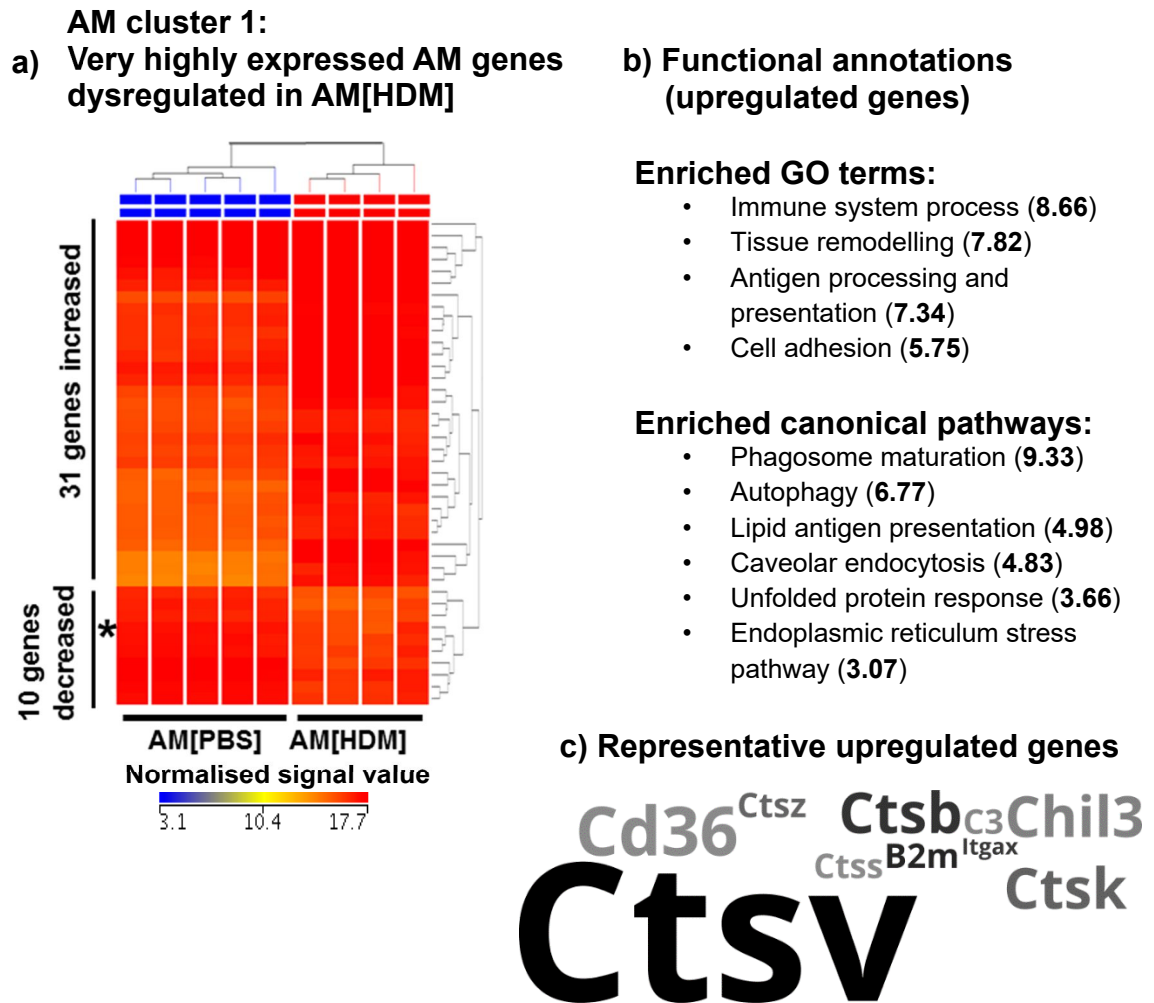


Figure 5.9. AM[HDM] cluster 1: abundant AM genes that are differentially expressed during AAD

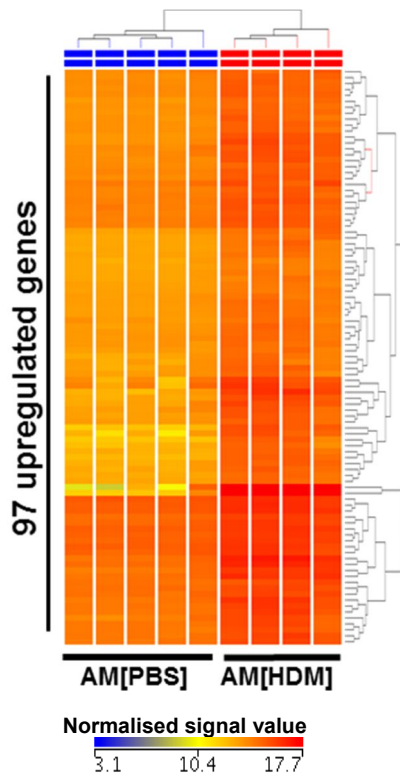
a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 1. * indicates the position of *Axl* among downregulated genes. **b)** A representative list of the most highly enriched GO terms and canonical pathways based on upregulated genes in cluster i (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of upregulated genes responsible for the annotations in **(b)**. Sizes of gene names are proportional to the $\log_2$ fold change in expression in AM[HDM] (range: 1-4.2).

Similarly to cluster 1, cluster 3 contained genes with high resting expression in AM[PBS], all of which were significantly increased in AM[HDM] (**Fig. 5.10a**). Consistent with this similar expression pattern, cluster 2 was also enriched for GO term and canonical pathway annotations broadly related to antigen presentation to T cells ('antigen processing and presentation', T helper cell differentiation, OX40 signalling') and acidification of the phagolysosome during phagocytosis ('metabolism', 'ATP hydrolysis-coupled proton transport', 'phagosome maturation'; **Fig. 5.10b**). Antigen presentation and T cell activation annotations were driven by increased expression of multiple components of the murine H2 complex, including *H2-Aa*, *H2-Ab1* and *H2-Eb1*, which encode components of the MHC-II protein, along with the shared IL-4/IL-13 receptor *Il4ra*, a marker and driver of alternative macrophage activation (**Fig. 5.10c**). Phagolysosome-related annotations were principally driven by increased expression of genes encoding components of the vacuolar type H⁺ ATPase (V-ATPase), which is involved in acidification of lysosomes and phagolysosomes³⁷⁴, cathepsin B (*Ctsb*) and *Lamp2*, a receptor for selective uptakes of proteins into lysosomes (**Fig. 5.10c**).

Taken together, data in clusters 1 and 3 suggest that AM express markers of alternative activation at steady state (e.g. *Cd36*, *Chil3*, *Il4ra*), along with relatively high levels of genes related to their phagocytic and antigen processing functions. These genes are upregulated by AM during HDM-driven AAD, potentially enhancing the ability of AM[HDM] to perform these core macrophage functions.

a) AM Cluster 3:

Increased from high baseline expression in AM[HDM]



b) Functional annotations

Enriched GO terms:

- Antigen processing and presentation (11.6)
- Defence response (7.32)
- Metabolic process (5.29)
- Respiratory burst (5.04)
- ATP hydrolysis-coupled proton transport (5.41)

Enriched canonical pathways:

- Antigen presentation (8.26)
- Autoimmune thyroid disease (5.97)
- T helper cell differentiation (5.91)
- Graft vs host disease (5.85)
- Phagosome maturation (5.17)
- B cell development (4.98)
- Calcium-induced T cell apoptosis (4.92)
- OX40 signalling (4.65)
- IL-4 signaling (4.16)
- Th2 pathway (4.16)
- Nur77 signaling in T cells (3.86)
- Type I diabetes signalling (3.74)

c) Representative upregulated genes

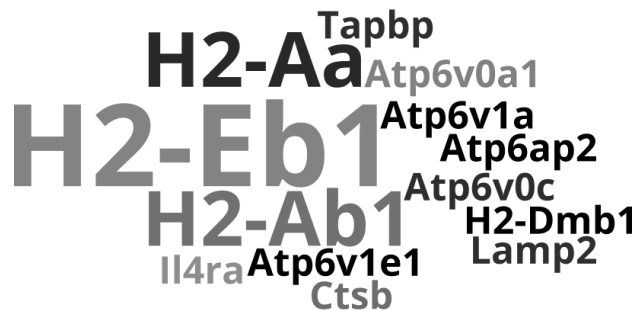


Figure 5.10. AM[HDM] cluster 3: upregulated genes related to antigen presentation and phagosome maturation

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 3. **b)** A representative list of highly enriched GO terms and canonical pathways in cluster 3 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative upregulated genes driving the annotations in **(b)**. Sizes of gene names are proportional to the log2 fold increase in AM[HDM] (range:1.1-3).

5.6.2 AM express genes associated with leukocyte recruitment, tissue repair and oxidative metabolism during allergic airway disease

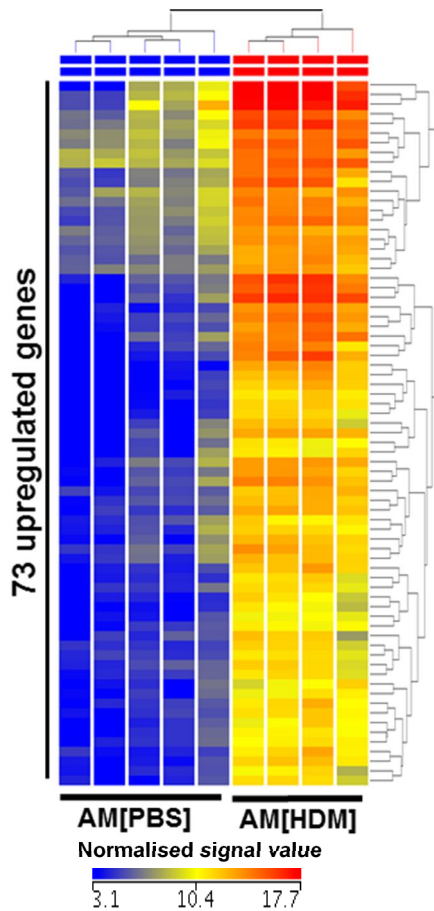
Additional clusters revealed genes with low to moderate expression in AM from PBS control mice, expression of which was significantly elevated in AM[HDM]. These expression patterns were suggestive of gene signatures strongly induced in AM[HDM] from relatively low initial levels and were likely to reflect substantial functional changes to AM during AAD.

Genes in clusters 7 and 8 showed the highest fold increases of all genes in AM[HDM] (**Fig. 5.8**), due to their expression generally being close to or below the lower threshold for gene expression in AM[PBS] (**Fig. 5.8a**). Cluster 7 contained the most strongly upregulated genes in AM[HDM] (**Fig. 5.11a**), which were highly enriched for GO terms and canonical pathways related to complement system, tissue repair ('response to wounding'), and chemotactic cell recruitment ('locomotion', 'granulocyte/agranulocyte adhesion and diapedesis'; **Fig. 5.11b**). Enrichment of the 'complement system' canonical pathway was dependent on large fold increases in expression of the classical complement activation components *C1qa*, *C1qb* and *C1qc*, while cell recruitment annotations were driven by high levels of expression of a number of chemokines, in particular the eosinophil chemoattractant *Ccl24* and monocyte and T cell chemokine *Ccl8* (**Fig. 5.11c**). Notably, a number of genes in cluster 7 were reported IL-4 targets (**Fig. 5.11d**), including *Arg1* (arginase) and *Retnla* (RELM α), suggesting that the major changes to gene expression in AM[HDM] were at least partially IL-4-driven. However, in addition to *Arg1*, AM[HDM] also displayed significantly increased expression of *Nos2* (iNOS; **Fig. 5.11c**), a classical macrophage activation gene, the product of which functionally antagonises Arginase in macrophages by competition for cellular arginine³⁷⁵ and induction of which is suppressed by IL-4 in BMDM¹⁶⁸. These data support a model in which AM integrate signals from IL-4 with those from other cytokines in the airway milieu during HDM-driven AAD.

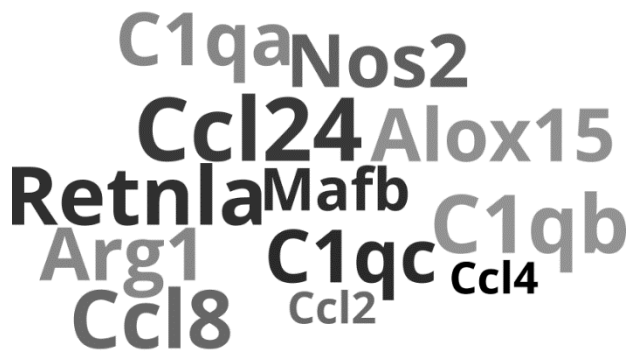
Genes in cluster 8 showed smaller fold increases than those in cluster 7, but were still among the most substantially induced AM[HDM] genes (**Fig. 5.12a**). Like cluster 7, these genes were enriched for the 'granulocyte adhesion and diapedesis' canonical pathway (**Fig. 5.12b**), in part due to upregulation of the monocyte chemokine *Ccl7*⁵⁸ and the CCL24 receptor gene *Ccr3* (**Fig. 5.12c**), expression of which has been observed previously on a subset of CD11c⁺ pulmonary macrophages during AAD³⁷⁶. This suggests that AM increase expression of both chemokines and their receptors during HDM-driven AAD and that chemokine signalling is likely to be involved in functions of AM in the allergic lung. Cluster 8 was also enriched for tissue repair genes ('hepatic fibrosis' canonical pathway), owing to increased expression of genes such as *Edn1* (endothelin 1), which has been implicated in airway remodelling during AAD¹³⁷, the pro-fibrotic mediator *Pdgfa* (platelet-derived growth factor-A) and *Sdc1* (syndecan-1), a cell surface proteoglycan implicated in tissue repair³⁷⁷ as well as protective sequestration of chemokines during AAD³⁷⁸. Together these data suggest that AM may be an active source of mediators involved in the tissue remodelling response to chronic allergen exposure.

a) AM Cluster 7:

Strongly increased from little to no baseline expression in AM[HDM]



c) Representative upregulated genes



b) Functional annotations

Enriched GO terms:

- Regulation of immune system process (14.68)
- Response to wounding (8.68)
- Locomotion (7.35)
- Developmental process (5.38)
- Positive regulation of cellular component movement (12.14)

Enriched canonical pathways:

- Granulocyte adhesion and diapedesis (8.11)
- Agranulocyte adhesion and diapedesis (6.65)
- Complement system (5.12)
- Atherosclerosis signalling (4.11)
- Inhibition of matrix metalloproteases (3.41)
- Osteoarthritis pathway (2.96)
- Leukocyte extravasation signalling (2.94)
- Role of tissue factor in cancer (2.93)
- Superpathway of citrulline metabolism[†] (2.89)

d) IL-4-inducible genes

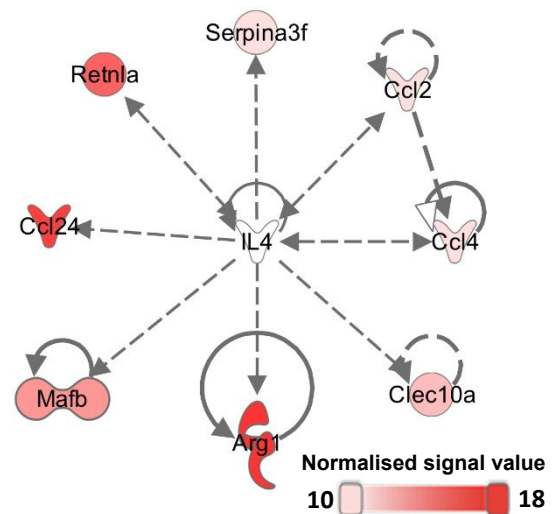
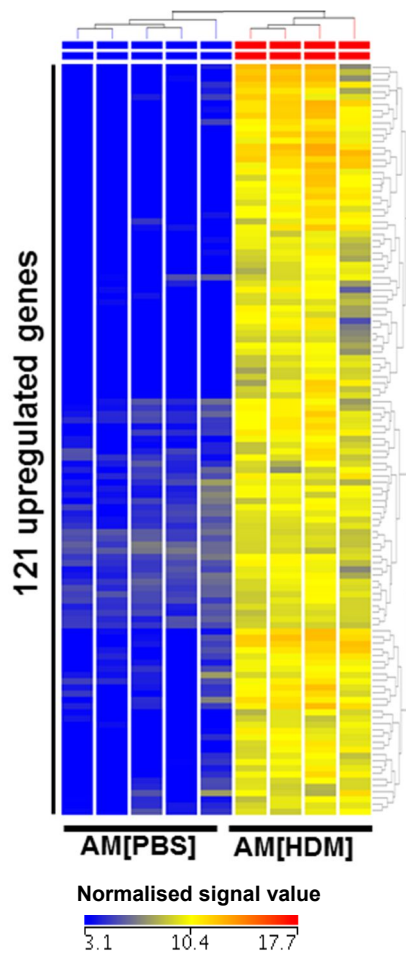


Figure 5.11 AM[HDM] cluster 7: upregulated IL-4-inducible and complement pathway genes

a) Sub-tree of heat map shown in Fig. 5.8, showing all genes in cluster 7. **b)** A representative list of highly enriched GO terms and canonical pathways in the cluster (all $p \leq 0.01$, Fisher's exact test). [†] Note that the 'Superpathway of citrulline metabolism' pathway was enriched on the basis of only 2 genes (*Arg1* and *Nos2*). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative upregulated genes driving the annotations in (b). Sizes of gene names are proportional to the $\log_2$ fold increase in AM[HDM] (range:6.1-12.34). **d)** Interaction map of genes in cluster 7 reportedly induced by IL-4, based on data in the Ingenuity Knowledge Base. Arrows indicate positive regulatory relationships between genes. Colour coding indicates the normalised expression of genes in AM[HDM].

a) AM Cluster 8:

Strongly increased from little to no baseline expression in AM[HDM]



b) Functional annotations

Enriched GO terms:

- Regulation of multicellular organismal process (6.68)
- Regulation of immune system process (6.35)
- Defence response (6.32)
- Positive regulation of phosphorylation (5.81)

Enriched canonical pathways:

- Hepatic fibrosis (4.92)
- Th2 pathway (4.74)
- Atherosclerosis signalling (4.17)
- Th1 and Th2 cell activation pathway (4.13)
- Granulocyte adhesion and diapedesis (3.44)
- TREM1 signalling (3.09)
- MAPK signalling in pathogenesis of influenza (3.09)

c) Representative upregulated genes

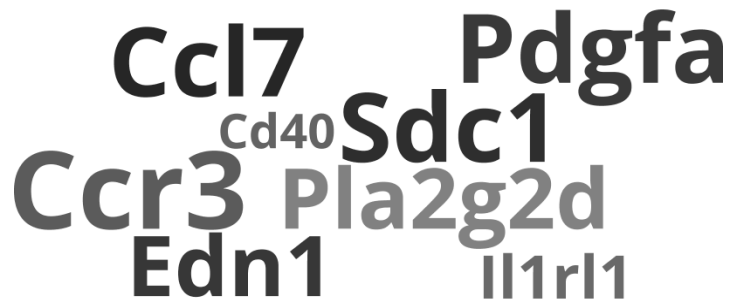


Figure 5.12 AM[HDM] cluster 8: upregulated immune activation and tissue repair genes

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 8. **b)** A representative list of highly enriched GO terms and canonical pathways in cluster 8 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative upregulated genes driving the annotations in **(b)**. Sizes of gene names are proportional to the log2 fold increase in AM[HDM] (range: 4.7-9).

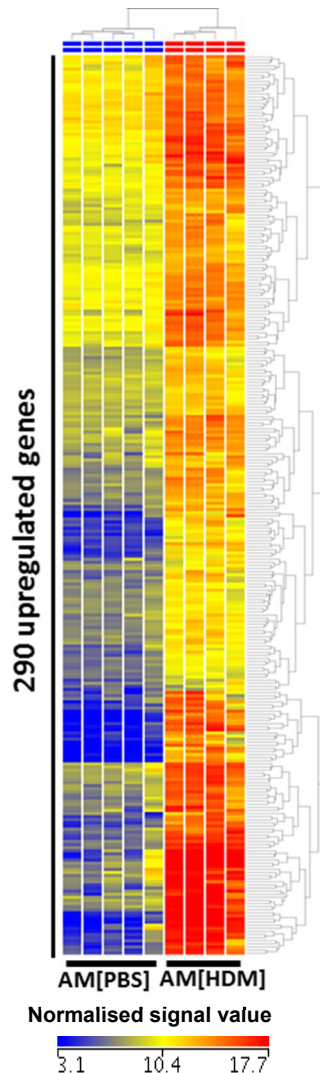
Cluster 8 also heavily featured functional annotations related to activation of T helper cells (e.g. GO term: 'regulation of immune system process', canonical pathway: 'Th2 pathway'; **Fig. 5.12b**), due to increased expression of co-stimulatory molecule *Cd40* along with the IL-33 receptor *Il1rl1* (ST2), which mediates signals promoting Th2 cell generation *in vivo*³⁷⁹ and has been reported to drive alternative activation of AM during type two airway inflammation²⁸. These data suggest that AM, which are poor antigen presenting cells at steady state¹⁵², may become more capable of locally providing activation signals T cells during AAD.

Cluster 9 highlighted another set of genes with low to moderate baseline expression that was induced in AM[HDM] (**Fig. 5.13a**). The most significantly enriched GO terms in this cluster were related to cytokine responses ('defence response', 'response to cytokine', 'response to stress'; **Fig. 5.13b**), which were driven by presence of a large number of interferon-stimulated genes (ISGs), including *Gbp4*, *Gbp6*, *Ifi44*, *Ifitm2*, *Isg15*, *Rsad2*, *Mx1* and *Oas1* (**Fig. 5.13c**). These data suggest that AM receive either autocrine or paracrine type I or III interferon signalling during AAD, as has recently been demonstrated for dendritic cells during type 2 inflammation³⁸⁰. Canonical pathway analysis was dominated by a number of related pathways involved in degradation of small molecules (e.g. 'ethanol degradation', 'tryptophan degradation'; **Fig. 5.13b**), due to upregulation of a number of aldehyde dehydrogenase genes (*Aldh1a2*, *Aldh1b1*, *Aldh7a1*; **Fig. 5.13c**), suggesting that the stress response occurring in AM during HDM-driven AAD may be accompanied by increased metabolism via these enzymes. Increased *Aldh1a2* expression is of interest, since this gene encodes the retinoic acid synthesis enzyme retinal dehydrogenase 2 (RALDH2), which is widely implicated in immune regulation by macrophages^{153,381,382}. Increased *Aldh1a2* expression in AM[HDM] may therefore indicate a potential immunoregulatory mechanism of AM during established AAD.

AM cluster 4 consisted of a large number of genes (295) with moderate expression in AM from PBS control mice and showing relatively subtle increases AM[HDM] (**Fig. 5.14a**). However, these genes were very highly enriched for mitochondrial electron transport chain (ETC) and oxidative phosphorylation functions (**Fig. 5.14b**), strongly suggesting increased usage of oxidative metabolism in AM[HDM]. These annotations were driven by expression of components of the F-ATPase (*Atp5* genes), which generates ATP by proton gradient-driven rotation at the end of the mitochondrial ETC³⁸³, along with the majority of components of ETC complexes I (e.g. NADH-ubiquinone reductase genes *Ndufa1* and *Ndufb5*), II (e.g. succinate dehydrogenase subunit genes *Sdhb* and *Sdhc*), III (e.g. ubiquinone-cytochrome C reductase genes *Uqcr10* and *Uqcr11*) and IV (e.g. cytochrome C oxidase subunit genes *Cox5b* and *Cox7b*; **Fig. 5.14c**). Overlaying these gene expression data with the subcellular locations of this pathway clearly showed upregulation of multiple genes in all mitochondrial ETC complexes (**Fig. 5.15**). Gene expression data therefore strongly suggested that activation of AM during HDM-driven AAD was accompanied by increased usage of oxidative phosphorylation.

a) AM Cluster 9:

Increased from low to moderate baseline expression in AM[HDM]



b) Functional annotations

Enriched GO terms:

- Defence response (6.56)
- Response to cytokine (5.56)
- Response to biotic stimulus (5.57)
- Response to stress (5.58)

Enriched canonical pathways:

- Ethanol degradation (4.12)
- Histamine degradation (4.06)
- Noradrenaline/adrenaline degradation (3.92)
- Fatty acid β -oxidation (3.76)
- Putrescine degradation (3.68)
- Tryptophan degradation via tryptamine (3.52)
- TWEAK signalling (3.86)
- TNFR1 signalling (3.20)

c) Representative upregulated genes

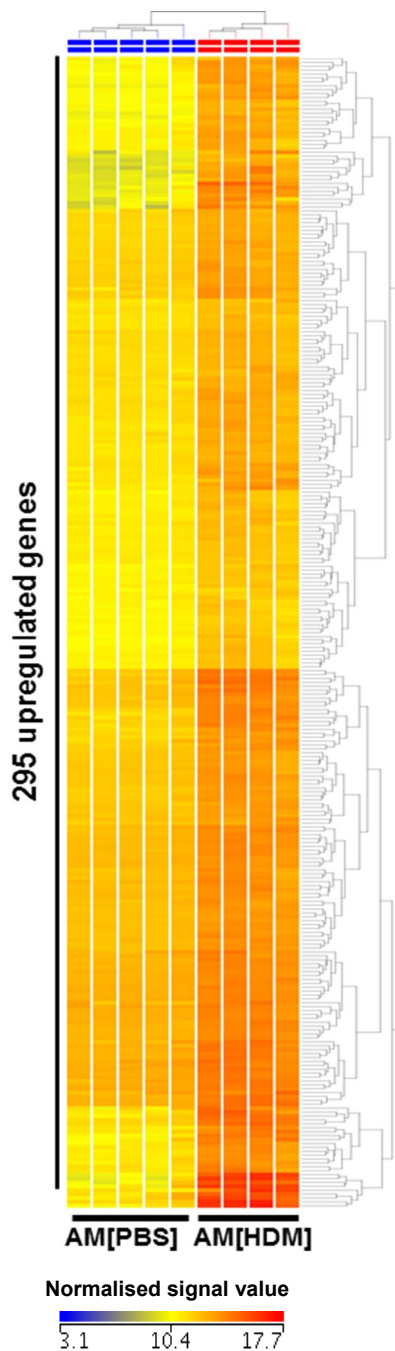


Figure 5.13. AM[HDM] cluster 9: upregulated interferon-stimulated and aldehyde dehydrogenase genes

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 9. **b)** A representative list of the most highly enriched GO terms and canonical pathways among genes in the cluster (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of genes upregulated in AM[HDM] representative of functional annotations in **(b)**. Sizes of gene names are proportional to the $\log_2$ fold increase in AM[HDM] (range:1.2-4.57).

a) AM Cluster 4:

Increased from moderate baseline expression in AM[HDM]



b) Functional annotations

Enriched GO terms:

- Electron transport chain (14.67)
- Hydrogen ion transmembrane transport (15.38)
- Generation of precursor metabolites and energy (11.93)
- Immune response (5.04)

Enriched canonical pathways:

- Oxidative phosphorylation (24.30)
- Mitochondrial dysfunction (19.90)
- Interferon signalling (5.17)
- Antigen presentation (4.21)
- Type I diabetes signaling (3.74)

c) Representative upregulated genes

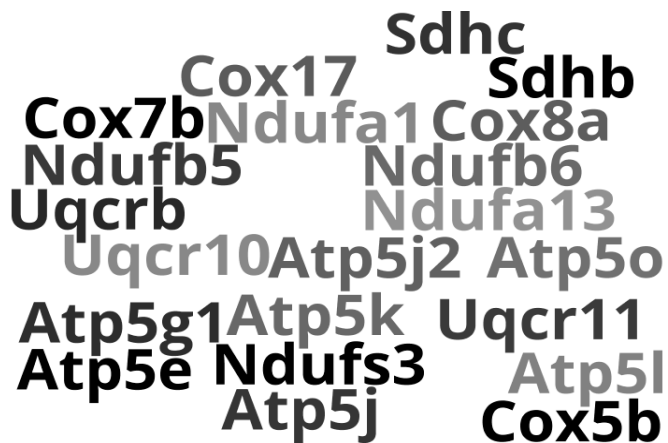


Figure 5.14. AM[HDM] cluster 4: heightened oxidative phosphorylation gene expression

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 4. b) A representative list of highly enriched GO terms and canonical pathways in cluster 4 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. c) Word cloud visualisation of representative upregulated genes driving the annotations in (b). Sizes of gene names are proportional to the $\log_2$ fold increase in AM[HDM] (range:1-1.5).

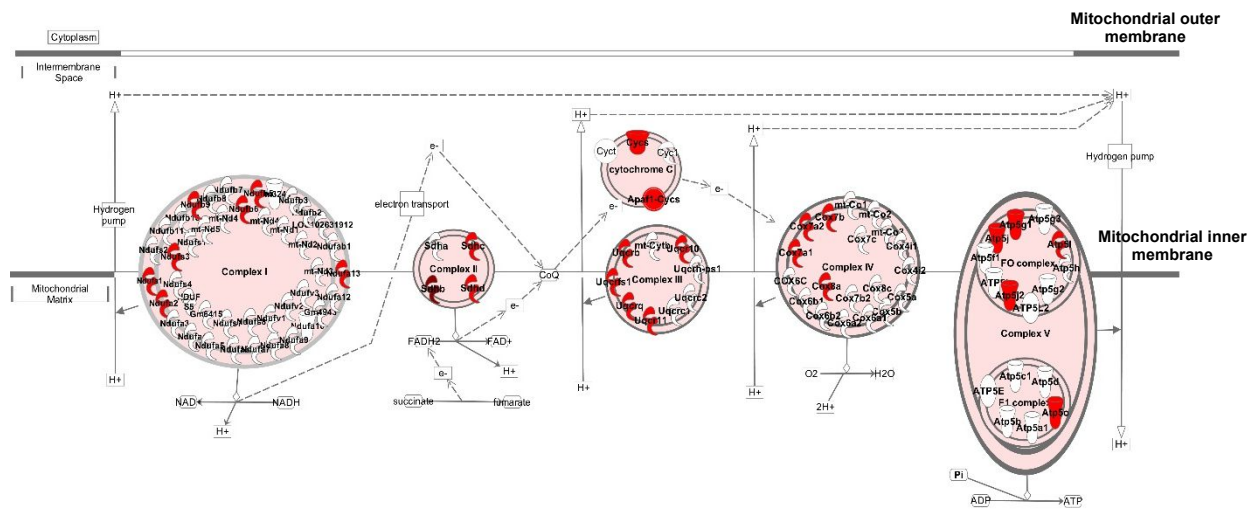


Figure 5.15. Increased expression of oxidative phosphorylation pathway genes in AM[HDM]
Diagram of the ‘oxidative phosphorylation’ canonical pathway from the Ingenuity Knowledge Base, indicating the subcellular locations of gene products. Complexes with upregulated components are coloured pink and individual molecules are coloured red if significantly upregulated in AM[HDM] ($p < 0.01$, $FC \geq 2$).

5.6.3 Decreased expression of a subset of genes involved in pathogen sensing and cytoskeletal dynamics by AM during allergic airway disease

Among clusters of genes downregulated in AM[HDM], clusters 2 and 5 were similar in having initially high expression in AM[PBS] (**Figs. 5.8, 5.16, 5.17**), suggesting that these genes are relatively abundant in naïve AM at steady state and are reduced during activation in HDM-driven AAD. Genes in these clusters could therefore provide insights into potential losses of function in AM[HDM].

Cluster 2 was highly enriched for GO terms and canonical pathways related to dynamics and interactions of integrins, membrane proteins and the actin cytoskeleton, including 'cell junction assembly and organisation' and 'regulation of cellular mechanics by calpain' (**Fig. 5.16a**). Related genes included *Tln1* (Talin-1) and *Vasp* (vasodilator-stimulated phosphoprotein, VASP), which regulate actin filament assembly downstream of integrins and other membrane proteins³⁸⁴, and the DOCK family guanine nucleotide exchange factors (GEFs) *Dock8* and *Dock10*, which activate Rac and/or CDC42 GTPases to perform diverse cellular functions via regulation of actin dynamics³⁸⁵ (**Fig. 5.16c**). Similarly, cluster 5 was enriched for genes related suggestive to dynamics of subcellular components (GO terms 'organelle organisation' and 'localisation', **Fig. 5.17b**), including DOCK family members *Dock4* and *Dock11*, the Rho and Rac GEF *Vav2*³⁸⁶ $\alpha 4$ integrin (*Itga4*) and microtubule actin cross-linking factor 1 (*Macf1*; **Fig. 5.17c**). Together the data in these clusters support widespread reduction in expression of genes involved in cytoskeletal dynamics in AM[HDM], which may have implications for macrophage functions reliant on cytoskeletal remodelling, such as phagocytosis³⁸⁷.

Clusters 2 and 5 also highlighted decreases in expression of constitutively expressed immunological genes in AM during HDM-driven AAD. Consistent with flow cytometry data described in Chapter 3, cluster 2 showed *Siglec5* (Siglec F) to be significantly downregulated in AM[HDM], along with the related sialic acid-binding lectin, *Siglec1* (CD169; **Fig. 5.16c**). Also downregulated in cluster 2 was *Tlr2* (**Fig. 5.16c**), as reported in a similar model of AAD¹⁷⁹, highlighting consistency between this and previous work. Cluster 5 demonstrated downregulation of scavenger receptor *Marco* and both formyl peptide receptors, *Fpr1* and *Fpr2* (**Fig. 5.17c**), which recognise diverse bacterial and endogenous ligands and trigger inflammatory responses³⁸⁸. The pro-inflammatory cytokine *Il18* was also downregulated in AM[HDM] (**Fig. 5.17c**, contributing to the enrichment of the 'Th1' canonical pathway among downregulated genes in cluster 5 (**Fig. 5.17b**). Thus, despite displaying marked overall increases in pro-inflammatory gene expression, AM downregulated a distinct subset of pattern recognition and immune response genes during HDM-driven AAD.

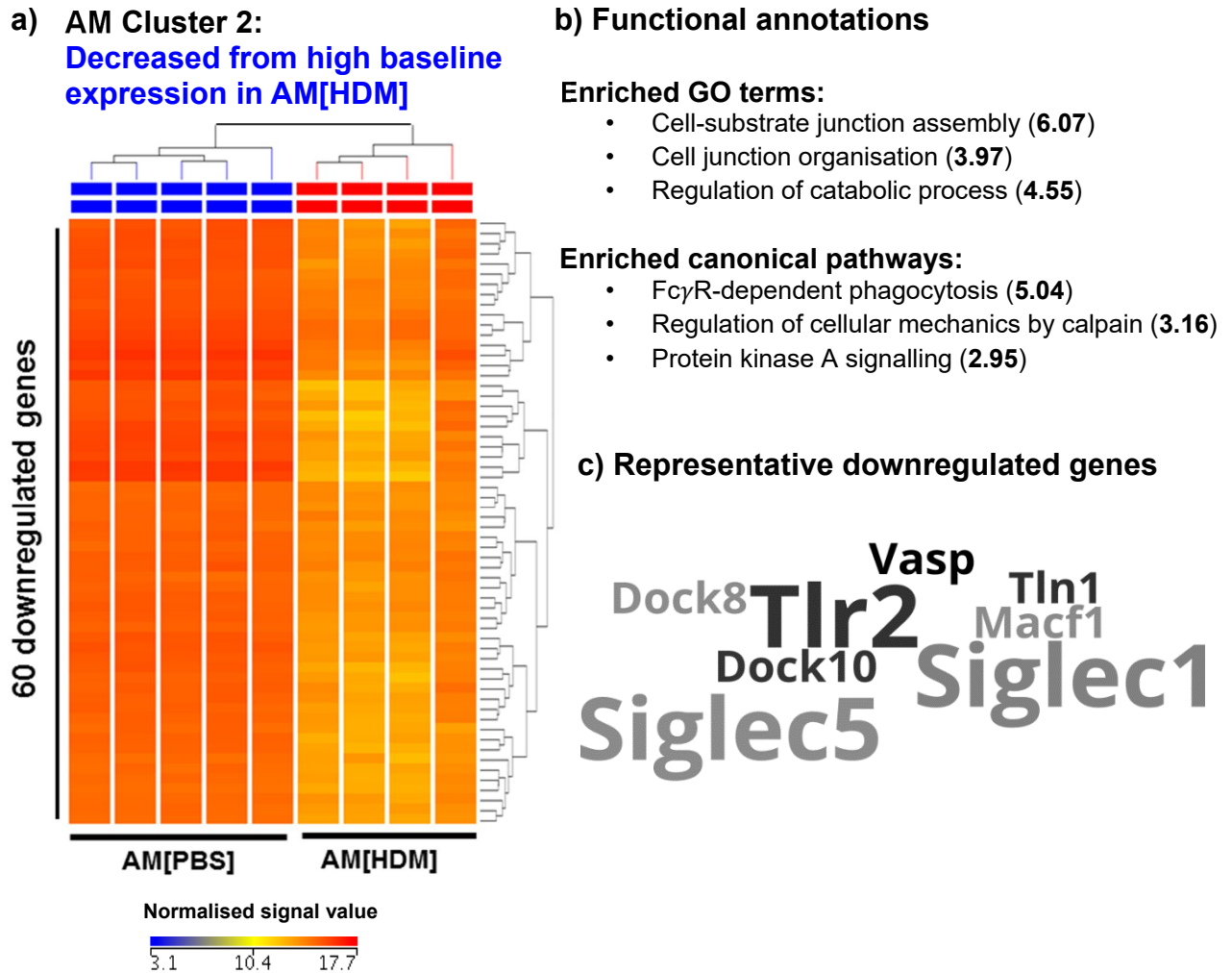
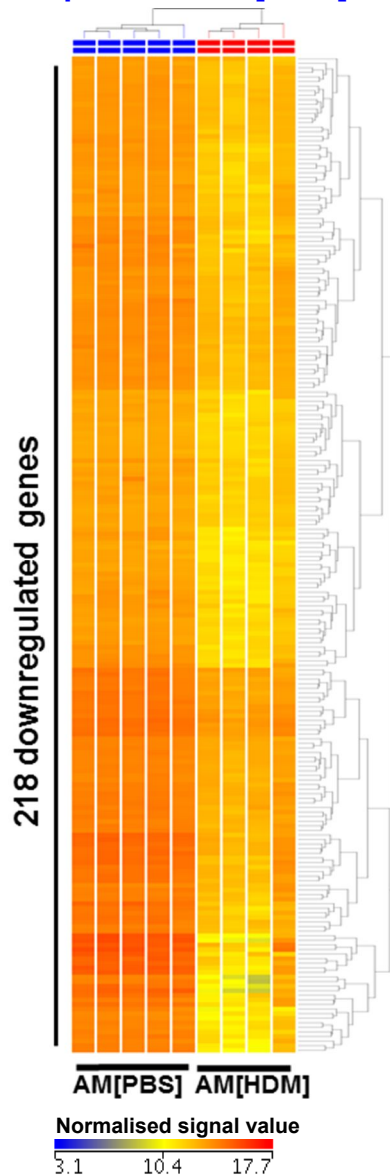


Figure 5.16. AM[HDM] cluster 2: downregulation of *Tlr2*, sialic acid-binding lectins and genes related to actin dynamics

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 2. **b)** A representative list of highly enriched GO terms and canonical pathways in cluster 2 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative downregulated genes driving the annotations in **(b)**, along with additional genes of interest: *Siglec1*, *Siglec5* and *Tlr2*. Sizes of gene names are proportional to the $\log_2$ fold decrease in AM[HDM] (range:1-2.5).

a) AM Cluster 5:

Decreased from high baseline
expression AM[HDM]



b) Functional annotations

Enriched GO terms:

- Organelle organisation (9.78)
- Regulation of macromolecule metabolic process (9.25)
- Cell cycle (6.56)
- Localisation (5.29)

Enriched canonical pathways:

- ERK/MAPK signalling (4.85)
- Gαq signalling (4.82)
- Th1 pathway (4.07)
- Tec kinase signalling (3.91)
- Leukocyte extravasation signalling (3.86)
- B cell receptor signalling (3.62)
- T cell receptor signalling (3.59)
- Glucocorticoid receptor signalling (3.41)
- fMLP signalling in neutrophils (3.31)

c) Representative downregulated genes



Figure 5.17. AM[HDM] cluster 5: downregulated genes related to actin dynamics and innate immunity

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 5. b) A representative list of highly enriched GO terms and canonical pathways in cluster 5 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. c) Word cloud visualisation of representative downregulated genes driving the annotations in (b). Sizes of gene names are proportional to the $\log_2$ fold decrease in AM[HDM] (range:1.1-4.5).

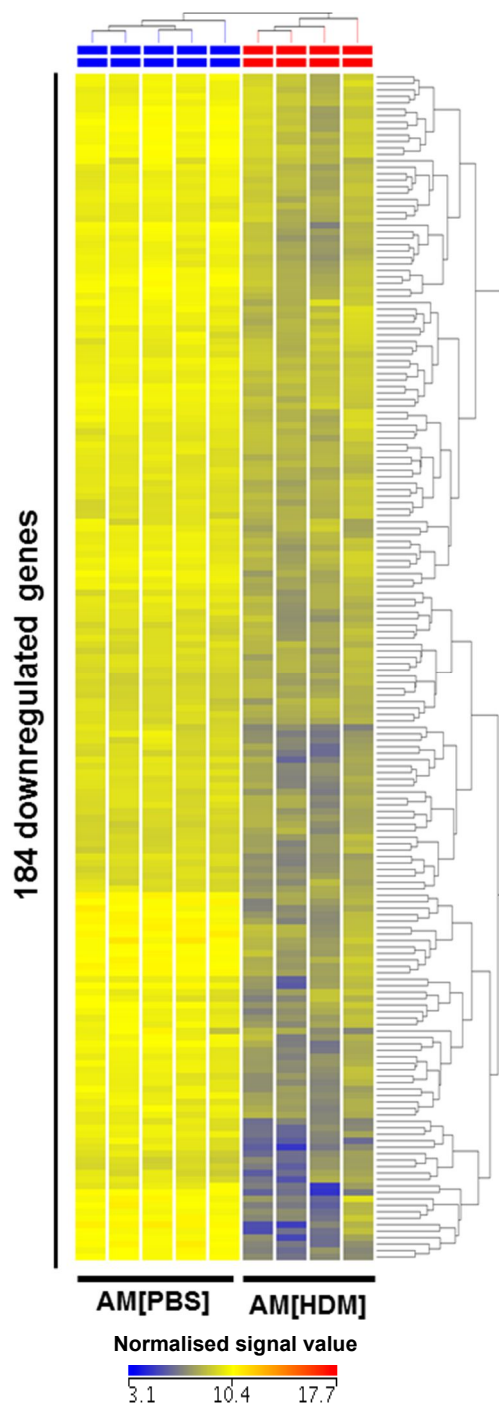
5.6.4 Evidence of decreased cell cycle and DNA repair capacity in AM[HDM], along with decreased expression of a number of innate immune response genes

Gene expression clusters 12 and 13 were among the most markedly downregulated in AM[HDM], consisting of genes expressed at moderate levels in AM[PBS] but decreased during HDM-driven AAD (**Figs. 5.8, 5.18, 5.19**). This pattern is suggestive of reduction in homeostatic functions during allergic inflammation. Genes in cluster 12 were highly enriched for GO terms and canonical pathways related to DNA replication and repair (**Fig. 5.18b**), owing to the presence of cell cycle and DNA replication genes such as minichromosome maintenance 10 replication initiation factor (*Mcm10*), cyclin E2 (*Ccne2*) and the transcription factor cell-division cycle-associated 2 and 7 (*Cdca2*, *Cdca7*), along with genes involved in repair of DNA crosslinks and double-strand breaks, including *Fancd2*, *Rad51*, *Rad52* and *Brca2*³⁸⁹ (**Fig. 5.18c**).

Cluster 13 showed related functional annotations to cluster 12, although related more to the cell division cycle than DNA damage repair (**Fig. 5.19b**). Annotations included the 'cell cycle' and 'cell division' GO terms and the 'mitotic roles of polo-like kinase (PLK)' canonical pathway and were driven by the presence of genes such as cyclin-dependent kinase 6 (*Cdk6*), Cyclins B1 and B2 (*Ccnb1* and *Ccnb2*) and centrosome protein E (*Cenpe*) which regulate distinct phases of the cell division cycle³⁹⁰ (**Fig. 5.19c**). DNA polymerase α 1 (*Pola1*), which is essential for initiation of DNA replication³⁹¹, was also present in cluster 13 (**Fig. 5.19c**). Collectively, gene expression in clusters 12 and 13 suggested that the homeostatic cell cycling and associated DNA metabolism known to occur in AM⁶¹ at steady state may be reduced during allergic airway inflammation.

Aside from cell cycle-related genes, cluster 13 also highlighted a number of downregulated immune response genes (**Fig. 5.19**), as described above for clusters 2 and 5. Immunological genes were much less enriched within this cluster compared to cell cycle-related genes, so did not lead to annotation of cluster 13 with immune-related GO terms or canonical pathways. However, a number of genes of interest were downregulated in this cluster, including the myeloid cell activating receptor *Trem1*³⁹², the membrane-bound and soluble monocyte chemokine *Cx3cl1*³⁹³ and the pro-inflammatory cytokine *Tnf* (**Fig. 5.19b**), which was shown previously in this thesis to be potently induced in AM by LPS (see Chapter 4). Also downregulated were the genes *Trim56* and *Trim65*, which encode tripartite motif family ubiquitin ligases that promote innate immune responses to viral stimuli^{394,395}. Therefore, these data support those in clusters 2 and 5 in showing that, despite substantial overall upregulation of pro-inflammatory genes, the AM[HDM] transcriptome represents a distinct state in which a number of innate immune mediators are in fact downregulated.

a) **AM Cluster 12:**
Decreased from moderate baseline
expression in AM[HDM]



b) **Functional annotations**

Enriched GO terms:

- Cell cycle (18.61)
- Nuclear division (15.88)
- DNA repair (9.16)
- Heterocycle metabolic process (9.25)

Enriched canonical pathways:

- Role of BRCA1 in DNA damage response (4.07)
- DNA repair by homologous recombination (3.58)

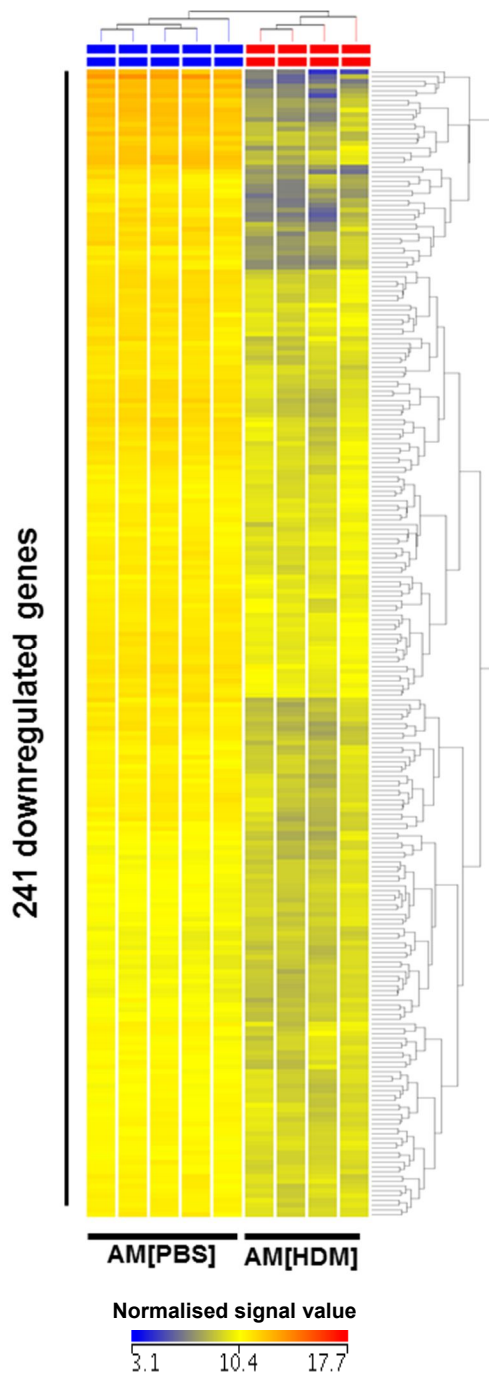
c) **Representative downregulated genes**



Figure 5.18. AM[HDM] cluster 12: downregulated DNA repair and cell cycle genes

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 12. b) A representative list of highly enriched GO terms and canonical pathways in cluster 12 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. c) Word cloud visualisation of representative downregulated genes driving the annotations in (b). Sizes of gene names are proportional to the $\log_2$ fold decrease in AM[HDM] (range:1-2.3).

a) **AM Cluster 13:**
Decreased from moderate baseline
expression in AM[HDM]



b) **Functional annotations**

Enriched GO terms:

- Cell cycle (11.57)
- Protein localisation (17.30)
- Macromolecule localisation (18.70)
- Cellular component organisation or biogenesis (24.05)

Enriched canonical pathways:

- Mitotic roles of polo-like kinase (4.82)
- Cell cycle control of chromosomal replication (3.44)

c) **Representative downregulated genes**



Figure 5.19. AM[HDM] cluster 13: downregulated cell cycle and innate immune response genes

a) Sub-tree of heat map shown in **Fig. 5.8**, showing all genes in cluster 13. b) A representative list of highly enriched GO terms and canonical pathways in cluster 13 (all $p \leq 0.01$, Fisher's exact test). Numbers in brackets are $-\log_{10} p$ values of enrichment. c) Word cloud visualisation of representative downregulated genes driving the annotations in (b) and additional genes of interest *Cx3cl1*, *Trem1*, *Trim56*, *Trim65* and *Tnf*. Sizes of gene names are proportional to the $\log_2$ fold decrease in AM[HDM] (range:1.2-5.2).

5.6.5 Functional annotation of total differentially expressed genes in AM[HDM] yields comparable results to cluster-based approach

A potential caveat of functional annotation of distinct gene expression clusters is that genes with similar functions but different patterns of up- or downregulation will be analysed separately, which may cause certain pathways to be missed. This was exemplified by the detection of downregulated innate immune response-related genes in AM[HDM] across 4 distinct expression clusters in the above analysis (**Figs. 5.16-5.19**), as well as the lack of significant enrichment of functional annotations in 3 of the 13 clusters defined from the AM dataset. To ensure that the cluster-based analysis had not substantially affected results, functional annotations were repeated on entire lists of up- and downregulated genes.

Global analysis of upregulated genes revealed enrichment of the same highly overrepresented GO terms assigned to upregulated gene clusters (**Fig. 5.20a**). These included terms related to inflammation and cell recruitment ('defence response', 'immune system process', 'localisation' and 'locomotion') and oxidative metabolism ('hydrogen transport' and 'electron transport chain'). The most highly enriched canonical pathways from analysis of individual upregulated gene clusters were also prominent hits when all upregulated genes were analysed together (**Fig. 5.20b**). 'Oxidative phosphorylation' and 'antigen presentation' were the most highly enriched canonical pathways, along with pathways related to cell recruitment ('granulocyte/agranulocyte adhesion and diapedesis'), tissue repair ('hepatic fibrosis', 'atherosclerosis signalling'), phagosome maturation, complement activation and response to type I/III interferons (**Fig. 5.20b**).

Analysis of genes downregulated in AM[HDM] also showed agreement between functional annotation of total genes and individual clusters. Annotation of all genes downregulated in AM[HDM] demonstrated marked enrichment of GO terms and canonical pathways associated with cell cycling and DNA replication and repair (**Fig. 5.20c-d**), as observed in analysis of clusters 12 and 13. 'Cell cycle', 'DNA replication' and 'regulation of phosphate metabolic process' were the major GO terms representative of nodes of highly similar terms following reductive analysis (**Fig. 5.20c**). Similarly, 'control of chromosomal replication' and 'cyclins and cell cycle regulation' were among the most highly enriched canonical pathways, owing to downregulation of DNA repair genes such as DNA polymerase E (*Pole*) and topoisomerase 2a (*Top2a*) and a number of cyclin and cyclin-dependent kinase genes (**Fig. 5.20d**). Canonical pathways also included 'TREM1 signalling' and 'PRRs in recognition of bacteria/viruses', driven by downregulation of innate immune response genes also identified by cluster-based analysis, including *Tlr2*, *Tlr5* and *Trem1* (**Fig. 5.20d**). Consistent with the decreased expression of genes related to actin dynamics observed in clusters 2 and 5, total downregulated genes were enriched for the 'Fc γ R-mediated phagocytosis' canonical pathway due to decreased expression of the actin remodelling-related genes *Rac3*, *Tln1*, *Vav2* and *Vasp*, as well as signalling kinase genes such as haematopoietic cell kinase (*Hck*; **Fig. 5.20d**), suggesting that decreased expression of these genes may impact the phagocytic capacity of AM[HDM].

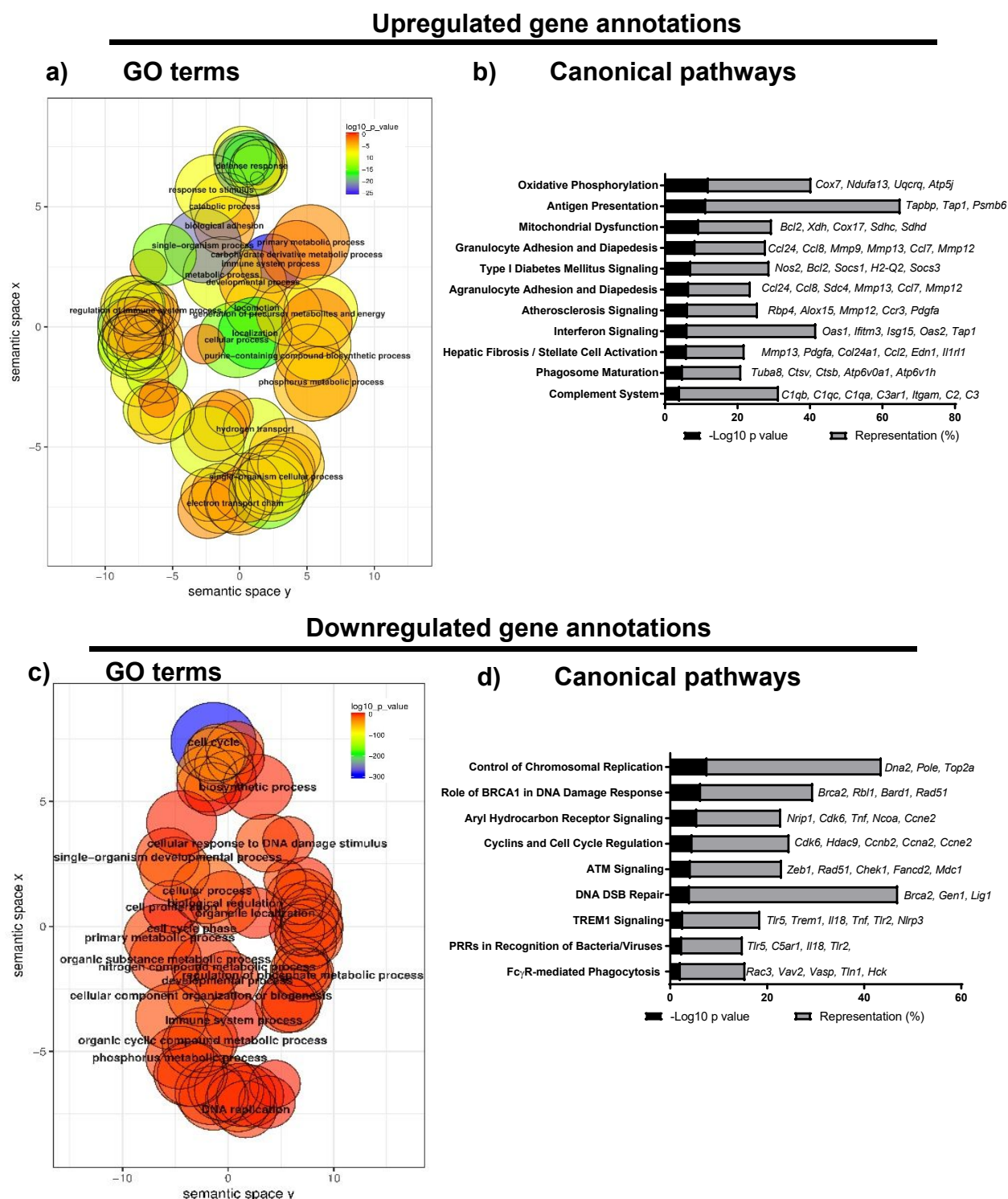


Figure 5.20. Functional annotation of total genes differentially expressed in AM[HDM]

Upregulated (1143) and downregulated (1150) genes in AM[HDM] were subjected GO enrichment analysis and Ingenuity Pathway Analysis. **a)** REViGO-filtered GO terms enriched among upregulated genes. **b)** Canonical pathways enriched in upregulated genes. **c)** REViGO-filtered GO terms among downregulated genes. **d)** Canonical pathways enriched in downregulated genes. REViGO plots in **(a)** and **(c)** show the most significantly enriched biological process GO terms, filtered to remove highly similar redundant terms and clustered in semantic space based on similarity and colour-coded based on transformed p values. Sizes of circles are proportional to the size (i.e. generality) of the GO term. Bar plots in **(b)** and **(d)** show transformed Fisher's exact test p values and percentage of genes in pathways present in the query gene list (i.e. % coverage), with representative genes displaying the largest fold changes in AM[HDM] listed adjacent to bars.

Overall, annotation of differentially expressed genes suggested that oxidative phosphorylation, chemokine production, antigen presentation, phagosome maturation and tissue repair functions were increased in AM[HDM], accompanied by decreased cell cycling, reduced expression of a subset of innate immune response genes and potentially defective actin reorganisation. Implementation of a hierarchical clustering approach had the added benefit of identifying groups of genes with similar expression patterns highly enriched for these functions.

5.7. AM upregulate both classical and alternative activation genes during HDM-driven AAD

Macrophage activation has historically been broadly split into 'classical' activation, driven by bacterial products, such as LPS, or pro-inflammatory cytokines, such as IFN γ , and 'alternative' activation by the type 2 cytokines IL-4 and IL-13⁸⁰. Initial analysis showed AM to express high levels of a number of genes associated with alternative activation, such as *Cd36*, *Chil3* and *Il4ra*, which were further elevated during HDM-driven AAD (**Figs. 5.9-5.10**), along with upregulation of a number of known IL-4-inducible genes (**Fig. 5.11.**), suggestive of M[IL-4]-like activation of AM[HDM]. Since the classical/alternative activation dichotomy is now known to be an oversimplification, with macrophages displaying a spectrum of activation phenotypes depending on integration and accumulation of signals^{80,167}, it was of interest to evaluate how far the activation state of AM during HDM-driven AAD truly fit with that of alternative activation.

To address this question, publically available microarray data were obtained from work published by Jablonski *et al.*, in which murine bone marrow-derived macrophages (BMDM) were stimulated with either IFN γ or IL-4 to generate classically-activated M[IFN γ] and alternatively-activated M[IL-4], respectively⁷⁹. Lists of significantly upregulated genes in M[IFN γ] and M[IL-4] compared with unstimulated BMDM ($FC \geq 2$, $p \leq 0.05$, unpaired t test with Benjamini-Hochberg adjustment) were obtained from supplementary data from the published study⁷⁹. M[IFN γ] and M[IL-4] gene lists were then filtered to remove genes upregulated in both conditions, to focus on genes associated with polarised macrophage activation. Resultant gene lists were then examined in the AM dataset by unsupervised hierarchical clustering.

Of 354 M[IL-4] genes, a cluster of 17 genes showed no detectable expression in either AM[PBS] or AM[HDM], indicating that the transcriptional signature of AM[HDM] does not completely match that of IL-4-polarised BMDM (**Fig. 5.21a-b**). However, this was unsurprising given the stark differences in gene expression responses to inflammatory stimuli between AM and BMDM discussed in chapter 4. Most of the remaining M[IL-4] genes showed either upregulation in AM[HDM] or high resting expression in AM[PBS], suggesting a strong M[IL-4]-like signature in AM from HDM-driven AAD. One cluster of 20 genes displayed expression in the upper quartile of normalised expression in AM[PBS], with some increases in AM[HDM] (**Fig. 5.21a,c**), highlighting a steady state M[IL-4]-like gene signature in AM. This cluster included *Chil3* (also known as *Chi3l3* and *Ym1*), as noted in previous analysis (**Fig. 5.9**), along with *Cd44* and Transglutaminase 2 (*Tgm2*; **Fig. 5.21c**), which encode proteins that have previously been suggested to cooperate in apoptotic cell uptake by macrophages³⁹⁶. A further cluster of 40 genes showed little to no

expression in resting AM, but were highly upregulated AM[HDM] (**Fig. 5.21d**). This cluster showed substantial overlap with the previously noted cluster 7 (**Fig. 5.11**), including the IL-4-inducible genes *Ccl24*, *Retnla* and *Arg1* (**Fig. 5.21d**), supporting induction of a strong M[IL-4]-like gene programme in AM following 3 weeks of HDM inhalation.

Despite displaying a clear M[IL-4]-like gene expression signature, hierarchical clustering revealed a substantial subset of M[IFN γ] genes to also be constitutively expressed at a high level in AM[PBS] or upregulated in AM[HDM] (**Fig. 5.22a**). As observed for M[IL-4] genes, a subset of M[IFN γ] genes were undetectable in both AM[PBS] and AM[HDM], including the genes encoding both subunits of the key Th1 cell differentiation cytokine, IL-12 (*Il12a* and *Il12b*; **Fig. 5.22b**). The most substantially induced M[IFN γ] genes in AM[HDM] fell into two major clusters. The first consisted of genes with moderate expression in AM[PBS] that were upregulated in AM[HDM], including matrix metalloproteinase (MMP) 14 (*Mmp14*) and Arginosuccinate synthase 1 (*Ass1*; **Fig. 5.22c**), which is required for sustained nitric oxide production by classically activated macrophages during tuberculosis infection³⁹⁷. The second cluster consisted of 21 genes with low to moderate expression in AM[PBS] that were expressed at moderate to high levels in AM[HDM], which were highly enriched for ISGs and the ADP-ribosyl cyclase CD38, which Jablonski and colleagues validated in their study as a specific marker of M[IFN γ] in the case of bone marrow-derived cells⁷⁹.

Altogether, this comparison to published data further underscores differences between AM and macrophages generated from BM cells *in vitro* and demonstrates that while AM[HDM] show a strong M[IL-4]-like gene signature, they also express a number of genes typical of macrophage activation by IFN γ .

5.8. Transcriptomic data suggest that AM integrate multiple cytokine signals during HDM-driven AAD

The presence of gene expression patterns associated with both IL-4 and IFN γ stimulation in AM[HDM] suggested that, at least at the population level, gene expression in these cells is coordinated by parallel sensing of multiple cytokine signals with the potential to cross-regulate one another¹⁶⁸. To gain further insight into the likely signals underlying the observed gene expression changes in AM[HDM], differentially expressed genes were subjected to Ingenuity upstream regulator analysis, which calculates likely molecules upstream of observed gene expression changes based on published literature. The analysis was limited to cytokines and growth factors and highlighted 14 such molecules for which significant directional changes in activity (Fisher's exact test, $p \leq 0.001$ with absolute Z score ≥ 2) were predicted (**Table 5.3**).

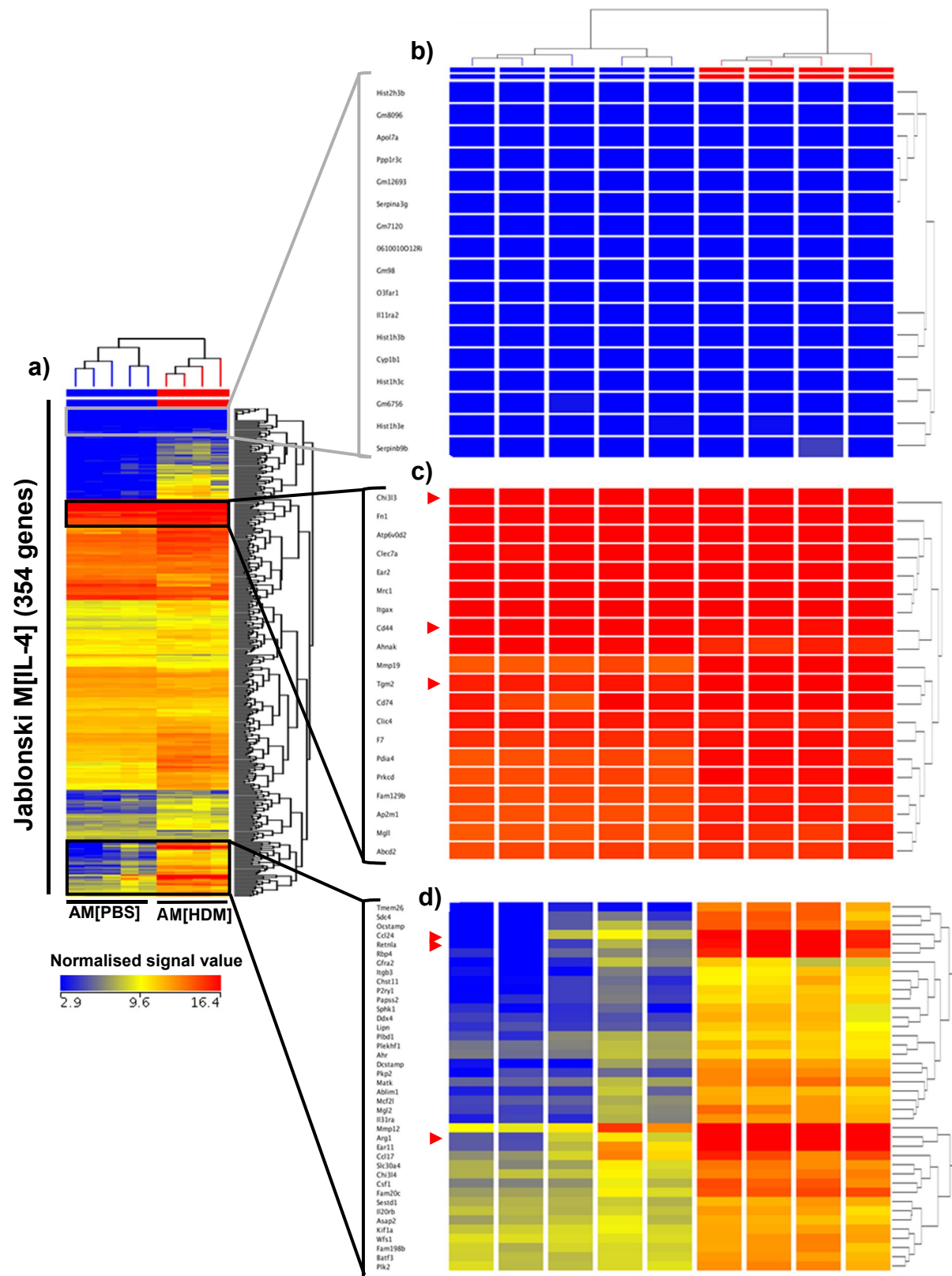


Fig. 5.21. Expression of alternative M[IL-4] activation genes in AM[HDM]

a) Normalised expression values of genes determined to be significantly upregulated by IL-4 but not IFN γ in BMDM by Jablonski *et al* (2015) were compared in the AM dataset by unsupervised hierarchical clustering. **b-d)** Detailed views of representative gene clusters discussed in the text. Red arrowheads mark representative genes from detailed clusters discussed in the text.

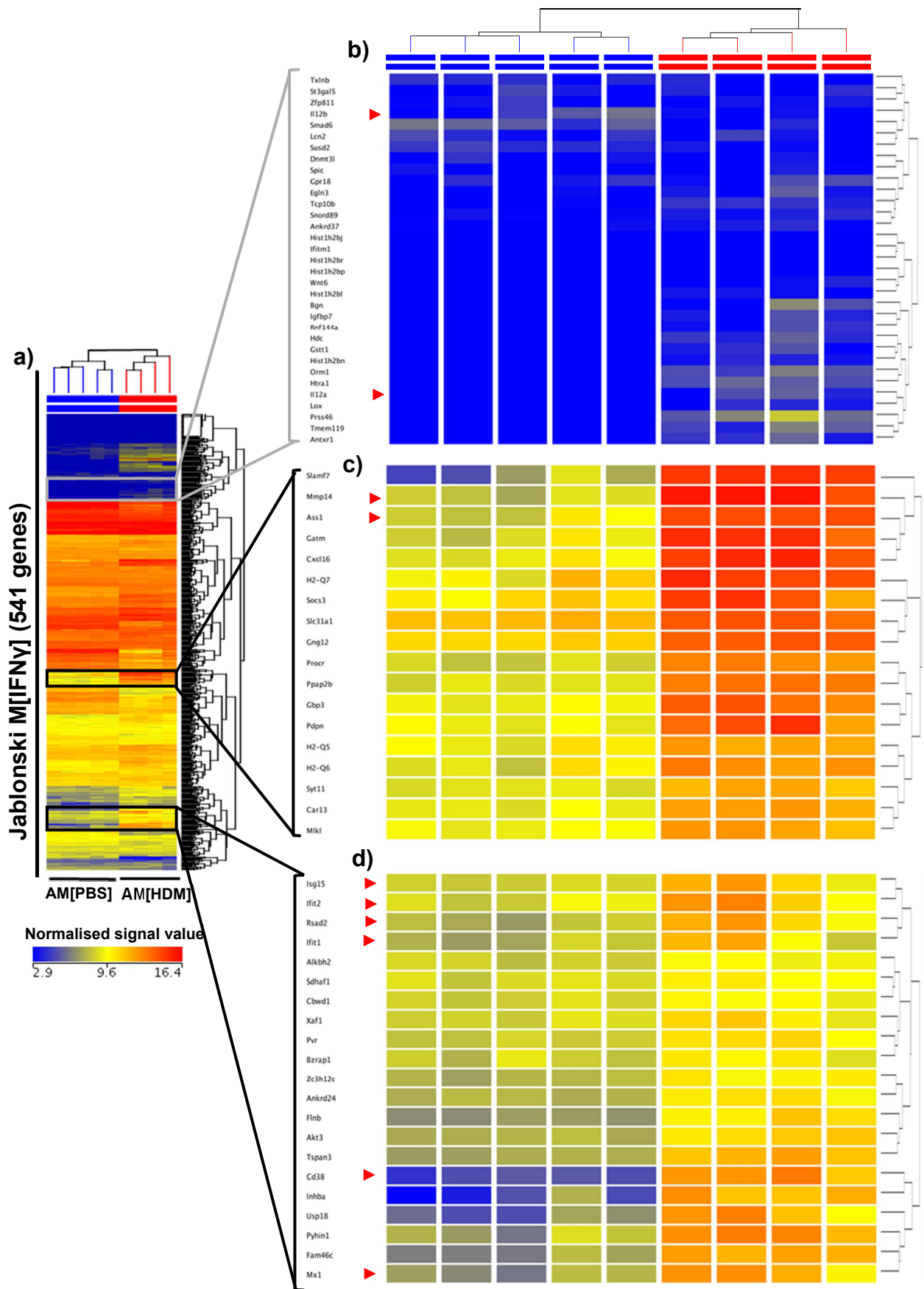


Fig. 5.22. Expression of classical M[IFN γ] activation genes in AM[HDM]

a) Normalised expression values of genes determined to be significantly upregulated by IFN γ but not IL-4 in BMDM by Jablonski *et al* (2015) were compared in the AM dataset by unsupervised hierarchical clustering. **b-d)** Detailed views of representative gene clusters discussed in the text are shown in the right hand panels. Red arrowheads indicate representative genes from detailed clusters discussed in the text.

Protein	Predicted activity on AM[HDM]	Z (directionality) score	-log ₁₀ <i>p</i> value
GM-CSF	Decreased	-3.8	30.9
IFN γ	Increased	5.4	20.3
IL-4	Increased	2.4	14.0
IL-5	Increased	3.0	8.3
TNF α	Increased	2.7	7.7
IFN β	Increased	3.5	7.0
IL-13	Increased	3.0	6.8
IL-1 β	Increased	2.1	6.6
TWEAK	Increased	2.9	5.3
CCL2	Increased	2.7	4.2
IL-6	Increased	3.0	4.2
OSM	Increased	2.4	3.0
CTGF	Increased	2.2	2.9
IL-33	Increased	2.5	2.9

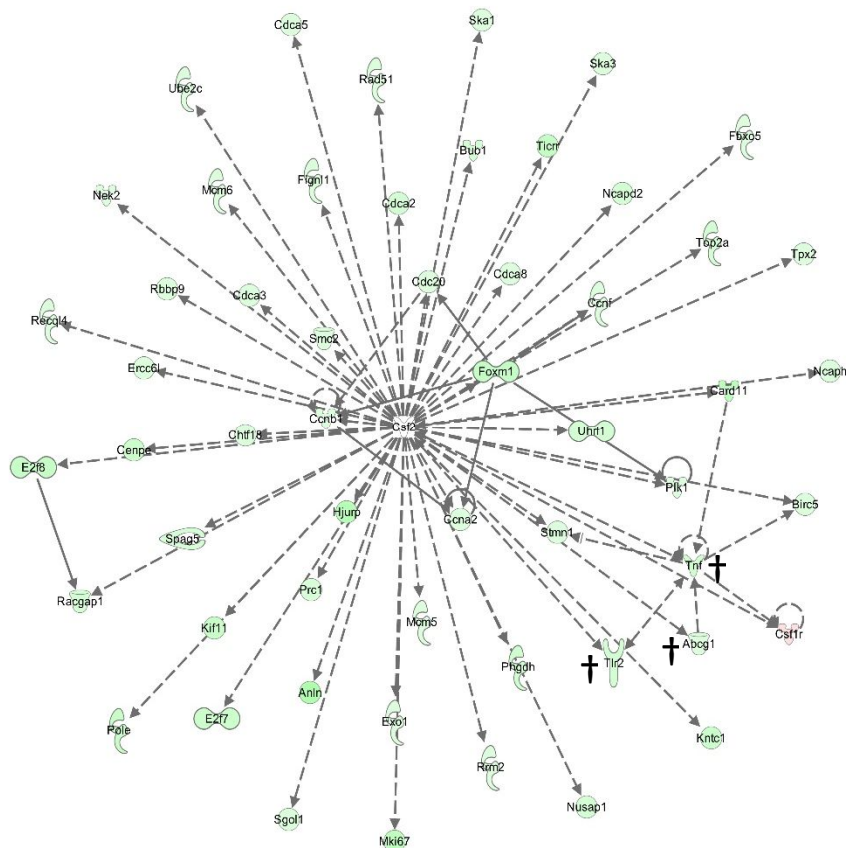
Table 5.3. Predicted cytokine activity upstream of differential gene expression in AM[HDM]

List calculated from agreement of genes differentially expressed in AM[HDM] with the expected effects of cytokines and growth factors based on the Ingenuity Knowledge Base. The most significant hits with a Z score of $\pm \geq 2$ are shown in order of statistical significance. *P* values were calculated based on Fisher's exact test of observed gene expression changes against predicted changes in response to the cytokine/growth factor.

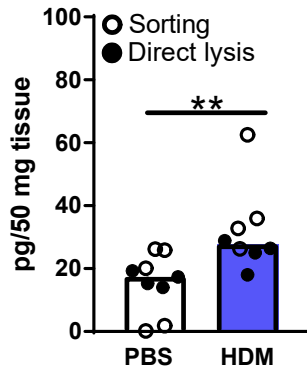
The top predicted differentially active cytokine signal to AM[HDM] was GM-CSF, which was also the only cytokine from which signalling was predicted to be decreased, rather than increased (**Table 5.3**). This was potentially of interest since GM-CSF is critical for maturation and homeostatic function of AM^{24,398,399}. However, manual inspection of published observations underlying this prediction revealed that it was largely dependent a single published study describing a role for GM-CSF in driving expression of cell cycle genes in intestinal crypt epithelial cells⁴⁰⁰. As described in section 5.6.4, a large number of cell cycle-related genes were downregulated in AM[HDM], resulting in the highly significant result for GM-CSF (**Fig. 5.23a**). However, the prediction was also partially based on significantly reduced expression of *Tnf*, *Tlr2* and the lipid transporter, ATP-binding cassette transporter 1 (*Abcg1*), which have been reported to be GM-CSF-induced in AM and BMDM († in **Fig 5.23a**)^{398,401-404}. To determine the potential for reduced GM-CSF signalling to AM during HDM-driven AAD, the concentration of GM-CSF was quantified in lungs of the mice used for RNAseq and shown to be slightly but significantly elevated in the HDM-treated group (**Fig. 5.23b**). Although this does not exclude the possibility that GM-CSF signalling to AM[HDM] is reduced due to inaccessibility of this pulmonary GM-CSF to AM, or by competing or antagonistic signals from other mediators, these data do not provide compelling evidence for reduced GM-CSF signalling to AM during HDM-driven AAD. Further experiments would therefore be necessary to evaluate the extent of GM-CSF signalling to AM during HDM-driven AAD.

Consistent with the earlier comparisons to published macrophage stimulation data, the most strongly predicted increased cytokine signals to AM[HDM] were IL-4 and IFN γ (**Table 5.3**). Accordingly, both of these cytokines were significantly more concentrated in the lungs of HDM-treated mice than PBS controls (**Fig. 5.23c,d**). IL-4 was detected at around a 10-fold higher concentration than IFN γ in HDM-treated mice, in accordance with the known bias of type 2 over type 1 immunity in this model³¹⁷. In line with the substantial induction of M[IL-4]-related genes in AM[HDM] and the known effects of repeated HDM inhalation on increasing pulmonary type 2 cytokine levels³¹⁷, increased signalling of IL-5, IL-13 and IL-33 to AM[HDM] was also highly predicted from transcriptomic data (**Table 5.3**). Predicted IFN γ signalling was accompanied by predicted increased responses of AM[HDM] to other classical pro-inflammatory cytokines, including TNF α , IL-1 β , IL-6 and IFN β (**Table 5.3**), the latter expected given the strong ISG signature in AM[HDM] (**Figs. 5.13, 5.22**). Together, these results support a model in which AM integrate signals from both type 2 and classical pro-inflammatory cytokines during HDM-driven AAD.

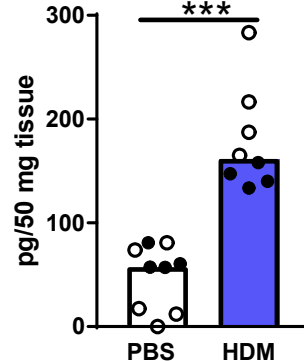
a) GM-CSF-regulated genes changed in AM[HDM]



b) Lung GM-CSF



c) Lung IL-4



d) Lung IFN γ

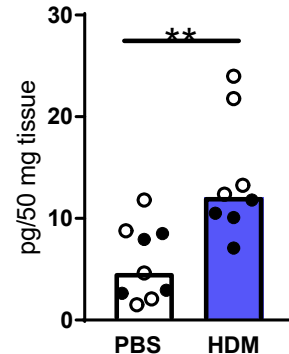


Figure 5.23. Altered pulmonary cytokine environment for AM during AAD

a) Interaction network of genes showing an expression pattern in AM[HDM] in agreement with decreased GM-CSF signalling, according to the Ingenuity Knowledge Base. Arrows indicate experimentally observed interactions (activation or downregulation) between gene products. Genes coloured in red and green are up- and downregulated, respectively, in AM[HDM]. †, indicates genes for which GM-CSF regulation has been demonstrated in murine macrophages (see text for references). **b-d)** ELISA data showing concentrations of GM-CSF (**b**), IL-4 (**c**) and IFN γ (**d**) in lungs of HDM-treated mice and PBS controls. Scatter/bar graphs show medians of pooled data from 3 independent experiments used to collect cells sorting and for direct lysis of BAL, as shown by shading of symbols (total 8-9 per group). Statistics: Mann-Whitney test. **, $p \leq 0.01$; ***, $p \leq 0.001$.

5.9. Minimal changes to gene expression of canonical IL-10 and TGF- β 1 signalling pathway components in AM from HDM-treated mice

Notably absent from the list of predicted differentially active cytokines were IL-10 and TGF- β 1 (**Table 5.3**). However, since many of the effects of these cytokines are anti-inflammatory, it is challenging to isolate the effects of these cytokines from transcriptomic data in an inflamed setting where there is a large net increase in pro-inflammatory gene expression. In a different approach, biased analysis was performed on a list of IL-10 and TGF- β signalling pathway genes, to look for gene expression changes suggestive of altered capacity of AM[HDM] to signal via these pathways. Lists of genes for each pathway were obtained from the Ingenuity Knowledge Base and their expression in the AM dataset compared by unsupervised hierarchical clustering, to determine whether individual or groups of genes were differentially expressed in AM[HDM]. In parallel, differential expression analysis results were overlaid onto diagrams of molecular interactions in each pathway, to examine potential changes in particular nodes of these canonical pathways.

5.9.1. The TGF- β pathway

For simplicity, analysis of TGF- β signalling was restricted to the canonical Smad2/3 pathway, which acts downstream of the TGF- β and Inhibin families of molecules⁴⁰⁵. Among cytoplasmic transducers and modulators of canonical TGF- β /Inhibin signalling, the receptor-Smad (R-Smad), *Smad2* and co-Smad, *Smad4* clustered closely together, with high expression in AM[PBS] that was not significantly changed with HDM treatment (**Fig 5.24a**). In contrast, expression of the R-Smad, *Smad3*, was below the imposed lower detection limit in all samples (**Fig 5.24a**), consistent with the low expression of this gene observed by qPCR in experiments described in Chapter 4. The negative regulator of Smad2/3 signalling, *Smad7*²²⁷, was expressed at low levels, close to the lower threshold of detection, in both AM[PBS] and AM[HDM] (**Fig. 5.24a**), consistent with the low expression of *Smad7* observed in AM compared to other murine macrophages in work described in Chapter 4. Expression of the E3 ubiquitin ligase and negative regulator of TGF- β receptor signalling, *Smurf1*⁴⁰⁶, was significantly reduced in AM[HDM], however the functionally similar ubiquitin ligase *Smurf2*⁴⁰⁶ was significantly decreased in these cells with a comparable absolute fold change (+2.48 for *Smurf1* and -2.25 for *Smurf2*, **Fig. 5.24a-c**). These data therefore did not provide evidence for a shift in expression of genes favouring or limiting TGF- β signal transduction in the cytoplasm.

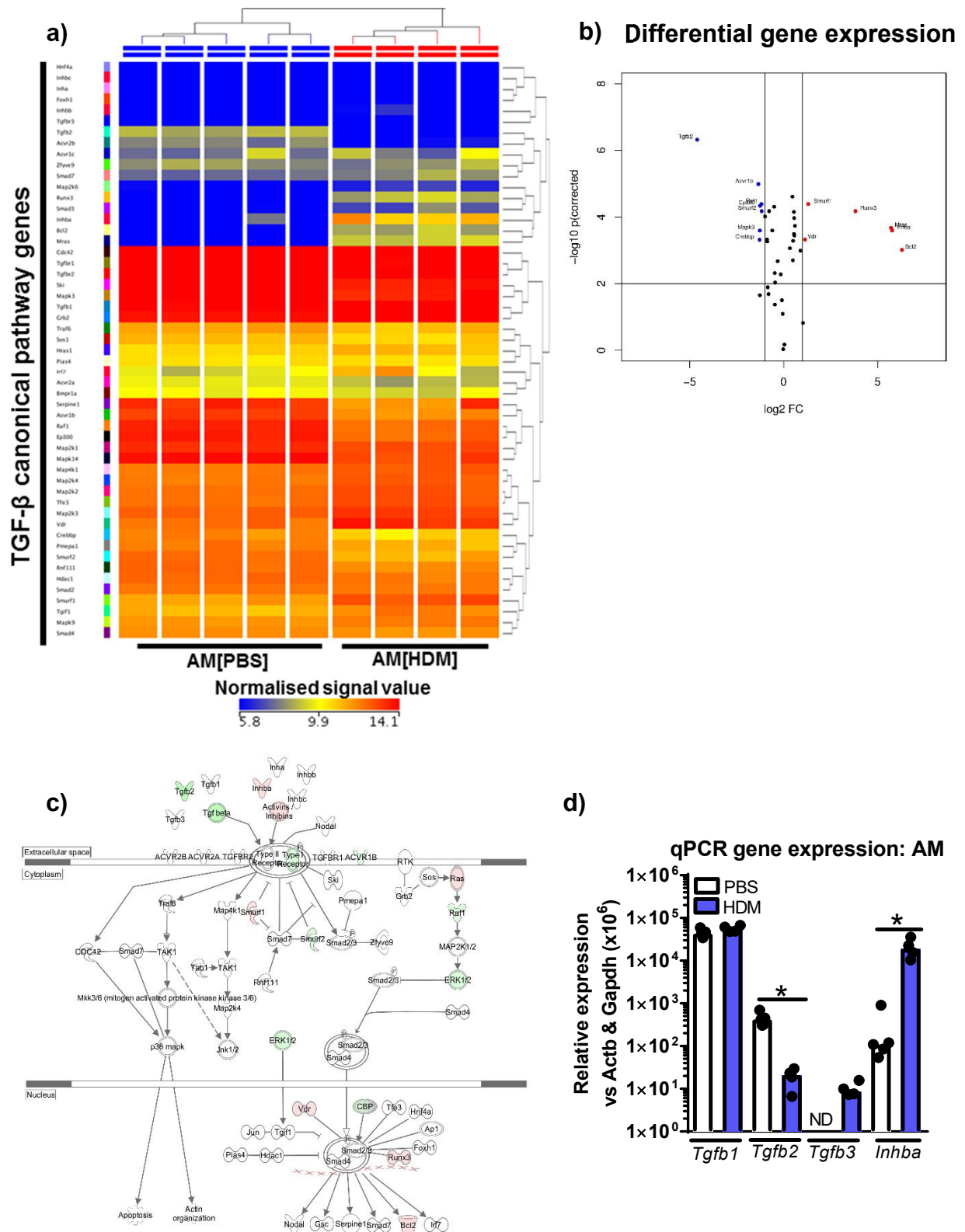


Figure 5.24. Expression of TGF-β pathway genes in AM[HDM]

a) Hierarchical clustering of all 'TGF-β signalling' Ingenuity canonical pathway genes based on expression pattern in AM[HDM]. **b)** Volcano plot showing differential expression analysis (moderated t -test with Benjamini-Hochberg correction) of TGF-β pathway genes in AM[HDM]. Significantly up- and down-regulated genes ($FC \geq 2$, $p \leq 0.01$) are marked in red and blue, respectively. **c)** Interactions and cellular locations of analysed genes. Genes coloured in red and green were up- and downregulated, respectively, in AM[HDM]. **d)** Confirmatory qPCR data showing expression of *Tgfb1*, *Tgfb2* and *Inhba* mRNA relative to housekeeping genes. Data shown are medians of 4-5 mice per group. Statistics: Mann-Whitney test. *, $p \leq 0.05$.

Of the known nuclear regulators of Smad2/3 signalling tested in this analysis, the transcription factors *Crebbp* and *Ep300* were significantly downregulated in AM[HDM] (**Fig. 5.24a-c**), although levels of both remained relatively high (normalised expression > 10; **Fig. 5.24a**). *Crebbp* (Creb-binding protein) and *Ep300* (p300) encode members of transcriptional co-activator family known to promote Smad2/3-mediated gene expression⁴⁰⁷. In contrast, gene expression of the vitamin D3 receptor *Vdr*, which can enhance Smad-dependent TGF- β signalling⁴⁰⁸, was increased in AM[HDM], as was *Runx3* (**Fig. 5.24a-c**), which cooperates with p300 and Smad2/3 to enhance TGF- β signalling⁴⁰⁹⁻⁴¹¹. Altogether, while significant positive and negative gene expression changes were observed in known nuclear modulators of canonical TGF- β signalling, no overall net change in TGF- β signalling capacity could be inferred from these gene expression data.

Of the TGF- β family ligands upstream of Smad2/3 signalling, *Tgfb1* was the most highly expressed in AM under both conditions and showed no significant change in AM[HDM] (**Fig. 5.24a**), consistent with data discussed in Chapter 4. *Tgfb2* was expressed at relatively low levels in AM[PBS] and significantly decreased to below the lower analysis threshold in AM[HDM] (**Fig. 5.24a-c**), while *Tgfb3* was below the lower expression limit in all samples (**Fig. 5.24a**). In contrast to TGF- β isoforms, expression of *Inhba*, encoding Activin A, was significantly elevated in AM[HDM] (**Fig. 5.24a-c**). *Acvr1b*, the type 1 receptor for Activin A, was significantly downregulated in AM[HDM], while levels of the type two Activin receptor genes *Acvr2a* and *Acvr2b* were non-significantly reduced and undetectable in AM[HDM], respectively. The TGF- β receptor genes *Tgfr1* and *Tgfr2* were highly expressed in AM[PBS] and did not change significantly with HDM treatment (**Fig. 5.24a**). Overall, this analysis showed minimal expression changes to canonical TGF- β pathway gene expression, with increased *Inhba* and decreased *Tgfb2* expression being the most substantial differences.

Since *Tgfb2* and *Inhba* expression levels in AM[PBS] were close to the imposed lower expression limit of this analysis (normalised signal value ≥ 8) and were decreased and increased, respectively, these genes were used to verify RNAseq results and threshold choice by qPCR. RNA from the same samples used for RNAseq was converted to cDNA and *Tgfb1*, *Tgfb2*, *Tgfb3* and *Inhba* expression determined by qPCR. As observed by RNAseq, *Tgfb1* was the most abundant of the TGF- β isoforms in both AM[PBS] and AM[HDM] and its expression did not differ significantly between these groups (**Fig. 5.24d**). Consistent with their near-threshold detection in AM RNAseq libraries, *Tgfb2* and *Inhba* showed low relative expression by qPCR (values between around $1-4 \times 10^2$) and were significantly decreased and increased in AM[HDM], respectively (**Fig. 5.24d**). *Tgfb3* was undetectable in AM[PBS] and showed some signal close to the detection limit of the qPCR assay (raw threshold cycle > 35) in AM[HDM] (**Fig. 5.24d**), supporting the lack of detection of this isoform in any of the samples by RNAseq (**Fig. 5.24a**). Thus, qPCR of TGF- β superfamily ligands showed identical expression patterns to those obtained by RNAseq and suggested that the lower detection threshold for RNAseq analysis allowed similar discrimination of detectable genes above potential noise to conventional qPCR.

5.9.2. The IL-10 Pathway

Targeted analysis of the canonical IL-10 signalling pathway showed no clear net differences in expression patterns between AM[HDM] and AM[PBS]. Samples from PBS and HDM-treated mice incompletely segregated by hierarchical clustering of 20 IL-10 pathway-related genes, with one AM[PBS] sample clustering with those from the AM[HDM] group (**Fig. 5.25a**), highlighting the lack of major differences in expression of IL-10 pathway components occurring in AM during AAD. Importantly, *Il10* itself was below the lower detection limit in AM from either experimental group (**Fig. 5.25b**), consistent with data described in Chapter 4 in suggesting that AM do not produce readily detectable levels of IL-10 at homeostasis or during HDM-driven allergic inflammation.

A lack of major transcriptional differences in IL-10 pathway components was supported by differential expression analysis on the 13 IL-10 pathway genes detected above threshold in the AM dataset (**Fig. 5.25c**). Expression of IL-10 receptor subunits *Il10ra* and *Il10rb* was significantly decreased and increased, respectively, by around two-fold in AM[HDM], although both remained at relatively high levels (normalised signal > 11; **Fig. 5.25a**), such that the relevance of these changes to overall IL-10 signalling capability is unclear. Similarly, downstream signalling molecules *Jak1*, *Tyk2* and *Stat3* were all highly expressed by AM and were not significantly changed after HDM treatment (**Fig. 5.25a,c,d**), suggesting that cytoplasmic transduction of IL-10 signals was intact in AM[HDM]. Together these results confirm that AM are not a major source of IL-10 during AAD and suggest that there is little net difference in gene expression of IL-10 receptors or downstream signalling molecules in this setting.

Analysis of the IL-10 canonical pathway showed the IL-10-inducible genes *Il4ra*, *Socs3*, *Fcgr2*, *Ccr1* and *Ccr5* to be significantly upregulated in AM[HDM] (**Fig. 5.25a,c,d**). These changes might reflect increased IL-10 signalling to AM during AAD, a notion supported by detection of elevated IL-10 levels in lungs of HDM-treated mice (**Fig. 5.25e**). However, these genes can also be induced by cytokines other than IL-10, including induction of *Socs3* by IL-4⁴¹², so it is unclear from these data alone whether their increased expression in AM[HDM] can be attributed to heightened IL-10 signalling. Indeed, any IL-10-induced gene expression changes in AM[HDM] would be likely to integrate with other cytokine signals, as exemplified by the cooperative induction of CCL24 by IL-4 and IL-10 in BMDM⁴¹³. Better understanding of IL-10 signalling to AM during AAD will require conditional knockout experiments in which IL-10 signalling is defective in AM, as discussed in Chapters 6 and 8 of this thesis.

Altogether these analyses suggested that the core machinery of TGF- β and IL-10 signalling pathways was intact in AM[HDM], consistent with AM remaining receptive to IL-10 and TGF- β signals during HDM-driven AAD. No net changes in signalling capacity of either pathway were obvious from gene expression data, although AM[HDM] showed significant induction of Activin A gene expression. Further, more direct, studies will be required to determine the functional integrity and activity of these pathways *in vivo* during HDM-driven AAD and their importance in the regulation of inflammation by AM.

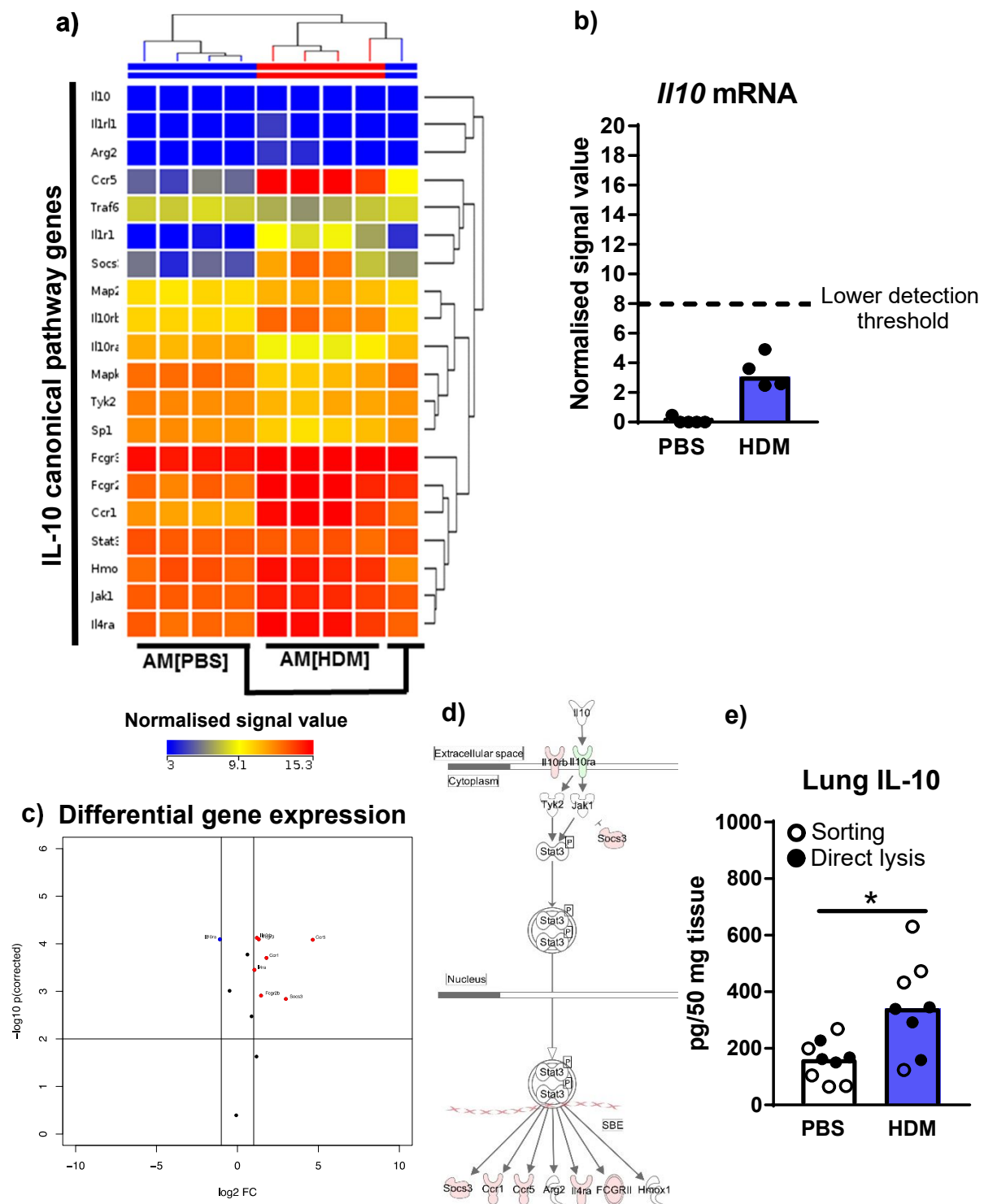


Figure 5.25. Expression of IL-10 pathway genes in AM[HDM]

a) Heatmap showing hierarchical clustering of all 'IL-10 signalling' Ingenuity canonical pathway genes based on expression pattern in the AM dataset. **b)** DESeq normalised expression value of *Il10* in AM dataset, shown relative to minimum expression threshold. **c)** Volcano plot of differential expression analysis (moderated *t*-test with Benjamini-Hochberg correction) of IL-10 pathway genes in AM[HDM]. Significantly up- and down-regulated genes ($FC \geq 2$, $p \leq 0.01$) are coloured red and blue, respectively. **d)** IL-10 signalling pathway diagram. Molecules coloured in red and green were up- and downregulated, respectively, in AM[HDM]. **e)** IL-10 concentrations in lung homogenates of mice used for sorted AM and whole BAL (direct lysis) RNA. Scatter/bar plots show medians of 4-9 mice per group. Statistics: Mann-Whitney test, *, $p < 0.05$.

5.10. Comparison of gene expression in sorted AM and total BAL cells during HDM-driven allergic airway disease

Having demonstrated profound gene expression changes in AM during HDM-driven AAD, it remained to be determined whether these gene expression patterns could also be detected in unenriched BAL cells, which comprised a mixture of eosinophils, lymphocytes and other inflammatory cells and in which AM were a minority cell population (**Fig. 5.2**). Increased expression of genes upregulated by AM[HDM] in BAL of HDM-treated mice would indicate either common genes expressed by multiple cell types in the allergic airway, or AM[HDM] signatures of sufficient magnitude to be readily monitored in unrefined BAL. In contrast, genes showing more subtle induction in AM[HDM] and no increase in BAL of HDM-treated mice would likely relate to pathways and processes increased in AM, but not other cell types, during HDM-driven AAD. To this end, AM and whole BAL datasets from PBS and HDM-treated mice were compared.

5.10.1. Differential gene expression in unsorted BAL cells of HDM-treated mice

BAL RNAseq data were normalised and filtered using identical parameters to those applied to the AM dataset, removing genes with normalised expression values of less than 8 in both experimental groups. This left 10 960 genes above threshold in the BAL genes for analysis (**Fig. 5.26a**). As expected, given the increase in inflammatory cells during HDM-driven inflammation (**Fig. 5.2**), gene expression in BAL[HDM] differed markedly from PBS controls, clustering separately in two principal components, accounting in total for around 91 % of variation in the dataset, with no clear outliers in either experimental group (**Fig. 5.26b**). Differential expression analysis using parameters identical to those applied to the AM dataset revealed 1496 upregulated and 1312 downregulated genes in BAL[HDM] compared with BAL[PBS] (**Fig. 5.26c**). As expected, given the combination of activated AM, lymphocytes and eosinophils in the airway lumen of HDM-treated mice (**Fig. 5.2**), upregulated genes in BAL[HDM] were highly enriched for GO terms related to cell recruitment and cell activation in response to cytokines and antigens, including 'immune system process', 'locomotion' and 'regulation of response to stimulus' (**Fig. 5.26d**). GO annotations for downregulated genes in BAL[HDM] were similar to those obtained for sorted AM (compare with **Fig. 5.20c**), largely dominated by cell cycle and DNA replication-related terms such as 'cell cycle process', 'cell cycle phase' and 'organelle fission' (**Fig. 5.26e**). This suggests that decreased expression of cell cycle-related genes in AM[HDM] is not compensated for by recruited cells during AAD, causing these genes to remain decreased when unsorted BAL from HDM-treated mice was analysed.

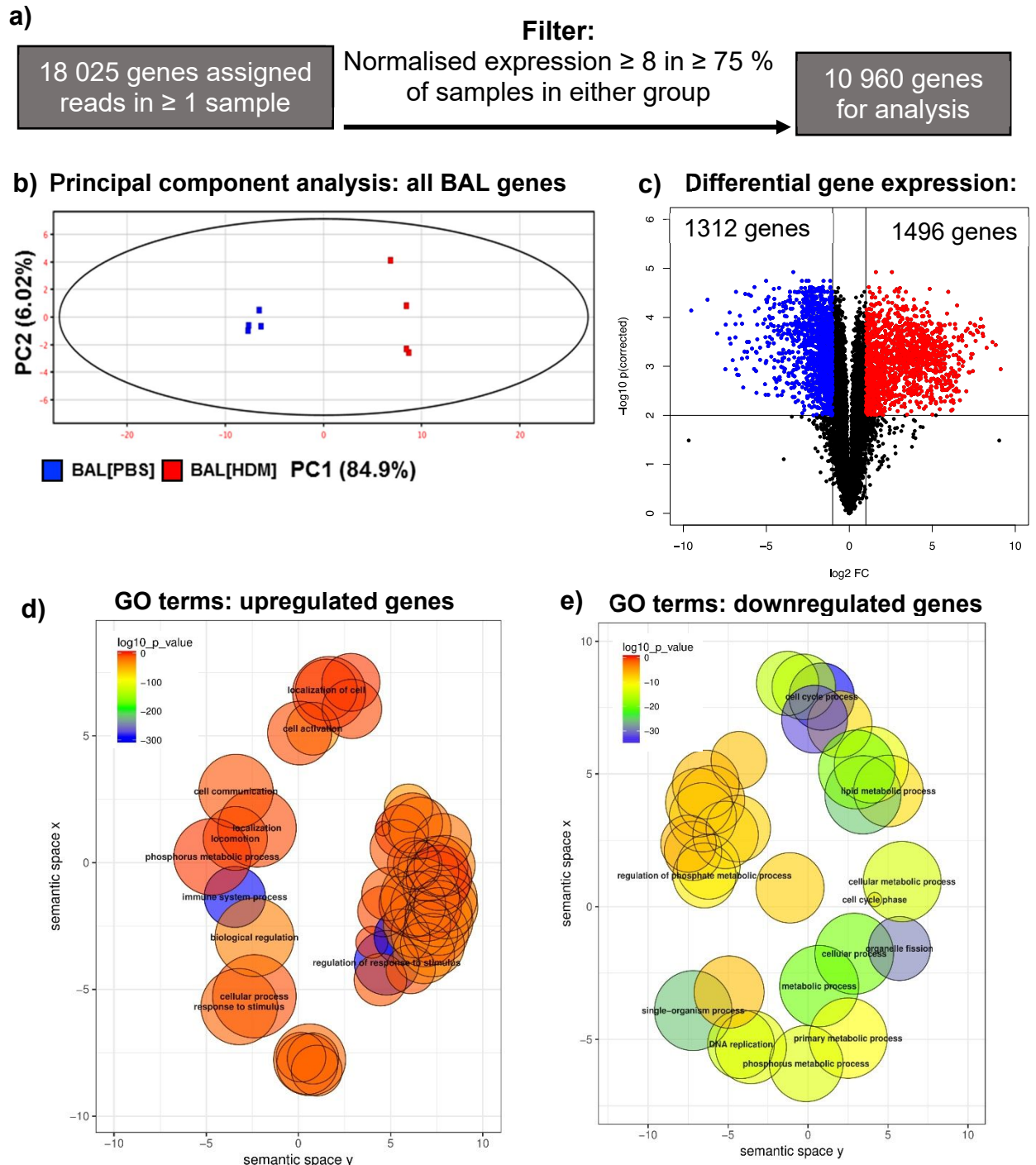


Figure 5.26. Gene expression in whole BAL cells from HDM-treated mice

a) Transcripts detected in whole BAL cell libraries of HDM and PBS treated mice were filtered to remove those with a DESeq normalised expression value of ≤ 8 in $\geq 75\%$ of samples in either group. **b)** PCA showing variation in gene expression between all BAL[PBS] and BAL[HDM] samples. **b)** Volcano plot showing transformed fold changes and p values resulting from moderated t -tests of gene expression in BAL[HDM] vs BAL[PBS] after Benjamini-Hochberg correction ($n=4$ per group). Upregulated genes are shown in red, downregulated genes in blue. **c-d)** REVIGO-filtered GO terms enriched in gene lists upregulated (**c**) and downregulated (**d**) in BAL[HDM], clustered in semantic space based on similarity and colour-coded based on significance. Sizes of circles are proportional to the size (i.e. generality) of the GO term. Names of GO terms representative of the reduced clusters are shown.

5.10.2. A subset of AM[HDM] gene signatures are detectable in unsorted BAL cells

To begin to dissect the contribution of AM[HDM] gene expression relative to other cell types in the allergic airway lumen, genes upregulated in AM[HDM] compared with AM[PBS] were interrogated in the whole BAL dataset. Of 1143 genes significantly upregulated in AM[HDM], 1089 were detectable above the imposed lower threshold in BAL from PBS and/or HDM-treated mice (**Fig. 5.27a**). Unsupervised hierarchical clustering of these genes based on normalised expression values in the BAL dataset (hereafter referred to as AM → BAL clustering) clearly separated PBS and HDM-treated BAL samples and revealed 10 major clusters, labelled i-x (**Fig. 5.27b**). Of these clusters, three were selected for detailed analysis on the basis of having differential expression (HDM vs PBS) patterns of interest when compared to the AM dataset, as shown by paired volcano plots in **Fig. 5.27c**.

Cluster i consisted of genes highly expressed in BAL cells of non-allergic PBS-treated mice, which were significantly upregulated in AM[HDM] (**Fig. 5.27c**), but few of which were significantly upregulated in BAL[HDM] (**Fig. 5.27c, Fig. 5.28a**). This pattern suggested that expression of these genes among airway leukocytes was mostly restricted to AM both at steady state and during HDM-driven AAD, such that increased expression was not readily detected in unsorted BAL cells of HDM-treated mice where AM were a minority population. In support of this, cluster i was dominated by genes related to macrophage function in immune responses. Genes in this cluster were enriched for GO term and canonical pathway annotations included 'antigen presentation' (**Fig. 5.28b**), driven by presence of MHC-II genes such as *H2-Aa*, *H2-Ab1* and *H2-Eb1* (**Fig. 5.28c**) and 'phagosome maturation' (**Fig. 5.28b**), owing to genes such as *Ctsk* and V-ATPase components *Atp6v0c* and *Atp6v1b2* (**Fig. 5.28c**). AM → BAL cluster i also included the macrophage-restricted enzyme MMP12^{244,414} (*Mmp12*) and *Chil3/Ym1* (**Fig. 5.28c**), of which AM are a major source among airway leukocytes during allergic inflammation¹⁷³, consistent with predominant expression of genes in this cluster by AM rather than other airway leukocytes.

Cluster iii consisted of genes expressed at a moderate level in BAL of PBS-treated mice, which displayed little to no upregulation in BAL[HDM] (**Fig. 5.27c, Fig. 5.29a**). These genes showed relatively modest upregulation in AM[HDM] (log2 fold change < 4; **Fig. 5.27c**), suggesting that their induction during HDM-driven AAD was largely restricted to AM and was of insufficient magnitude for detection in unenriched BAL. This cluster was highly enriched for GO terms and canonical pathways related to oxidative phosphorylation (e.g. 'hydrogen ion transport', 'generation of precursor metabolites and energy', 'oxidative phosphorylation', 'TCA cycle'; **Fig. 5.29b**) and contained many of the ETC-related genes determined to be upregulated in AM[HDM], including several *Atp5*, *Cox* and *Sdh* subunit genes (**Fig. 5.29c**). These data therefore suggest that the increased oxidative phosphorylation gene expression signature is not a general feature of leukocytes in the allergic airway and may instead be restricted to AM.

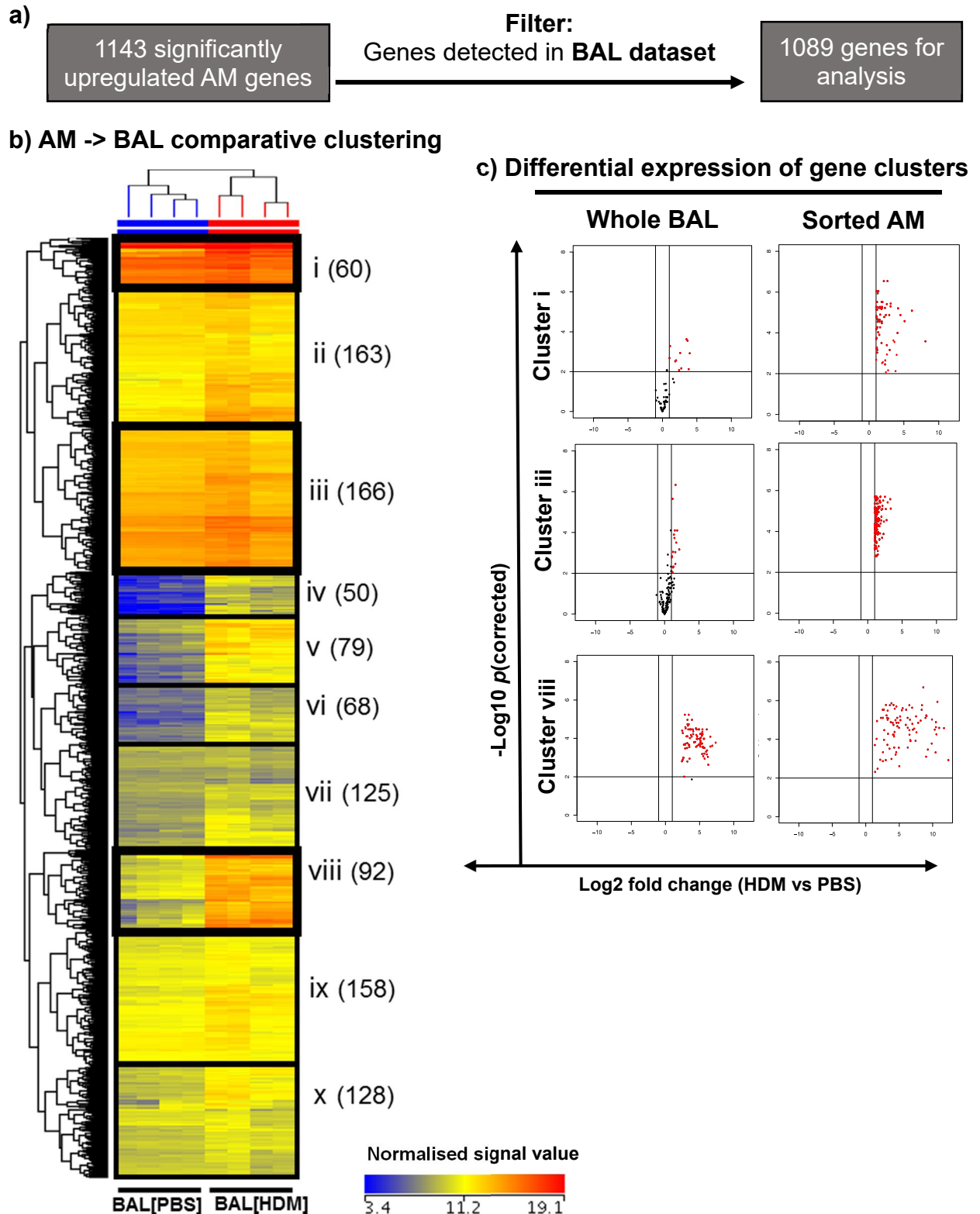


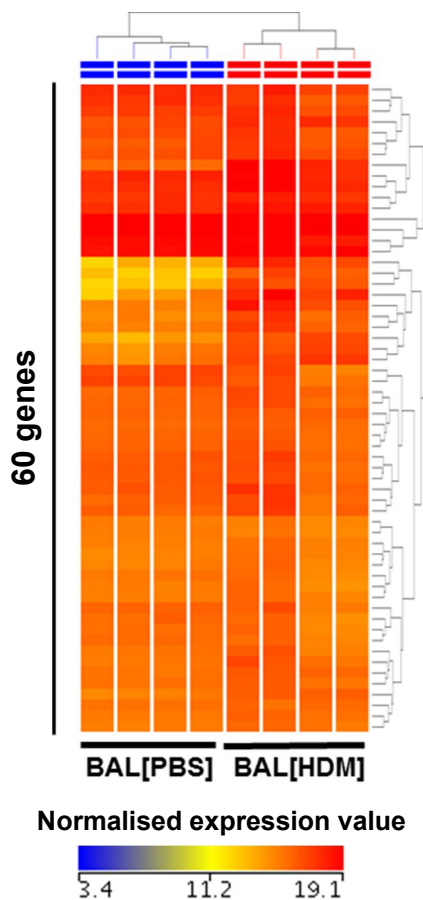
Figure 5.27. Interrogation of AM[HDM] gene signatures in unsorted BAL cells

a) All significantly upregulated genes in AM[HDM] were filtered to remove those below threshold (normalised signal ≤ 8 in ≥ 75 % of samples in both PBS and HDM groups) in the BAL dataset. **b)** The remaining 1089 genes were subjected to unsupervised hierarchical clustering in the BAL dataset, manually selecting 10 major clusters with distinct gene expression patterns from the resultant dendrogram. Numbers in brackets indicate numbers of genes in clusters. **c)** Volcano plots showing results of differential expression analysis (moderated t -test with Benjamini-Hochberg correction) of selected gene clusters from (**b**), comparing whole BAL and sorted AM. All volcano plots are on the same scale to allow comparison between clusters and datasets.

a) AM -> BAL Cluster i

BAL[HDM]: little to no increase from high baseline

AM[HDM]: Increase from high baseline



b) Functional annotations

Enriched GO terms:

- Antigen processing and presentation (11.93)
- Immune system process (10.66)
- Defense response (10.58)
- Response to stress (9.601)
- Response to wounding (6.82)
- Regulation of biological quality (5.19)
- Tissue remodeling (4.25)

Enriched canonical pathways:

- Phagosome maturation (12.29)
- Antigen presentation (11.58)
- Autophagy (7.49)
- Lipid antigen presentation (6.35)
- Allograft rejection signalling (6.06)

c) Representative genes



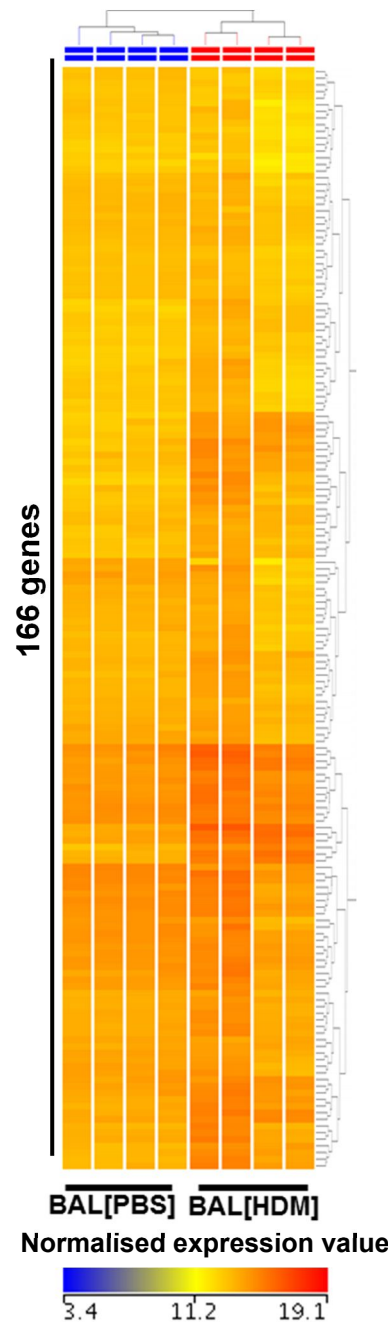
Figure 5.28. A set of abundant AM genes show little upregulation in BAL of HDM-treated mice

a) Sub-tree of heat map shown in **Fig. 5.27**, showing hierarchical clustering of cluster i genes in BAL samples. **b)** A representative list of the most highly enriched GO terms and canonical pathways (all $p \leq 0.01$, Fisher's exact test) in cluster i. Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative genes driving the annotations in (b). Sizes of words are relative to the log2 fold change in AM[HDM] vs AM[PBS]; range: 1.08-8.1.

AM -> BAL Cluster iii

BAL[HDM]: little to no increase from moderate baseline

AM[HDM]: Increase from moderate baseline



b) Functional annotations

Enriched GO terms:

- Hydrogen ion transmembrane transport (12.82)
- Single organism metabolic process (7.37)
- Localisation (6.56)
- Generation of precursor metabolites and energy (5.64)
- TCA cycle (5.27)

Enriched canonical pathways:

- Oxidative phosphorylation (16.73)
- Mitochondrial dysfunction (14.37)
- TCA cycle (eukaryotic) (7.51)
- Antigen presentation (6.83)
- T helper cell differentiation (6.83)
- Interferon signalling (3.80)

c) Representative genes

H2-Aa

Atp5g1 Ndufa13 Uqcrh Uqcrb

Atp6v1e1 Atp6v1a Atp5o

Atp6ap2 H2-Dmb1 Ifngr2

Atp5j2 Cox6c

Atp5e Atp5j Sdhb Cox7a2

Atp5l Sdhc Cox5b

Cox7b

Figure 5.29. Little net upregulation of oxidative phosphorylation genes in total BAL cells of HDM-treated mice

a) Sub-tree of heat map shown in **Fig. 5.27**, showing expression of cluster iii genes in BAL samples. **b)** A representative list of the most highly enriched GO terms and canonical pathways (all $p \leq 0.01$, Fisher's exact test) in cluster iii, in order of increasing p value. Numbers in brackets are $-\log_{10} p$ values of enrichment. **c)** Word cloud visualisation of representative genes driving the annotations in **(b)**. Sizes of words are relative to the $\log_2$ fold change in AM[HDM] vs AM[PBS]; range: 1-3.9.

The final AM -> BAL analysis cluster of interest, cluster viii, contained genes displaying the greatest fold increases in BAL[HDM], which showed even larger fold increases in AM[HDM] (**Fig. 5.27c, Fig. 5.30a**). These patterns indicated that expression of this cluster in BAL[HDM] was substantially driven by gene expression changes in AM, but could not exclude contributions from other cell types in the allergic airway. Consistent with an AM-dominated signature, cluster viii was highly enriched for genes associated with M[IL-4]-like macrophage activation, including *Arg1*, *Retnla*, arachidonate 15-lipoxygenase (*Alox15*) and *Ccl24*^{80,321} along with associated GO and canonical pathway annotations such as 'response to wounding', 'granulocyte/agranulocyte adhesion and diapedesis' and 'eicosanoid signalling' (**Fig. 5.30b,c**). AM -> BAL cluster viii was also highly enriched for the complement system genes *C1qa*, *C1qb*, *C1qc*, *Itgam* (CD11b) and *C3ar1*, resulting in significant overrepresentation of the 'complement system' canonical pathway (**Fig. 5.30b,c**). Thus, cluster viii represents a strong AM[HDM] gene expression signature dominated by signatures of alternative macrophage activation and complement function, readily detectable in unsorted BAL of HDM-treated mice.

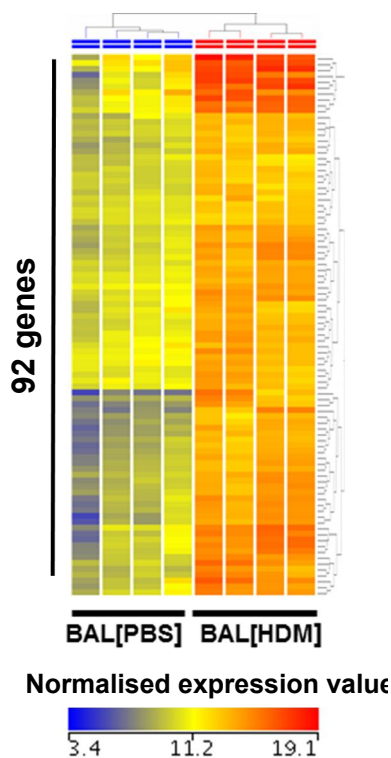
5.10.3. Identification of an AM[HDM] gene signature highly detectable in unsorted BAL

To further focus on a gene expression signature of AM[HDM] that could be detectable in unsorted BAL samples, a reciprocal comparative analysis was performed- clustering genes upregulated in BAL[HDM] in the AM dataset. It was reasoned that genes detectable by both comparative analyses would be robust markers of AM[HDM] gene expression detectable in unenriched BAL cells. Of 1496 genes significantly upregulated in BAL[HDM], 701 were detectable above threshold in the AM dataset (**Fig. 5.31a**). Unsupervised hierarchical clustering of these genes in the AM dataset (hereafter referred to as BAL -> AM clustering) clearly segregated AM[PBS] and AM[HDM] samples and revealed clusters of genes with distinct expression patterns between the two conditions (**Fig. 5.31b**). To focus on BAL[HDM] genes with the strongest AM contribution, a single gene cluster of 34 genes was manually selected which displayed the most substantial fold changes in AM[HDM], showing little to no expression in AM from PBS control mice, but high expression in AM[HDM] (**Fig. 5.31b**). The BAL->AM cluster showed an equivalent expression pattern to cluster viii from the previous AM->BAL analysis (**Fig. 5.30**), suggesting that together these clusters contain the AM[HDM] genes most readily detectable in whole BAL. Comparison of these two clusters revealed an overlap of 16 genes (**Fig. 5.31c**), including *Ccl24*, *Arg1*, *C1q* subunit genes, the IL-7 receptor (*Il7r*), self-liganded cell surface receptor SLAM family member 7 (*Slamf7*) and disintegrin and metalloproteinase domain-containing protein (*Adam8*). These results suggest that high expression of these genes in BAL cells of HDM-treated mice may be of use as an indicator of AM dysfunction during allergic inflammation.

a) AM -> BAL Cluster viii

BAL[HDM]: Large increase from low baseline

AM[HDM]: Large increase from low baseline



b) Functional annotations

Enriched GO terms:

- Regulation of immune system process (13.31)
- Immune system process (12.15)
- Defense response (7.78)
- Response to wounding (7.78)
- Inflammatory response (6.96)
- Regulation of cell migration (5.94)

Enriched canonical pathways:

- Granulocyte adhesion and diapedesis (8.38)
- Complement system (6.28)
- Agranulocyte adhesion and diapedesis (5.85)
- Atherosclerosis signalling (4.70)
- Leukocyte extravasation (4.19)
- Eicosanoid signalling (3.57)

c) Representative genes



Figure 5.30. AM->BAL cluster viii: genes highly upregulated in sorted AM and whole BAL of HDM-treated mice

a) Sub-tree of heat map shown in **Fig. 5.27**, showing expression of cluster viii genes in BAL samples.

b) A representative list of the most highly enriched GO terms and canonical pathways (all $p \leq 0.01$, Fisher's exact test) in cluster viii. Numbers in brackets are $-\log_{10} p$ values of enrichment.

c) Word cloud visualisation of representative genes driving the annotations in (b). Sizes of words are relative to the $\log_2$ fold change in AM[HDM] vs AM[PBS]; range: 2-1-12.3.

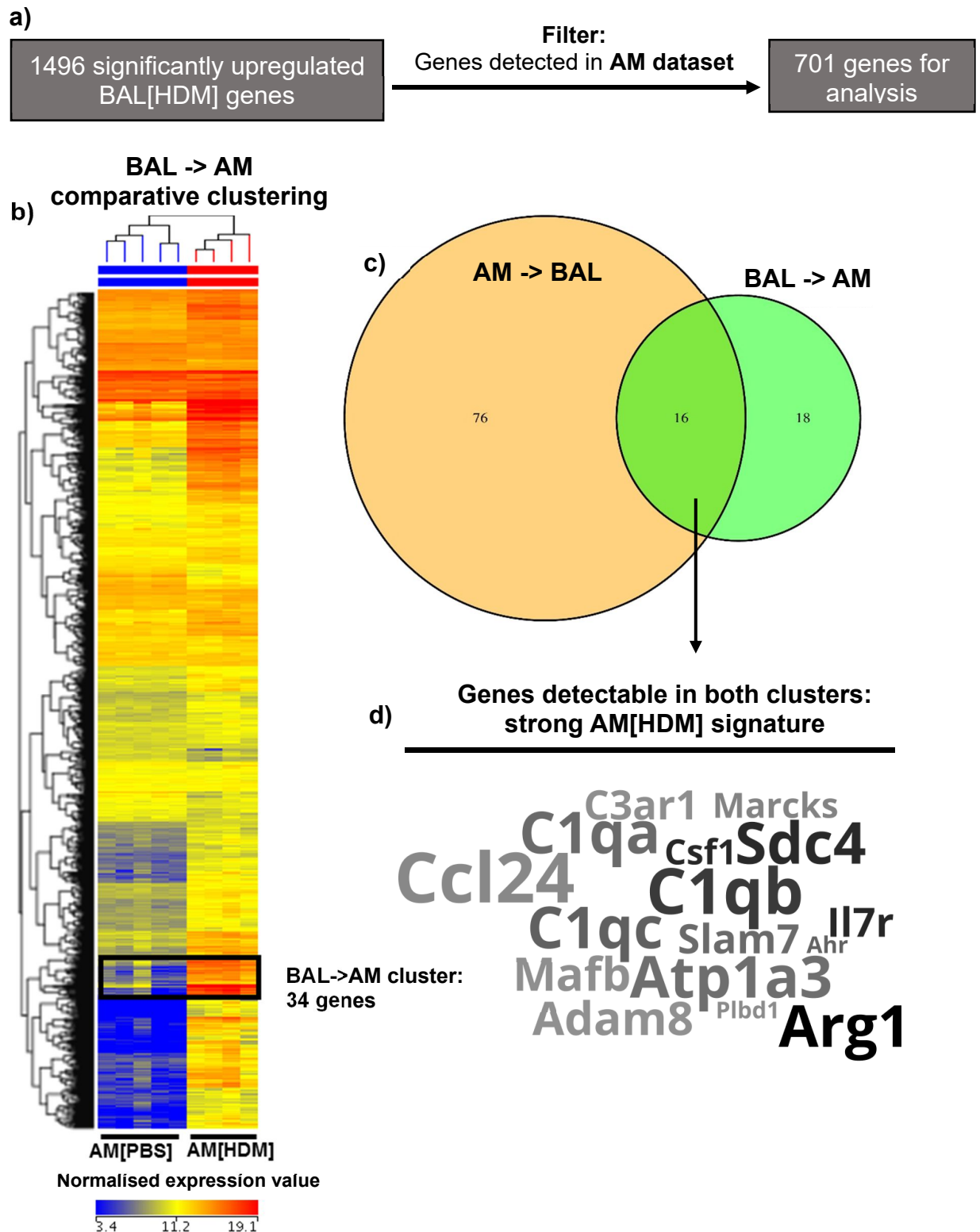


Figure 5.31. A subset of AM[HDM] genes highly detectable in unsorted BAL

a) 1497 genes significantly upregulated in BAL[HDM] were filtered to leave only the 701 also detectable above threshold in the AM dataset (normalised signal ≥ 8 in ≥ 75 % of samples in PBS and/or HDM groups). **b)** Unsupervised hierarchical clustering of 701 BAL[HDM] genes in the AM dataset, with a cluster of genes showing the largest fold increase in AM[HDM] highlighted. **c)** Venn diagram showing number of overlapping genes between the cluster highlighted in (**b**; BAL->AM) and AM -> BAL cluster viii (**Fig. 5.30**). **d)** Word cloud visualisation of all overlapping genes from the highly upregulated AM -> BAL and BAL -> AM gene clusters. Sizes of words are relative to the log₂ fold change expression in AM[HDM] vs AM[PBS]; range: 4-12.34.

5.11. Discussion

In this chapter, a transcriptomic approach was used to determine global gene expression changes in AM during HDM-driven AAD, with the aim of better understanding how AM contribute to immunopathology of this disease and assessing the state of the IL-10 and TGF- β immunoregulatory signalling pathways. Comparative analysis was also used to gauge the relative contribution of AM to expression of gene subsets compared to other airway leukocytes. These results offer insights into potential pathogenic and regulatory mechanisms of AM during AAD and provide considerations for future experiments.

5.11.1. Critical appraisal of methodology of RNAseq project

The flow cytometry sorting method employed for AM yielded high numbers of cells from individual mice, from which high-integrity RNA was consistently obtained. However, the purity of resultant AM samples was sub-optimal, resulting in one sample being excluded from analysis. The large size of AM and their tendency to bind to other cells in suspension meant that a 100 μ m nozzle was used for sorting with a relatively low drop rate, resulting in an increased chance of contaminants compared to smaller cells such as lymphocytes. Although purity sort settings were used in an attempt to circumvent this problem, in which drops with potential contaminant cells were discarded at the expense of cell yield, this does not appear to have been sufficient to ensure optimal purity of sorted AM. As a result of relatively low purity, a stringent minimum expression limit was required to filter contaminant reads, which could conceivably have removed some genes truly expressed at low levels by AM. In future experiments, it will be imperative to improve purity of AM sorts to increase the resolution of gene expression analysis. More stringent (i.e. tighter) gating around positive populations and inclusion of further negative selection markers to more effectively exclude cells such as lymphocytes may help achieve this. Another option is to re-sort AM a second time to ensure maximal removal of contaminant cells, a method employed by the Immunological Genome Project consortium for transcriptomic analysis of macrophage populations⁴¹⁵.

5.11.2. Perspectives from genes upregulated by AM during AAD

Genes related to a number of diverse biological functions and canonical pathways were increased in AM[HDM], offering insights into the function of these cells in the allergic airway. AM[HDM] expressed increased levels of genes associated with antigen presentation to T cells, a function thought to be relatively poor in AM compared with other macrophage subsets^{151,152}. AM can present antigen *in vitro*⁴¹⁶ and their ability to stimulate T cell proliferation and cytokine production is enhanced by cytokines such as IL-4, GM-CSF and TNF α ^{417,418}, so the cytokine milieu of the allergic airway could conceivably increase the antigen presenting cell (APC) function of AM[HDM].

Several other strong gene expression signals in AM[HDM] related to recruitment of leukocytes. Most highly expressed among these genes was the eosinophil chemokine *Ccl24*, which has previously been reported to be upregulated by AM in models of AAD^{28,157} and to be induced by *Brugia (B.) malayi* nematode infection in peritoneal macrophages (PM) in an IL-4R-dependent

manner⁴¹⁹. *Ccl8*, encoding a chemokine involved in monocyte and T cell recruitment^{420,421} and increased in the IL-4R-dependent nematode model reported by Thomas *et al*⁴¹⁹, was significantly elevated in AM[HDM]. Together these results suggest that IL-4-driven chemokine expression is a major feature of AM during HDM-driven AAD. However, not all chemokine expression in AM[HDM] may be IL-4-dependent, since *Ccl7*, which showed IL-4R-dependent downregulation in PM in the *B. malayi* infection model, was significantly elevated in AM[HDM]. Chemokine expression by AM is therefore likely to be a result of integration of multiple signals in the allergic lung.

In addition to chemokines, AM[HDM] increased expression of a number of MMPs, including *Mmp12* and *Mmp14*, which can facilitate cell recruitment by several mechanisms, including ECM degradation and generation of chemokine gradients⁴²². A model can therefore be envisioned in which AM are key drivers of cell recruitment to the allergic airway through production of molecules such as chemokines and MMPs. This may seem paradoxical given that clodronate-mediated depletion of AM during AAD actually increases airway eosinophilia¹⁴⁴. However, crude depletion of AM does not dissect apart their pro-inflammatory and regulatory capacity, such that the loss of other AM functions may mask the lack of AM contribution to leukocyte recruitment in this model. Directed experiments examining the AM-specific roles of chemokines and MMPs will therefore be required to better understand the function of AM in leukocyte recruitment *in vivo*, but were outside the scope of this thesis.

Along with the increased expression of a number of putatively IL-4 induced chemokines, AM[HDM] expressed a number of genes associated with IL-4/IL-13-dependent alternative activation. These genes are thought to have functions in tissue repair and promotion of type 2 immune responses⁸⁰ and include *Arg1*, *Tgm2*, *Chil3* and *Retnla*. The precise functions of these molecules in AM during AAD are incompletely understood. For example, elevated *Chil3*/Ym1 expression by macrophages has been reported in multiple models of parasitic infection and allergic inflammation, but its function in these settings is unknown⁴²³. Paradoxical effects for *Retn1a*/Relm α expression *in vivo* have been reported. Some studies have shown Relm α expression to dampen type 2 inflammation and fibrosis^{424,425}, while others have suggested a role in promoting vascular remodelling in the allergic lung⁴²⁶. Despite this ambiguity, there is clearly a strong alternative, M[IL-4]-like, activation component to the AM[HDM] transcriptome, which is likely to have consequences for the function of these cells *in vivo*.

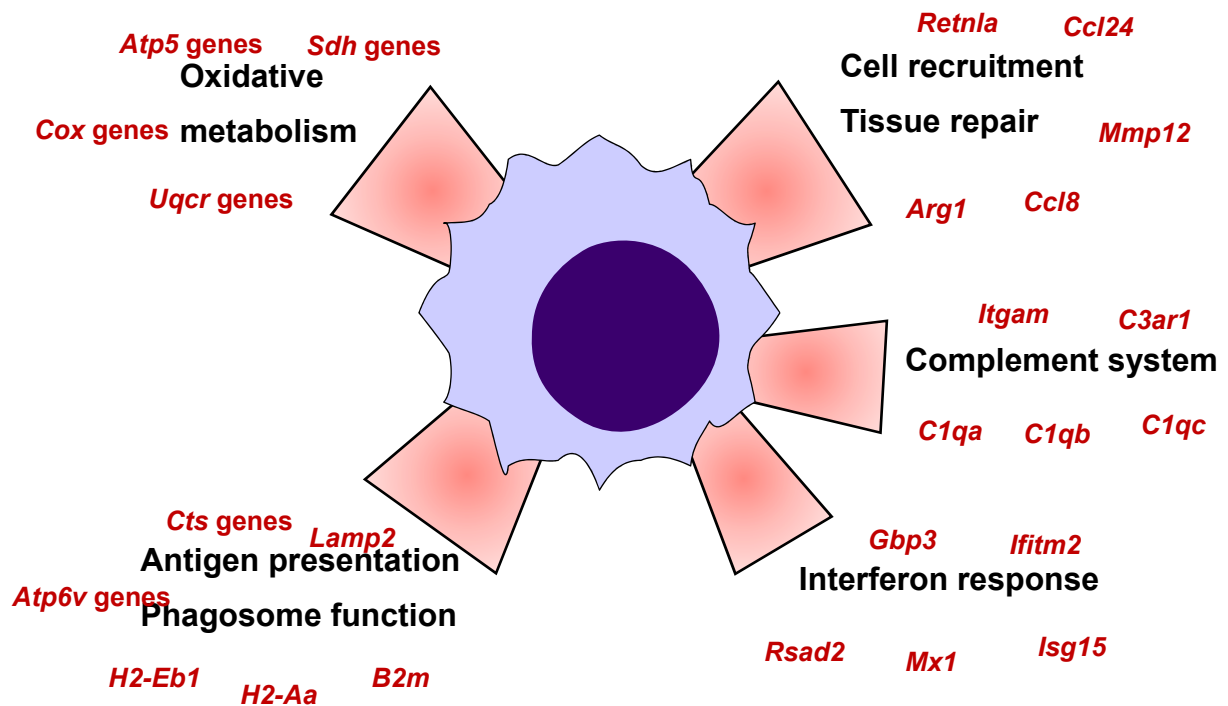
Gene expression data suggested that the AM[HDM] phenotype was accompanied by increased expression of a large proportion of ETC genes, and therefore presumably a shift towards ATP generation by oxidative phosphorylation. Oxidative phosphorylation is a more efficient and durable method of ATP generation than glycolysis and is associated with macrophage activation by IL-4 and/or IL-13^{427,428} and reduced inflammatory cytokine production by macrophages⁴²⁹. Deviation towards oxidative phosphorylation has also been demonstrated as a mechanism by which IL-10 dampens pro-inflammatory cytokine production in macrophages⁴³⁰. As suggested for

other gene signatures in AM[HDM], the shift towards oxidative phosphorylation genes is therefore likely to be driven by the cytokine milieu of the allergic lung, particularly IL-4. Indeed, a number of the ETC genes induced in AM[HDM] were also elevated in PM following nematode infection, in an IL-4R-dependent manner⁴¹⁹. The link between oxidative phosphorylation and the IL-4-dependent alternative activation phenotype in macrophages appears to be bidirectional, since inhibition of mitochondrial function prevents the anti-inflammatory effects of IL-4 on macrophages⁴²⁹. Consequently, if determined to be beneficial to the outcome of AAD, it may be possible to reduce the effects of IL-4 on AM by therapeutic deviation of metabolism away from oxidative phosphorylation.

Despite the strong evidence for an IL-4-driven component, the AM[HDM] transcriptional profile was not indicative of purely alternatively activated, M[IL-4]-like macrophages. As discussed above, AM[HDM] showed some key transcriptional differences to the IL-4R-dependent signature induced by nematode infection in PM⁴¹⁹, including expression of *Ccl7* in AM[HDM]. Direct comparison to published M[IFN γ] and M[IL-4] transcriptome data⁷⁹ also demonstrated that, while AM[HDM] showed substantial expression of M[IL-4]-associated genes, they also expressed a proportion of M[IFN γ] activation genes, suggesting an intermediate phenotype, driven by IL-4, IFN γ and other signals in the allergic lung. In support of a such a signal integration model in response to mucosal allergen exposure, AM[HDM] showed increased expression of a number of interferon-stimulated genes, suggesting that they are exposed to either autocrine or paracrine type I or III IFN signalling during HDM-driven AAD. Type I IFNs have recently been shown to promote DC migration during initiation of a Th2 response to *Schistosoma mansoni*³⁸⁰, supporting a potential role in type 2 immunity. Since AM are not thought to migrate to draining lymph nodes^{19,20}, the effects of type I IFN signalling in these cells during type 2 immunity is likely to be distinct to that in DC and will require further investigation.

Altogether, AM[HDM] showed elevated expression of a number of distinct clusters of genes, suggesting increased capacity for cell recruitment, tissue repair, antigen presentation and phagosome functions during HDM-drive AAD (**Fig. 5.32a**). These changes appeared to be driven by multiple cytokine signals and not just IL-4, illustrating the likely integration of type 2 cytokines and classical pro-inflammatory cytokines by AM in response to mucosal HDM exposure.

a) Increased in AM[HDM]



b) Decreased in AM[HDM]

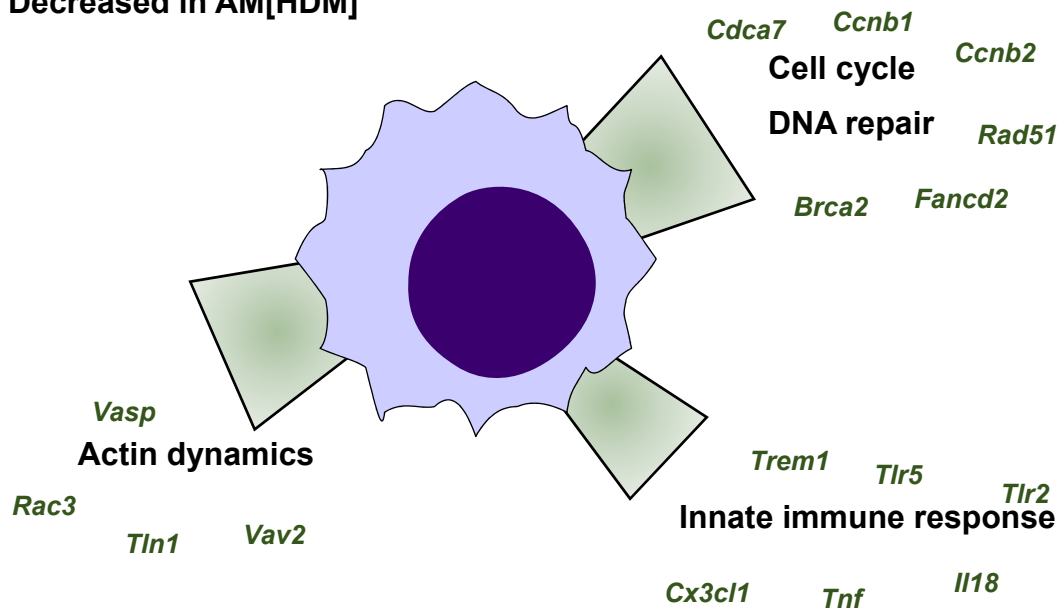


Figure 5.32. Major gene expression changes in AM[HDM]

Summary of the major biological processes predicted to be altered in AM from HDM-treated mice, based on functional annotation of hierarchically clustered upregulated (a) and downregulated (b) genes. Genes representative of each process are shown in red (upregulated) and green (downregulated).

5.11.3. Perspectives from genes decreased or unchanged in AM during AAD

Despite marked upregulation of a number of pro-inflammatory cytokine genes in AM[HDM], including *Il1a*, *Il1b* and several chemokines, a minority of pro-inflammatory genes were decreased in AM from HDM-treated mice. These included *Tnf*, which was shown earlier in this thesis to be expressed by resting murine AM and substantially induced by stimulation with TLR agonists, including in the presence of IL-4 (see Chapter 4). This further supports the activated phenotype of AM[HDM] arising from complex integration of local signals, rather than being dominated by a response to endotoxin present in HDM preparations, or IL-4 in the type 2 immune environment. IL-4R-dependent downregulation of *Tnf* has been reported in PM of *B. malayi*-infected mice, indicating that IL-4/IL-13 signalling *in vivo* can dampen *Tnf* expression in macrophages⁴¹⁹. It remains to be seen whether decreased *Tnf* expression in AM[HDM] depends on IL-4R signalling.

Changes to pattern recognition signalling molecules in AM[HDM] were also varied. While significant decreases were observed in expression of *Tlr2*, *Tlr5* and the TLR signal-enhancing receptor *Trem1*, levels of many other key pattern recognition genes, including *Tlr3*, *Tlr4*, *Tlr7* and *Myd88*, were unchanged in AM[HDM]. Thus, AM[HDM] display specific reductions in selected pattern recognition signalling molecules, rather than a general loss of expression in these genes. Reduced TLR2 expression by AM in HDM-driven AAD has previously been demonstrated and suggested to increase susceptibility of mice to subsequent respiratory *Streptococcus pneumoniae* infection¹⁷⁹, as is known to occur in asthma⁴³¹. It will be interesting to more thoroughly examine the impact of the altered pattern recognition receptor repertoire of AM[HDM] on their responsiveness to microbial infection.

In addition to innate immune signalling genes, a number of genes related to remodelling of the actin cytoskeleton were significantly downregulated in AM[HDM], including small GTPase genes such as *Rac3* and genes encoding proteins that anchor surface receptors to the cytoskeleton, such as *Tln1/Talin-1*³⁸⁴. Because of the fundamental role of the cytoskeleton in multiple aspects of cell biology, it is difficult to infer the likely functional outcomes, if any, of these decreases in gene expression. However, canonical pathway analysis of downregulated genes in AM[HDM] suggested that the Fc γ receptor-mediated phagocytosis pathway was reduced in these cells. Capacity for phagocytosis has previously been shown to be reduced in AM from experimental AAD models¹⁴⁶ and in severe asthma patients^{432,433}. It would therefore be interesting to determine the impact of the observed decreases in expression of actin remodelling genes on the phagocytic ability of AM[HDM].

The most significantly enriched functional annotations for downregulated genes in AM[HDM] were related to cell cycle and DNA replication and repair processes. This suggests that cell cycle-dependent turnover of AM, a feature of these cells at homeostasis⁶¹, is reduced in AM[HDM]. This is in contrast to the known effects of IL-4 in driving proliferation of macrophages, including AM, *in vivo*^{138,434} and suggests that integration of signals in the allergic airway may result in reduction of AM self-renewal by proliferation, even in the context of IL-4 signalling. It is also possible that a

subset of AM are derived from monocytes during AAD and express lower levels of cell cycle-related genes than true resident, self-renewing, cells, contributing the net decrease observed compared to resident AM from PBS control mice. The effects of HDM-driven AAD on proliferation and cell cycling of AM has not previously been reported and would need to be examined experimentally, for example by flow cytometry staining for Ki67 and DNA content⁴³⁵, to verify these findings.

Overall, in parallel to marked upregulation of pro-inflammatory genes in AM[HDM], a subset of immune response genes were decreased, along with a gene expression pattern suggestive of reduced cell cycling capacity (**Fig. 5.32b**).

5.11.4. Perspectives from comparison of sorted AM and whole BAL transcriptomes

Following identification of signatures of AM[HDM] gene expression, comparative analysis was performed to determine the expression of these genes in a mixed population of airway lumen leukocytes. A limitation of these analyses is that it is difficult to truly attribute expression of certain genes in BAL to AM[HDM] without first understanding the gene expression of all other major cell populations. Such an approach, sorting major pulmonary cell populations and comparing to whole lung, has proven useful in attributing a gene signature associated with lethality to neutrophils in mouse models of influenza infection⁸. A more powerful comparative analysis of AM and BAL of HDM-treated mice could therefore be performed by sorting of other major leukocyte populations from the allergic airway, particularly eosinophils and lymphocytes, which make up around 50-60 % and 10-20 % of BAL cells, respectively, during HDM-driven AAD.

Despite this limitation, it was possible to infer the relative restriction of certain gene sets to AM[HDM], which showed increased expression in AM[HDM] but not in unsorted BAL of HDM-treated mice. This expression pattern suggested a lack of upregulation by other cell types in BAL, such that the AM-derived signal was drowned out in unsorted cell preparations. By this reasoning, expression of a number of phagosome function and antigen presentation genes, including cathepsins and MHC-II genes, were determined to be predominantly expressed by AM in BAL of HDM-treated mice. This supports AM[HDM] being superior to other airway lumen leukocytes in terms of antigen presentation and phagosome function, despite the potential role for eosinophils in antigen presentation during AAD⁴³⁶. It is notable however that many highly competent phagocytes and APC, including dendritic cells, monocyte-derived cells and interstitial macrophages, are predominantly located in lung tissue rather than the airway lumen^{31,40,214}, so the relative contribution of AM to pulmonary antigen presentation during AAD will require experimental assessment. At least at steady state, AM are highly sessile and predominantly located in alveoli²⁰, away from the peribronchial inflammation site of allergic inflammation. However, altered AM dynamics and localisation, along with T cell motility in the lung, could facilitate local T cell re-stimulation by AM during AAD.

In addition, comparative analysis suggested that increased expression of oxidative phosphorylation and ETC genes appeared to largely derive from AM in BAL of HDM-treated mice.

This fits with the known shift towards glycolysis as the means of ATP generation in activated effector T cells⁴³⁷, suggesting that infiltrating CD4⁺ T cells in the airway would not be expected to contribute high levels of ETC expression. Generation of reactive oxygen species via NADPH oxidase is central to the function of eosinophils in tissues⁵⁵. However, the ETC activity of eosinophils during AAD has not been reported, although the data in this chapter would suggest that they do not express sufficiently high levels of these genes in the allergic airway to result in increased detection in BAL[HDM]. Nonetheless, these data suggest that the mechanisms of ATP generation by AM[HDM] and effector T helper cells in the allergic airway are likely to be highly distinct.

By comparing gene clusters obtained from reciprocal comparison of sorted AM and whole BAL transcriptomes, a signature of 16 AM[HDM] genes was identified that was readily detectable in the unsorted BAL of HDM-treated mice. These included *Arg1*, a well-established indicator of alternative macrophage activation⁸⁰, detection of which in BAL could be used to infer the presence of cells with the AM[HDM] phenotype. *Arg1* was also shown to be expressed by ILC2s, but not eosinophils or CD4⁺ T cells, in a mouse AAD model⁴³⁸, suggesting that detection of high levels of *Arg1* expression in BAL cells is likely to be indicative of AM dysfunction during HDM-driven AAD. However, expression of other components of this gene signature may derive from a combination of AM and other airway leukocyte populations. For example, metalloproteinase Adam8 (*Adam8*) negatively regulates allergic airway inflammation, but is known to be expressed by both AM and eosinophils⁴³⁹. Nevertheless, the detectability of these AM[HDM] genes in whole BAL may be useful for monitoring AM dysfunction in AAD models without cell sorting.

The 16 gene AM-BAL crossover signature was dominated by complement-related genes *C1qa-c* and *C3ar*, highlighting these genes as some of the strongest signals of AM[HDM]. However, the function of this increased complement gene expression by AM in AAD is not clear. *C1q* genes have been shown to be increased in an IL-4Ra-dependent manner in peritoneal macrophages following *B. malayi* nematode infection, indicating that C1q expression can be a feature of the type 2 immune environment⁴¹⁹. C1q has also recently been shown to synergise with IL-4 in induction of proliferation and alternative activation of peritoneal and liver macrophages¹²⁹, highlighting a key local role of these genes in conditioning macrophages during type 2 immunity. However, C1q was shown to be redundant for these effects in AM, with SP-A instead performing the equivalent function¹²⁹, so it is unlikely that the increased expression detected in AM[HDM] reflects such a mechanism. Increased C1q expression may promote beneficial efferocytosis by opsonisation of apoptotic cells, as has been shown previously in macrophages^{440,441}. In contrast, the observed increased expression of *C3ar1* could conceivably promote inflammatory cytokine production by AM, since C3a has been shown to drive inflammasome activation in human monocytes via C3aR1⁴⁴². The functional implications of increased complement gene expression by AM during AAD are therefore likely to be complex and will require focussed experiments to dissect.

While AM->BAL comparative clustering analysis allowed AM gene expression to be compared to that broadly displayed by other cells in the airway lumen, this approach did not include parenchymal leukocytes or pulmonary stromal cells. The latter cells are crucial for airway remodelling, which is largely driven by dysregulation of epithelial cells, fibroblasts and smooth muscle⁴⁴³. The relative contribution of AM to pulmonary expression of remodelling genes upregulated following HDM exposure, such as *Pdgfa* and *Edn1*, could therefore not be estimated. Despite this, elevated PDGF production has been observed by AM from idiopathic pulmonary fibrosis patients, with potential to drive smooth muscle cell recruitment and fibroblast growth⁴⁴⁴, suggesting that AM-derived PDGF may be a common mediator in multiple chronic lung diseases. Conditional knockout approaches targeting these mediators in AM would therefore be required to evaluate the role of AM-derived PDGF and endothelin-1.

5.12.5. Activity of TGF- β and IL-10 pathways in AM[HDM]

In addition to unbiased analyses of the AM[HDM] transcriptome, the TGF- β and IL-10 signalling pathways were examined in detail. No clear net increases in expression of components of these pathways was apparent in AM[HDM], suggesting that AM remain capable of receiving signals from TGF- β and IL-10 during AAD and that these pathways are therefore likely to regulate AM function during HDM-driven AAD.

In the case of the TGF- β pathway, few changes in gene expression were observed. *Tgfb2* was significantly decreased to levels below the lower detection threshold in AM[HDM]. This was in stark contrast to *in vitro* stimulation of AM with LPS or RSV, which increase *Tgfb2* mRNA expression (see Chapter 4), suggesting that the allergic airway microenvironment has distinct effects on regulation of *Tgfb2* than other pro-inflammatory stimuli. Notably, the resting levels of *Tgfb2* mRNA in AM[PBS] in this study and naïve AM from previous work (Chapter 4) were very low relative to *Tgfb1*, so the *in vivo* relevance of decreased expression in AAD is unlikely to be of major importance. The foremost change in TGF- β family gene expression was in *Inhba*/activin A, which may be of interest given the role of activin A for airway remodelling *in vivo*^{96,136} and the increased expression of the activin A receptor on bronchial epithelial cells of asthmatics following allergen challenge¹³⁵. Assessment of the specific contribution of AM-derived activin A to airway remodelling during AAD is therefore an interesting future avenue for study.

Although no net changes were observed in components of the IL-10 signalling pathway, IL-10-inducible genes, such as *Socs3* were elevated in AM[HDM], which may indicate increased IL-10 signalling to AM during HDM-driven AAD. However, it is not possible from these data to separate the effects of IL-10 from other cytokines with overlapping effects on gene expression, particularly IL-4. IL-10 and IL-4 can synergise to induce expression of genes such as *Ccl24* in macrophages⁴¹³ and both IL-4 and IL-10 can drive macrophages towards oxidative phosphorylation^{427,430}, such that the gene expression profile of AM[HDM] could feasibly reflect integration of IL-4 and IL-10 signals. However, as discussed later in this thesis, it will be necessary to specifically interfere with IL-10 signalling to AM to test this hypothesis.

Despite the focus of this thesis on TGF- β and IL-10, the possibility remains that AM employ other immunoregulatory mechanisms during AAD. Production of retinoic acid by AM has been shown to promote Treg generation in co-culture systems^{153,445}. The increased expression of the *Aldh1a2*/RALDH2 by AM[HDM] observed in the present work suggests that RA production by AM may increase during AAD. The effects of AM-derived RA on regulation of the pulmonary immune response to allergen may therefore be of interest in future studies.

5.11.6. Potential future work

A caveat of this study is that, due to time and resource limitations, AM were only studied at 24 hours post final allergen challenge, a timepoint at which airway inflammation is still highly active in the HDM AAD model³¹⁷. It is possible that the marked upregulation of pro-inflammatory genes in AM[HDM] may have masked gene expression related to immunoregulatory functions of these cells. HDM-induced AAD substantially resolves in mice in the two weeks following cessation of allergen exposure, in a manner dependent on AM¹⁴⁴, suggesting that expression of immunoregulatory genes by AM may be higher at later timepoints following allergen challenge. These timepoints may also better model stable AAD, rather than an acute response to allergen and allow better resolution of inflammatory and regulatory gene expression by AM. With this in mind, future study of the transcriptomes of AM[HDM] should be examined throughout the resolution of disease following final HDM challenge, to better understand how these cells contribute to immunoregulation and resolution of allergic inflammation.

Another intriguing possibility is that the AM[HDM] analysed in this study consisted of two or more subpopulations, potentially differing based on being initially tissue-resident or derived from recruited monocytes, as has been shown for peritoneal macrophages⁴⁴⁶ and for AM during fibrosis¹⁴⁰. It can be speculated that the increased expression of both classical and alternative activation genes in AM[HDM] could reflect the presence of two or more distinct AM populations. Specific labelling or chimaera-based approaches to distinguish tissue-resident versus recruited AM prior to transcriptomic analysis would be useful in addressing this question, possibly along with single cell RNAseq. Such analysis would allow better understanding of heterogeneity within AM[HDM] and their developmental relationship to precursor cells, as has recently been described for human dendritic cell subsets⁴⁷.

5.12. Conclusions

Overall, transcriptomic analysis of AM from a mouse model of AAD driven by repeated mucosal allergen exposure revealed a number of gene expression signatures indicative of pro-inflammatory and tissue repair processes. These gene expression changes highlight potential contributions of AM to the immunopathogenesis of AAD, which warrant further investigation. Notably, strong evidence of increased metabolism by oxidative phosphorylation was also found to accompany these changes. No dysregulation or heightened activation of the IL-10 and/or TGF- β pathways was apparent from these transcriptomic data alone, indicating that more direct approaches will be required to understand the function of these pathways in AM *in vivo*, as discussed in chapters 6 and 8.

**6. Dissecting the role of airway macrophages in
regulation of allergic airway inflammation by
interleukin 10 and TGF- β 1**

6.1. Introduction

The cytokines IL-10 and TGF- β 1 are central to the complex regulatory networks controlling pulmonary immune responses, such as those driving the immunopathogenesis of allergic airway disease (AAD). Both IL-10 and TGF- β 1 are widely implicated in airway tolerance to inhaled antigens and in regulation of AAD, particularly production of these cytokines by thymic and peripherally-generated regulatory T cells (Tregs)^{101,102,104,357,447}. However, as established in the literature and assessed in Chapter 4 of this thesis, both IL-10 and TGF- β 1 can derive from diverse cellular sources *in vivo* and drive distinct responses in different cell types^{106,181,218}. Dissecting the functions of specific cell types in the action of IL-10 and TGF- β 1 *in vivo* is therefore crucial to understanding regulatory immune interactions in the lung.

Given the apparent tolerogenic capacity of airway macrophages (AM) at homeostasis and during AAD^{19,144,145,147,148,448}, it is possible that TGF- β 1 and IL-10 are involved in their immunoregulatory function; however direct evidence for this is lacking. Although the work described in Chapter 4 and in published literature suggests that AM are minor pulmonary IL-10 producers compared with T cells¹⁰⁸, interstitial macrophages (IM) and inflammatory monocytes and macrophages^{31,156,214}, it remains possible that low-level IL-10 production by AM may regulate lung immunity. More conceivably, IL-10 from other cellular sources may promote a regulatory phenotype of AM *in vivo*, as described recently for resident macrophages in the intestine^{205,206}.

Although AM are just one of many sources of TGF- β 1 in the resting and inflamed lung (see Chapter 4 and references^{272,273}), this does not preclude a non-redundant immunoregulatory role for AM-derived TGF- β 1 in certain contexts, particularly given the unique spatial niche of these cells *in vivo*^{19,20}. TGF- β is essential for induction of FoxP3 in naïve T cells by AM in co-culture experiments¹⁵³, suggesting a potential role for AM-derived TGF- β in regulating T cell phenotype. However the extent to which this occurs *in vivo* is unclear, particularly given the sessile, non-motile behaviour of these cells in the lung, which is unlikely to favour frequent contact with naïve T cells²⁰. During preparation of this thesis, work was published describing an essential requirement for signalling via TGF- β R2 in the development and maintenance of AM and suggesting that the TGF- β required for this process is at least partially produced by AM themselves²⁸². Thus, AM may signal via TGF- β 1 in a paracrine manner to neighbouring leukocytes and stromal cells, as well as in an autocrine manner to regulate their own survival and phenotype.

Cytokines can be highly pleiotropic in function, driving distinct responses depending on the cellular source, local microenvironment and the responding cell. This is well exemplified by TGF- β 1, where global or T cell-restricted deletion results in fatal inflammatory disease²⁵⁰⁻²⁵², but deletion in bronchiolar epithelial club cells causes no adverse phenotype at baseline, but limits the inflammatory response to inhaled allergen²⁷³. In the case of IL-10, global knockout causes spontaneous inflammatory bowel disease, due in part to the requirement for IL-10 for suppression of antigen-presenting cell (APC) function and dampening of CD4⁺ T cell responses^{195,196,449}.

However, IL-10 can also promote generation of antibody-producing plasma cells from germinal centre B cells⁴⁵⁰ and its production by T follicular regulatory cells is crucial for optimal germinal centre formation following acute infection⁴⁵¹, highlighting its potential to both positively and negatively regulate immune responses.

These cell type-specific functions of regulatory cytokines are not merely of academic interest, since they can drastically impact the effects of therapeutic interventions. Global blockade of TGF- β 1, 2 and 3 with a pan-TGF- β antibody had conflicting effects on murine models of AAD, depending on whether antigen sensitisation was performed by injection into the peritoneal cavity or via inhalation^{279,280}: two models that employ distinct immunological mechanisms and, presumably, result in differential utilisation of TGF- β signalling. These contrasting findings clearly illustrate how globally blocking cytokines *in vivo* will not discriminate between cellular sources and so may mask effects of cytokine derived from particular cell types. With this in mind, conditional knockout mice present a more refined approach for specifically studying the role of cytokines derived from cells relevant to the model of interest. Such approaches have been useful in defining a critical role for T cell-derived IL-10 in regulating the immune response to RSV infection¹⁰⁸ and demonstrating an unexpected pro-inflammatory function of bronchiolar epithelial cell-derived TGF- β 1 in promoting innate lymphoid cell recruitment to the airway upon allergen inhalation²⁷³.

Cre-Lox technology has emerged as the gold standard method for spatially and/or temporally-specific study of mammalian gene function⁴⁵². However, conditional knockout of genes in individual cell types is limited by the requirement for a specific and robustly activated promoter in the cell of interest from which to drive Cre recombinase expression. This is particularly problematic when studying AM, for which no single defining marker currently exists¹⁹, meaning that partially specific conditional knockout approaches are required. Both the *Lyz2/LysM-Cre*⁴⁵³ and *Itgax/CD11c-Cre*²⁸⁴ mouse lines have been used for conditional gene knockout in AM^{25,283}, with up to 100 % recombination efficiency reported⁴⁵⁴. However, these lines also drive recombination in dendritic cells (DC; CD11c-Cre) and neutrophils, monocytes and other macrophage subsets (LysM-Cre)⁴⁵⁴, so results obtained must be interpreted with caution. A recent study showed LysM-Cre to drive inferior gene deletion in AM to CD11c-Cre, with the two Cre lines yielding different results when used to delete *Pparg*/PPAR γ , a key transcription factor for AM maturation²⁵. Further, since the focus of the present study was on regulatory cytokines, broad targeting of macrophage subsets by LysM-Cre would be likely to drive gene deletion in CD11c⁻ LysM⁺ IM, which can regulate pulmonary immunity via IL-10^{31,214}, potentially complicating interpretation of results. With these factors in mind, CD11c-Cre was selected as the best currently available tool for knockout of AM-derived IL-10 and TGF- β 1 *in vivo*.

In this chapter, conditional knockout and antibody blockade approaches were used to study the role of AM-derived IL-10 and TGF- β 1 in pulmonary immunity and to examine the effects of IL-10 from other major cellular sources on AM activation and the immune response to inhaled allergen.

6.2. Hypothesis and aims

Hypotheses

- 1) Based on data in Chapter 4, it was hypothesised that AM-derived IL-10 is not required to regulate pulmonary inflammation, but that IL-10 from other cellular sources controls AM phenotype in the allergic lung
- 2) AM-derived TGF- β 1 exerts a non-redundant role in regulating the pulmonary immune response to inhaled allergen

Aims

- 1) Validate a Cre-Lox system for conditional gene deletion in AM
- 2) Determine the effects of AM-specific IL-10 knockout on pulmonary inflammation
- 3) Determine the effects of IL-10 from other cellular sources on AM phenotype and development of AAD
- 4) Determine the effects of AM-specific TGF- β 1 deletion on development of pulmonary inflammation

6.3. CD11c-Cre: a partially specific murine system for conditional *in vivo* gene deletion in airway macrophages

To evaluate the efficiency of CD11c-Cre as a driver of recombination in AM and other cell types at homeostasis and during inflammation, CD11c-Cre mice were crossed to the *R26R^{eYFP}* reporter strain, which produce enhanced yellow fluorescent protein (eYFP) under control of the near-ubiquitously expressed *Rosa26* promoter in cells expressing Cre recombinase²⁸⁵. Resultant mice, expressing both CD11c-Cre and *R26R^{eYFP}*, were subjected to a 3 week house dust mite (HDM) exposure model of AAD and eYFP signal in lung leukocytes assessed by flow cytometry (**Fig. 6.1a**).

Consistent with effective recombination in AM, around 80 % of AM showed positive eYFP signal in CD11c-Cre x *R26R^{eYFP}* mice that received either PBS control or HDM (**Fig. 6.1b**). Recombination was also detected in the majority of classical DC (cDC) in PBS and HDM-treated mice, although efficiency was unexpectedly low at around 60 % in CD103⁺ CD11b⁻ cDC1 and close to 50 % in CD11b⁺ CD64⁻ CD103⁻ cDC2 (**Fig. 6.1b,c**). Recombination efficiency was lower still in HDM-elicited CD64⁺ CD11b⁺ monocyte-derived DC (moDC), at less than 40 % (**Fig. 6.1b**), consistent with these cells having likely only recently upregulated CD11c upon differentiation from monocytes in the lung⁴⁰. This relatively low recombination efficiency in pulmonary DC is not entirely surprising, since although this CD11c-Cre line is well established to efficiently function in splenic DC^{284,454}, the original publication describing these mice referred briefly to lung DC being only 'mostly eYFP⁺' when crossed to *R26R^{eYFP}* mice²⁸⁴.

Low levels of CD11c-cre-mediated recombination were observed in pulmonary lymphoid cells, including CD4⁺ T cells (7-13 %), CD8⁺ T cells (6-11 %) and NKp46⁺ lymphoid cells (natural killer and natural killer T cells, 9-25 %), while less than 10 % of eosinophils and less than 3 % of neutrophils showed positive eYFP signal (**Fig. 6.1b,c**). These low levels of recombination in lymphoid cells are consistent with a published resource⁴⁵⁴. To examine the distribution of CD11c-Cre-mediated recombination outside of the lung, eYFP expression was also quantified in peripheral blood leukocytes. Recombination was detected in 40-50 % of circulating MHC-II⁺ CD11c⁺ DC (**Fig. 6.1d,e**), as well as around 30 % of CD64⁺ CD11b⁺ Siglec F⁻ Ly6G⁻ monocytes (**Fig. 6.1d**), specifically on a proportion of these monocytes also staining positively with an anti-CD11c antibody (**Fig. 6.1e**). Small proportions of peripheral blood lymphoid cells showed positive eYFP signal, as observed in the lung (**Fig. 6.1d**). Notably, HDM treatment had minimal effects on the proportions of eYFP expression by any cell populations in either lung or blood, with the exception of moDC, which, being inflammatory cells, were not detected at sufficient levels for reliable analysis in lungs of PBS-treated mice (**Fig. 6.1b**).

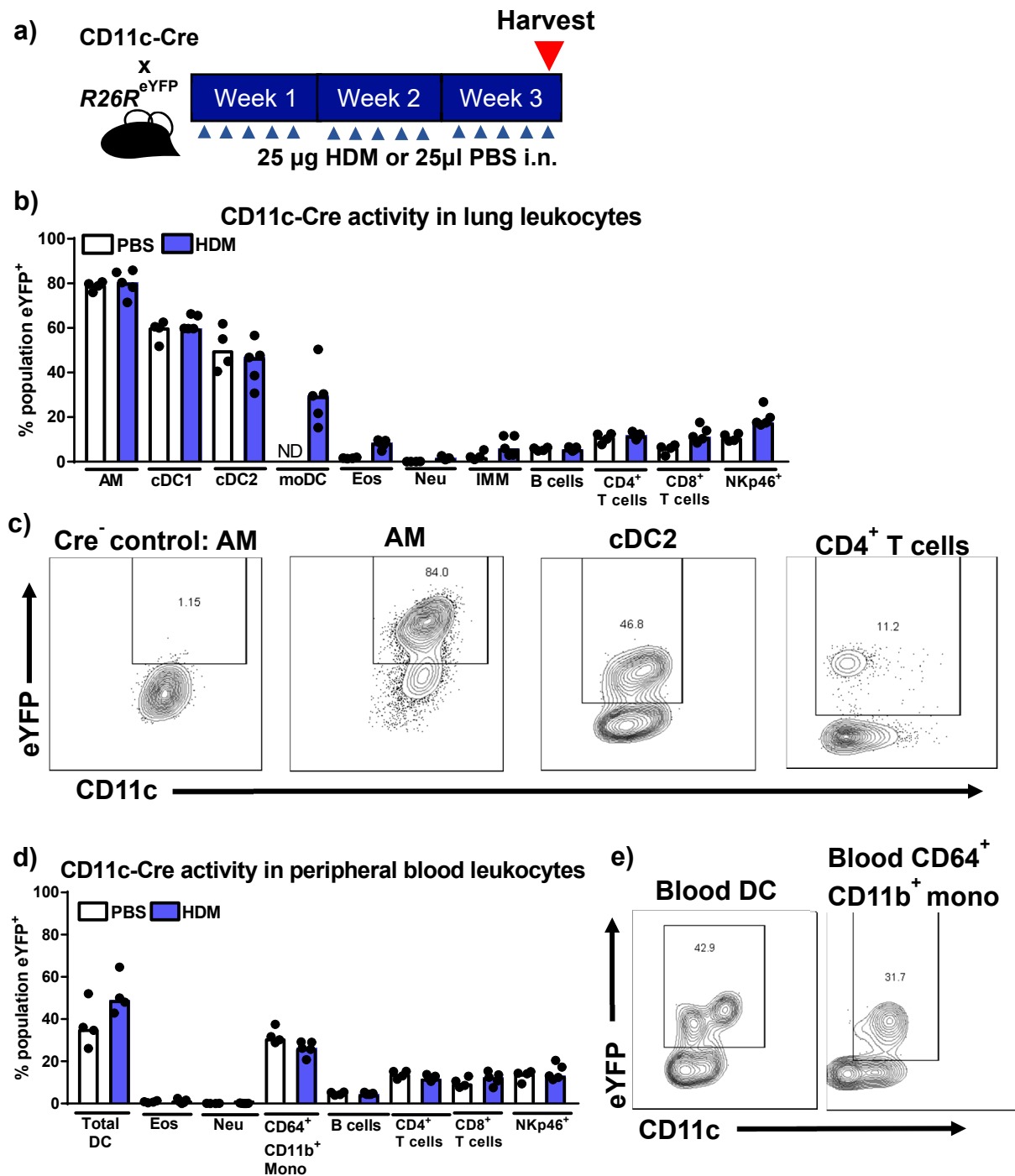


Figure 6.1. CD11c-Cre system for conditional gene deletion in airway macrophages

a) CD11c-Cre-reporter mice were generated by crossing CD11c-Cre mice to homozygous $R26R^{eYFP}$ mice. Reporter mice were exposed intranasally to HDM or PBS control for 3 weeks and tissues harvested 24h after final allergen challenge. **b)** Proportions of lung leukocyte populations with positive CD11c-Cre-reporter signal, as determined by flow cytometry. **c)** Representative flow cytometry plots from data shown in **(b)**. AM from a Cre-negative mouse are shown as a negative control. **d)** Proportions of peripheral blood leukocytes with positive CD11c-Cre reporter signal, as determined by flow cytometry. **e)** Representative plots of major reporter-expressing populations in peripheral blood. Scatter/bar plots show medians of 4-5 replicate mice per group from a single experiment. Numbers on flow cytometry plots show percentages of events within gates. Eos, eosinophils; Neu, neutrophils; IMM, interstitial/inflammatory monocytes/macrophages; mono, monocytes; NKp46⁺, NKp46⁺ lymphoid cells. ND, not detectable.

To examine the location of CD11c-Cre signal *in situ* in the lungs of mice during AAD, precision cut lung slices were prepared from HDM-treated CD11c-Cre x *R26R^{eYFP}* mice and stained with antibodies to CD11c, Siglec F and EpCAM to visualise the distribution of CD11c-Cre activity relative to AM (CD11c⁺ Siglec F⁺), DC (CD11c⁺ Siglec F⁻), eosinophils (Siglec F⁺ CD11c⁻) and epithelial cells (EpCAM⁺). CD11c-Cre reporter signal was localised to large cells with round morphology, the majority of which stained positively for CD11c and Siglec F, consistent with AM (white pseudocolour in **Fig. 6.2**). Importantly, no eYFP signal was observed in eosinophils or epithelial cells, indicating that there was no off-target Cre activity in these cells (**Fig. 6.2**). Due to their relative scarcity in lung tissue, recombination in DC populations could not be reliably assessed by this method.

Overall, CD11c-Cre was shown to be active in the vast majority of AM at homeostasis and during allergic inflammation and AM were shown by microscopy to be the most abundant cells to have undergone Cre-mediated recombination in the allergic lung. Lesser, but still substantial, recombination was detected in pulmonary DC, while granulocytes and lymphoid cells were mostly spared from recombination, consistent with a partially specific Cre system mostly targeting AM and cDC in the lung.

6.4. AM-derived IL-10 is dispensable for regulation of allergic lung inflammation

Although AM were shown previously to produce little to no IL-10 in response to pathogen-associated molecular patterns or during allergic pulmonary inflammation (see Chapter 4 and reference²¹⁴), it remained possible that low levels of AM-derived IL-10, near the limits of detection of the methods used in this thesis, could locally influence immune responses *in vivo*. To directly assess the importance of IL-10 production by AM, a conditional CD11c-driven IL-10 knockout mouse strain on a C57BL/6 genetic background, generated by crossing CD11c-Cre and *Il10^{fl/fl}* mice²⁸⁶ (hereafter CD11c^{Il10}), was employed (**Fig. 6.3a**).

As in previous studies in wild type mice (see Chapter 4), LPS stimulation of AM obtained from bronchoalveolar lavage (BAL) of *Il10^{fl/fl}* control animals induced a low level of *Il10* mRNA, which was undetectable in resting cells (**Fig. 6.3b**). However, this induction was not observed in AM from CD11c^{Il10} mice, consistent with effective IL-10 knockout in these cells. IL-10-deficient bone marrow-derived macrophages (BMDM) show enhanced production of pro-inflammatory cytokines such as TNF α in response to LPS stimulation *in vitro*, indicative of autocrine regulation by macrophage-derived IL-10 in this system²⁰⁶. To determine whether such autocrine regulation also occurred in AM cultures, pro-inflammatory cytokine gene expression was quantified in IL-10 deficient and control AM following LPS stimulation. LPS stimulation caused substantial increases in levels of *Tnf* and *Il1b* mRNA in both control and CD11c^{Il10} AM, with no differences observed in the IL-10-deficient cells (**Fig. 6.3b**), despite expression of both of these cytokine genes being suppressed by addition of exogenous IL-10 to AM (see Chapter 4). This suggests that the low level of IL-10 produced by AM does not regulate the pro-inflammatory LPS response in an autocrine manner *in vitro*.

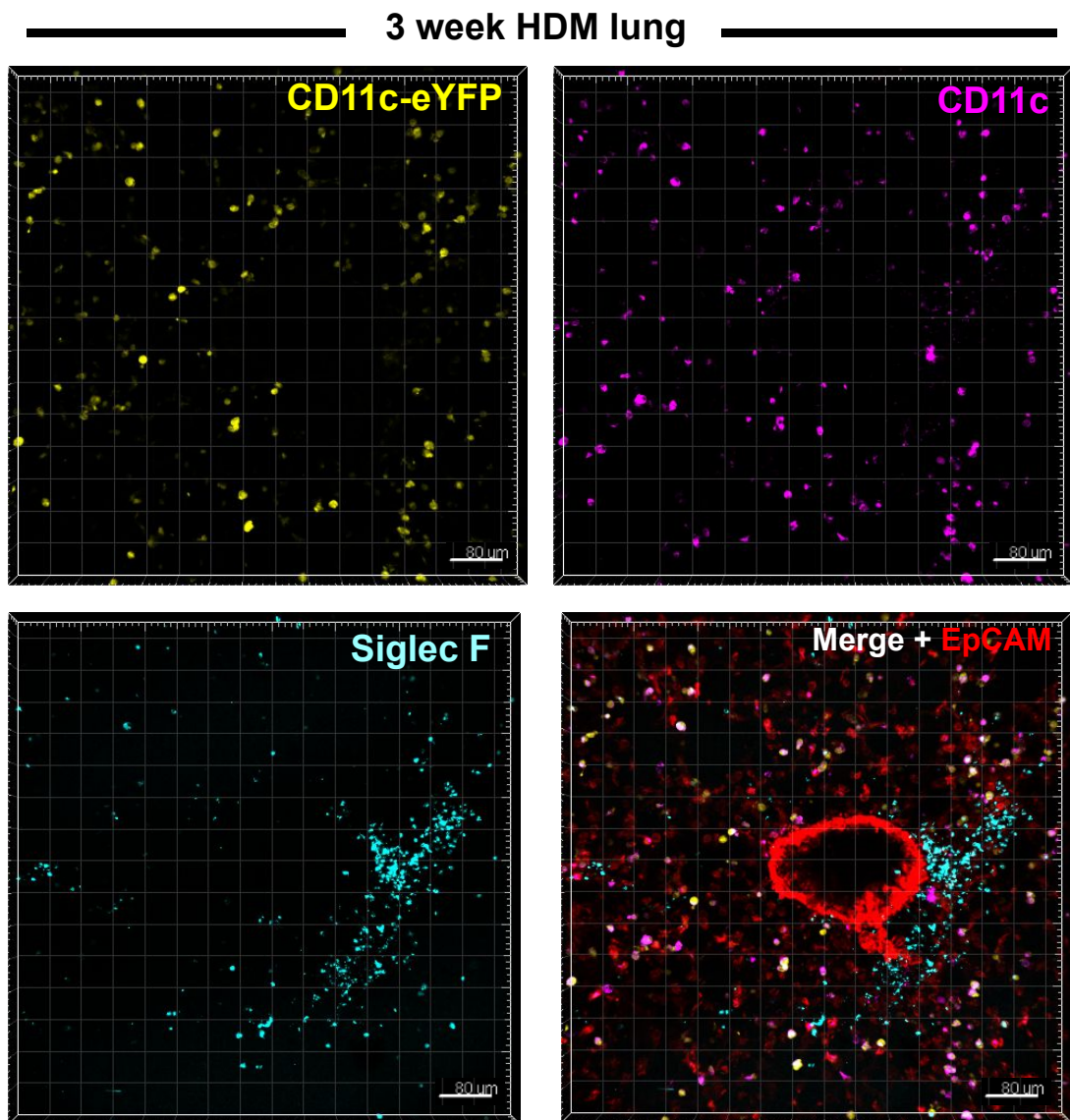


Figure 6.2. Detection of CD11c-Cre reporter signal in AM *in situ*

HDM-driven AAD was induced in CD11c-eYFP reporter mice as described in **Fig. 6.1.** and 200 μm precision-cut lung slices prepared and stained with anti-CD11c-APC (magenta) anti-Siglec F-BV421 (cyan) and anti-EpCAM-PE (red). A representative pseudocolour image is shown to demonstrate eYFP reporter expression relative to AM (Siglec F⁺ CD11c⁺), DC (CD11c⁺ Siglec F⁻) and eosinophils (Siglec F⁺ CD11c⁻). Individual fluorescent parameters are shown and merged with EpCAM staining to visualise overlap. eYFP⁺ cells with positive staining for CD11c and Siglec F (i.e. AM) appear white in the merged pseudocolour image.

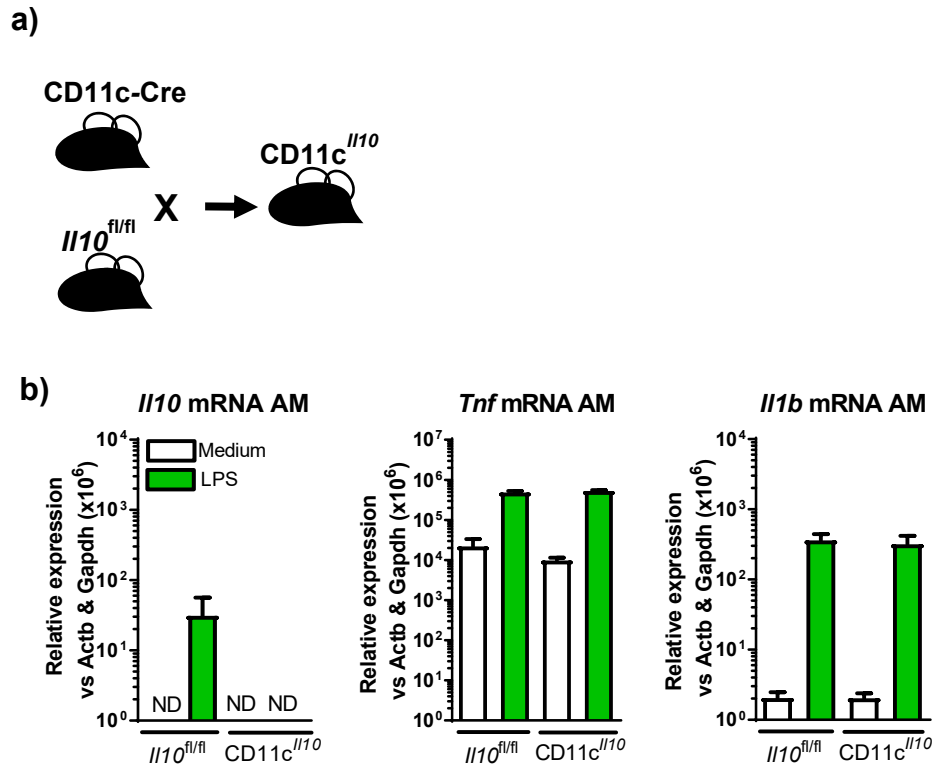


Figure 6.3. *In vitro* characterisation of AM from CD11c-conditional IL-10 knockout mice

a) CD11c ^{fl10} conditional knockout mice were generated by inter-crossing CD11c-Cre and $Il10^{fl/fl}$ mice for ≥ 2 generations to obtain CD11c-Cre hemizygous, $Il10^{fl/fl}$ mice (CD11c ^{fl10}) and Cre-negative littermate controls. b) AM from BAL of CD11c ^{fl10} mice and littermate controls were purified by adhesion and stimulate with LPS for 8h, before assessing *Il10*, *Tnf* and *Il1b* mRNA expression in cell lysates by qPCR relative to housekeeping genes. Data shown are means of 3 cell cultures from individual mice $\pm$ SEM and are representative of two independent experiments. ND, not detectable.

Having confirmed IL-10 knockout in AM from CD11c^{fl10} mice, the role of AM-derived IL-10 during AAD was assessed *in vivo*. AAD was induced in CD11c^{fl10} and littermate *Il10^{fl/fl}* controls by repeated exposure to HDM extract via the airways over 3 weeks (**Fig. 6.4a**). IL-10 was detectable in the lungs of non-inflamed PBS control and HDM-treated mice and, in both cases, concentrations did not differ between CD11c^{fl10} mice and controls (**Fig. 6.4b**). This supports the data described in Chapter 4 in suggesting that AM are not a major source of pulmonary IL-10 at homeostasis or during AAD.

Consistent with CD11c^{fl10} mice displaying no defect in overall IL-10 levels following HDM-treatment, no augmentation of AAD parameters was observed in these mice. HDM treatment induced substantial pulmonary inflammation, as shown by increased leukocyte counts in lung tissue, BAL and mediastinal lymph nodes (mLN), but numbers were comparable in CD11c^{fl10} and control mice (**Fig. 6.4c**). Similarly, HDM-induced airway hyperresponsiveness (AHR) to methacholine and decreased baseline pulmonary compliance were comparable in the conditional knockout and control mice (**Fig. 6.4d**). The composition of airway inflammatory infiltrates was also unchanged in the absence of AM-derived IL-10. AM numbers in BAL were consistent in PBS and HDM treated mice and did not differ between CD11c^{fl10} and control mice after either treatment (**Fig. 6.4e**), indicating that neither Cre expression nor IL-10 deletion impacted AM numbers in the airway lumen. The HDM regimen used in these studies induces mixed eosinophilic and neutrophilic airway inflammation in mice on a C57BL/6 background; however, numbers of neither cell type were significantly different from controls in HDM-treated CD11c^{fl10} mice (**Fig. 6.4e**). Thus, the overall phenotype and severity of AAD in HDM-treated mice was unaffected by a lack of IL-10 production from AM.

It was crucial to assess the effects of CD11c Cre-driven IL-10 deletion on DC subsets and T cell phenotypes following HDM exposure, as CD11c-Cre was active in a large proportion of lung DC (**Fig. 6.1b,c**), which are the major T cell activators during HDM-driven AAD⁴⁰. Moreover, IL-10 can directly regulate T helper cell function and survival *in vivo*^{212,455}, so lack of DC-derived IL-10 at the interface with CD4⁺ T cells could feasibly impact their phenotype. HDM treatment resulted in increased numbers of the cDC1, cDC2 and moDC in lung tissue, of which CD64⁺ CD11b⁺ moDC were the most abundant subset, however no differences in abundance of these cells were observed between CD11c^{fl10} and control mice (**Fig. 6.5a**). Similarly, quantifying total MHC-II⁺ CD11c⁺ DC in mLN as an indicator of DC migration from the lung showed no differences between conditional knockout mice and controls (**Fig. 6.5b**). These data were consistent with lung DC numbers and migration being unaffected by IL-10 knockout in AM and DC.

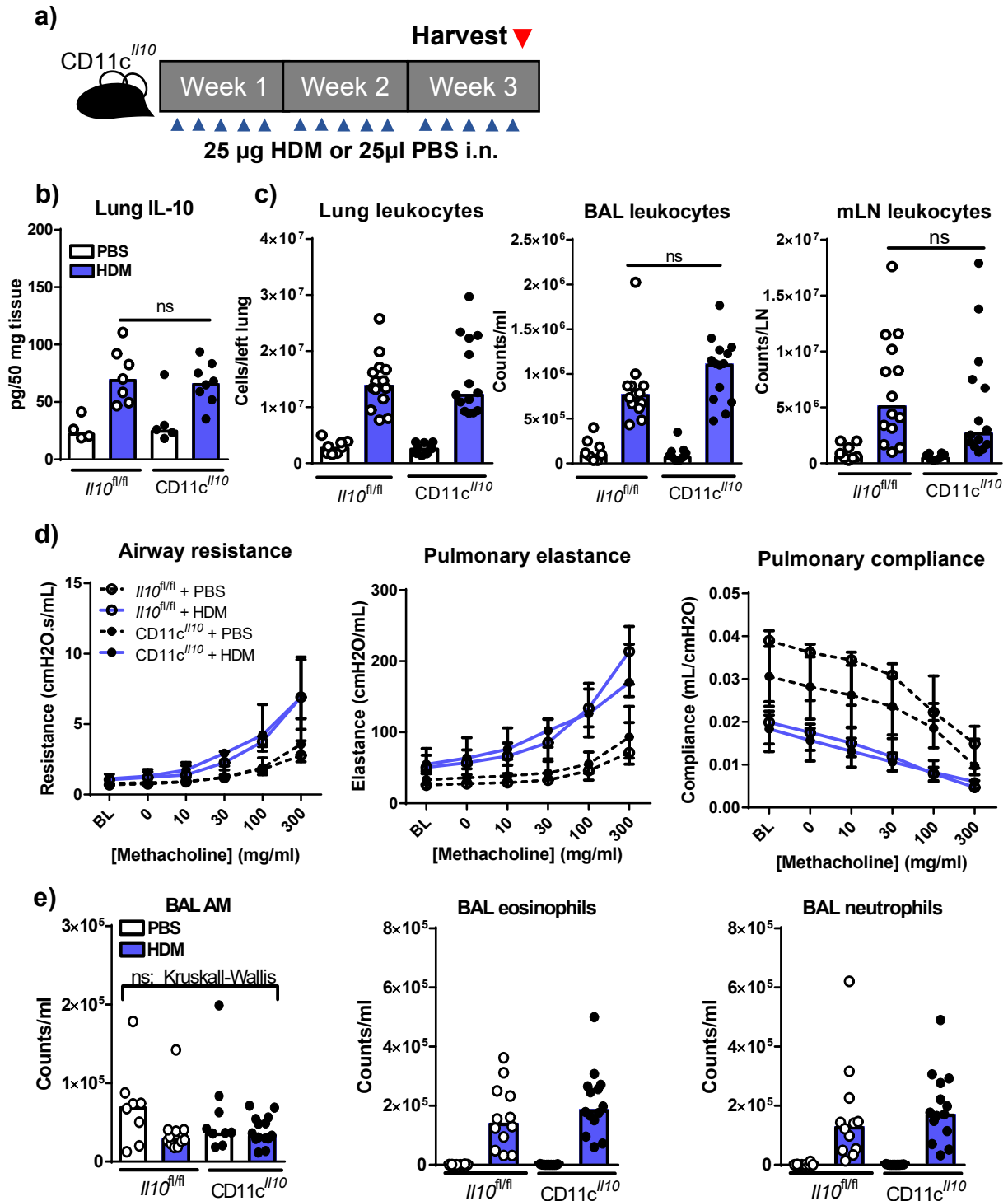


Figure 6.4. HDM-driven allergic airway disease is unchanged in CD11c^{Il10} mice

a) CD11c^{Il10} and Il10^{fl/fl} control mice were exposed to intranasal HDM or PBS control for 3 weeks (blue arrowheads) and analysed at 24h after final allergen challenge. **b)** Concentration of IL-10 in lung homogenates as determined by ELISA. **c)** Total crystal violet cell counts of leukocytes in lung digest, BAL and mLN preparations. **d)** FlexiventTM lung function measurements or airway resistance, pulmonary elastance and compliance at baseline (BL) and in response to increasing concentrations of inhaled methacholine. Graphs show medians $\pm$ IQR of 12-14 mice per group pooled from three independent experiments. **e)** Absolute numbers of AM, eosinophils and neutrophils in BAL as determined by flow cytometry. Scatter/bar plots show medians of 4-14 mice pooled from one (**b**) or two (**c** and **e**) independent experiments. Statistics: Kruskal-Wallis test comparing all groups, followed by Mann-Whitney tests between indicated groups; ns, not-significant.

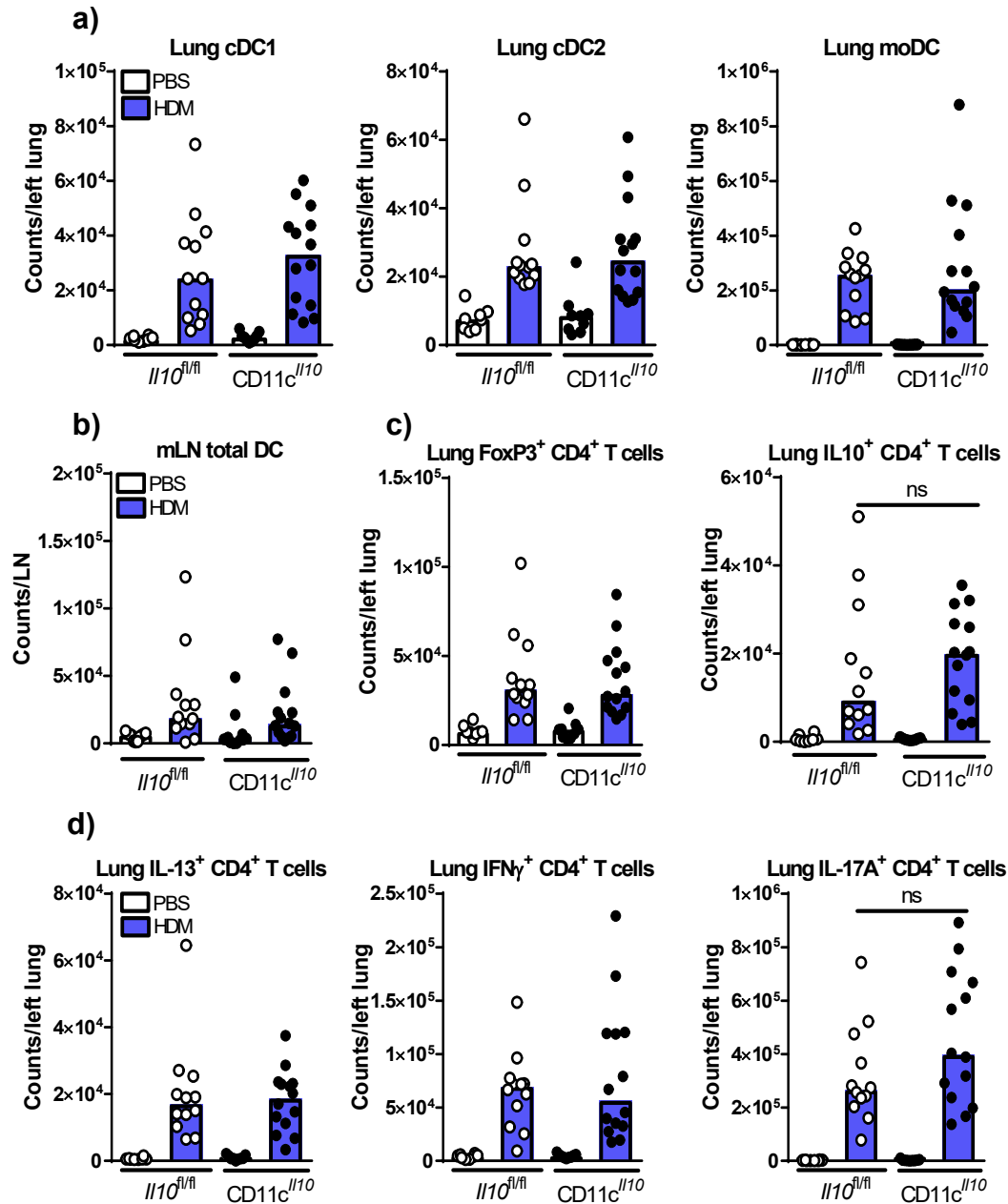


Figure 6.5. Adaptive immune response to HDM is unchanged in CD11c^{Il10} mice

Absolute numbers of immune cell populations as determined by flow cytometry, **a)** Numbers of cDC1 (CD103⁺ CD11b⁻ CD64⁻), cDC2 (CD11b⁺ CD64⁻ CD103⁻) and moDC (CD64⁺ CD11b⁺ CD103⁻) in lung digests. **b)** Total DC (MHC-II⁺ CD11c⁺ Ly6G⁻ Siglec F⁻ CD19⁻ CD90.2⁻ NKp46⁻) in mLN cell suspensions. **c)** Numbers of CD4⁺ T cells positive for intranuclear FoxP3 or intracellular IL-10 in lung digests. **d)** Numbers of CD4⁺ T cells positive for intracellular IL-13, IFN γ or IL-17A in lung digests. Graphs show medians of 8-14 replicate mice per group, pooled from two independent experiments. Statistics: Kruskal-Wallis test between all groups, followed by Mann-Whitney tests between indicated groups; ns, not-significant.

IL-10 can maintain FoxP3 expression and the immunosuppressive function of Tregs in tissues⁴⁵⁶ and can promote induction of IL-10 and regulatory capacity in CD4⁺ T cells by antigen-presenting cells (APC)^{457,458}. HDM-treated mice showed increased numbers of pulmonary FoxP3⁺ Tregs and IL-10⁺ CD4⁺ T cells, but numbers were not significantly different between littermate controls and CD11c^{fl10} mice (**Fig. 6.5c**). Similarly, no differences were observed in expression of the hallmark effector cytokines of the Th1, Th2 and Th17 lineages by CD4⁺ T cells (**Fig. 6.5d**). The HDM regimen used in these experiments induced a mixed effector T-helper cell response in the lungs, with induction of IL-13, IFN γ and IL-17A production by CD4⁺ T cells when assayed by *ex vivo* stimulation with PMA and ionomycin, none of which were detected at altered levels in HDM-treated CD11c^{fl10} mice than in littermate controls (**Fig. 6.5d**).

Overall, IL-10 expression by AM and DC was redundant for regulation of HDM-induced T cell responses and for controlling the overall severity of AAD.

6.5. AM-derived IL-10 is dispensable for regulation of the early immune response to RSV infection

Given that the AAD phenotype was unaffected by IL-10 deletion in AM, it was important to determine whether this lack of effect was specific to allergic inflammation. Experiments described in Chapter 4 demonstrated superior induction of *Il10* mRNA in AM *in vitro* by RSV stimulation compared with LPS, as well as a small proportion of AM with IL-10 reporter signal following *in vivo* RSV infection. Although these levels of IL-10 production were low compared to other cell types tested, it remained possible that AM-derived IL-10 regulated the immune response to RSV, particularly since AM are crucial cytokine producing cells in the early stages of infection with this virus^{60,154}.

To assess the importance of AM-derived IL-10 in regulating the early immune response to RSV, CD11c^{fl10} mice and littermate *Il10*^{fl/fl} controls were infected with RSV-A2 or virus-free cell lysate control and disease monitored over 4 days post-infection (dpi) before harvesting tissues (**Fig. 6.6a**). 4 dpi was selected as a timepoint at which the earliest pulmonary lymphocyte infiltration is detected in RSV-infected mice³⁵², to allow assessment of the role of AM-derived IL-10 during the innate immune response and T cell priming stages of disease. Focussing on this early timepoint would also avoid potential confounding of results by emergence of CD8⁺ T cells expressing CD11c, which have been reported to have a key antiviral function at later timepoints in experimental RSV infection⁴⁵⁹.

RSV infection caused a characteristic reduction in body weight of mice at 1 dpi, however this transient anorexia did not differ between CD11c^{fl10} and control mice (**Fig. 6.6b**). IL-10 was detectable in lung tissue in both uninfected and infected mice but was not significantly increased upon infection and not reduced in CD11c^{fl10} mice (**Fig. 6.6c**), consistent with little contribution of AM to total pulmonary IL-10 levels during the innate response to infection. No IL-10 protein was detected in BAL fluid in RSV-infected mice of either genotype (limit of detection: 31.25 pg/ml; not shown), supporting a lack of IL-10 release into the airway lumen by AM.

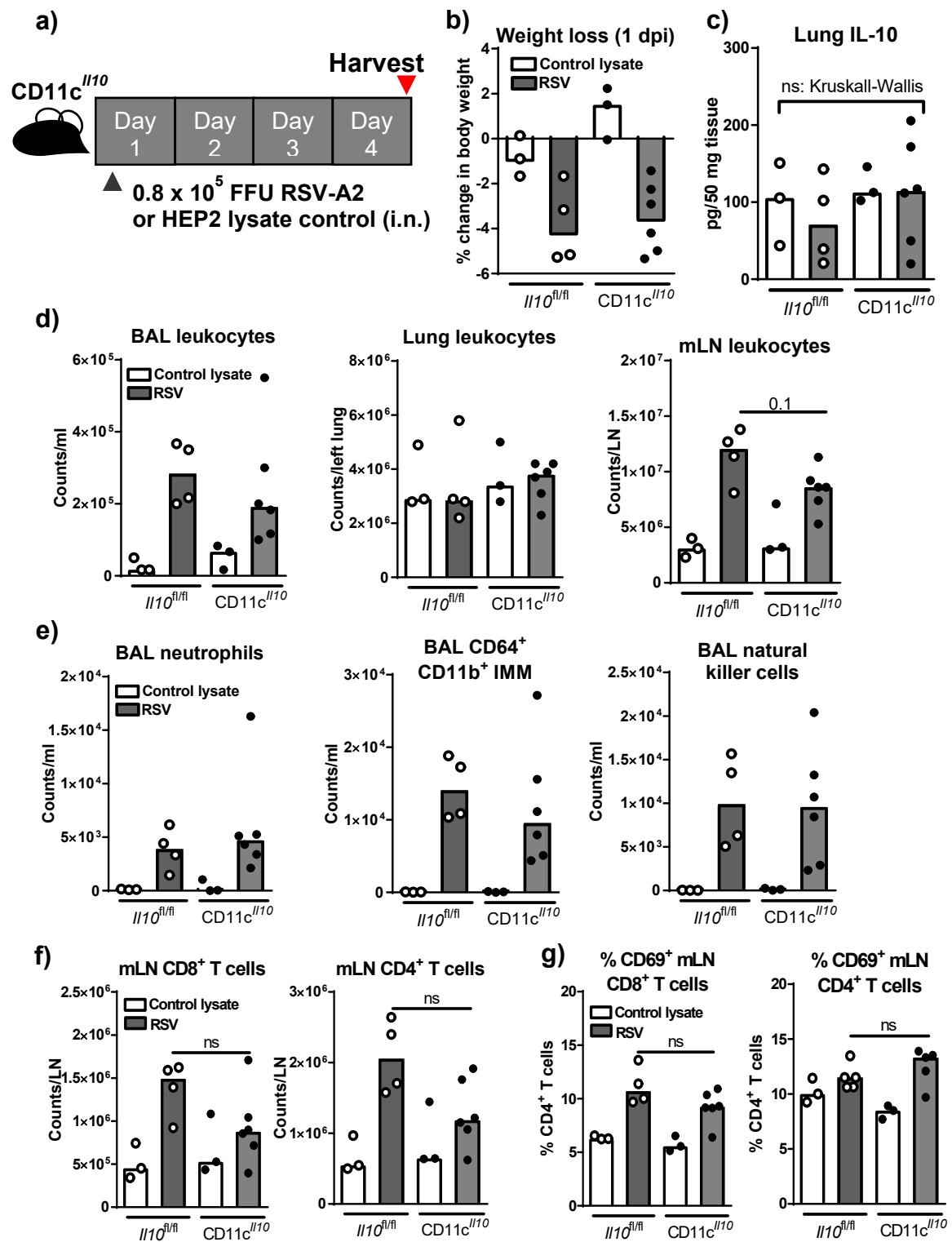


Figure 6.6. The early immune response to RSV infection unaffected in CD11c^{Il10} mice

a) CD11c^{Il10} mice and littermate controls were infected with RSV-A2 or HEP2 lysate control, weighed daily and culled at 4 dpi. **b)** Percentage change in body weight at 1 dpi from pre-infection weight. **c)** Concentration of IL-10 in lung homogenates, as determined by ELISA. **d)** Total crystal violet counts from BAL, lung digest and mLN preparations. **e-g)** BAL and mLN cells were analysed by flow cytometry. **e)** Total neutrophil, interstitial/inflammatory monocytes/macrophages and natural killer cells in BAL. **f)** Total numbers of CD8⁺ and CD4⁺ T cells in mLN. **g)** Percentages of CD8⁺ and CD4⁺ T cells in mLN with positive surface CD69 staining. Graphs show medians of the indicated number of 3-6 replicate mice per group. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ns, not-significant.

RSV infection caused inflammation in the alveolar spaces, as evidenced by increased leukocyte counts in BAL but not lung tissue (**Fig. 6.6d**). Leukocyte counts in mLN were also elevated in infected mice (**Fig. 6.6d**), consistent with the T cell response to virus having been initiated by this timepoint³⁵². However, neither BAL nor mLN leukocyte counts were significantly different in CD11c^{fl10} mice compared with controls (**Fig. 6.6d**), suggesting that AM-derived IL-10 is not required for regulating airway inflammation or early lymphocyte expansion in lymph nodes. Accordingly, numbers of neutrophils, interstitial/inflammatory monocytes/macrophages (IMM) and natural killer cells in BAL were comparable in RSV-infected CD11c^{fl10} and control mice (**Fig. 6.6e**) and CD8⁺ and CD4⁺ T cell counts in mLN were not significantly different between these mouse strains (**Fig. 6.6f**). Increased expression of the early lymphocyte activation marker CD69³⁰⁸ was apparent on CD8⁺ T cells and, to a lesser extent, CD4⁺ T cells, in mLN but the proportion of either subset expressing CD69 was not affected by IL-10 knockout in AM (**Fig. 6.6g**). Thus, AM-derived IL-10 is not required for regulating the early immune response to RSV, consistent with IL-10 production not being a typical mechanism by which AM regulate pulmonary inflammation.

6.6. Pulmonary IL-10 signalling regulates the immune response to inhaled HDM

Having established that AM-derived IL-10 had no major regulatory function during AAD driven by repeated HDM inhalation, it was necessary to determine the overall role of pulmonary IL-10 in this system, to allow dissection of the mechanisms by which IL-10 mediates cellular interactions in the allergic lung. Genetic ablation of IL-10 (*Il10*^{-/-} mice) has been shown previously to exacerbate eosinophilic and neutrophilic inflammation, respectively, in mice sensitised and challenged to OVA²¹⁰ or repeatedly exposed to intranasal HDM for 2 weeks²¹¹. However, the effects of pulmonary IL-10 in a 3 week model of HDM exposure, a duration known to be required for establishment of AHR and other canonical AAD features in mice³¹⁷, has not been previously reported.

To determine the effects of ablation of pulmonary IL-10 signalling in HDM-driven AAD, C57BL/6 mice were administered a blocking antibody to the IL-10RB subunit of the IL-10 receptor (hereafter α IL10R), or isotype control, one day prior to the first HDM exposure and at twice-weekly intervals throughout the HDM protocol (**Fig. 6.7a**). Blocking antibody was administered intranasally to specifically target blockade to the lungs and minimise impact on IL-10 signalling in other tissue sites.

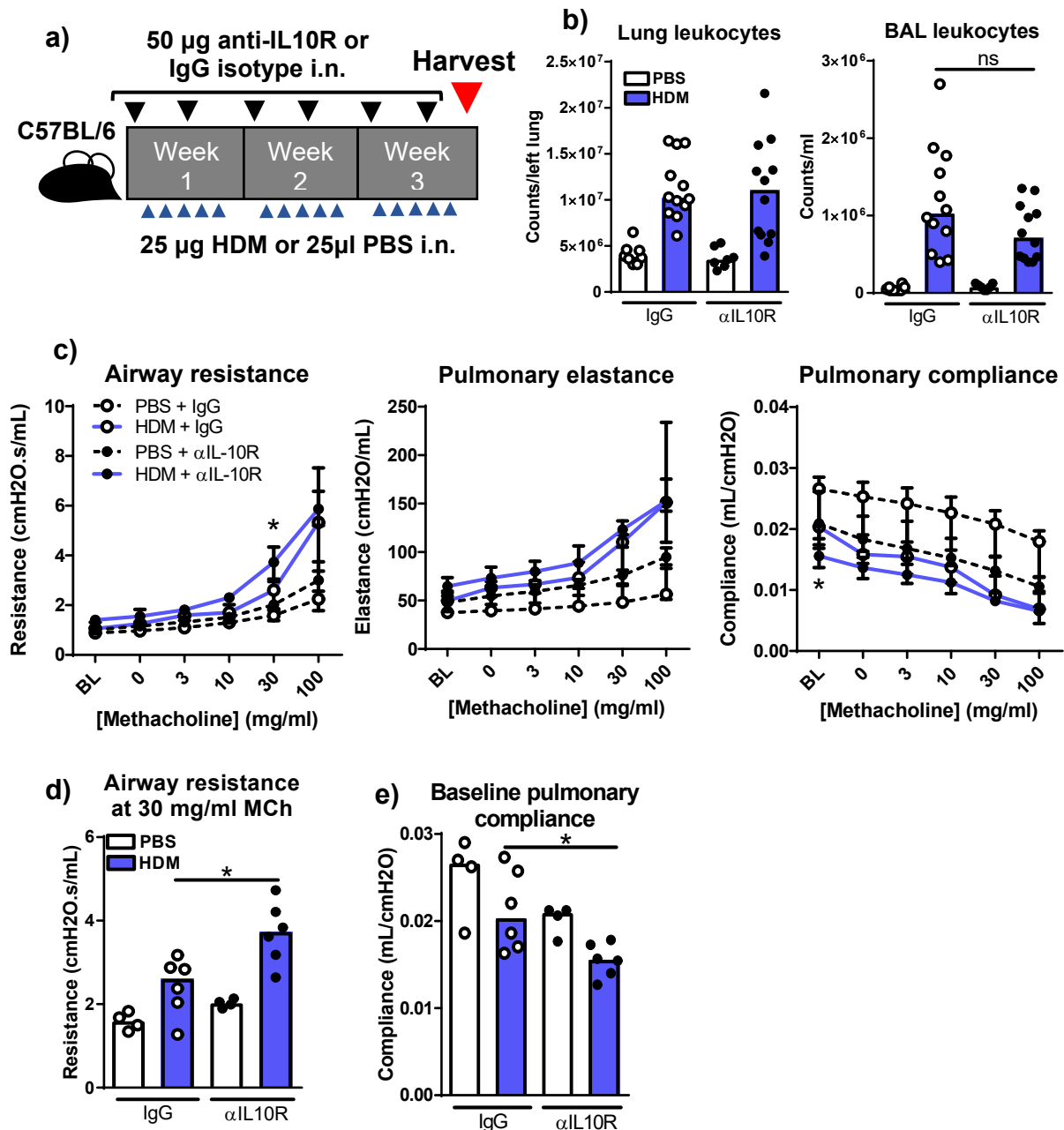


Figure 6.7. Pulmonary IL-10R blockade worsens HDM-driven allergic airway disease

a) Schematic of experimental protocol. C57BL/6 mice were administered intranasal α IL10R or rat IgG isotype control (black arrowheads) prior to and throughout intranasal HDM or PBS exposure (blue arrowheads). Lung function measurements and tissue harvests were performed at 24h after final HDM challenge. **b)** Total crystal violet cell counts in lung digests and BAL. **c)** Flexivent[™] lung function measurements or airway resistance, pulmonary elastance and compliance at baseline (BL) and in response to increasing concentrations of inhaled methacholine. **d-e)** Data from selected points in (c) showing resistance at 30 mg/ml methacholine (MCh, **d**) and compliance at baseline (**e**). Scatter/bar plots show medians of 4-12 replicate mice per group pooled from 2 (**b**) or one (**d-e**) independent experiment(s). Curves show medians $\pm$ IQR of 4-6 mice per group. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; ns, non-significant. Symbols on line graphs indicate significance between α IL10R and IgG control-treated HDM groups.

As expected, HDM exposure resulted in inflammation in the lung parenchyma and bronchoalveolar spaces, as shown by increased total leukocyte counts in lung digests and BAL (**Fig. 6.7b**). These counts were not altered by intranasal α IL10R (**Fig. 6.7b**), suggesting that the total extent of pulmonary inflammation was not enhanced in the absence of IL-10 signalling. Despite a comparable overall level of inflammation, α IL10R-treated mice showed slightly enhanced AHR following HDM-treatment, detectable as increased airway resistance in response to 30 mg/ml of inhaled methacholine (**Fig. 6.7c,d**). HDM treatment also increased the extent of methacholine-induced pulmonary elastance and decreased pulmonary compliance, but these parameters were not significantly different with the addition of IL-10R blockade (**Fig. 6.7c**). However, baseline pulmonary compliance (prior to methacholine challenge), a measurement of the ability of the lungs to expand during ventilation, was significantly decreased in HDM-exposed mice treated with α IL10R (**Fig. 6.7c,e**), suggesting that HDM-induced changes to pulmonary physiology may be enhanced in the absence of IL-10R signalling.

To determine the potential causes underlying impaired lung function resulting from IL-10R blockade during HDM exposure, the cellular immune response was examined by flow cytometry. As noted previously (**Fig. 6.4e**), total AM numbers obtained in BAL were not significantly different from PBS controls following 3 weeks of HDM exposure and were unaffected by α IL10R treatment under both conditions (**Fig. 6.8a**). However, eosinophils in BAL were present in significantly reduced numbers in BAL of mice treated with HDM and α IL10R than those receiving HDM and isotype control (**Fig. 6.8a**). Reduced eosinophilia was accompanied by a trend towards increased total neutrophil numbers (Mann-Whitney $p = 0.14$), but no change in interstitial and inflammatory monocyte and macrophage numbers (IMM; **Fig. 6.8a**). Examining airway eosinophilia and neutrophilia as percentages of total airway leukocytes highlighted a decrease in the proportion of eosinophils and an increase in the proportion of neutrophils with IL-10R blockade in HDM-treated mice (**Fig. 6.8b,c**). As a result, HDM-induced airway cytology changed from being highly dominated by eosinophils to containing an eosinophil to neutrophil ratio of only approximately 2:1 (**Fig. 6.8b,c**). Decreased eosinophilia was also apparent in lung digests of allergic α IL10R-treated mice, but no increase in neutrophil numbers was observed (**Fig. 6.8d**), consistent with decreased eosinophil numbers, rather than increased neutrophils, being the major driver of the altered pulmonary granulocyte profile achieved with α IL10R treatment. Interestingly, α IL10R-treated PBS control mice displayed a small but significant decrease in numbers of pulmonary neutrophils (**Fig. 6.8d**), however the reasons for this were unclear. No difference in numbers of IMM was observed in lung tissue with α IL10R treatment in PBS or HDM-exposed mice (**Fig. 6.8d**).

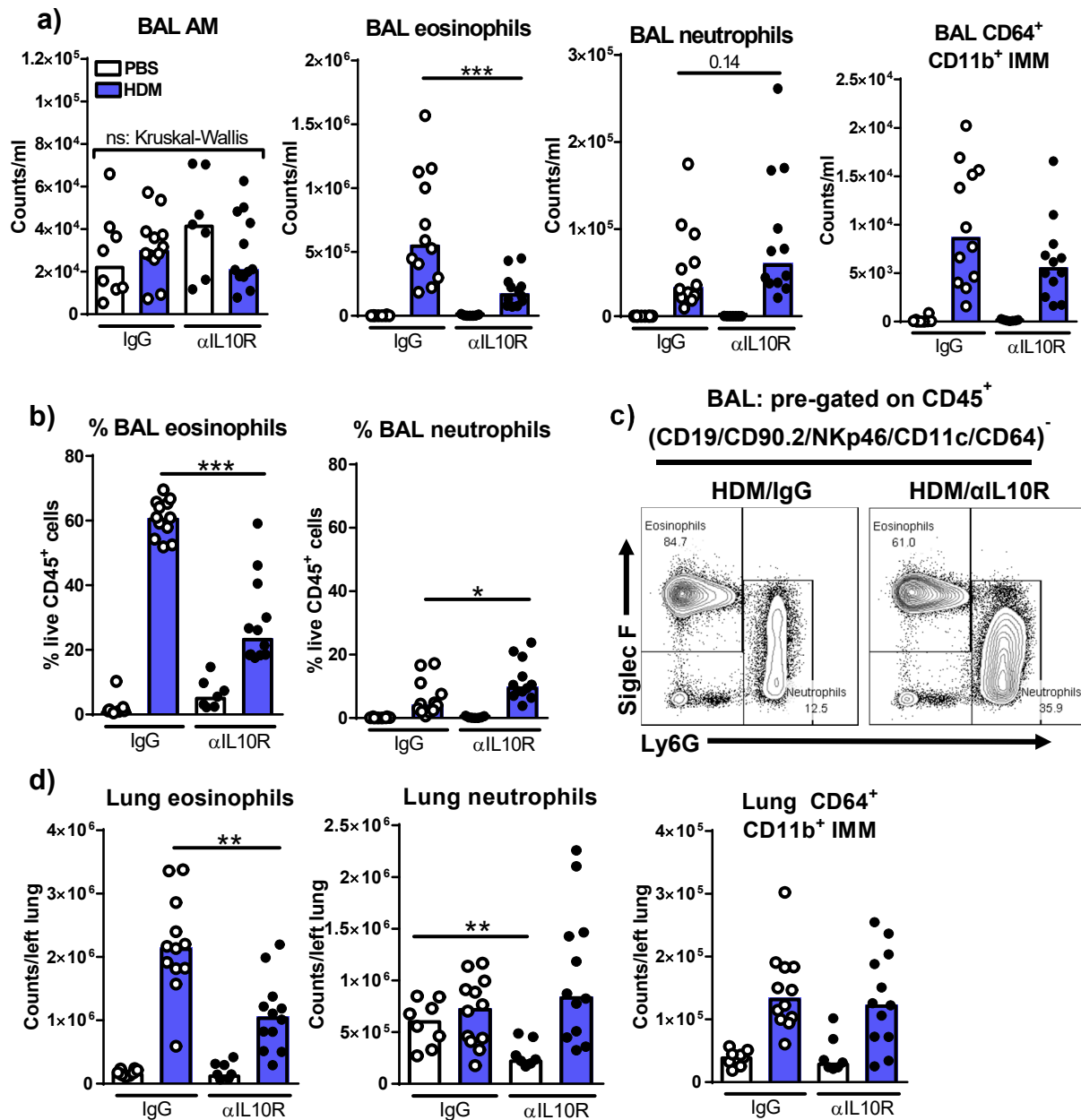


Figure 6.8. Altered pulmonary granulocyte profile in allergic airway disease with IL-10R blockade

Leukocyte populations in BAL and lung digests were enumerated by flow cytometry. **a)** Total numbers of AM, eosinophils, neutrophils and interstitial/inflammatory monocytes and macrophages (IMM) in BAL. **b)** BAL eosinophil and neutrophil numbers expressed as a proportion of total live, single, CD45⁺ leukocytes in BAL cell preparations. **c)** Representative flow cytometry plots of BAL preparations showing Siglec F⁺ eosinophils and Ly6G⁺ neutrophils, after excluding lymphocytes and CD11c⁺ CD64⁺ AM. Number show percentages of events within gates **d)** Total numbers of eosinophils, neutrophils and IMM in lung digests. Scatter/bar plots show medians of 7-12 replicate mice per group, pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between the indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant. Where relevant, p values are stated between groups.

The decreased ratio of eosinophils to neutrophils observed in HDM-driven AAD with IL-10R blockade was suggestive of a skewing away from a type 2 immune response. As expected from the allergen regimen used in these experiments, HDM exposure resulted in a mixed T helper cell response, in which pulmonary CD4⁺ T cell subsets producing IL-13, IL-17A or IFN γ are apparent following *ex vivo* stimulation with PMA and ionomycin (**Fig. 6.9a**). Consistent with skewing away from a type 2 T helper cell response, α IL10R treatment significantly reduced the proportion of IL-13⁺ CD4⁺ T cells in lungs of HDM-exposed mice to levels observed in PBS-treated controls (**Fig. 6.9a**). In parallel, the proportion of pulmonary IFN γ ⁺ CD4⁺ T cells was significantly increased in HDM-treated mice that received α IL10R (**Fig. 6.9a,b**), consistent with a shift from Th2 to Th1 cells in the allergic lung where IL-10R was neutralised. A substantial proportion of lung CD4⁺ T cells were IL-17A⁺ in HDM-exposed mice, which showed a trend towards increase with IL-10R blockade (Mann-Whitney $p = 0.11$, **Fig. 6.9a**). However, when total numbers were calculated, IFN γ ⁺, but not IL-17A⁺, CD4⁺ T cells were significantly increased in lungs of HDM-treated mice that received α IL10R (**Fig. 6.9c**), consistent with the major skewing with IL-10R neutralisation being towards Th1 cells.

Further supporting a skewing from a type 2 towards a type 1 immune response to inhaled HDM where IL-10R was blocked, pulmonary concentrations of type 2 cytokines IL-13, IL-4 and IL-33 were decreased in HDM-treated mice that received α IL10R (**Fig. 6.9d**). Levels of CCL24, a type 2-related chemokine and attractant for eosinophils⁴⁶⁰, were also significantly reduced in the lungs of α IL10R-treated mice (**Fig. 6.9d**), consistent with the dampened eosinophilia observed in this treatment group (**Fig. 6.8**). Conversely, IFN γ concentrations increased by more than two-fold in lung tissue of α IL10R-treated allergic mice (**Fig. 6.9d**), consistent with type 1 skewing in this model. In line with intracellular cytokine staining data, pulmonary IL-17A was detected at comparable concentrations in lungs of α IL10R and isotype control-treated mice that received HDM (**Fig. 6.9d**), suggesting that IL-17A was not responsible for the distinct AAD phenotype driven by α IL10R blockade. Similarly, concentrations of the neutrophil chemokine CXCL1 were comparable in lung tissue in HDM-treated mice receiving α IL10R compared with those receiving isotype control (**Fig. 6.9d**), consistent with the minor overall increase in airway neutrophilia in these mice (**Fig. 6.8a**)

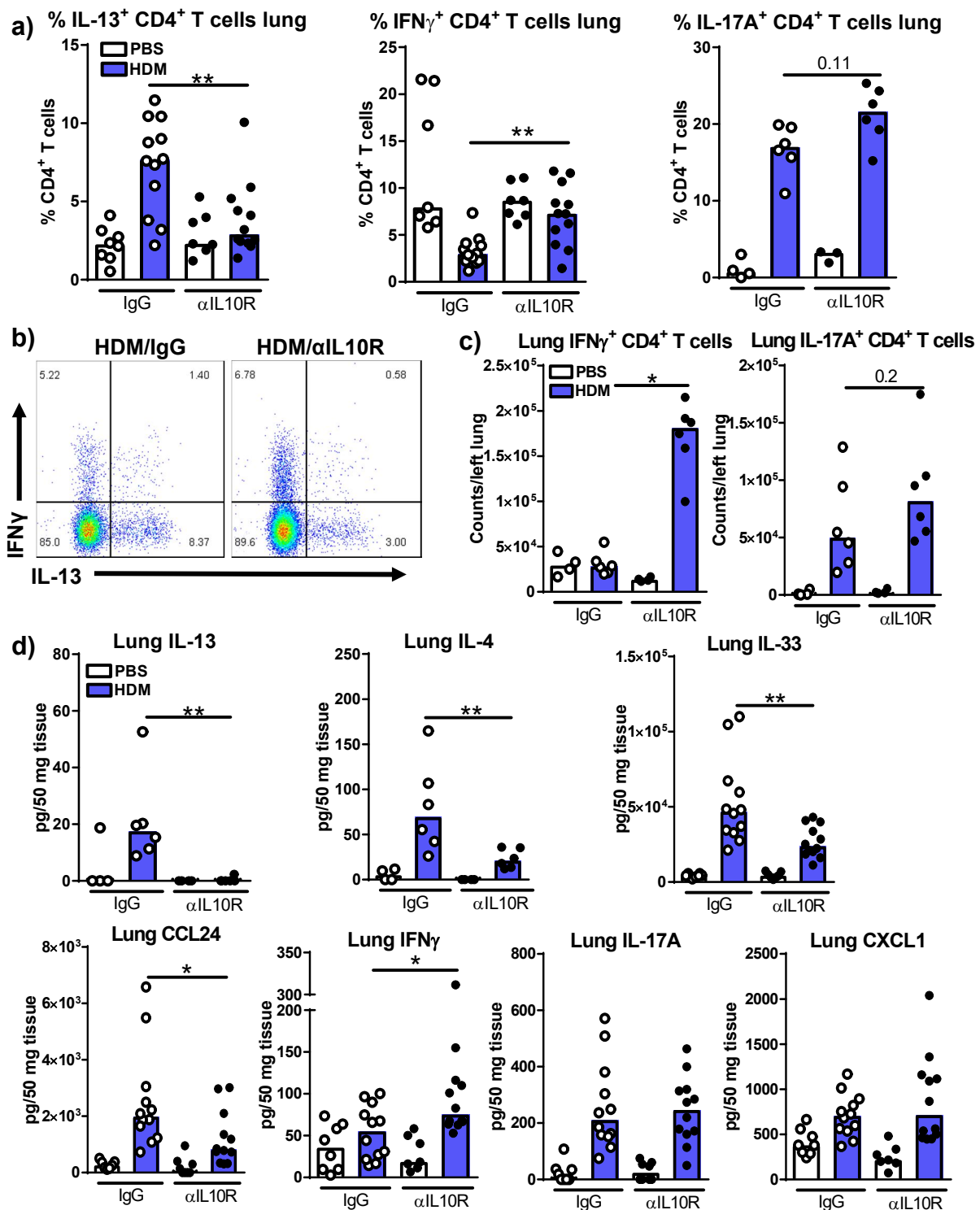


Figure 6.9. Altered T helper cell response to HDM with IL-10R blockade

a) Percentages of lung CD4⁺ T cells with positive intracellular cytokine staining determined by flow cytometry. Data shown are medians of 3-12 mice from two pooled experiments (IL-13, IFN γ) or one of two representative experiments (IL-17A). **b)** Flow cytometry plots representative of data shown in (a), pre-gated on CD4⁺ T cells. Numbers indicate percentages of events in each quadrant. **c)** Total IFN γ ⁺ and IL-17A⁺ CD4⁺ T cells in lung digests, medians of 4-6 mice per group, representative of two independent experiments. **d)** Concentrations of IL-13, IL-4, IL-33, CCL24, IFN γ , IL-17A and CXCL1 in lung homogenates. Data shown are medians of 3-12 biological replicates from one (IL-13, IL-4) or two (IL-33, CCL24, IFN γ , IL-17A, CXCL1) independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between the indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$. Where relevant, p values are stated between groups.

The changes observed in CD4⁺ T cell phenotypes in lungs of mice treated with HDM while blocking IL-10R were suggestive of impaired regulation of pulmonary T helper cells. To determine whether this was associated with reduced regulatory capacity of CD4⁺ T cells themselves, IL-10 and FoxP3 expression was assessed in T cells. Intranasal α IL10R treatment did not impact the proportion of lung CD4⁺ T cells that produced IL-10 upon *ex vivo* stimulation during HDM-driven AAD (**Fig. 6.10a**). Pulmonary IL-10 concentrations in PBS and HDM-treated mice were not significantly decreased in mice receiving α IL10R (**Fig 6.10b**), consistent with this antibody blocking IL-10 signalling rather than depleting IL-10 itself. In contrast, α IL10R administration throughout HDM exposure significantly reduced the absolute number of FoxP3⁺ CD4⁺ T cells in lung tissue (**Fig. 6.10c**), suggesting that expansion or maintenance of the pulmonary Treg compartment was impaired when local IL-10 signalling was blocked. The proportion of CD4⁺ cells that were FoxP3⁺ was also reduced in lungs of α IL10R-treated allergic mice, where they generally comprised less than 5 % of CD4⁺ T cells (**Fig. 6.10d**). The Treg deficiency in α IL10R-treated mice appeared to be restricted to the lungs, since no difference was observed in the proportion of FoxP3⁺ CD4⁺ T cells in mLN (**Fig. 6.10e**). Thus, the observed increase in Th1 cells with IL-10R neutralisation in HDM-driven AAD was accompanied by a reduction in pulmonary FoxP3⁺ Tregs.

Having demonstrated a profound skewing of the pulmonary immune response to HDM with local IL-10R blockade to a Th1^{hi}, Th2/Treg^{low} phenotype, the effects of this altered inflammatory milieu on AM phenotype were assessed. HDM-induced expression of activation marker CD11b on AM was comparable in α IL10R and isotype control-treated mice, with the majority of AM showing positive staining (see representative plots in **Fig. 6.11a**). However, AM from α IL10R-treated mice displayed markedly enhanced upregulation of MHC-II following 3 weeks of HDM exposure, with close to 100 % of cells showing positive staining, compared with 40-50 % in isotype control-treated mice (**Fig. 6.11a,b**). An increased proportion of MHC-II⁺ cells was also observed among the mixed IMM population in lung tissue in HDM- but not PBS-treated mice (**Fig. 6.11b**), suggesting that activation of multiple subsets of macrophages is augmented by IL-10R blockade during AAD. In contrast, lung DC, all showing positive MHC-II expression by definition, did not differ in their intensity of MHC-II staining in mice treated with α IL10R, although the average intensity of MHC-II staining was reduced in both HDM groups (**Fig. 6.11c**). Thus, the skewed immune response to HDM during IL-10R blockade appeared to be accompanied by increased activation of pulmonary macrophages, but not DC.

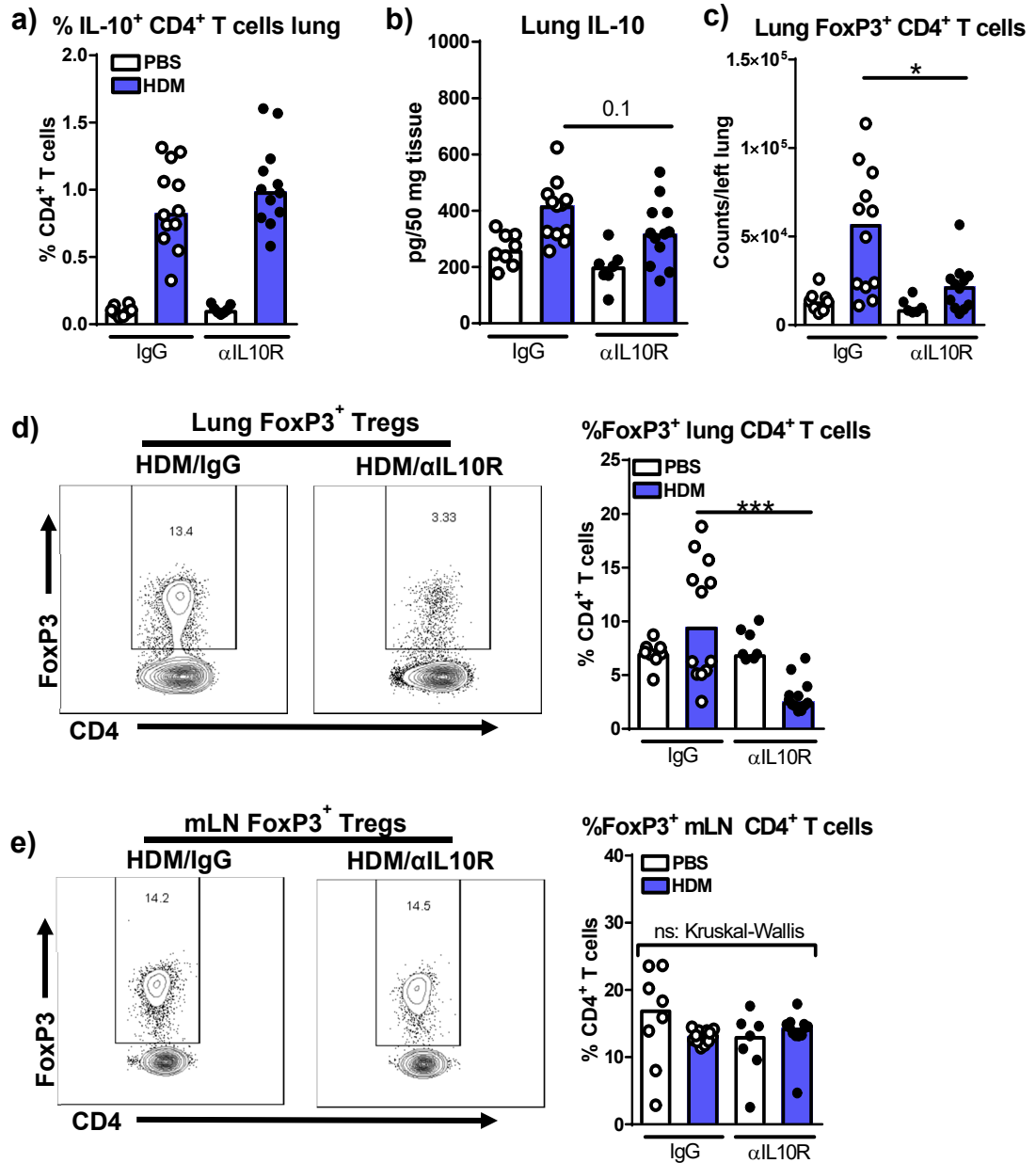


Figure 6.10. Pulmonary FoxP3⁺ Tregs in allergic airway disease reduced with IL-10R blockade

a) Percentage of CD4⁺ T cells positive for intracellular IL-10 in lung digests as determined by flow cytometry. **b)** Concentrations of IL-10 in lung homogenates as determined by ELISA. **c)** Number of FoxP3⁺ CD4⁺ T cells in lung tissue as determined by flow cytometry. **d-e)** Representative flow cytometry plots (left) and enumeration of percentages of FoxP3⁺ cells (right) among CD4⁺ T cells in lung (**d**) and mLN (**e**). Numbers on flow cytometry plots show the percentage of positive cells. Scatter/bar plots show medians 7-12 replicate mice per group, pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; ***, $p \leq 0.001$; ns, non-significant. Where relevant, p values are stated between groups.

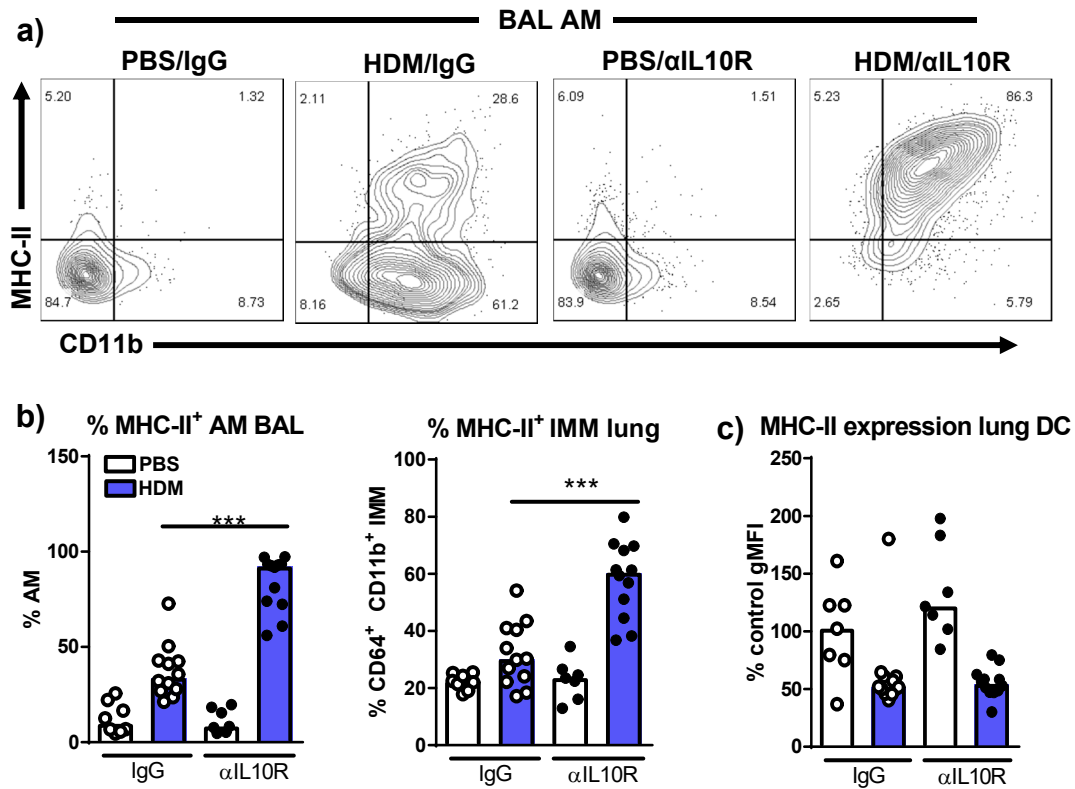


Figure 6.11. Increased HDM-driven activation of pulmonary macrophages with IL-10R blockade
a) Representative flow cytometry plots showing expression of CD11b and MHC-II activation markers on AM in BAL obtained from the indicated experimental groups. Numbers indicate percentages of events in each quadrant. **b)** Percentages of AM (left) and CD64⁺ CD11b⁺ Siglec F⁻ IMM (right) with positive MHC-II staining. **c)** gMFI of MHC-II staining on lung CD11c⁺ MHC-II⁺ DC, shown as a percentage of mean gMFI in the PBS/IgG negative control group. Scatter/bar plots show medians of 7-12 replicate mice per group pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ***, $p \leq 0.001$.

6.7. CD4⁺ T cells are the functionally dominant source of IL-10 in the lung during HDM-driven allergic airway disease

The Th1^{hi}, Th2/Treg^{low} AAD phenotype observed with IL-10R blockade clearly demonstrated an important role for pulmonary IL-10 signalling in regulating the immune response to inhaled HDM. However, these studies did not discriminate between cellular sources of IL-10 in the lung. Since reporter mouse studies described in Chapter 4 had revealed CD4⁺ T cells to be a major IL-10-producing population that emerged in the lungs following sustained HDM exposure, the role of CD4⁺ T cell-derived IL-10 during HDM-driven AAD was assessed.

To study AAD in a system with T cells lack IL-10, conditional knockout mice were generated by crossing *Il10^{fl/fl}* mice to a CD4-Cre line²⁸⁶ (hereafter CD4^{Il10}) and subjected to the 3 week HDM exposure protocol (**Fig. 6.12a**). IL-10 expression in CD4⁺ T cells, as measured by intracellular staining and flow cytometry, was ablated in CD4^{Il10} mice (**Fig. 6.12b**), demonstrating high efficiency of the conditional knockout. IL-10 concentrations in lung tissue were comparable in PBS- and HDM-treated *Il10^{fl/fl}* mice (**Fig. 6.12c**), indicating that HDM treatment did not induce a net increase in pulmonary IL-10 levels in this strain of mice. CD4^{Il10} mice that received PBS vehicle treatment showed no difference in IL-10 levels from *Il10^{fl/fl}* controls (**Fig. 6.12c**), supporting the IL-10 expression analysis in Chapter 4 in suggesting that T cells are not major contributors to pulmonary IL-10 levels in the steady state. In contrast, lung tissue IL-10 concentrations were significantly reduced in HDM-treated CD4^{Il10} mice compared to controls and were decreased by more than 2-fold from PBS-treated CD4^{Il10} mice (**Fig. 6.12c**). These data suggest a shift towards T cells as an IL-10 source during HDM-driven AAD, which was successfully ablated in CD4^{Il10} mice.

Total lung leukocyte counts were comparable between CD4^{Il10} mice and controls, while BAL cellularity was around 1.5-fold higher in HDM-treated CD4^{Il10} mice (**Fig. 6.12d**), suggesting that overall allergic airway inflammation was enhanced in the absence of T cell-derived IL-10. Increased airway inflammation was accompanied by significantly increased airway resistance in response to intermediate concentrations of inhaled methacholine in HDM-treated CD4^{Il10} mice, consistent with worsened AHR in the absence of T cell-derived IL-10 (**Fig. 6.12e**). Pulmonary elastance and compliance were reciprocally increased and decreased, respectively, in HDM-treated CD4^{Il10} mice compared with controls at baseline and at lower concentrations of inhaled methacholine (**Fig. 6.12e**), suggesting that stiffness of lung tissue following repeated HDM inhalation was enhanced in the absence of T cell-derived IL-10.

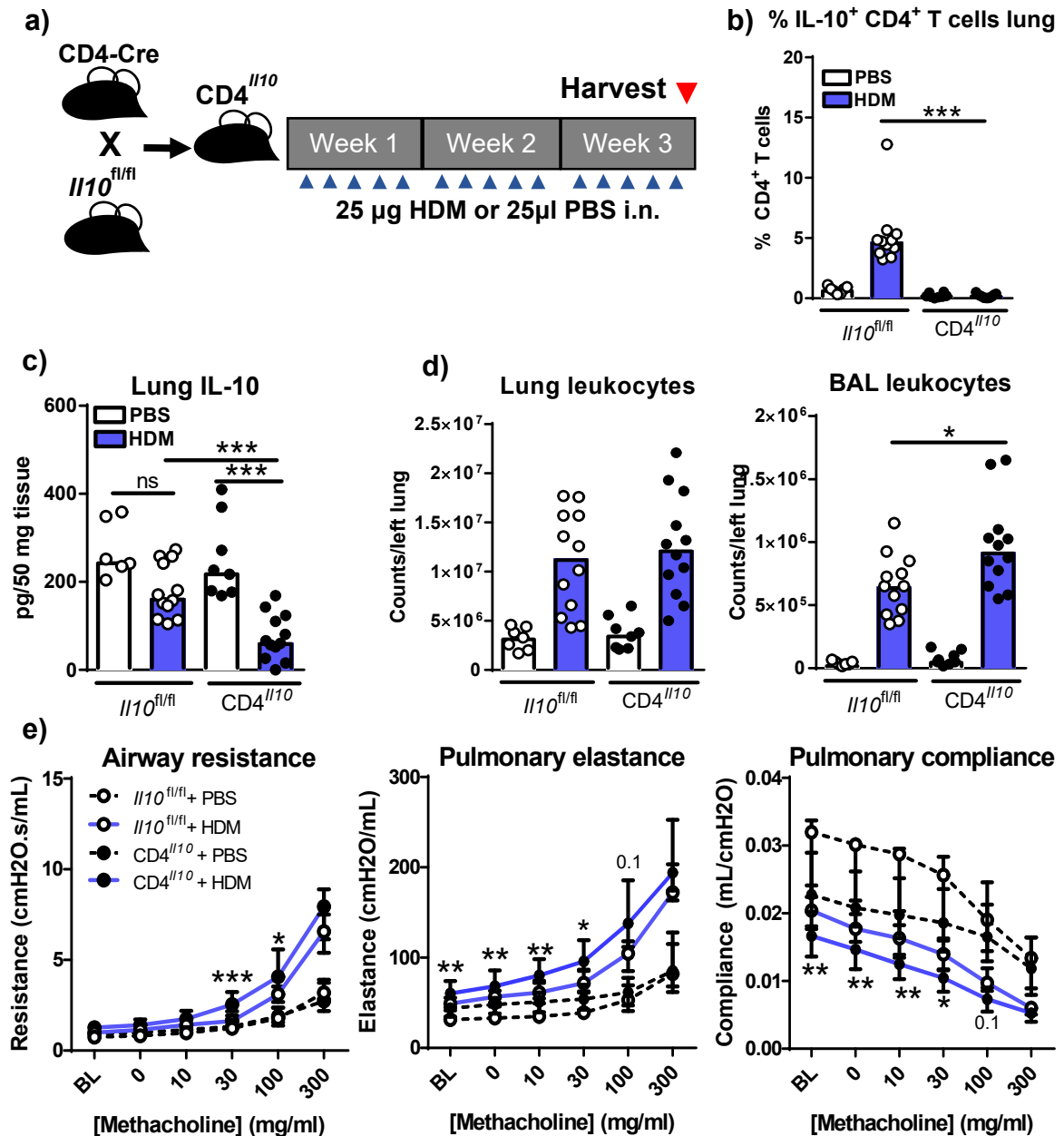


Figure 6.12. More severe allergic airway disease in CD4-conditional IL-10 knockout mice

a) CD4 $^{fl/fl}$ conditional knockout mice were generated by inter-crossing CD4-Cre and $Il10^{fl/fl}$ mice for ≥ 2 generations to obtain CD4-Cre hemizygous, $Il10^{fl/fl}$ mice (CD4 $^{fl/fl}$) and Cre-negative littermate controls. Mice were exposed to intranasal HDM or PBS vehicle control for 3 weeks and measurements made 24h after final challenge. **b)** Percentage of lung CD4 $^{+}$ T cells positive for intracellular IL-10 staining, as determined by flow cytometry. **c)** Concentrations of IL-10 in lung homogenates, as determined by ELISA. **d)** Total crystal violet cell counts from lung and BAL cell preparations. **e)** Line graphs showing median $\pm$ IQR FlexiventTM measurements of airway resistance and pulmonary elastance and compliance of 7-12 mice per group pooled from two independent experiments. Scatter/bar plots show medians of 7-12 replicate mice pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant. Where relevant, corrected p values are shown above data points. Symbols in **(e)** show statistical comparison of CD4 $^{fl/fl}$ and $Il10^{fl/fl}$ HDM-treated groups.

Assessment of the nature of airway inflammation by flow cytometry showed no changes in AM numbers obtained in BAL in the absence of T cell-derived IL-10 (**Fig. 6.13a**), but a skewing of the eosinophil to neutrophil ratio in BAL, with significantly decreased eosinophil numbers and increased neutrophil counts in HDM-treated CD4^{Il10} mice (**Fig. 6.13a**), similar to that observed with IL-10R blockade (**Fig. 6.8**). HDM-treated CD4^{Il10} mice displayed decreased lung eosinophil counts, but no difference in pulmonary neutrophils, compared to littermate controls (**Fig. 6.13b**). Notably, while lung IMM numbers were increased as expected in *Il10^{fl/fl}* mice during AAD compared with PBS-treated controls, this population was not significantly elevated above PBS control levels in CD4^{Il10} mice (**Fig. 6.13b**), suggesting that the altered pulmonary immune environment in the absence of T cell-derived IL-10 was less permissive to expansion of this particular population.

Despite neither population increasing in absolute number following HDM treatment in CD4^{Il10} mice, a significantly increased proportion of BAL AM and lung IMM expressed MHC-II in these mice, compared to littermate controls (**Fig. 6.13c,d**), recapitulating the effects of α IL10R in this model (**Fig. 6.11b**). Interestingly and in contrast to IL-10R blockade, a significantly increased proportion of AM expressed MHC-II in PBS-treated CD4^{Il10} mice, suggesting that low levels of T cell-derived IL-10 present prior to allergen exposure may homeostatically condition AM to limit their activation (**Fig. 6.13c,d**).

To determine whether HDM-treated CD4^{Il10} mice also recapitulated the Th1 skewing observed with global IL-10R blockade, T helper cell phenotypes were assessed by intracellular cytokine staining. As observed with α IL10R treatment (**Fig. 6.9**), a reduced proportion of CD4⁺ T cells in HDM-exposed CD4^{Il10} mice displayed IL-13 production and a significantly increased proportion produced IFN γ , compared to littermate controls (**Fig. 6.14a,b**). No change in the proportion of IL-17A⁺ CD4⁺ T cells was observed in HDM-treated CD4^{Il10} mice (**Fig. 6.14a**). Similarly, total numbers of IFN γ ⁺, but not IL-17A⁺, CD4⁺ T cells were increased in lungs of HDM-treated CD4^{Il10} mice compared with littermate controls (**Fig. 6.14c**), consistent with Th1 cells being the major T helper cell population to be enhanced in the absence of T cell-derived IL-10 during AAD.

Consistent with Th2 to Th1 skewing, and in line with IL-10R antibody blocking experiments, HDM-exposed CD4^{Il10} mice displayed decreased pulmonary concentrations of type 2-related cytokines IL-33 and CCL24, but increased levels of IFN γ (**Fig. 6.14d**). In contrast to IL-10R blockade, T cell-specific IL-10 deletion also resulted in small (around 1.5-fold) but significant increases in pulmonary concentrations of IL-17A and CXCL1 in HDM-treated mice (**Fig. 6.14d**). Since these mediators can both promote neutrophil recruitment⁴⁶¹, their increased production in HDM-treated CD4^{Il10} mice may explain the enhanced airway neutrophilia in these mice compared with IL-10R blockade (compare median count 1.2×10^5 in **Fig. 6.13a** with 0.6×10^5 in **Fig. 6.8a**). However, despite small differences, the HDM-induced pulmonary T helper cell and cytokine profile of CD4^{Il10} mice largely phenocopied that achieved with local non-specific blockade of IL-10 signalling.

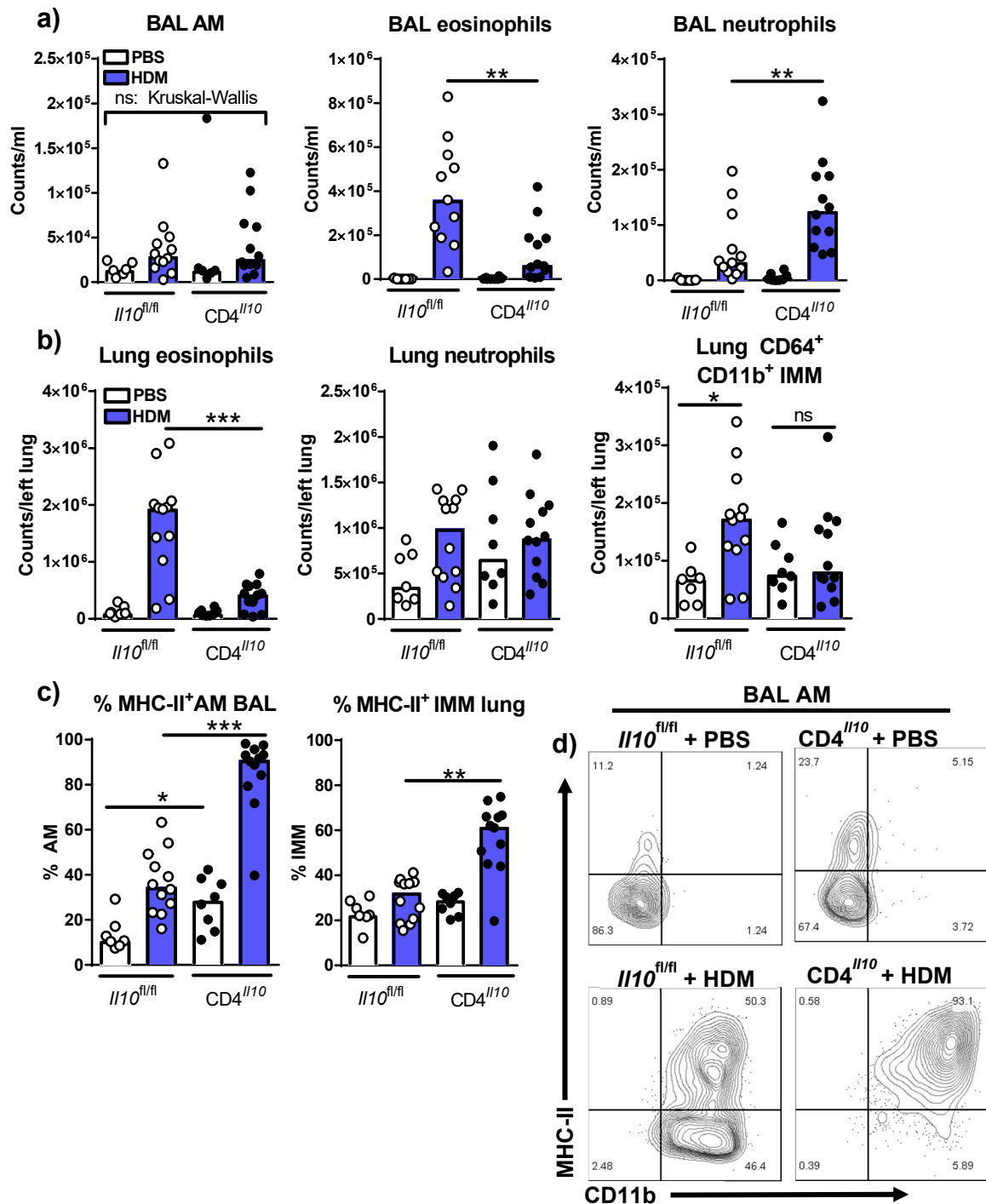


Figure 6.13. Altered HDM-driven pulmonary myeloid cell profile and increased macrophage activation in mice lacking T cell-derived IL-10

Myeloid cell number and phenotype in BAL and lung digests were determined by flow cytometry **a)** Numbers of AM, eosinophils and neutrophils in BAL. **b)** Numbers of eosinophils, neutrophils and IMM in lung digests, **c)** Percentages of BAL AM(left) and lung IMM (right) with positive MHC-II surface staining. **d)** Representative flow cytometry plots of AM data shown in (c). Numbers indicate percentages of events in quadrants. Scatter/bar plots show medians of 7-12 replicate mice pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant.

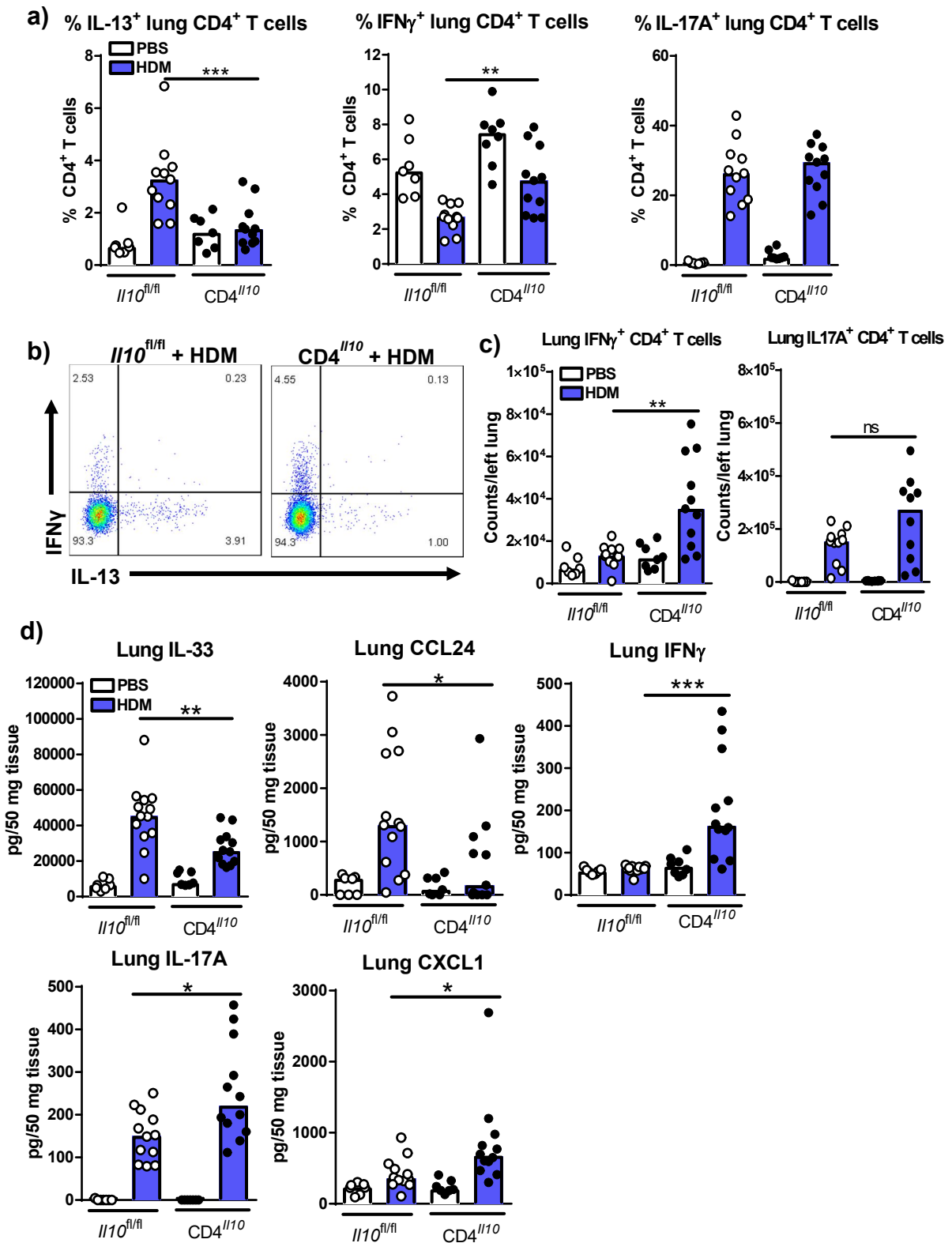


Figure 6.14. Skewed T cell response to HDM in the absence of T cell-derived IL-10

a-c) Lung CD4⁺ T cells were analysed by flow cytometry. **a)** Proportions of lung CD4⁺ T cells with positive intracellular staining for IL-13, IFN γ or IL-17A. **b)** Representative flow cytometry plots showing IL-13 and IFN γ expression by lung CD4⁺ T cells. Numbers indicate percentages of events in quadrants. **c)** Numbers of IFN γ ⁺ and IL-17A⁺ CD4⁺ T cells in lung digests. **d)** Concentrations of IL-33, CCL24, IFN γ , IL-17A and CXCL1 in lung tissue, as quantified by ELISA. Scatter/bar plots show medians of 7-12 replicate mice per group pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant.

Finally, the relative deficiency in HDM-elicited pulmonary FoxP3⁺ CD4⁺ T cells observed with IL-10R blockade (**Fig. 6.10**) was also recapitulated in CD4^{Il10} mice. FoxP3⁺ Tregs were significantly decreased as a percentage of lung CD4⁺ T cells (**Fig. 6.15a**), indicating that these cells were not generated and/or maintained proportionately to effector T helper cell subsets in the absence of T cell-derived IL-10. Despite a downward trend, no significant decrease was observed in total FoxP3⁺ CD4⁺ T cell numbers in lungs of HDM-treated CD4^{Il10} mice compared to littermate controls (**Fig. 6.15b**). However, closer inspection of the data revealed a strong inverse correlation between FoxP3 expression by CD4⁺ T cells and total CD4⁺ T cell numbers in lungs of HDM-treated mice, such that mice with the lowest proportions of FoxP3⁺ CD4⁺ T cells generally had the highest numbers of total CD4⁺ T cells (Spearman $r = -0.64$, $p = 0.0008$; **Fig. 6.15c**), largely masking the relative deficiency in Tregs. Thus, lung FoxP3⁺ Treg deficiency in HDM-treated CD4^{Il10} mice reflects a decrease proportionally compared with effector T cells, rather than a decrease in total numbers. As observed with IL-10R antibody blockade, FoxP3⁺ Tregs were not deficient in mLN of HDM-treated CD4^{Il10} mice. However, in contrast to the earlier study, there was a small but significant increase in the proportion of FoxP3⁺ CD4⁺ T cells in the mLN of HDM-treated conditional knockout mice (**Fig. 6.15d**), the cause of which was unclear from the available data.

Overall, the near-complete recapitulation of the effects of non-selective blockade of pulmonary IL-10 signalling by specific deletion of IL-10 in T cells, suggests that CD4⁺ T cells are the functionally dominant cellular source of IL-10 required to control the balance of effector T cell subsets and the nature of inflammation during HDM-driven AAD.

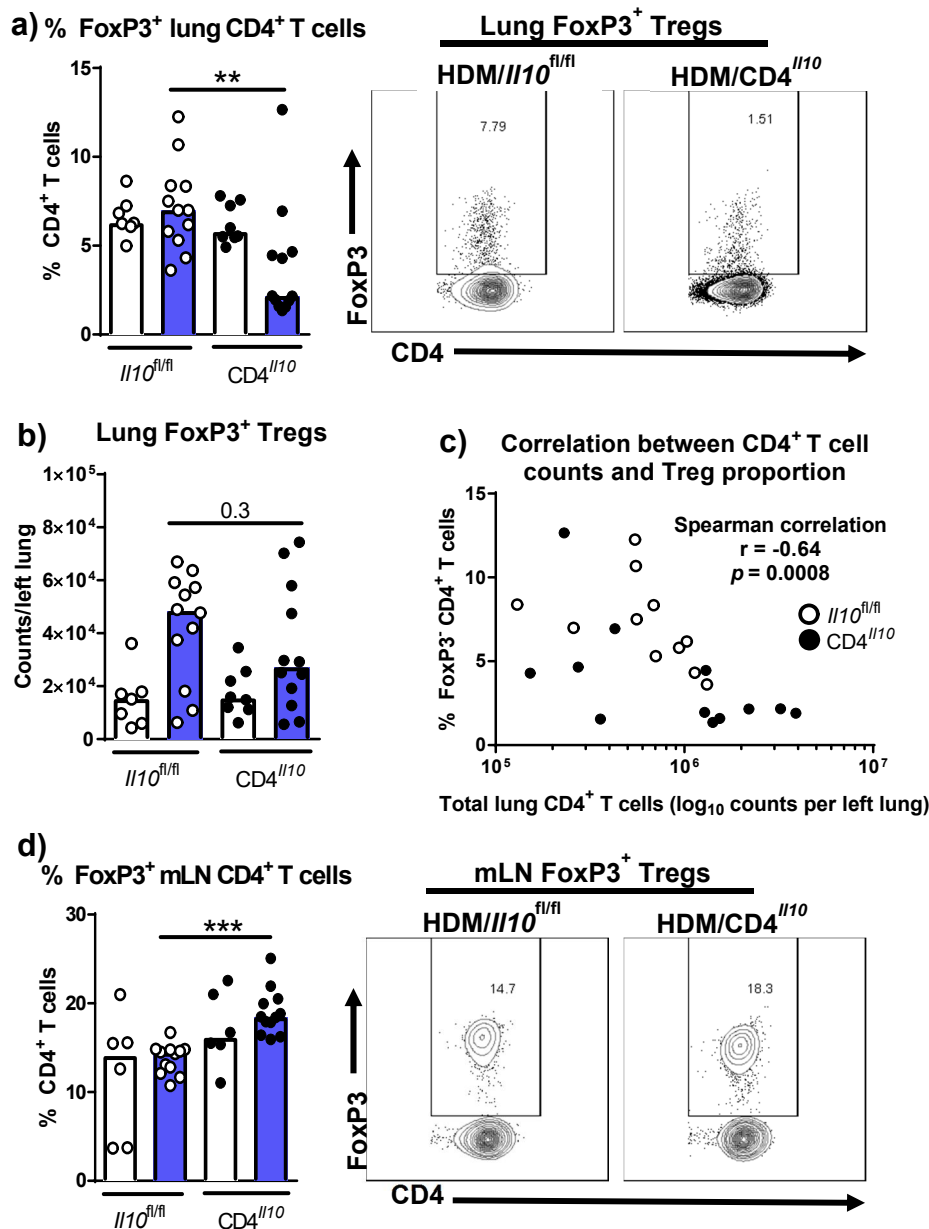


Figure 6.15. Reduced proportion of lung FoxP3⁺ Tregs in allergic airway disease in the absence of T cell-derived IL-10

FoxP3⁺ Tregs were analysed in lung digests and mLN by flow cytometry. **a)** Percentages of CD4⁺ T cells in lung tissue with positive FoxP3 intranuclear staining (left) and representative flow cytometry plots gated on CD4⁺ CD3⁺ T cells (right). **b)** Absolute FoxP3⁺ CD4⁺ T cell numbers in lung tissue. **c)** Spearman correlation between total lung CD4⁺ T cell counts and percentages of FoxP3⁺ lung CD4⁺ T cells in HDM-treated *CD4^{Il10}* and littermate control mice. **d)** Percentage of CD4⁺ T cells in mLN with positive FoxP3 intranuclear staining (left) and representative flow cytometry plots gated on CD4⁺ CD3⁺ T cells (right). Scatter/bar plots show medians of 7-12 replicate mice per group pooled from two independent experiments. Numbers on flow cytometry plots indicate percentages of events in gates. Statistics for scatter/bar graphed data: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups, **, $p \leq 0.01$; ***, $p \leq 0.001$. Where relevant, p values are stated between groups.

6.8. Generation of CD11c-conditional *Tgfb1* knockout mice

Experiments manipulating different cellular sources of IL-10 *in vivo*, together with earlier studies demonstrating minimal IL-10 production by AM *in vitro* and *in vivo* (see Chapter 4), strongly suggested that AM do not utilise IL-10 as a major immunoregulatory mechanism. AM are therefore likely to rely on other regulatory cytokines for their tolerogenic function, with TGF- β 1 being a major candidate.

To allow assessment of the role of AM-derived TGF- β 1 *in vivo*, CD11c-Cre mice were intercrossed with *Tgfb1*^{fl/fl} mice²⁸⁸ for ≥ 2 generations to generate CD11c-Cre hemizygous *Tgfb1*^{fl/fl} mice (hereafter CD11c^{Tgfb1}) and Cre-negative littermate controls (hereafter *Tgfb1*^{fl/fl}) on a mixed genetic background (C57BL/6, 129, Swiss Black)²⁸⁸. AM enriched from BAL of CD11c^{Tgfb1} mice by adhesion under serum-free conditions showed a significant > 2.5 -fold reduction in *Tgfb1* mRNA expression compared with littermate controls (**Fig. 6.16**), consistent with the knockout of *Tgfb1* in the majority of AM expected from the around 80 % efficiency of CD11c-Cre observed in these cells (**Fig. 6.1**). *Tgfb1* expression was assessed by qPCR amplification around the exon 6-7 boundary, chosen as the *Tgfb1*^{fl/fl} allele used in this line has LoxP sites flanking exon 6²⁸⁸. The specificity of knockout to CD11c⁺ macrophages was confirmed by differentiating BMDM from CD11c^{Tgfb1} and control bone marrow and showing no difference in *Tgfb1* mRNA expression (**Fig. 6.16b**).

To our knowledge, the only previous study of lung immunology in CD11c^{Tgfb1} mice at the time of writing is that of Yu et al, published during preparation of this thesis²⁸². In the published work, mice with CD11c-Cre-driven deletion of TGF- β 2 developed very few AM due to failure to receive TGF- β signals. A similar, but less severe phenotype was observed in CD11c^{Tgfb1} mice, suggesting that the TGF- β required for AM development was at least partially derived from AM themselves²⁸². However, in the present work, no decrease in AM numbers was observed in BAL or lung tissue of adult CD11c^{Tgfb1} mice, after either 3 weeks of PBS vehicle treatment or induction of AAD with HDM (**Fig. 6.16c**). Ageing naïve CD11c^{Tgfb1} mice in specified pathogen-free conditions for up to 7 months did not change AM numbers in lungs compared to littermate controls (**Fig. 6.16d**). These data suggest that, in our animal facilities, sufficient TGF- β is available in airways of CD11c^{Tgfb1} mice for AM development and maintenance. This is likely due to a combination of incomplete deletion of *Tgfb1* in 100 % of AM (see 80 % Cre efficiency in **Fig. 6.1b**), plus production by compensatory non-AM sources, such as epithelial cells. However, the discrepancies between this and the published data will be addressed in future work.

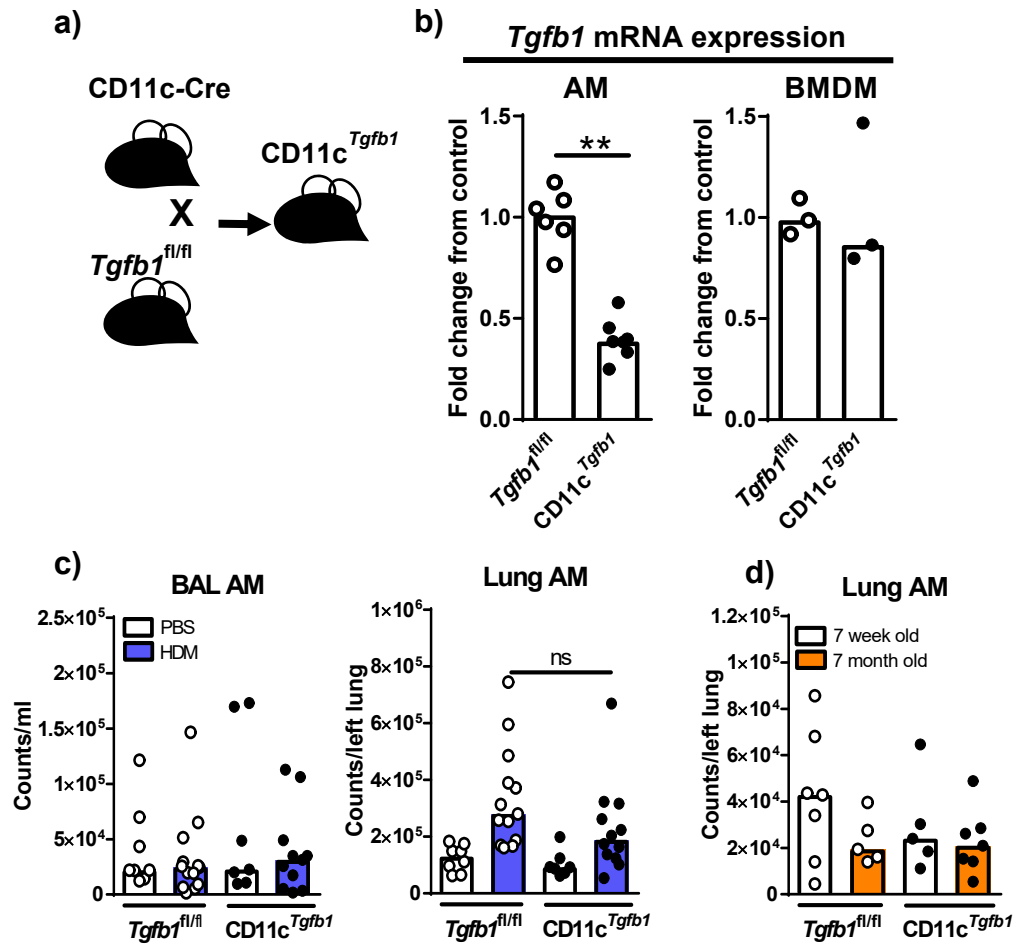


Figure 6.16. Conditional reduction of TGF- β 1 expression in AM in CD11c^{Tgfb1} mice

a) CD11c-conditional TGF- β 1 knockout (CD11c^{Tgfb1}) mice were generated by crossing CD11c-Cre and *Tgfb1*^{fl/fl} mice for ≥ 2 generations. **b)** qPCR data showing *Tgfb1* (exon 6/7 boundary) mRNA expression in AM and BMDM relative to *Gapdh* and *Actb* housekeeping genes and expressed as fold changes from *Tgfb1*^{fl/fl} littermate controls. AM data are median fold changes pooled from 6 mice per group from two independent experiments. BMDM data are median fold changes of 3 cell cultures established from bone marrow of a single mouse per genotype. **c-d)** Numbers of AM in BAL and lung tissue from 8-10 week old mice after 3 weeks of HDM or PBS treatment (**c**) and in lungs of 7 week and 7 month old naive mice (**d**), as determined by flow cytometry. Data shown are medians of 5-8 mice from two (**c**) or one (**d**) independent experiment(s). Statistics: Kruskal-Wallis (where required), followed by Mann-Whitney tests between the indicated pairs of groups; **, $p \leq 0.01$; ns, non-significant.

As CD11c^{Tgfb1} mice in our experiments displayed normal numbers of AM, but substantially decreased *Tgfb1* gene expression in AM, it was necessary to determine whether this reduced TGF-β1 expression resulted in baseline autoimmunity, as occurs with global or T cell-restricted TGF-β1 knockout²⁵⁰⁻²⁵². When breeding Cre hemizygous and negative *Tgfb1*^{fl/fl} mice, offspring genotypes were obtained in the expected Mendelian ratio of approximately 1:1 (**Fig. 6.17a**; Chi-square $p = 0.223$), indicating that CD11c^{Tgfb1} mice had no intrinsic health defect to select against their survival *in utero*. Of the first 44 litters of CD11c^{Tgfb1} mice born, pre-weaning mortality of 12 % was observed (242 animals total), which was comparable to other transgenic lines in our breeding colony. Autoimmune disease in global TGF-β1 knockout mice begins shortly after weaning at around 6 weeks of age²⁵⁰, however no mice died or showed signs of ill health at this time of life. Indeed aging of CD11c^{Tgfb1} mice up to 7 months did not reveal any evidence of spontaneous autoimmunity and no mice died unexpectedly during this time. Spleen leukocyte counts were not significantly different between CD11c^{Tgfb1} and littermate controls at 7 weeks or 7 months of age (**Fig. 6.17b**), consistent with a lack of systemic autoimmunity. Global and T cell-restricted knockout mice show spontaneous intestinal inflammation^{250,252}, however no macroscopic intestinal pathology (swelling, redness) was observed in 7 week or 7 month old CD11c^{Tgfb1} mice. No significant differences in length of the intestinal tract, which can indicate gut remodelling during inflammation, were apparent between 7 week or 7 month old CD11c^{Tgfb1} mice and controls (**Fig. 6.17c**).

Despite reduced TGF-β1 expression in AM, CD11c^{Tgfb1} mice displayed no evidence of baseline lung inflammation, as judged by total leukocyte counts in enzymatically digested lung tissue (**Fig. 6.17d**) and examination of haematoxylin and eosin-stained lung sections (**Fig. 16.7e**). Consistent with a lack of lung inflammation, there was no increase in CD4⁺ T cell numbers in lungs of 7 week or 7 month old CD11c^{Tgfb1} mice (**Fig. 6.17f**), nor was there any increase in the proportion of these cells that produced IFN γ upon *ex vivo* stimulation with PMA and ionomycin (**Fig. 6.17f**). A small proportion (7-10 %) of pulmonary CD4⁺ T cells in *Tgfb1*^{fl/fl} mice expressed FoxP3 and this was not altered in CD11c^{Tgfb1} mice of either age group (**Fig. 6.17g**). Overall, CD11c^{Tgfb1} mice did not exhibit a baseline inflammatory phenotype.

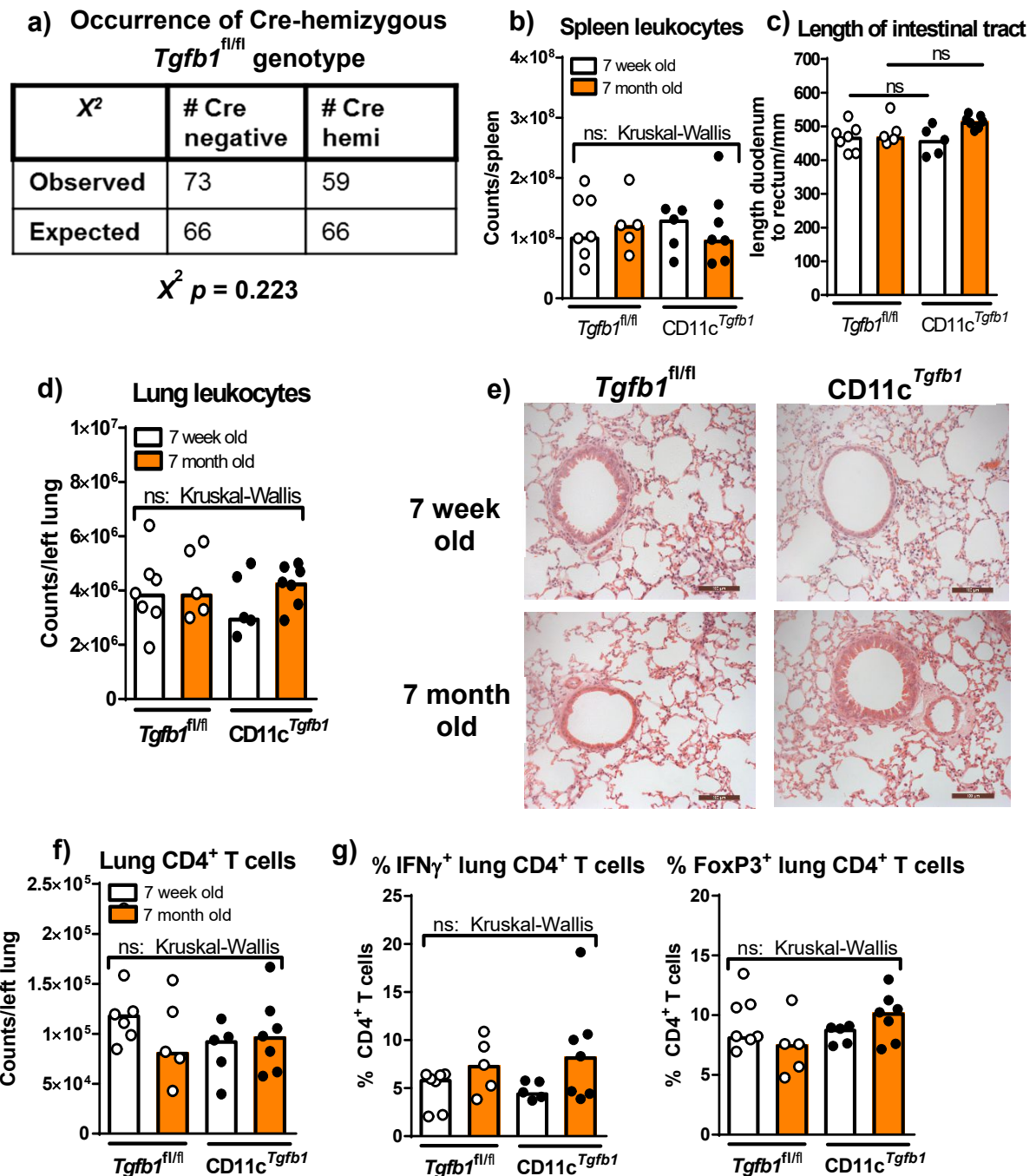


Figure 6.17. CD11c^{Tgfb1} mice show no steady state inflammatory disease

a) Results of Chi-square (χ^2) test comparing observed offspring genotype outcomes of breeding CD11c-Cre-hemizygous (hemi) and negative $Tgfb1^{fl/fl}$ mice with the expected 1:1 Mendelian ratio. **b)** Total crystal violet cell counts of spleen preparations. **c)** Lengths of intestinal tracts measured from duodenum to rectum. **d)** Total crystal violet cell counts in lung digests. **e)** Representative images of haematoxylin & eosin stained lung sections at 200x magnification, showing conducting airways, lung parenchyma and blood vessels after BAL. **f)** Total CD4⁺ CD3⁺ T cells in lung cell preparations as determined by flow cytometry. **g)** Percentages of lung CD4⁺ T cells with positive intracellular IFN γ and intranuclear FoxP3 staining determined by flow cytometry. Scatter/bar plots show medians of 5-6 replicate mice per group. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ns, non-significant.

6.9. Enhanced type 2 immune response to inhaled HDM in the absence of CD11c⁺ cell-derived TGF-β1

Baseline data suggested that AM-derived TGF-β1 was not required for preventing immunopathology at the steady state. To assess the role of AM-derived TGF-β1 in response to an inflammatory stimulus, CD11c^{Tgfb1} mice and littermate controls were exposed to inhaled HDM for 3 weeks and AAD parameters assessed (**Fig. 6.18a**). As described previously²⁷³, repeated HDM exposure resulted in increased levels of TGF-β bioactivity in the airway lumen, detectable in BAL fluid 24h after final allergen challenge (**Fig. 6.18b**). Airway TGF-β bioactivity levels were comparable between CD11c^{Tgfb1} mice and controls treated with either PBS or HDM (**Fig. 6.18b**), suggesting that AM were not a major contributor to the pool of bioactive TGF-β detectable in BAL fluid at this timepoint. These data are consistent with previous work in this model, which showed bronchiolar epithelial club cells to be the major source of bioactive TGF-β detected in BAL of HDM-treated mice²⁷³.

No statistically significant differences in total pulmonary inflammation, measured as leukocyte counts of lung digests, BAL and mLN, were observed between HDM-treated CD11c^{Tgfb1} and control mice (**Fig. 6.18c**). However, HDM-treated CD11c^{Tgfb1} mice showed a trend towards increased lung leukocyte counts (**Fig. 6.18c**; 1.3-fold change, Mann-Whitney $p = 0.08$), suggestive of a subtle increase in AAD severity in the absence of CD11c⁺ cell-derived TGF-β1. Accordingly, airway resistance in HDM-treated CD11c^{Tgfb1} mice was significantly higher than in *Tgfb1*^{fl/fl} controls in response to 100 mg/ml of inhaled methacholine (**Fig. 6.18d**), consistent with a modest increase in AHR. Parameters reflecting reduced ease of lung expansion, elastance and compliance, increased and decreased, respectively, in HDM-treated mice, but did not differ significantly between CD11c^{Tgfb1} mice and controls (**Fig. 6.18d**).

Considering the modestly increased airway resistance observed in HDM-treated CD11c^{Tgfb1} mice and the central role for type 2 immunity, in particular IL-13, in driving allergen driven AHR⁸⁴, the nature of the pulmonary immune response to allergen in these mice was assessed. HDM exposure increased eosinophil, but not neutrophil, numbers in lung tissue of both mouse strains, consistent with induction of allergic eosinophilic pulmonary inflammation (**Fig. 6.19a**). Notably, numbers of eosinophils in lung tissue of HDM-treated CD11c^{Tgfb1} mice were significantly around 50 % greater than those in littermate controls (**Fig. 6.19a**), suggesting that reduced TGF-β1 production from CD11c⁺ cells results in heightened eosinophilic lung inflammation in response to HDM inhalation. HDM-induced BAL eosinophil and neutrophil numbers were not different in CD11c^{Tgfb1} mice and *Tgfb1*^{fl/fl} controls (**Fig. 6.19b**), suggesting that the enhanced eosinophilia observed was localised to lung tissue rather than the airway lumen.

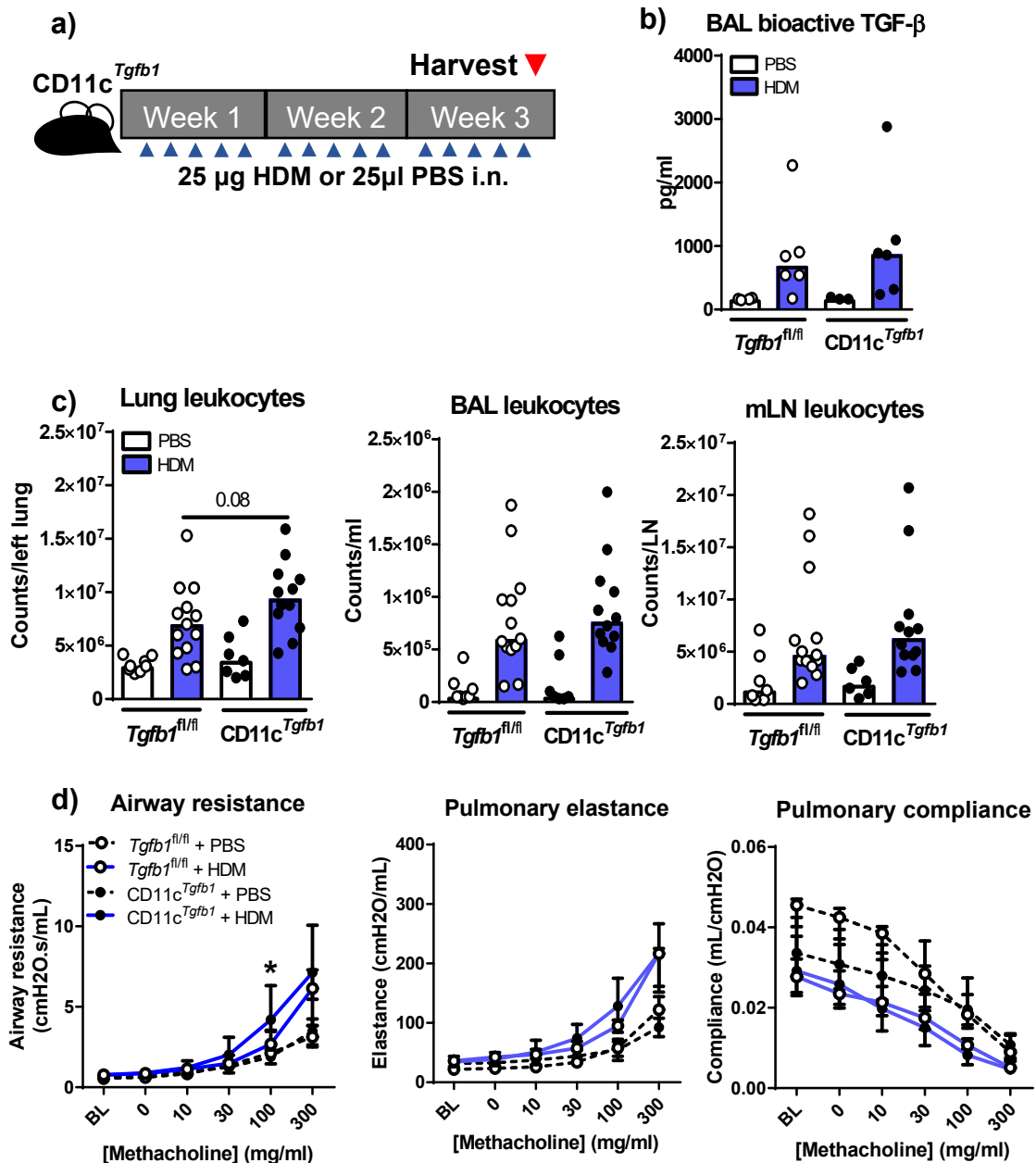


Figure 6.18. Increased HDM-driven airway hyperresponsiveness in CD11c^{Tgfb1} mice

a) CD11c^{Tgfb1} mice and Tgfb1^{fl/fl} littermate controls were exposed to intranasal HDM or PBS 5 times per week for 3 weeks and measurements made 24h after final challenge. **b)** Concentration of bioactive TGF-β in BAL fluid as determined by standardised Smad reporter bioassay. **c)** Total crystal violet counts of cells in lung digest, BAL and mLN preparations. **d)** Line graphs showing median ± IQR Flexivent[™] measurements of airway resistance and pulmonary elastance and compliance of 12-13 mice per group pooled from three independent experiments. Scatter/bar plots show medians of 4-13 replicate mice per group from one (b) or two (c) independent experiment(s). Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; *, $p \leq 0.05$. Where relevant, p values are shown between groups. Symbols above line graphs indicate significance between Tgfb1^{fl/fl} and CD11c^{Tgfb1} HDM-treated groups.

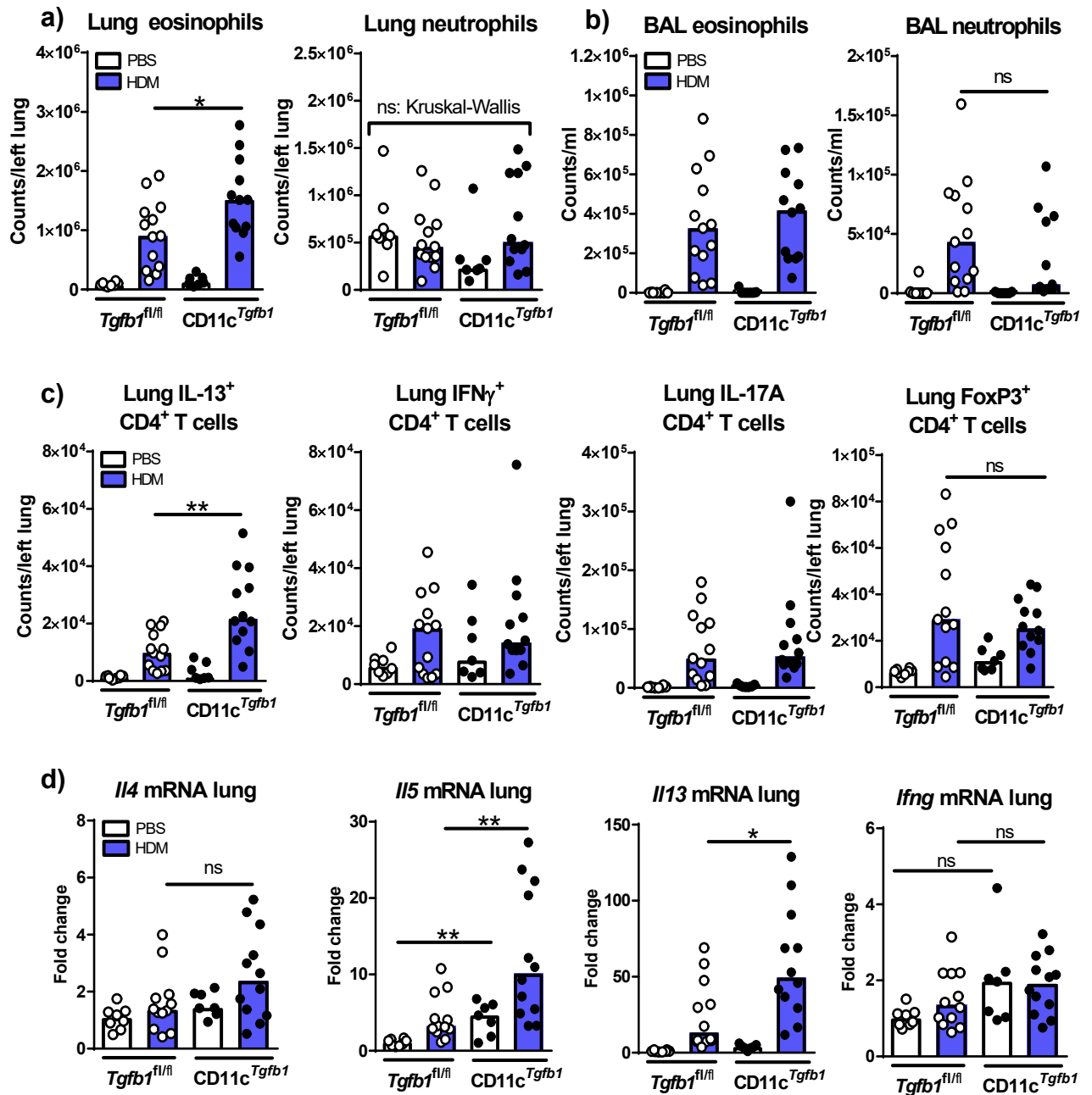


Figure 6.19. Enhanced type 2 immune response to HDM in lungs of CD11c^{Tgfb1} mice

a-c) Leukocyte populations were quantified by flow cytometry. **a-b)** Total numbers of eosinophils and neutrophils in lung digests (**a**) and BAL (**b**). **c)** Total numbers of CD4⁺ T cells with positive staining for IL-13, IFN γ , IL-17A or FoxP3 in lung digests. **d)** qPCR data showing levels of *Il4*, *Il5*, *Il13* and *Ifng* mRNA in lung tissue relative to *Gapdh* and *Actb* housekeeping genes and expressed as fold changes from PBS-treated *Tgfb1*^{fl/fl} mice. Scatter/bar plots show medians of 7-13 replicate mice per group pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant.

Consistent with increased pulmonary eosinophilia, which suggested an enhanced type 2 immune response, numbers of IL-13⁺ CD4⁺ T cells were increased in lungs of HDM-treated CD11c^{Tgfb1} mice compared with controls (**Fig. 6.19c**). In contrast, numbers of CD4⁺ T cells positive for IFN γ , IL-17A or FoxP3⁺ were comparable in lungs of HDM-treated CD11c^{Tgfb1} and control mice (**Fig. 6.19c**), suggesting an enhancement of Th2 cell generation, recruitment or maintenance, rather than a general dysregulation of the T helper cell response. In support of an enhanced pulmonary Th2 response to HDM where TGF- β 1 is deleted in CD11c⁺ cells, relative abundance of mRNA for the type 2 cytokines *Il5* and *Il13* was significantly increased in lungs of HDM-treated CD11c^{Tgfb1} mice (**Fig. 6.19d**). Gene expression changes appeared to be specific to certain cytokines, since relative expression of *Il4* and the type 1 cytokine gene *Ifng* was not significantly different between CD11c^{Tgfb1} and control mice (**Fig. 6.19d**). Notably, *Il5* mRNA expression was significantly increased by around 4-fold in PBS-treated CD11c^{Tgfb1} mice, suggesting that this conditional TGF- β 1 knockout may predispose to production of type 2 cytokines even prior to allergen exposure.

Th2 skewing in CD11c^{Tgfb1} mice appeared to be restricted to lung tissue rather than the site of T cell priming, since numbers of IL-13⁺, IFN γ ⁺, IL-17A⁺ and FoxP3⁺ CD4⁺ T cells in mLN of HDM-treated CD11c^{Tgfb1} mice and controls were not different (**Fig. 6.20**). This suggests that the defect in regulation of pulmonary Th2 cell numbers in HDM-treated CD11c^{Tgfb1} knockout mice was dependent on signals at the site of inflammation, rather than the location of T cell priming.

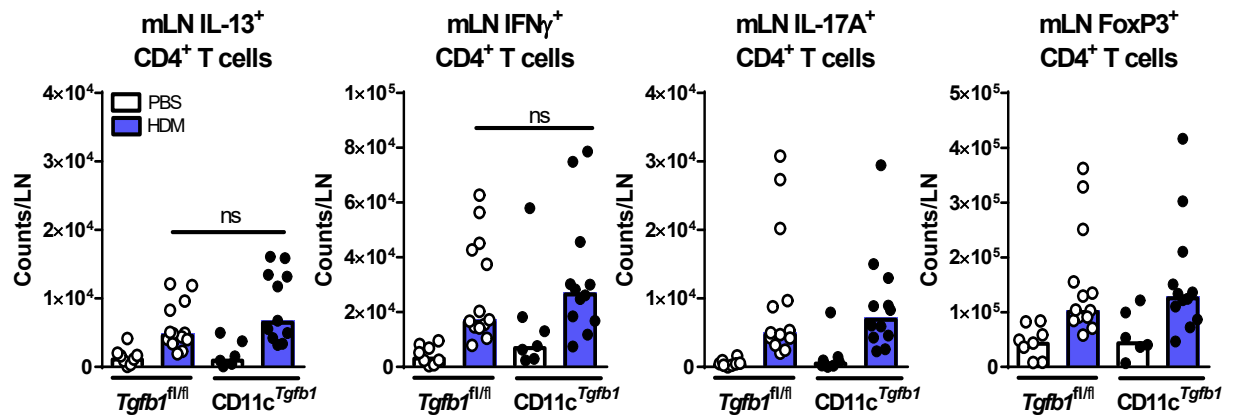


Figure 6.20. No change in mediastinal lymph node T cell populations in *CD11c*^{Tgfb1} mice during HDM-driven allergic airway disease

Total numbers of CD4⁺ T cells with positive staining for IL-13, IFN γ , IL-17A or FoxP3 in mLN, as determined by flow cytometry. Scatter/bar plots show medians of 7-12 replicate mice per group, pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ns, non-significant.

6.10. Evidence of enhanced recruitment of monocyte-derived cells to lungs of CD11c^{Tgfb1} mice following HDM exposure

The increased pulmonary Th2 response observed in CD11c^{Tgfb1} mice, in which TGF-β1 deletion is targeted to selected myeloid cell subsets, was suggestive of altered communication between innate and adaptive immunity. Given the activity of CD11c-Cre in a subset of DC (**Fig. 6.1**), which could feasibly alter their *in vivo* function by deletion of TGF-β1, it was necessary to assess the quantity and distribution of DC in HDM-treated CD11c^{Tgfb1} mice. DC numbers in mLN were increased by HDM exposure in both CD11c^{Tgfb1} mice and controls, but did not differ between the two strains (**Fig. 6.21a**), suggesting that net migration of DC from lungs to mLN was not affected by conditional *Tgfb1* knockout. Similarly, numbers of cDC1 and cDC2, did not differ between HDM-treated CD11c^{Tgfb1} mice and controls (**Fig. 6.21b**). However, CD64⁺ CD11b⁺ moDC, the most abundant DC-like cells in lungs of HDM-treated mice, were present in significantly higher numbers in lungs of HDM-treated CD11c^{Tgfb1} mice compared to controls (**Fig. 6.21b**). Extension of analysis to other likely monocyte-derived cells in the allergic lung revealed a significant increase of around two-fold in numbers of pulmonary CD64⁺ CD11b⁺ Siglec F⁺ IMM in CD11c^{Tgfb1} mice (**Fig. 6.21c,d**). Thus, the enhanced pulmonary type 2 immune response in HDM-exposed CD11c^{Tgfb1} mice was accompanied by increased numbers of inflammatory monocyte-derived cells in lung tissue.

Increased numbers of IMM in lungs of CD11c^{Tgfb1} mice did not appear to reflect selective expansion of IMM in which TGF-β1 is conditionally deleted due to upregulation of CD11c driving Cre expression, since an equal proportion of IMM showed positive CD11c expression in lungs of CD11c^{Tgfb1} and control mice (**Fig. 6.21e**). To address the alternative possibility of increased monocyte recruitment to lungs in HDM-treated CD11c^{Tgfb1} mice, chemokine levels in lung tissue were quantified. Consistent with increased monocyte recruitment to lungs, CCL2, a chemokine for monocytes, among other cell types such as natural killer cells⁵⁸, was increased by around two-fold in lungs of HDM-treated CD11c^{Tgfb1} mice (**Fig. 6.21f**). The concentration of CCL2 was not increased above PBS control levels in lung tissue of *Tgfb1*^{fl/fl} mice during AAD (**Fig. 6.21f**), suggesting that its increased production following HDM exposure in these mice was dependent on deletion of TGF-β1 in CD11c⁺ cells. The increased pulmonary CCL2 concentration did not reflect a general increase in chemokine levels in HDM-treated CD11c^{Tgfb1} mice, since no differences in the concentrations of CCL24 or CXCL1 were observed in lungs of these mice compared to *Tgfb1*^{fl/fl} controls (**Fig. 6.21f**).

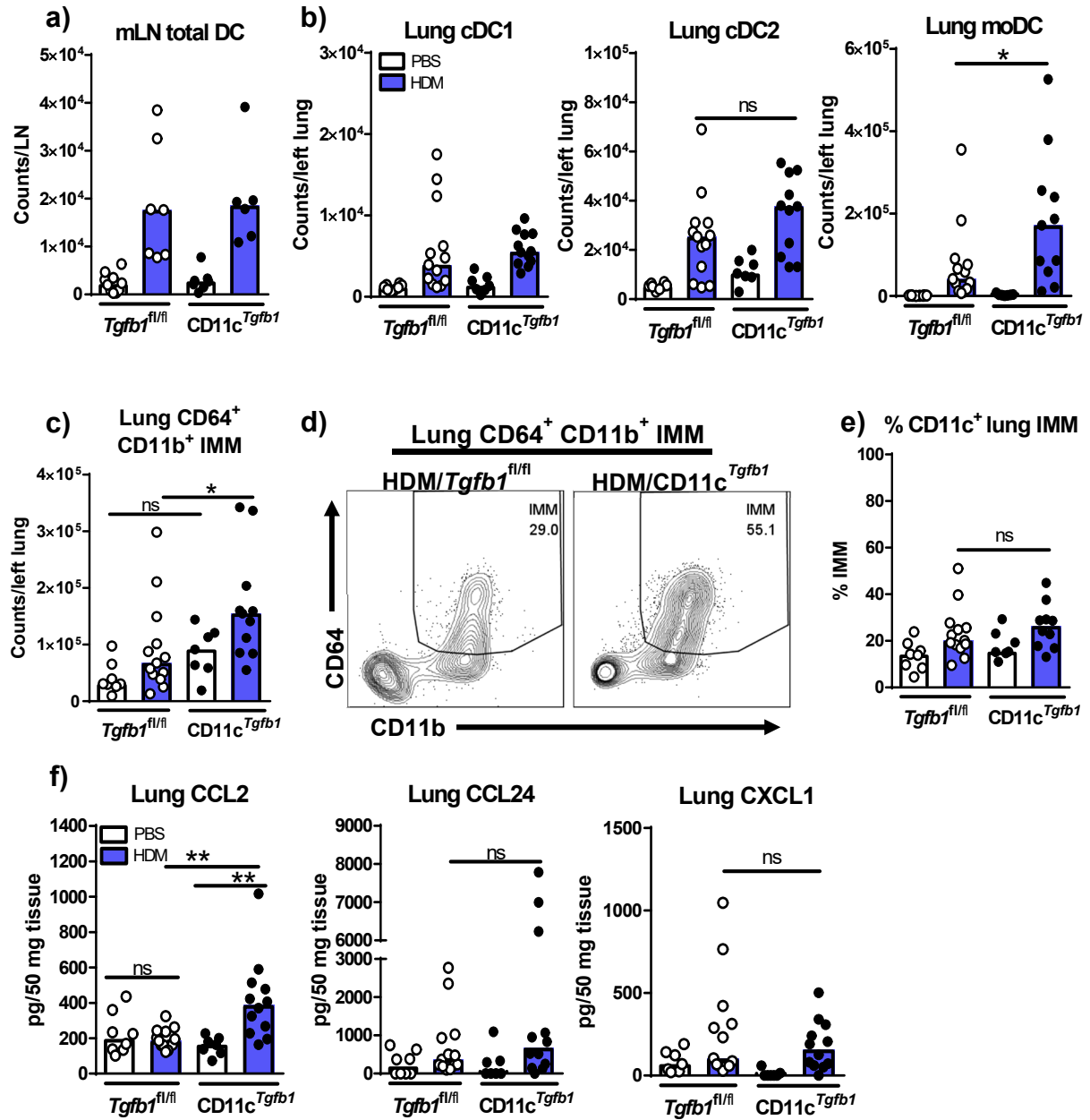


Figure 6.21. Increased monocyte-derived cell numbers in lungs of HDM-treated *CD11c*^{*Tgfb1*} mice
a-e) Cells in lung digest and mLN preparations were analysed and quantified by flow cytometry. **a)** Total *CD11c*⁺ *MHC-II*⁺ *CD90.2/CD19/NKp46/Siglec F/Ly6G*-negative DC numbers in mediastinal lymph nodes. **b)** Total cDC1, cDC2 and moDC numbers in lung digests. **c)** Total *CD64*⁺ *CD11b*⁺ IMM counts in lung digests. **d)** Representative flow cytometry plots showing IMM as proportions of live, single, *CD45*⁺ cells after excluding *CD90.2/CD19/NKp46/Siglec F/Ly6G*-positive cells and cells double-positive for *MHC-II* and *CD11c* (i.e. DC). **e)** Percentages of lung IMM with positive surface staining for *CD11c*. **f)** Concentrations of CCL2, CCL24 and CXCL1 in lung homogenates as determined by ELISA. Scatter/bar plots show medians of 7-12 replicate mice per group, pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant.

6.11. TGF- β 1-deficient AM overproduce CCL2 *in vitro*

The increased levels of CCL2 in lung tissue, congruent with elevated pulmonary IMM and moDC numbers in HDM-treated CD11c^{Tgfb1} mice raised questions surrounding the cellular source of this chemokine. AM can produce CCL2 during acute lung inflammation^{462,463} and, as shown by RNAseq data in Chapter 5, upregulate *Ccl2* mRNA during HDM-driven AAD. Since the CD11c-conditional knockout of TGF- β 1 is targeted to AM, it was hypothesised that in the absence of autocrine TGF- β 1, AM are hyperactive and overproduce pro-inflammatory mediators such as CCL2.

To assess AM activation *in vivo*, proportions of MHC-II and CD11b-expressing cells were determined as markers of AM activation. Around 20-30 % of 'steady state' AM from PBS-treated *Tgfb1*^{fl/fl} control mice expressed MHC-II, and this frequency was not altered in conditional knockout mice (**Fig. 6.22a**). A modest but significant increase in the proportion of CD11b⁺ steady state AM was observed in CD11c^{Tgfb1} knockout mice (**Fig. 6.22a**; median 4.4, range 0-6 vs 2.2, range 0-15). However, the overall numbers of CD11b⁺ AM were very low in these mice, accounting for a small number of flow cytometry events (**Fig. 6.22b**), so the observed change in CD11b expression did not strongly suggest heightened AM activation in the absence of intrinsic TGF- β 1. As expected, 'inflamed' AM from HDM-treated mice showed much higher proportions of positive MHC-II and CD11b staining than those from mice receiving PBS. However no significant differences in percentages of positive cells were observed between CD11c^{Tgfb1} mice and controls (**Fig. 6.22c**). A non-significant trend towards a slightly greater proportion of CD11b⁺ AM was observed in HDM-treated AM (median 64 % vs 54 % $p = 0.09$; **Fig 6.22c**), which was also apparent when normalised gMFI of CD11b staining was assessed (median 132 % vs 100 %; $p = 0.052$; **Fig. 6.22d**). However, these changes were very subtle, so altogether there was little evidence from surface marker expression of heightened AM activation in CD11c^{Tgfb1} mice.

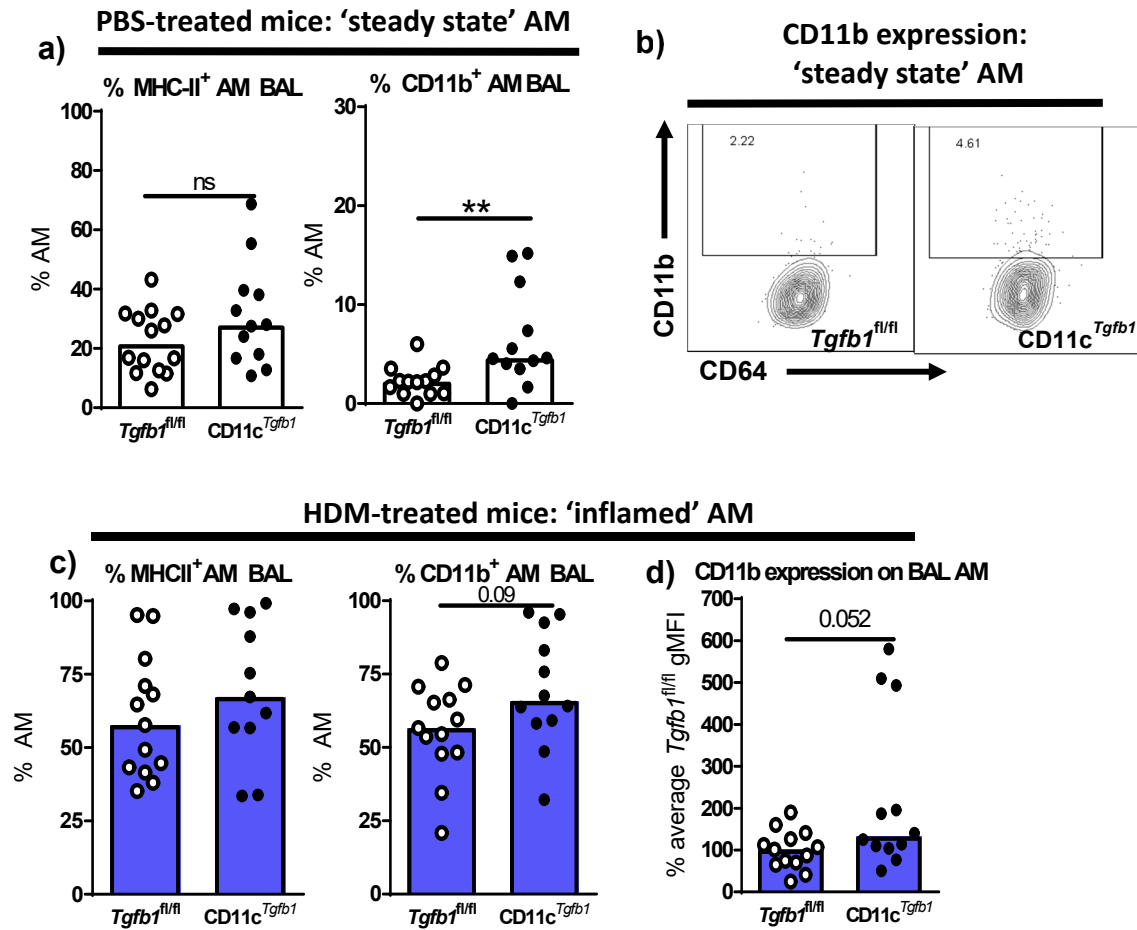


Figure 6.22. AM activation in *CD11c*^{*Tgfb1*} mice

Cells from *CD11c*^{*Tgfb1*} and *Tgfb1*^{fl/fl} control mice exposed to intranasal PBS ('steady state') or HDM ('inflamed') for 3 weeks were analysed by flow cytometry. **a)** Proportions of steady state AM with positive surface staining for MHC-II and CD11b. **b)** Representative flow cytometry plots of CD11b expression data from (a). **c)** Proportions of inflamed AM with positive surface staining for MHC-II and CD11b. **d)** Geometric mean fluorescence intensity of CD11b staining on BAL AM, expressed as a percentage of the mean value obtained in HDM-treated *Tgfb1*^{fl/fl} mice in individual experiments. Numbers on flow cytometry plots indicate the percentage of events in gates. Scatter/bar plots show medians of 12-13 replicate mice per group, pooled from three (a) or two (c-d) independent experiments. Statistics: Mann-Whitney test; **, $p \leq 0.01$; ns, non-significant. Where relevant, p values are shown between groups.

To determine the propensity of TGF- β 1-deficient AM to produce pro-inflammatory cytokines and chemokines, AM were obtained by BAL of naïve CD11c^{Tgfb1} and Tgfb1^{fl/fl} control mice and stimulated with LPS in the absence of exogenous TGF- β or serum. AM from CD11c^{Tgfb1} mice did not express significantly different *Tnf* mRNA levels to control cells (**Fig. 6.23a**), consistent with previous data showing no regulation of LPS-induced *Tnf* gene expression by exogenous TGF- β 1 in AM (see Chapter 4). However, LPS-induced expression of *Il27* mRNA, shown previously to be suppressed by exogenous TGF- β 1 in AM (see Chapter 4), displayed a trend towards increase (Mann-Whitney $p = 0.07$) in AM from CD11c^{Tgfb1} mice, suggestive of a loss of autocrine regulation by TGF- β 1 in these cells. Interestingly, TGF- β 1-deficient AM displayed significantly increased levels of *Ccl2* mRNA, both when cultured with serum-free medium alone and following LPS stimulation (**Fig. 6.23a**), suggesting that autocrine TGF- β 1 regulated *Ccl2* gene expression in AM in these conditions. Quantification of secreted protein supported these gene expression data, showing no change in LPS-induced TNF α levels in supernatants of CD11c^{Tgfb1} AM compared with controls, while AM from CD11c^{Tgfb1} mice displayed spontaneous secretion of CCL2 into supernatants and released significantly more CCL2 than control cells after LPS stimulation (**Fig. 6.23b**). These data suggest that AM are dysregulated in the absence of autocrine TGF- β 1 signalling, resulting in overproduction of CCL2, supporting the possibility that a similar mechanism may account for the increased pulmonary CCL2 levels observed following HDM exposure *in vivo*.

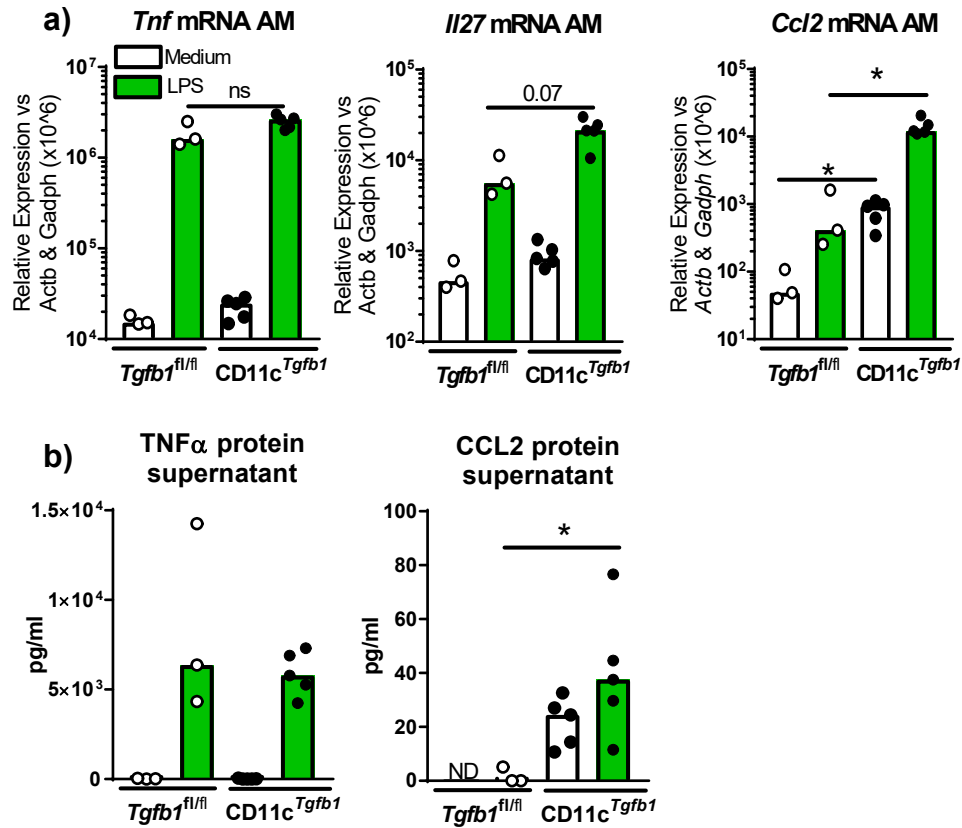


Figure 6.23. Dysregulated cytokine production by AM from CD11c^{Tgfb1} mice

AM from BAL of naïve CD11c^{Tgfb1} mice and *Tgfb1*^{fl/fl} controls were cultured in serum-free medium ± 500 ng/ml LPS for 8h. **a)** *Tnf*, *Ccl2* and *Il27* mRNA expression relative to housekeeping genes, as determined by qPCR. **b)** Concentrations of TNFα and CCL2 in culture supernatants, as determined by ELISA. Scatter/bar plots show medians of cultures of 3-5 individual mice per genotype from a single experiment. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; ns, non-significant. Where relevant, p values are shown between groups.

6.12. Discussion

The aim of this chapter was to determine how major cellular sources of the cytokines IL-10 and TGF- β 1 control immunity in the allergic lung, with a particular focus on the role of AM in the function of these cytokines. By combining experiments using conditional knockout mice and *in vivo* cytokine blockade, insights were gained into how IL-10 and TGF- β 1 regulate pulmonary immunity and how AM contribute to these mechanisms.

6.12.1. T cell-derived IL-10 regulates the balance between type 2 and type 1/3 immune responses to inhaled HDM, possibly by controlling macrophage activation state

Several lines of evidence in previous chapters strongly suggested that AM are minor contributors to pulmonary IL-10 levels during the immune response to inhaled HDM. However, as detection of genes and proteins is limited by the sensitivity of assays and timepoints of analysis, the possibility remained that low levels of AM-derived IL-10 could regulate the immune response to inhaled allergen. Through thorough analysis of AAD parameters in CD11c^{Il10} mice exposed to HDM, the low level of IL-10 produced by AM *in vivo* was shown to be redundant for regulation of the immune response to inhaled HDM.

An outstanding question is whether the lack of a regulatory role for AM-derived IL-10 observed in the HDM model more broadly reflects redundancy of these cells as an IL-10 source *in vivo*. This would have implications for measurement of IL-10 expression by AM as an indicator of regulatory capacity, as has been described in asthmatics^{156,216,337}. Results described in this chapter showing no effect of CD11c-Cre-driven IL-10 knockout on the early inflammatory response to RSV infection support this hypothesis. Similarly, a recent study reported minimal effects of IL-10 knockout driven by CD11c-Cre or LysM-Cre on the outcomes of *Mycobacterium tuberculosis* infection in mice, despite detection of IL-10 reporter signal in up to 40 % of AM¹⁹⁷, suggesting that even when produced by a much greater proportion of cells than observed in the HDM or RSV models (see Chapter 4), AM-derived IL-10 has minimal influence on the immune response *in vivo*. Parallels can be drawn between airway and intestinal macrophages. Despite intestinal macrophages producing high levels of IL-10⁴⁵⁶, macrophage-restricted IL-10 knockout does not recapitulate the spontaneous inflammatory bowel disease observed in global IL-10-null mice²⁰⁶, indicating that IL-10 from non-macrophage sources is sufficient to regulate immunity in the steady state gut.

Despite, the apparent redundancy of AM-derived IL-10 in the allergic lung, there is little doubt of a key role for IL-10 in regulating AAD. IL-10-null (*Il10*^{-/-}) mice show enhanced airway inflammation in models of systemic sensitisation and airway challenge with ovalbumin²¹⁰ or *Schistosoma mansoni* egg antigen (SEA)⁴⁶⁴, with elevated pulmonary type 2 cytokine levels detected in the case of SEA but not ovalbumin^{210,464}. Similarly, *Il10*^{-/-} mice displayed an enhanced eosinophilic inflammatory response following systemic sensitisation and inhalation challenge with the fungus *Aspergillus fumigatus*⁴⁶⁵. An essential regulatory role for IL-10 in regulating AAD is supported by the IL-10R blockade studies described in this work, with α IL10R administration throughout HDM exposure dampening eosinophilia, while augmenting the Th1 response. The intranasal antibody

approach had the added benefit of targeting IL-10 signalling specifically in the airways and only from the beginning of allergen exposure, rather than systemically and throughout life, as in the case of *Il10*^{-/-} mice. These data therefore suggest that IL-10 acts in the lungs during AAD and dictates the nature of the immune response to allergen. It remains to be seen whether the same augmentation of Th1 immunity would be observed if the α IL10R antibody was administered after allergic sensitisation, or whether blockade is required throughout HDM exposure.

In contrast to the previously published studies of AAD in *Il10*^{-/-} mice, which generally featured augmentation of type 2 immunity in the absence of IL-10^{210,464,465}, IL-10R blockade throughout the 3 week HDM inhalation model caused a reduction in IL-13⁺ CD4⁺ T cells and pulmonary eosinophilia, with a concomitant increase in IFN γ ⁺ CD4⁺ T cells, suggestive of a deviation towards a type 1 immune response. These different outcomes of impaired IL-10 signalling can likely be explained by the routes of sensitisation: inhalation versus systemic injection, the latter typically performed in combination with an adjuvant such as aluminium hydroxide, which promotes a Th2-biased response upon subsequent challenge. Mucosal sensitisation by repeated intranasal exposure to HDM drives a mixed granulocytic inflammation, in which eosinophilia is accompanied by a greater proportion of neutrophils than seen following systemic sensitisation and airway challenge protocols³¹⁷. Indeed, sensitisation to the same protein, ovalbumin, either via the airways with an LPS adjuvant or by systemic injection with an aluminium hydroxide adjuvant primes neutrophilic and eosinophilic inflammation, respectively³²⁹, illustrating the importance of the route of sensitisation and the presence of danger signals such as LPS for the nature of the immune response elicited. In the case of the HDM model used in the present work, the mixed inflammatory profile is likely due to both the repeated intranasal route of administration and the LPS present in the mite bodies and extracts, the importance of which demonstrated by the lack of sensitisation of mice lacking the LPS receptor TLR4 to inhaled HDM³⁶. In support of the findings in this chapter, *Il10*^{-/-} mice exposed to intranasal HDM 10 times over 2 weeks displayed reduced ratios of eosinophils to neutrophils in the airways compared with wild type controls²¹¹, independently verifying that IL-10 regulates the balance between eosinophil^{hi} and eosinophil^{low} inflammatory profiles in response to inhaled HDM following mucosal sensitisation.

Importantly, the effects of pulmonary blockade of IL-10R during HDM-driven AAD were recapitulated by genetic deletion of IL-10 from T cells, confirming that T cells are the dominant source of immunoregulatory IL-10 in this model. While the effects of T cell-restricted IL-10 deletion were highly similar to those of IL-10R blockade, CD4^{Il10} mice showed greater airway neutrophilia, lung IL-17A levels and more robust enhancement of lung function changes than mice treated with α IL-10R, potentially indicating that genetic deletion of the major cellular source is more effective at suppressing pulmonary IL-10 signalling than neutralisation of the receptor. The efficacy of CD4⁺ T cell-derived IL-10 in regulation of AAD has been shown previously by transfer of CD25^{hi} Tregs¹⁰² or transgenic antigen-specific CD4⁺ T cells overexpressing IL-10²¹³. However, these experiments demonstrate for the first time the importance of endogenous IL-10-producing CD4⁺ T cells in regulating the immune response to inhaled allergen.

The suggestion that CD4⁺ T cells are the major and dominant source of IL-10 during AAD contrasts with studies suggesting that IL-10 derived from IM and monocyte-derived macrophages (together equivalent to the IMM analysed in this work) regulates the immune response to inhaled ovalbumin³¹ or HDM²¹¹. However, these conclusions were largely based on transfer of IL-10-producing IMM into the airways of mice²¹¹ or focussed on the time of allergic sensitisation³¹, rather than the absence of IL-10 production from IMM during established disease. Transfer of IL-10-producing cells directly into the lungs is likely to dampen inflammation, as occurs when IL-10-producing T cells are transferred and traffic to the lungs^{102,213}, but this does not necessarily reproduce their function in an unmanipulated animal during AAD. It seems likely that, as suggested by Bedoret and colleagues, IMM-derived IL-10 is important for regulating DC function at homeostasis to prevent allergic sensitisation³¹, while the present work would suggest that CD4⁺ T cell-derived IL-10 is dominant during established AAD. Consistent with this hypothesis, no adverse phenotype or decrease in pulmonary IL-10 levels was observed in non-inflamed CD4^{fl/fl} mice treated with intranasal PBS, while IL-10 concentration in lungs was significantly decreased in HDM-treated CD4^{fl/fl} mice, to levels below that seen in PBS controls. IL-10 reporter mouse data described in Chapter 4 support this conclusion, as IM and lung monocytes were the major IL-10-competent cells in the absence of inflammation, with a large number of IL-10-competent CD4⁺ T cells detected in established HDM-driven AAD. Together these data fit with a model in which the major source of IL-10 shifts from IMM to CD4⁺ T cells during HDM-driven AAD.

The mechanisms underlying the enhanced severity of AAD in the absence of T cell-derived IL-10 are so far incompletely understood. The reduction in eosinophilia observed in these mice is likely to be secondary to an overall dampening of the type 2 immune response, in particular the reduction in the eosinophil chemokine, CCL24, which is required for maximal eosinophil recruitment to the lung downstream of IL-13 signalling⁴⁶⁶. However, a direct role of IL-10 in promoting type 2 immunity is unlikely, since IL-10 has been shown to negatively regulate Th2 cell survival during HDM-driven AAD²¹². More likely is that the lack of IL-10 signalling alleviates suppression of the type 1 immune response, which in turn dampens type 2 immunity and eosinophilia, as has been shown previously following overexpression of IFN γ in murine airways⁴⁶⁷. The effects on type 3 immunity (i.e. IL-17A-production) in this immune deviation process were less clear than the skewing from type 2 to type 1. The HDM model used in these studies elicited a substantial IL-17A⁺ CD4⁺ T cell response, but numbers or proportions of these cells in the lung did not change with IL-10R blockade or IL-10 knockout in T cells. However, the concentration of IL-17A in lung tissue was elevated in HDM-treated CD4^{fl/fl} mice compared to *fl/fl* controls, suggesting that overall production of this cytokine was increased. Absence of IL-10 from B cells has been shown to enhance Th1 and Th17 cell numbers in a mouse model of rheumatoid arthritis, indicating that IL-10 can concomitantly regulate these T cell phenotypes⁴⁶⁸, and *Il17a* mRNA was increased in lungs of HDM-treated *Il10*^{-/-} mice²¹¹. It remains to be seen whether the type 2 to type 1 skewing observed in the absence of T cell-derived IL-10 would occur in a model that does not generate a substantial Th17 response. This will be assessed in future experiments using the

Alternaria alternata fungal allergen inhalation model of highly type 2-biased AAD (as introduced in Chapter 3).

Both IL-10R blockade and CD4-conditional IL-10 knockout during HDM-driven AAD also resulted in a decrease in the proportion of pulmonary FoxP3⁺ Tregs relative to cytokine-producing effector T helper cells. It is possible that this imbalance in Treg to effector ratio contributed to worsened disease in the absence of T cell-derived IL-10, given the capacity of Tregs to dampen allergic inflammation¹⁰² and their requirement for limiting the severity of an ovalbumin-driven systemic sensitisation and airway challenge model of AAD⁴⁶⁹. However, it is unclear from these data whether the absence of IL-10 directly results in the relative reduction in FoxP3⁺ Tregs, owing to the potential for IL-10 to stabilise FoxP3 expression in CD4⁺ T cells⁴⁵⁶, or whether it is an indirect result of the substantial changes in the local cellular and cytokine milieu. What is clear from both models though is that reduced proportions of Tregs were only observed in lungs and not the mLN, suggestive of a dependence on local signals at the site of inflammation.

The precise link between the absence of T cell-derived IL-10 and dysregulation of T helper cell populations in the allergic lung remains to be determined, but may be related to the increased activation of both AM and IMM apparent in both the IL-10R blockade and CD4^{IL10} mouse models. IL-10 has long been understood to limit Th1 cell activation by suppression of APC activation and cytokine production^{195,196}, suggesting that T cell-derived IL-10 may function to limit further local T cell activation by pulmonary macrophages. In support of this, increased T cell co-stimulation capacity of AM from *IL10*^{-/-} mice has been reported⁴⁷⁰, suggesting that local IL-10 may condition AM to limit their ability to activate T cells. Such a model has been proposed in the intestine, in which T cell-derived IL-10 was shown to prevent spontaneous inflammation by limiting the activation of intestinal macrophages^{205,206}. The increased MHC-II expression on both AM and IMM in lungs of mice treated with HDM in the absence of intact IL-10 signalling was suggestive of classical, M[IFN γ]-like activation^{80,471}. Such classical activation, driven by the transcription factor IRF5, has been shown to promote Th1 and Th17 polarisation by monocyte-derived macrophages¹⁷⁵, offering a potential mechanistic link between macrophage dysfunction and the observed deviation of the T cell response in lungs of HDM-treated mice without intact IL-10 signalling. However, increased MHC-II expression on AM and IMM in HDM-treated mice without functional IL-10 signalling could be secondary to the increased levels of IFN γ , a major inducer of MHC-II in macrophages⁴⁷². Further studies will therefore be required to test for causal relationships between the enhanced type 1 immune response to HDM and augmented macrophage activation in the absence of an intact IL-10-IL10R axis. These will begin with experiments to determine whether conditional knockout of IL-10R on AM causes type 1/3 immune deviation in response to inhaled HDM.

It was interesting and unexpected to observe that genetic and antibody-mediated ablation of IL-10 signalling worsened AHR in HDM-treated mice, determined from the magnitude of increase in airway resistance in response to inhaled methacholine, despite a decrease in CD4⁺ T cell

production of IL-13, a key driver of AHR^{84,473}. Parallels can be drawn to a severe asthma mouse model, achieved by combining intranasal HDM with the bacterial second messenger cyclic-di-GMP, which featured elevated neutrophilia and worsened AHR, in a manner dependent upon IFN γ ⁴⁷⁴. With this in mind, the potential pathogenic function of IFN γ in the CD4^{IL10} model will be assessed in future experiments by therapeutic antibody blockade of IFN γ after establishment of Th1^{hi} AAD.

Overall, based on the data described in this chapter, a model is proposed in which sustained allergen exposure drives IL-10 production from CD4⁺ T cells, which acts to limit type 1 and 3 immunity, possibly via direct suppression of pulmonary macrophage activation and/or promotion of FoxP3⁺ Tregs (**Fig. 6.24**). In the absence of T cell-derived IL-10, increases in Th1 cells and IFN γ are likely to dampen type 2 immunity, resulting in a severe eosinophil^{low} Th1^{hi} AAD phenotype. Further study of this model will provide insights into non-type 2 drivers of AHR and interactions between T cells and macrophages in the allergic lung.

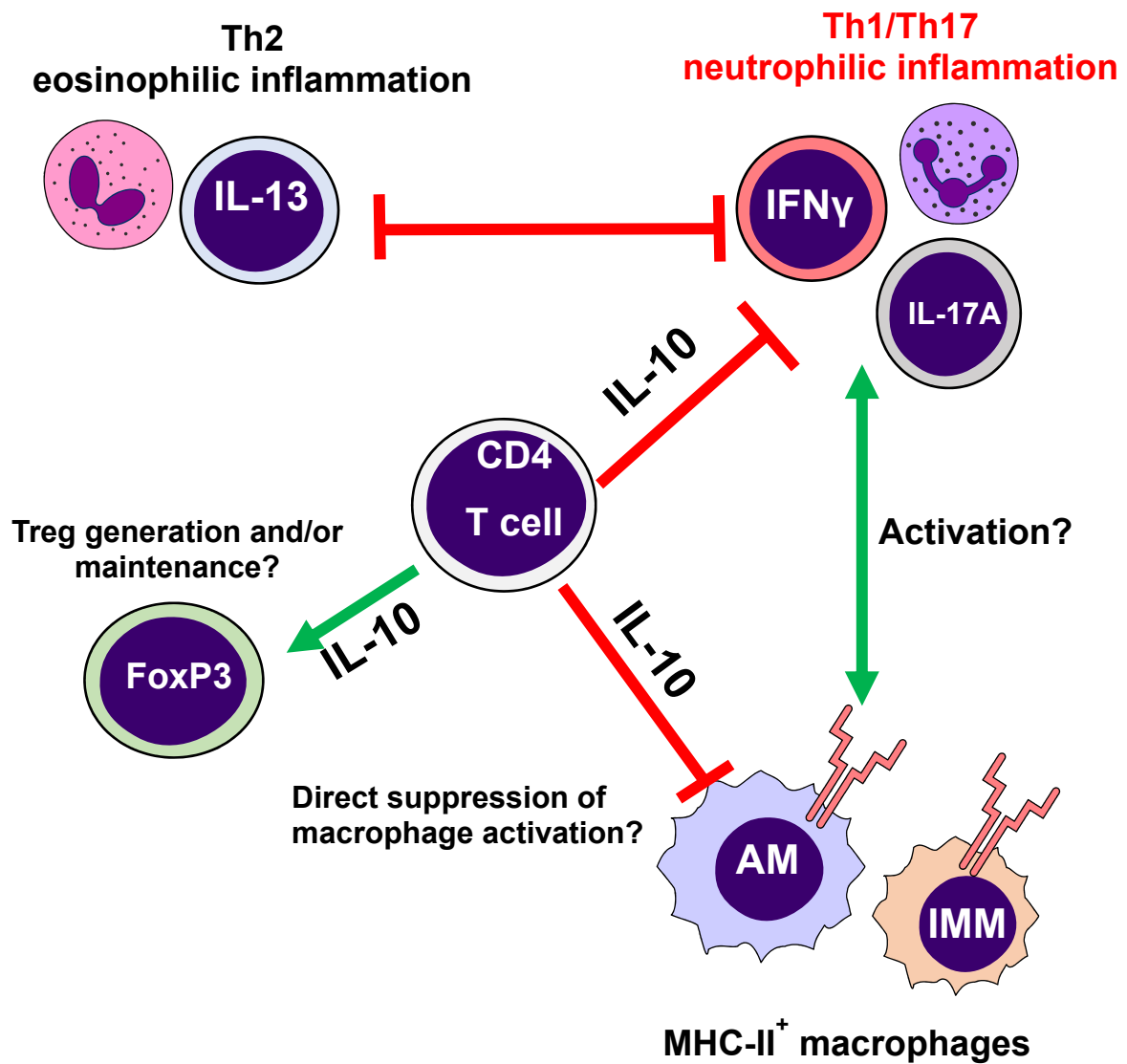


Figure 6.24. Model for IL-10 function in regulation of HDM-driven allergic airway inflammation

Proposed functions of IL-10 in cellular interactions in the lung of HDM-treated mice as suggested by data in this chapter. IL-10, predominantly derived from CD4⁺ T cells, favours Th2 cells and eosinophils over a neutrophilic Th1/Th17 immune response in the lungs, most likely by limiting Th1 and Th17 responses. IFN γ from Th1 cells is likely to promote activation of lung macrophages (AM and IMM), marked by increased MHC-II expression. CD4⁺ T cell-derived IL-10 may also directly promote generation and/or maintenance of FoxP3⁺ Tregs in the lung and limit activation of AM and IMM. Green arrows and red lines indicate proposed positive and negative interactions, respectively.

6.12.2. TGF- β 1 derived from CD11c⁺ cells regulates monocyte recruitment to the lung and the magnitude of the type 2 immune response to inhaled house dust mite

Unlike IL-10, there is strong evidence that AM are major producers of TGF- β 1 in both human and murine lung^{272,273}. However, until recently, no specific functions have been assigned to this source of TGF- β 1. Work from Yu and colleagues, published during preparation of this thesis, described a key role for TGF- β signalling to AM for their perinatal generation and maintenance in adulthood²⁸². This paper also suggested, based on experiments using CD11c^{Tgfb1} mice, that AM-derived TGF- β 1 was the dominant source of TGF- β required for this process²⁸². In contrast, despite using the same strains of CD11c-Cre and *Tgfb1*^{fl/fl} mice to generate conditional AM knockouts^{284,288} as the published work²⁸², no defect in AM numbers was observed in our CD11c^{Tgfb1} mice, even when aged for up to 7 months at steady state, or exposed to HDM for 3 weeks. There are a number of potential explanations for the discrepancies between data in this chapter and the work of Yu et al²⁸². Firstly, it is notable that the AM numbers reported in CD11c^{Tgfb1} mice were not as severely reduced as those in mice with CD11c-Cre-driven *Tgfb2* knockout, in which AM fail to receive TGF- β signals from all cellular sources²⁸². This suggests that compensatory sources of TGF- β are present in the airways of CD11c^{Tgfb1} mice, levels of which could conceivably vary depending on differences in microbiota and genetic drift within mouse colonies. It is therefore possible that these compensatory sources produce higher levels of TGF- β in our mice compared with those of Yu et al²⁸². Consistent with compensatory sources of TGF- β remaining in the CD11c^{Tgfb1} mice used in the present work, no decreases were observed in levels of TGF- β bioactivity in the BAL at steady state or during HDM-driven AAD, although this method does not necessarily reflect the local concentrations of TGF- β in the immediate vicinity of AM.

Another potential explanation for differences in the observed baseline phenotypes is that Cre-mediated *Tgfb1* deletion was less efficient in AM from our CD11c^{Tgfb1} mice than those of Yu et al²⁸². 80 % of AM showed evidence of CD11c-Cre-mediated recombination of the *R26R^{eYFP}* locus in the present studies, which was sufficient for a > 60 % reduction in *Tgfb1* mRNA levels in bulk AM preparations, although residual *Tgfb1* expression was still detectable. Yu et al did not report efficiency of CD11c-Cre or the extent of *Tgfb1* deletion in AM from their CD11c^{Tgfb1} mice²⁸², however it is possible that more efficient deletion of *Tgfb1* in the published work could explain the difference in baseline effects observed. If intrinsic TGF- β 1 indeed confers a developmental or survival benefit to AM, then cells escaping deletion early in life might have a selective advantage, resulting in a situation where AM consist of a mix of TGF- β 1-deficient and –proficient cells that together are sufficient to populate the airway niche. Such a mechanism was suggested by Yu et al based on their observations from deletion of *Tgfb2* in AM using LysM-Cre, in which hemizygous Cre expression was insufficient to cause a phenotype and likely led to population of the airways with AM that had escaped deletion. In contrast, homozygous expression of LysM-Cre in *Tgfb2*^{fl/fl} mice recapitulated the phenotype observed in mice expressing a single allele of the more efficient CD11c-Cre²⁸². To determine whether such a Cre dosage effect is observed in our Cre-

hemizygous *CD11c^{Tgfb1}* mice, AM numbers will be compared in *CD11c*-Cre-negative, -hemizygous and -homozygous *Tgfb1^{fl/fl}* mice. AM numbers will be analysed throughout neonatal development to specifically assess whether there are early defects in the AM compartment that are compensated for by cells escaping Cre-mediated deletion by the time mice reach adulthood. At this stage however, data with *CD11c^{Tgfb1}* mice must be interpreted with caution as they may reflect a situation in which a subset of TGF- β 1-proficient AM are present in the airways.

Regardless of the overall efficiency of deletion, *CD11c^{Tgfb1}* mice showed substantially and significantly reduced *Tgfb1* gene expression in AM and an enhanced type 2 immune response to repeated intranasal HDM exposure, accompanied by increased amounts of CCL2 and monocyte-derived cells in the lungs. This phenotype appeared to result in more severe disease, since airway resistance in response to inhaled methacholine was modestly but significantly elevated in HDM-treated *CD11c^{Tgfb1}* mice compared to controls. This contrasts with the effects of conditional deletion of TGF- β 1 in bronchiolar epithelial club cells, in which type 2 immunity was dampened due to impaired recruitment and activation of ILC2s²⁷³, illustrating the location-specific functions of this cytokine. Unlike conditional epithelial club cell knockout of TGF- β 1, which reduced the amount of bioactive TGF- β detected in BAL after 3 weeks of HDM exposures²⁷³, airway TGF- β bioactivity was unaffected by deletion in *CD11c⁺* cells. These opposing findings suggest that the bronchiolar epithelium is the major source of bioactive TGF- β 1 released into the airway lumen upon allergen challenge, while TGF- β 1 derived from AM acts more locally and therefore does not contribute substantially to the bioactive TGF- β detected in BAL fluid.

In support of a local function of AM-derived TGF- β 1, AM isolated from naïve *CD11c^{Tgfb1}* mice displayed enhanced spontaneous and LPS-induced production of *Ccl2* and *Il27* mRNA, suggesting that TGF- β 1 produced by AM acts in an autocrine manner to limit pro-inflammatory chemokine production. Given the proximity of AM to airway epithelial cells *in vivo*, which express integrins capable of activation of latent TGF- β 1^{240,244,475}, it can be speculated that AM-derived TGF- β 1 is rapidly activated by epithelial integrins or local proteases and acts in an autocrine manner to maintain the tolerogenic phenotype of AM by limiting pro-inflammatory cytokine production (**Fig. 6.25**).

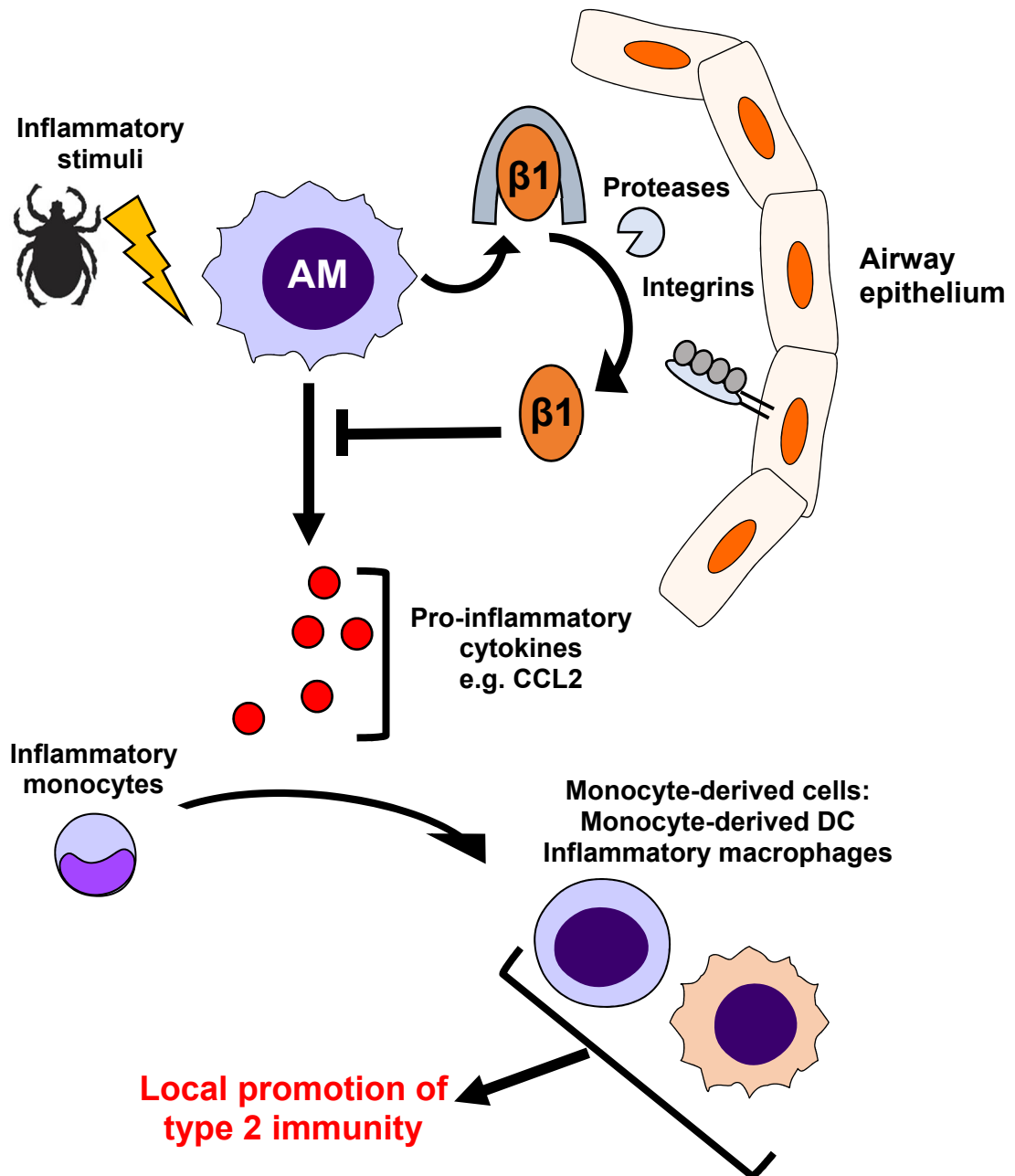


Fig. 6.25. Model for AM-derived TGF-β1 function in regulating allergic airway disease

Proposed autocrine role of AM-derived TGF-β in limiting allergic airway inflammation, as suggested by data in this chapter. TGF-β1 is produced constitutively by AM and is activated at homeostasis and during inflammation by proteases and epithelial integrins in the airway microenvironment. TGF-β1 signals in an autocrine manner to limit pro-inflammatory cytokine and chemokine production by AM in response to inhaled stimuli such as HDM. In the absence of AM-derived TGF-β1, increased chemokine production from AM, and potentially other cellular sources, enhances recruitment of monocytes to the lung, which differentiate into cells capable of locally promoting the type 2 immune response to allergen.

The increased CCL2 production observed in TGF- β 1-deficient AM offers a potential causal link between the CD11c^{Tgfb1} genotype and the increased pulmonary CCL2, IMM and moDC levels after HDM treatment. However, analysis of CCL2 expression was limited to naïve and *ex vivo* stimulated AM in this work, necessitating verification of these results in AM obtained directly from mice treated with HDM *in vivo*. AM are also not major producers of CCL2 in the lung compared with airway epithelial cells^{36,476}, suggesting that other cell types and/or monocyte chemokines may be involved in the link between TGF- β 1 knockout in AM and increased numbers of pulmonary monocyte-derived cells in HDM-treated CD11c^{Tgfb1} mice. In future work, production of CCL2 and other monocyte chemokines, such as CCL7 and CCL8⁵⁸, will be examined in lung tissue and sorted AM during HDM-driven AAD, to determine whether overproduction of chemokines by AM is relevant to the observed phenotype *in vivo*. Both CCL7 and CCL8 were determined to be substantially upregulated by AM from HDM-treated mice in the transcriptomic analysis described in Chapter 5 and are therefore promising candidate mediators for the proposed mechanism.

The increase in monocyte-derived cells in lungs of HDM-treated CD11c^{Tgfb1} mice may mechanistically explain the observed augmentation of the type 2 immune response in this model. CD64⁺ CD11b⁺ moDC are the major DC-like population in the HDM-allergic lung and play a role in driving the type 2 immune response to HDM⁴⁰. Although not essential for initial sensitisation, moDC are required for maximal magnitude of AAD, strongly suggestive of a role in local activation of T cells in lung tissue⁴⁰. Such a local effect fits with the observed phenotype of CD11c^{Tgfb1} mice following HDM treatment, in which type 2 immunity was augmented in lungs but not in the mLN. As well as moDC, other monocyte-derived cells are likely to promote type 2 immunity in the allergic lung, since ablation of all monocyte-derived cell recruitment to lungs during HDM-driven AAD, using intravenous clodronate liposomes dramatically reduced levels of type 2 cytokines and eosinophilia in the airways⁶³. Thus, increased monocyte recruitment and pulmonary IMM and moDC numbers due to dysregulated chemokine production from TGF- β 1-deficient AM may enhance local T cell activation and type 2 immunity in response to inhaled HDM (**Fig. 6.25**).

A caveat of the CD11c^{Tgfb1} mouse studies is the incomplete specificity of Cre expression to AM. In particular, Cre expression by DC, which are decisive in allergic sensitisation and the immune response to HDM challenge⁴⁰, has potential to confound results. However, several lines of evidence suggest that the observed phenotypes are not due to Cre-mediated TGF- β 1 knockout in DC. Reporting of CD11c-Cre efficiency using the *R26R^{eYFP}* mouse strain suggested that recombination was relatively poor in lung DC compared with AM (30-60 % compared with 80 %). Moreover, recombination was lowest in moDC among DC subsets and was largely absent (< 10 %) in IMM, only a proportion of which express CD11c, suggesting that increased numbers of moDC and IMM in lungs of HDM-treated CD11c^{Tgfb1} mice was unlikely to result from intrinsic loss of TGF- β 1 expression by these cells. Dysregulation of cDC populations in the absence of DC-derived TGF- β 1 might be expected to increase numbers of these cells or T cell activation in mLN; however neither of these features were observed in HDM-treated CD11c^{Tgfb1} mice. The

augmented type 2 immune response was instead restricted to lung tissue, suggestive of a dependency on lung-resident cells. In future experiments, both AM and DC from HDM-treated CD11c^{Tgfb1} mice will be sorted and levels of *Tgfb1* and pro-inflammatory genes assessed. Adoptive transfer of TGF-β1-replete AM to CD11c^{Tgfb1} recipient mice will also be used to confirm the role of AM in the enhanced type 2 immune response observed following HDM treatment.

Despite consistent effects on numbers of monocyte-derived cells and the type 2 immune response being observed in CD11c^{Tgfb1} mice across 2 independent experiments, the observed phenotype was rather subtle, with up to around 2-fold increases in the relevant cell types observed in lungs. Given the apparent skewing towards type 2 immunity in the HDM-treated CD11c^{Tgfb1} mice, it may be the case that the HDM exposure model used for these studies, which causes a mixed response with features of type 1, 2 and 3 immune responses, was not optimal for observing the effects of AM-derived TGF-β1 in this model. Experiments are therefore ongoing to determine the effects of repeated exposure to the type 2-dominant allergen *Alternaria alternata* in CD11c^{Tgfb1} mice, to more cleanly examine the effects on type 2 immunity. ILC2s will also be quantified in these studies, to determine whether enhanced type 2 immune response in CD11c^{Tgfb1} mice is restricted to T cells.

Additionally, given the proposed dependence of immune dysregulation in HDM-treated CD11c^{Tgfb1} mice on augmented monocyte recruitment to the lungs, it is possible that such a mechanism is also true in the case of non-allergic pulmonary inflammation and may be more apparent in the context of more severe monocyte recruitment. In future experiments, a model of RSV infection will be employed, in which AM are a major early chemokine source¹⁵⁴ and drive recruitment of antiviral monocytes⁶⁰, to more directly examine the effects of AM-derived TGF-β1 on inflammatory responses in the lung.

6.13. Conclusions

The data described here illustrate the concept that the cytokines IL-10 and TGF-β1 operate in cell type-specific niches to regulate immunity in the allergic lung. Pulmonary IL-10 function in HDM-driven AAD shows parallels to the intestine, with T cell-derived IL-10 being required for regulation of macrophage activation and AM-derived IL-10 being broadly redundant for immune regulation. In contrast, a fundamental role for AM-derived TGF-β1 is proposed for regulating monocyte recruitment and type 2 immunity in response to inhaled HDM, which will be investigated in greater detail in future work.

7. Determining the role of epithelial cell-derived TGF- β 1 in the immune response to respiratory infection

7.1. Introduction

In the previous chapter, a conditional knockout approach was used to outline a role for airway macrophage- (AM-) derived TGF- β 1 in regulating the immune response to inhaled house dust mite (HDM) allergen, possibly by autocrine regulation of inflammatory cytokine and chemokine production. However, AM are just one of several sources of TGF- β 1 in the lung and bronchiolar epithelial cells are also major producers at homeostasis and during inflammation^{272,273}. Non-ciliated, secretory club (formerly Clara¹⁵) cells are the major epithelial cell population in murine conducting airways⁴⁷⁷ and an abundant component of human bronchiolar epithelium⁴⁷⁸ and have been shown to release TGF- β 1 into the airway lumen following inhalation of allergen or the cytokine IL-33²⁷³. In previous work from our laboratory, club cell-derived TGF- β 1 was shown to be critical for enhancing IL-33-dependent recruitment and activation of type 2 innate lymphoid cells (ILC2s) in murine models of allergic airway disease (AAD), in a manner apparently independent of effects on AM or other myeloid cells²⁷³. However, the potential for club cell-derived TGF- β 1 production to regulate AM phenotype and recruitment and activation of inflammatory leukocytes in non-allergic inflammation has not previously been explored.

Research in recent years has altered our view of epithelial cells, showing them to be far more than a passive barrier in pulmonary immunity¹¹. Bone marrow chimera experiments have demonstrated a clear role for epithelial-intrinsic pattern recognition responses in initiation of immune responses to both inhaled allergen³⁶ and respiratory pathogens¹¹³. The bronchiolar epithelium is of particular importance during influenza infection, since these epithelial cells are the initial site of viral entry and replication¹²⁰ and produce a number of pro-inflammatory cytokines and chemokines upon infection¹²⁴. Bronchiolar epithelial cells also rapidly induce expression of type I interferons (IFNs), IFN α and IFN β and type III IFN, IFN λ ^{125,126}, which act on the IFN $\alpha\beta$ receptor (IFNAR) and the IL-28/IL-10RB complex, respectively, to induce antiviral interferon stimulated genes (ISGs) that restrict viral replication^{479,480}. As well as inducing an antiviral state, type I IFNs are known to promote inflammatory processes, such as monocyte recruitment, following respiratory infection^{60,481-483}. IFN λ is not thought to possess such pro-inflammatory functions⁴⁸¹, presumably owing to expression of the IFN λ receptor complex being largely restricted to epithelial cells⁴⁸⁴. As key sites of viral replication, IFN production and pro-inflammatory cytokine responses, bronchiolar epithelial cells are of substantial importance in understanding airway inflammation during influenza infection.

Influenza is also an interesting model for the study of TGF- β in regulating pulmonary immunity, since TGF- β signalling appears to be critical to the outcomes of infection. During the adaptive immune response to influenza, systemic pan-TGF- β blockade severely enhances disease severity²⁷⁵ and inducible deletion of TGF- β R2 ablates the protection against influenza offered by prior induction of AAD⁴⁸⁵, suggesting that TGF- β regulates the pulmonary T cell response to infection with this virus. Several strains of influenza have been shown to rapidly activate TGF- β from its latent extracellular form through enzymatic activity of viral neuraminidase, locally increasing TGF- β bioactivity at the site of infection^{275,486}, which is suggested to potentially favour

viral replication by dampening of host immunity⁴⁸⁶. Additionally, a recent study showed expression of $\alpha_v\beta_6$ integrin on airway epithelial cells to be required for activation of latent TGF- β upon influenza infection²⁴⁵. In contrast to global TGF- β blockade, knockout of this integrin protected mice from influenza-induced weight loss and death, in a manner proposed to depend upon reduced TGF- β activation in the airways²⁴⁵. Of particular importance to this thesis, protection of integrin β_6 knockout mice from influenza infection was accompanied by increased activation of AM, which showed evidence of enhanced type I IFN signalling that was proposed to restrict viral spread in the lungs²⁴⁵. Thus, TGF- β potentially mediates a regulatory axis of epithelial-AM communication that restricts early antiviral immunity and benefits influenza replication.

Respiratory syncytial virus (RSV) is another major epithelial-tropic respiratory virus, although in contrast to influenza, RSV principally infects alveolar, rather than conducting airway epithelium¹²¹. Based on *in vitro* studies, TGF- β has been suggested to limit early life adaptive immune responses to RSV infection⁴⁸⁷ and to enhance RSV replication in human epithelial cells by promoting cell cycle arrest^{488,489}. However, although RSV drives TGF- β production by human airway epithelial cells *in vitro*⁴⁸⁹, infection has not been reported to induce rapid TGF- β expression or activation *in vivo*, as described for influenza^{275,486}. TGF- β is therefore likely to differentially influence viral replication and host immune responses during RSV and influenza infections.

Multiple lines of evidence in published literature support potential for interactions between airway epithelial cells, AM and inflammatory leukocytes via TGF- β . However, the functions of TGF- β 1 derived from bronchiolar epithelial club cells during respiratory viral infection have not previously been described. In this chapter, an inducible, conditional knockout mouse system was employed to specifically determine the role of club cell-derived TGF- β 1 in regulating the immune response to influenza infection.

7.2. Hypothesis and aims

Hypothesis

TGF- β 1 is produced by conducting airway epithelial club cells during influenza A virus infection and regulates the immune response to infection.

Aims

1. Assess TGF- β 1 production by club cells during influenza infection using conditional knockout mice
2. Determine the effects of club cell-specific TGF- β 1 knockout on AM activation and the outcomes of influenza infection
3. Compare these effects to infection with respiratory syncytial virus, which is not thought to induce TGF- β activation *in vivo*

7.3. Influenza infection drives rapid release and *de novo* transcription of TGF- β 1 from bronchiolar epithelial club cells

Several strains of influenza A virus increase local TGF- β bioavailability, at least in part through direct cleavage of the latent TGF- β complex by the viral neuraminidase^{275,486}. However, it is unclear if bronchiolar epithelial club cells are a source of this rapidly activated TGF- β , as has been shown in response to airway challenge with HDM or recombinant IL-33²⁷³. To assess the contribution of club cells to influenza-induced TGF- β 1 release, an inducible, club cell-conditional TGF- β 1 knockout mouse line was employed, as described previously²⁷³. Briefly, these mice contain two LoxP-flanked *Tgfb1* alleles²⁸⁸, along with a CCSP (*Scgb1a1*) Tet-on system²⁸⁹, comprising a *Scgb1a1*-driven reverse tetracycline transactivator (*Ccsp*-rtTA) and a tetracycline response element-driven Cre recombinase (TRE-Cre; **Fig. 7.1a**). Mice with a complete Tet-on/*Tgfb1*^{fl/fl} genotype (hereafter: epTGF β 1KO) and littermate controls lacking either component of the Tet-on system (hereafter: control) were given a single injection of doxycycline, a protocol shown previously to induce Cre expression in CCSP⁺ club cells of epTGF β 1KO mice while leaving controls unaffected²⁷³ (**Fig. 7.1a**). Mice were then rested for 1 week without changing cages or bedding, to minimise disruption of the commensal microbiota following antibiotic treatment, before infection with 1.2×10^4 TCID₅₀ of mouse adapted H3N2 influenza X31 (**Fig. 7.1a**), a dose previously titrated to cause moderate disease in mice on this genetic background (Lloyd laboratory, unpublished observations).

Around 50 pg/ml of bioactive TGF- β was detected in bronchoalveolar lavage (BAL) fluid of both control and epTGF β 1KO mice prior to influenza infection, consistent with a homeostatic level of TGF- β production from non-club cell sources. Alternatively, baseline TGF- β bioactivity in epTGF β 1KO mice could potentially have been due to TGF- β 2 or TGF- β 3 isoforms, which are not deleted in epTGF β 1KO mice²⁷³ and are detected by the Smad reporter assay of TGF- β bioactivity used for this work⁴⁹⁰. As expected, influenza infection resulted in an increase in airway TGF- β bioactivity of around 1.8-fold (**Fig. 7.1b**). However, this increase was ablated in epTGF β 1KO mice, indicating that CCSP⁺ club cells are the major source of TGF- β 1 released into the airway lumen upon influenza infection (**Fig. 7.1b**). TGF- β bioactivity had not increased further in BAL of control mice at 3 days post-infection, but the concentration remained significantly lower in epTGF β 1KO mice at this time-point (**Fig. 7.1b**). Thus, epTGF β 1KO mice displayed a deficiency in early release of TGF- β 1 into the airway lumen following influenza infection.

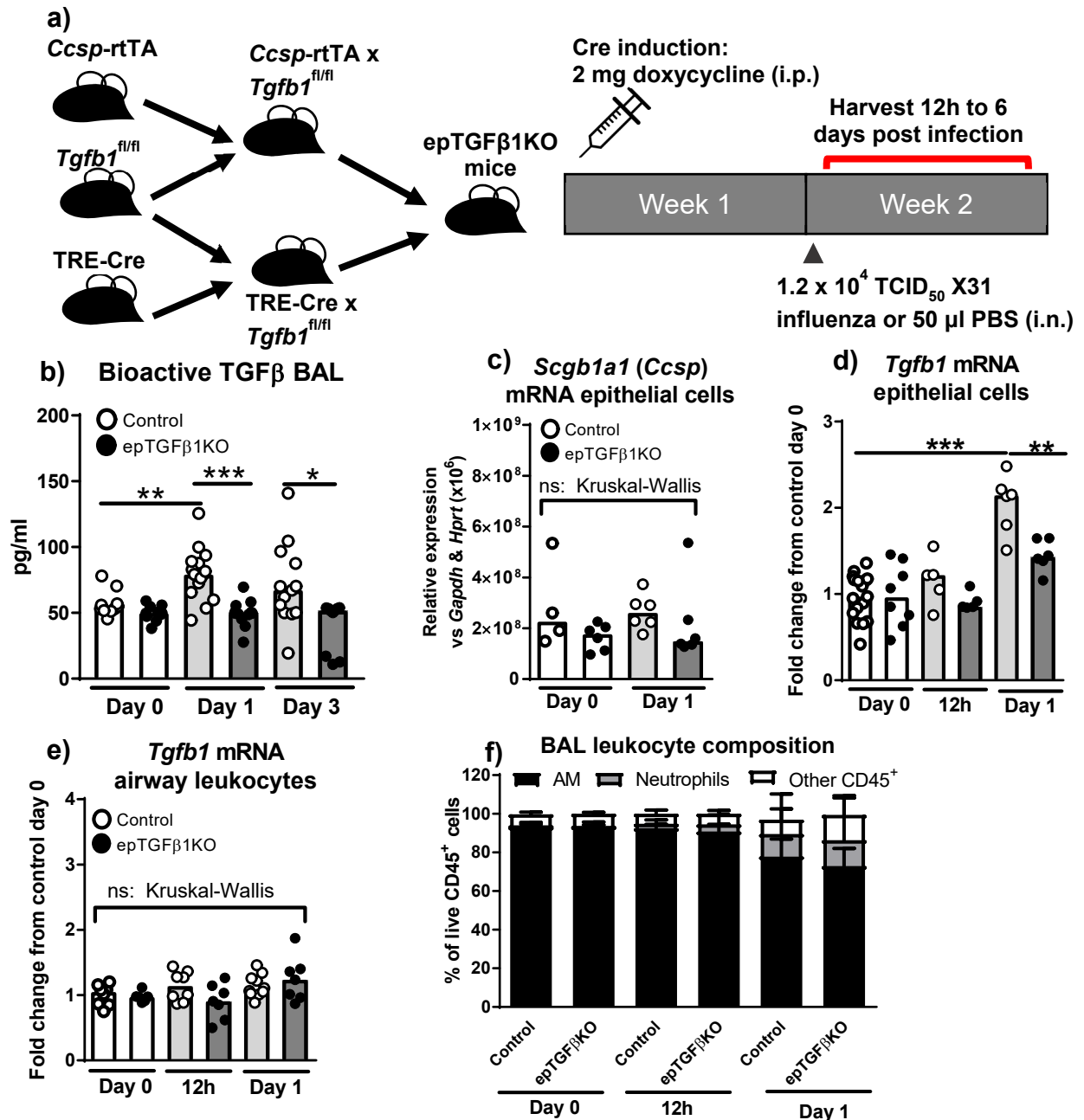


Fig. 7.1. Influenza infection drives rapid generation of bioactive TGF-β1 from club cells

a) Conditional deletion of *Tgfb1* in CCSP⁺ club cells. *Ccsp* reverse tetracycline transactivator (*Ccsp*-rtTA) and tetracycline response element Cre (TRE-Cre) mice were crossed to *Tgfb1*^{fl/fl} mice and intercrossed to generate triple transgenic mice (epTGFβ1KO). Littermate mice lacking either *Ccsp*-rtTA or TRE-Cre elements were used as controls. All mice received 2 mg intraperitoneal injection of doxycycline to induce Cre expression 1 week prior to intranasal influenza infection. Day 0 control mice received intranasal PBS. **b)** TGF-β bioactivity in BAL supernatant as detected by Smad reporter assay. **c)** *Scgb1a1* (*Ccsp*) mRNA expression in tracheal epithelial cells relative to housekeeping genes. **d-e)** Fold changes in *Tgfb1* mRNA expression relative to *Gapdh* and *Hprt* housekeeping genes in tracheal epithelial cells (**d**) and total BAL cells (**e**), normalised to control day 0 mice. **f)** BAL leukocyte populations as percentages of total CD45⁺ cells, as determined by flow cytometry. Stacked bar graphs show medians ± IQR of 7-16 mice per group pooled from two independent experiments. Scatter/bar graphs show medians of 5-10 mice per group pooled from two independent experiments. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant.

Previous reports of bioactive TGF- β generation in response to influenza infection have focussed on activation of preformed latent TGF- β by viral neuraminidase^{275,486}. To determine whether any of the club cell-derived TGF- β 1 release could be attributed to *de novo* production by club cells, primary epithelial cells were obtained by brushing tracheas of control and epTGF β 1KO mice. Tracheal cells were used due to the difficulties associated with obtaining primary bronchiolar epithelial cells from mice and the high degree of similarity in epithelium from the trachea and conducting airways in mice, both of which predominantly consist of club cells⁴⁹¹. Tracheal brushings expressed high levels of *Scgb1a1*/CCSP mRNA, which, importantly, did not change up to 24h after influenza infection (**Fig. 7.1c**), consistent with enrichment for club cells and a lack of contamination with inflammatory leukocytes upon infection. *Tgfb1* mRNA was significantly increased in epithelial cells at 1 day post-infection (dpi; **Fig. 7.1d**), coincident with the observed spike in TGF- β bioactivity in BAL (**Fig. 7.1b**). This increase did not occur in epTGF β 1KO mice (**Fig. 7.1d**), consistent with increased *Tgfb1* gene expression in response to influenza infection being attributable to CCSP⁺ club cells. In support of restriction of *de novo* TGF- β 1 production to epithelial cells, no change in *Tgfb1* gene expression was observed in airway leukocytes (**Fig. 7.1e**), which comprised ≥ 90 and ≥ 75 % airway macrophages at 12 and 24h post-infection, respectively (**Fig. 7.1f**).

These data show for the first time that club cells are the major source of TGF- β 1 released into the airway lumen following influenza A virus infection and that TGF- β 1 production occurs in part through *de novo* gene expression.

7.4. Lack of early epithelial TGF- β 1 release protects against influenza-induced weight loss and results in dampened innate and adaptive immune responses

Having shown that club cells release TGF- β 1 rapidly upon influenza infection, the effects of ablation of this specific pool of TGF- β 1 on the outcomes of infection were determined. Monitoring weight loss as a measure of disease severity up to 6 dpi showed control mice to lose around 15 % of their initial body weight, as expected from this moderate severity infection (**Fig. 7.2a**). In contrast, epTGF β 1KO mice were almost completely protected from influenza-induced weight loss (**Fig. 7.2a**), indicating that club cell-derived TGF- β 1 is required for maximal disease severity in this model. Protection in epTGF β 1KO mice was accompanied by a significant decrease in airway inflammation, as determined by total BAL leukocyte counts, at 3 and 6 dpi (**Fig. 7.2b**), suggesting that both the innate and adaptive immune responses to infection were dampened in the absence of club cell-derived TGF- β 1. In contrast to published work, in which epTGF β 1KO mice failed to develop AAD due to impaired recruitment and activation of ILC2s, the observed protection from influenza-induced airway inflammation was not due to differential ILC2 recruitment, since these cells were essentially undetectable in BAL of control and epTGF β 1KO mice before and after influenza infection (insufficient positive events for flow cytometry analysis, not shown).

Recruitment of virus-specific CD4⁺ and CD8⁺ T cells to the lung is crucial for clearance of influenza infection, but these cells also contribute to immunopathology⁷³, meaning that regulation of cell-mediated immunity is key to disease outcome. TGF- β 1 is important for regulation of T cells, limiting the generation of CD8⁺ effector cells and the Th1 and Th2 CD4⁺ T cell subsets^{492,493}, but promoting FoxP3 expression in Tregs⁹⁹. Moreover, increased morbidity and mortality of influenza infection after systemic pan-TGF- β blockade occurred from 6 dpi²⁷⁵, strongly suggestive of a role for TGF- β in regulating the T cell-mediated immune response to influenza. To determine whether protection of epTGF β 1KO mice from influenza could be attributed to an imbalance in airway T cell populations in the absence of club cell-derived TGF- β 1, T cells were quantified in BAL by flow cytometry. Although numbers of FoxP3⁺ Tregs in BAL were significantly reduced in epTGF β 1KO mice at 6 dpi, this was accompanied by decreases in effector IFN γ ⁺ CD8⁺ and CD4⁺ T cells (**Fig. 7.2c**), consistent with an overall dampening of the cell-mediated adaptive immune response to influenza, rather than an imbalance in the Treg to T effector ratio.

In accordance with a muted immune response to influenza infection, peak numbers of recruited innate myeloid cells in the airway lumen were significantly decreased in epTGF β 1KO mice. Neutrophil numbers in BAL peaked at 3 dpi and were more than 2-fold lower in epTGF β 1KO mice (**Fig. 7.2d**). As expected, CD64⁺ CD11b⁺ Siglec F⁻ non-DC cells, a mixed population of interstitial/inflammatory monocytes and macrophages (IMM), were recruited to airways with slower kinetics than neutrophils. The highest IMM numbers in BAL were observed at 6 dpi and were significantly lower in epTGF β 1KO mice than controls at this time-point (**Fig. 7.2d**). IFN γ ⁺ natural killer (NK) cell numbers increased in BAL between 1 and 3 dpi. However no differences in NK cell numbers were apparent between epTGF β 1KO and control mice and numbers of these cells were relatively low at all time-points compared to neutrophils and IMM (**Fig. 7.2d**).

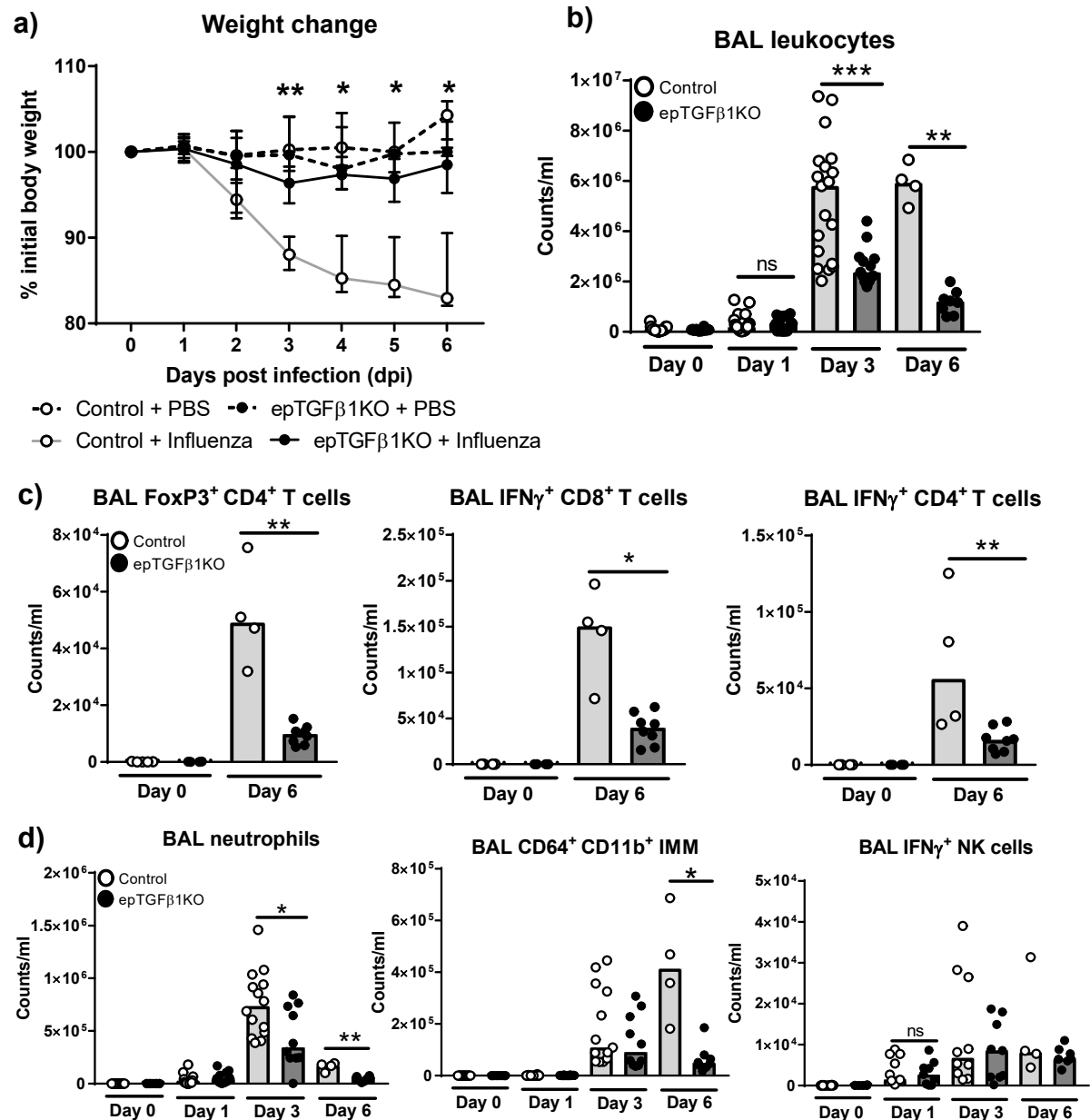


Fig. 7.2. EpTGFβ1KO mice are protected from weight loss and airway inflammation during influenza infection

a) Body weight changes over the course of influenza infection. Data shown are medians of 4-13 mice per group $\pm$ IQR; symbols indicate significant differences between control and epTGFβ1KO influenza groups. **b)** Total crystal violet cell counts in BAL. **c-d)** Flow cytometry data showing numbers of FoxP3⁺ CD4⁺ T cells and IFN γ ⁺ CD8⁺ and CD4⁺ T cells (**c**) and neutrophils, CD11b⁺ CD64⁺ Siglec F⁻ inflammatory monocytes and macrophages and IFN γ ⁺ natural killer cells (**d**) in BAL. Scatter/bar graphs show medians of 4-13 mice per group. Day 0-3 data are pooled from two to three independent experiments. Day 4-6 data are representative of two independent experiments that were not pooled due to use of different X31 batches. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ns, non-significant.

Given that the dampened cellular immune response to influenza in epTGF β 1KO mice was apparent from as early as 3 dpi (**Fig. 7.2**), potential chemokine determinants of innate cell recruitment to airways were examined in BAL to look for early differences linking club cell TGF- β 1 deficiency to reduced inflammation. Murine CXCL1 is a functional homologue of human CXCL1 and CXCL8 and can attract neutrophils by acting on the receptor CXCR2⁴⁹⁴. CCL2 is chemotactic for monocytes, among other cell types, and is required for recruitment of inflammatory monocytes to the airways during influenza infection⁵⁹. Both CXCL1 and CCL2 reached their maximum concentrations in BAL of control mice at 3 dpi and were significantly decreased in epTGF β 1KO mice (**Fig. 7.3**), suggesting a contribution of decreased chemokine levels to the reduced numbers of neutrophils and IMM observed in BAL of these mice. CXCL1, but not CCL2, levels were elevated above baseline at 1 dpi but were not decreased in epTGF β 1KO mice at this time-point (**Fig. 7.3**). These kinetics are consistent with the more rapid peak of neutrophils than IMM in this model and suggest that epTGF β 1KO mount a muted chemokine response to influenza infection, which is apparent at 1 dpi but less so by 3 dpi. Notably, the decreased levels of inflammatory mediators in BAL at 3 dpi was not restricted to chemokines, since levels of BAL IL-1 β , a robust feature of influenza infection¹⁵⁵, were also reduced to near-baseline levels at this time-point (**Fig. 7.3**). Thus, epTGF β 1KO mice show a generally dampened innate immune response to influenza infection, rather than specific impairment of chemokine production.

7.5. Dampened inflammation in influenza-infected epTGF β 1KO mice is not due to differential activation of airway macrophages

The general dampening of the immune response to influenza in the absence of club cell-derived TGF- β 1 was clearly apparent from 3 dpi, suggestive of altered behaviour of early responder cells. In the previous chapter, lack of intrinsic TGF- β 1 from AM was shown to enhance their chemokine production *in vitro*, raising the question of whether club cell-derived TGF- β 1 might regulate these cells during infection. TGF- β 1 activation by epithelial $\alpha_v\beta_6$ integrin has been shown to limit AM activation, as measured by CD11b expression, at homeostasis and after influenza infection, in support of such a mechanism²⁴⁵. Moreover, the reduced airway IL-1 β levels in epTGF β 1KO mice at 3 dpi (**Fig. 7.3**) were suggestive of altered AM activation in the absence of club cell-derived TGF- β 1, since AM are the dominant source of this cytokine in the early stages of X31 influenza infection¹⁵⁵. AM do not differ in baseline number or activation state in epTGF β 1KO mice (Laura Denney²⁷³ and unpublished observations). However, it remained possible that AM activation state was altered during influenza infection in the absence of club cell-derived TGF- β 1 and that this could contribute to the observed differences in the innate immune response in these mice.

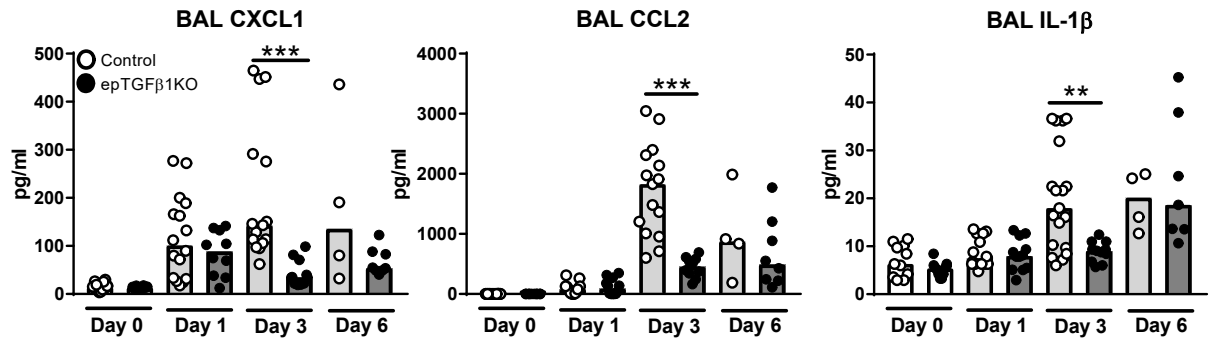


Fig. 7.3. Muted cytokine response in airways of influenza-infected epTGFβ1KO mice

Concentrations of CXCL1, CCL2 and IL-1β measured in BAL fluid by ELISA. Scatter/bar graphs show medians of 4-10 mice per group. Day 0-3 data are pooled from two to three independent experiments. Day 6 data are representative of two independent experiments that were not pooled due to use of different X31 batches. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; **, $p \leq 0.01$; ***, $p \leq 0.001$.

To assess the potential regulation of AM by club cell-derived TGF- β 1 during influenza infection, AM numbers and phenotype were determined by flow cytometry of BAL cells across the infection time-course. Total AM numbers in BAL were significantly elevated from 1 dpi (**Fig. 7.4a**), indicative of either proliferation of AM or, more likely, reduced adhesion of AM to airway epithelial cells during inflammation such that more cells were obtained by lavage. No significant differences in AM numbers were observed between epTGF β 1KO and control mice until 6 dpi, at which point significantly fewer AM were obtained in BAL of epTGF β 1KO (**Fig. 7.4a**), in line with the overall decrease in airway leukocyte counts at this time-point (**Fig. 7.2b**). Thus, protection of epTGF β 1KO mice from inflammation was not due to preferential expansion, death or reduced adhesion of AM in the early stages of influenza infection.

A relatively high proportion of AM from littermate control mice in this study expressed MHC-II in the absence of infection (median 35-40 %), while less than 10 % were CD11b⁺, as expected from naïve AM^{311,323} (**Fig. 7.4b,c**). These baseline proportions did not differ in epTGF β 1KO mice (**Fig. 7.4b,c**), suggesting that there is little to no regulation of AM phenotype by club cell-derived TGF- β 1 at homeostasis. Similarly, while proportions of CD11b⁺ AM significantly increased from 3 dpi and a trend towards increased MHC-II expression was apparent at 6 dpi, the percentage of AM expressing either marker did not differ between epTGF β 1KO and control mice (**Fig. 7.4b,c**). Thus, protection of epTGF β 1KO mice from infection was not accompanied by differential activation of AM. These data therefore contrast with those showing increased AM activation in integrin β_6 knockout mice²⁴⁵ and highlight differences between preventing integrin-mediated TGF- β activation throughout the airway lumen and specific ablation of the source of TGF- β 1 released rapidly upon infection.

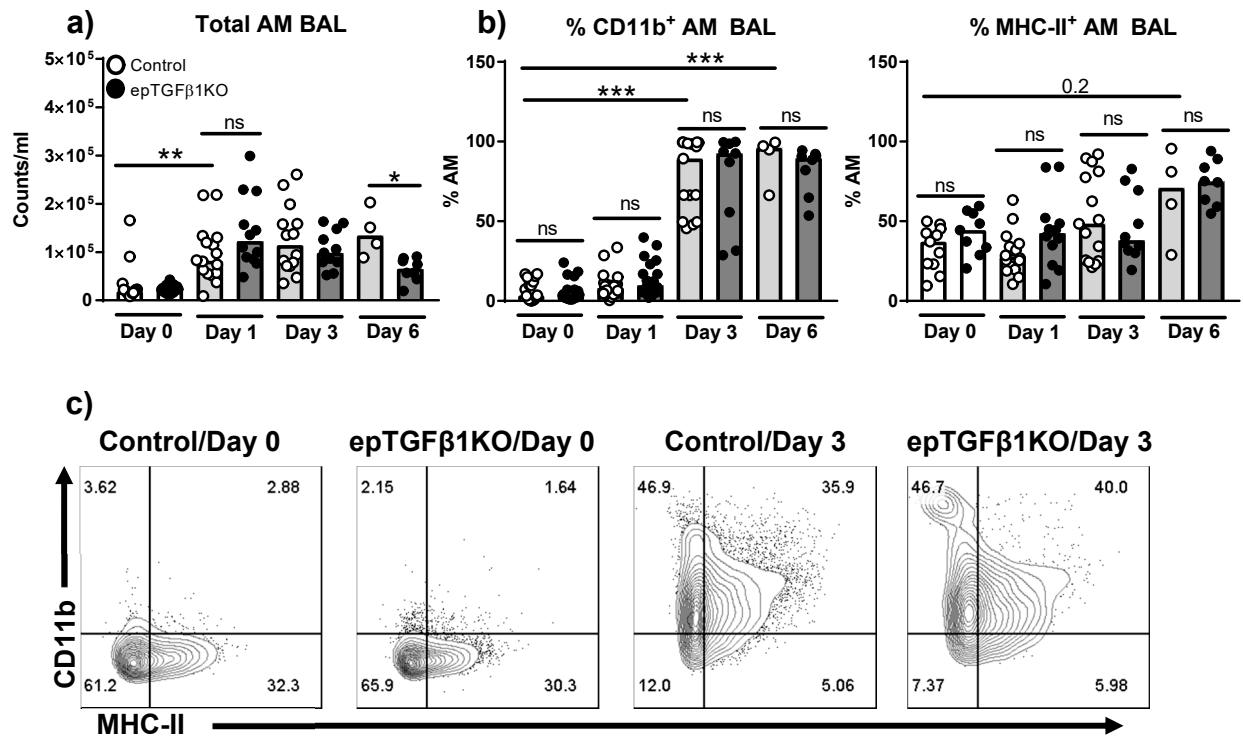


Fig. 7.4. No differential activation of airway macrophages in influenza-infected epTGFβ1KO mice

BAL cells were analysed by flow cytometry. **a)** Total CD11c⁺ CD64⁺ Siglec F⁺ AM in BAL. **b)** Percentages of AM with positive surface staining for CD11b and MHC-II in BAL. **c)** Representative flow cytometry plots of data shown in (b); numbers indicate percentages of events in gates. Scatter/bar graphs show medians of 4-10 mice per group. Day 0-3 data are pooled from two to three independent experiments. Day 6 data are representative of two independent experiments that were not pooled due to use of different X31 batches. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant. Where relevant, p values are shown between groups.

7.6. Respiratory syncytial virus does not drive TGF- β 1 release from club cells and causes equivalent disease in epTGF β 1KO mice and controls

Data to this point had suggested that the protection of epTGF β 1KO mice from influenza infection was an early event, arising due to changes occurring around the observed release of TGF- β 1 from club cells at 1 dpi. RSV infection drives many of the same airway inflammatory events as influenza, including rapid recruitment of neutrophils and inflammatory monocytes followed by virus-specific T cells^{60,352}. However, unlike influenza, RSV primarily infects alveolar, rather than bronchiolar, epithelial cells¹²¹ and has not been reported to rapidly activate latent TGF- β upon infection. It was therefore reasoned that if protection of epTGF β 1KO mice from influenza-induced inflammation was dependent on lack of early release of TGF- β 1 from club cells, then no protection would be observed from RSV-induced inflammation and weight loss.

To test this hypothesis, epTGF β 1KO and control mice were infected with the A2 strain of RSV and disease monitored up to 7 dpi (**Fig. 7.5a**), to allow analysis of both innate and adaptive immune responses to acute infection. In contrast to influenza, RSV infection did not increase TGF- β 1 bioactivity in BAL at 1 dpi (**Fig. 7.5b**). A trend towards increased TGF- β bioactivity was observed at 3 dpi, however this was not decreased in epTGF β 1KO mice (**Fig. 7.5b**), indicating that this TGF- β was not produced by club cells. RSV infection caused mild (~4 %) weight loss in control mice, apparent from 6 dpi, which occurred to a comparable extent in epTGF β 1KO mice (**Fig. 7.5c**). Consistent with comparable disease severity in mice of both genotypes, total cellular inflammation of the airways did not differ between epTGF β 1KO and control mice (**Fig. 7.5d**), with similar numbers of virus-specific CD8⁺ T cells detected in BAL of both groups at 7 dpi (**Fig. 7.5e**). Neutrophil numbers in BAL peaked at 1 dpi, while IMM numbers were increased from 3 dpi and highest at 7 dpi (**Fig. 7.5e**), consistent with sequential recruitment of these cells to the airways as infection progressed. In contrast to influenza infection, in which epTGF β 1KO mice showed decreased airway neutrophil and IMM counts (**Fig. 7.2d**), no differences were observed in numbers of these cells in BAL of RSV-infected epTGF β 1KO mice and controls (**Fig. 7.5e**).

Consistent with the comparable overall inflammation observed in RSV-infected epTGF β 1KO and control mice, no differences between genotypes was observed in total numbers of AM in BAL during infection (**Fig. 7.6a**), or their expression of activation markers CD11b and MHC-II, which was significantly increased from 3 and 7 dpi, respectively (**Fig. 7.6b**). Overall, these data suggest that conditional inducible knockout of club cell-derived TGF- β 1 does not protect from immunopathology of viral infections that do not drive TGF- β 1 release from club cells. This illustrates the importance of this specific source of TGF- β 1 for regulating the immune response to influenza.

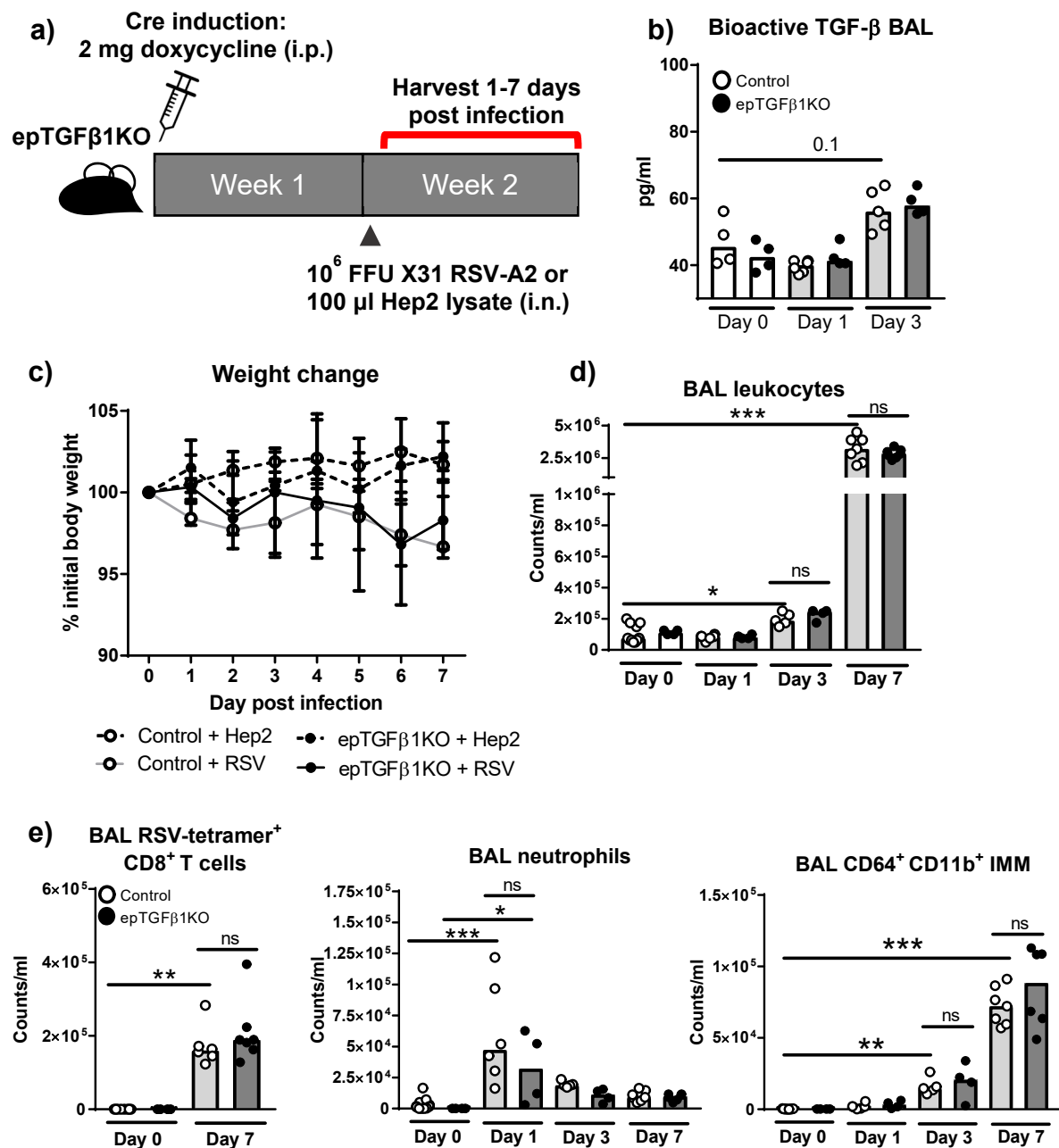


Fig. 7.5. EpTGFβ1KO mice are not protected from respiratory syncytial virus immunopathology

a) epTGFβ1KO mice and littermate controls were administered 10⁶ FFU RSV-A2 i.n. in the form of Hep2 cell lysate 1 week after induction of Cre expression. Day 0 controls received empty Hep2 lysate. b) Concentration of bioactive TGF-β in BAL fluid as determined by Smad reporter assay. c) Changes in bodyweight over the course of infection. Points show medians ± IQR of 4-7 mice per group d) Total crystal violet cell counts in BAL. e) Numbers of RSV tetramer (D^b RSV-M2₁₈₇₋₁₉₅)-positive CD8⁺ T cells, neutrophils and CD64⁺ CD11b⁺ Siglec F⁻ IMM in BAL as determined by flow cytometry. Scatter/bar graphs show medians of 4-10 mice per group. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant. Where relevant, p values are shown between groups.

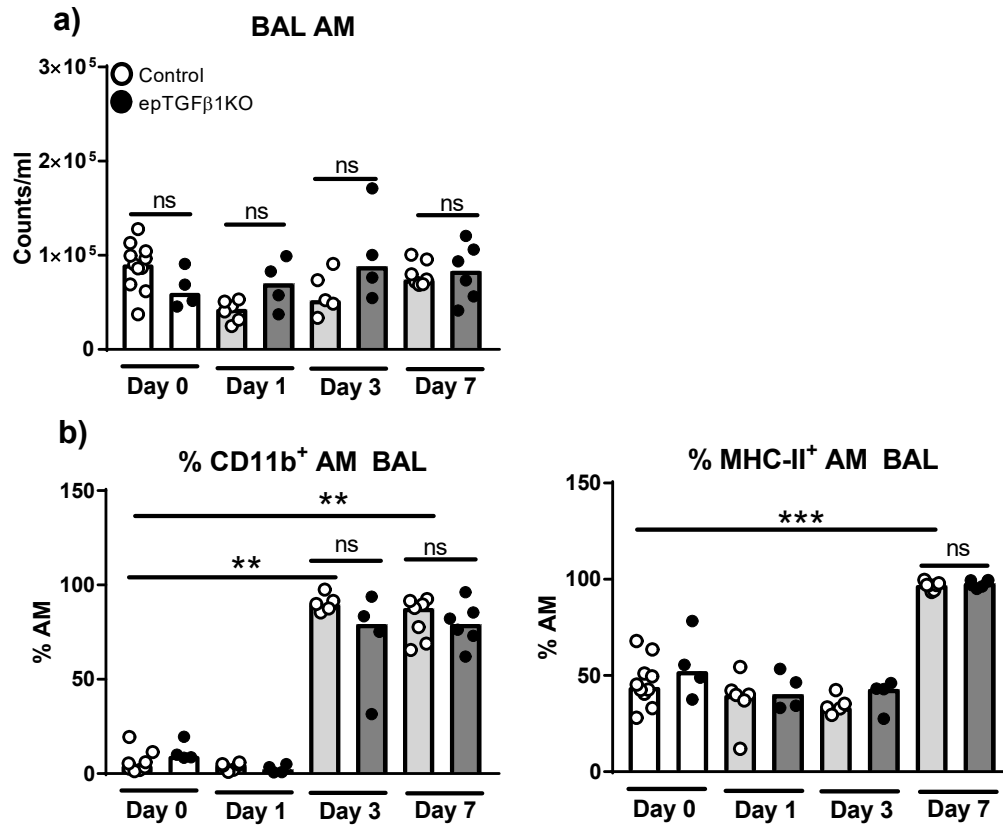


Fig. 7.6. No differential activation of AM during RSV infection in epTGFβ1KO mice

BAL cells were analysed by flow cytometry. **a)** Total numbers of CD64⁺ CD11c⁺ Siglec F⁺ AM in BAL. **b)** Percentages of AM with positive surface staining for CD11b and MHC-II. Data shown are medians of 4-10 mice per group. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant.

7.7. Influenza viral load is rapidly reduced in epTGFβ1KO mice

With kinetic data and comparisons to RSV suggesting that protection from influenza immunopathology in epTGFβ1KO mice began early in infection and was dependent on the absence of early club cell-derived TGF-β1 release, the mechanisms linking epithelial TGF-β1 deficiency and decreased inflammation remained to be determined. Since deletion of TGF-β1 from club cells could feasibly impact multiple aspects of the biology of the bronchiolar epithelium, it was reasoned that the permissiveness of epithelial cells to initial infection by influenza may be reduced in epTGFβ1KO mice. The ability of influenza to infect TGF-β1-deficient epithelium was therefore assessed by immunostaining of lung sections for influenza nucleoprotein (NP), a protein that acts at multiple stages of the influenza replication cycle⁴⁹⁵ and so marks both intact virions and replication intermediates. NP staining was predominantly detected in bronchiolar epithelial cells at all time points and its abundance and distribution were comparable at 1 dpi in epTGFβ1KO and control mice (**Fig. 7.7a,b**), indicating that influenza virus is able to infect TGF-β1-deficient epithelium upon initial inoculation. However, the degree of airway staining was significantly decreased by 3 dpi in epTGFβ1KO (**Fig. 7.7b**), suggesting that virus is cleared more rapidly in the absence of club cell-derived TGF-β1. This was confirmed by quantification of viral load in lung tissue by plaque forming assay. EpTGFβ1KO mice displayed significantly reduced pulmonary influenza titres than controls from 1 dpi (**Fig. 7.7c**). Moreover, plaque forming units were undetectable in 37.5 % (3 out of 8) of epTGFβ1KO mice, but none of the control mice, at 6 dpi (**Fig. 7.7c**). These data indicate that lack of club cell-derived TGF-β1 allowed more rapid clearance of influenza virus and strongly suggest that it was this early reduction in viral burden at 1 dpi that resulted in the decreased inflammation and immunopathology observed in epTGFβ1KO mice at later time-points.

7.8. EpTGFβ1KO mice display an enhanced early interferon β response to influenza infection

The rapidly reduced influenza viral load in epTGFβ1KO mice was suggestive of an enhanced early antiviral response in infected epithelial cells in the absence of club cell-derived TGF-β1. Type I IFNs, IFNα and IFNβ, are produced rapidly by both stromal and immune cells upon pathogen recognition and establish an antiviral state in host cells by inducing expression of ISGs⁴⁸⁰. Type III IFNs, the IFNλ isoforms, show comparable induction of the antiviral state to type I interferons, but are thought to act predominantly on epithelial cells⁴⁸¹. Since epithelial cells are a major source of type I and III IFNs during influenza infection¹²⁶, these mediators presented attractive candidate determinants of the rapid influenza clearance observed in epTGFβ1KO mice.

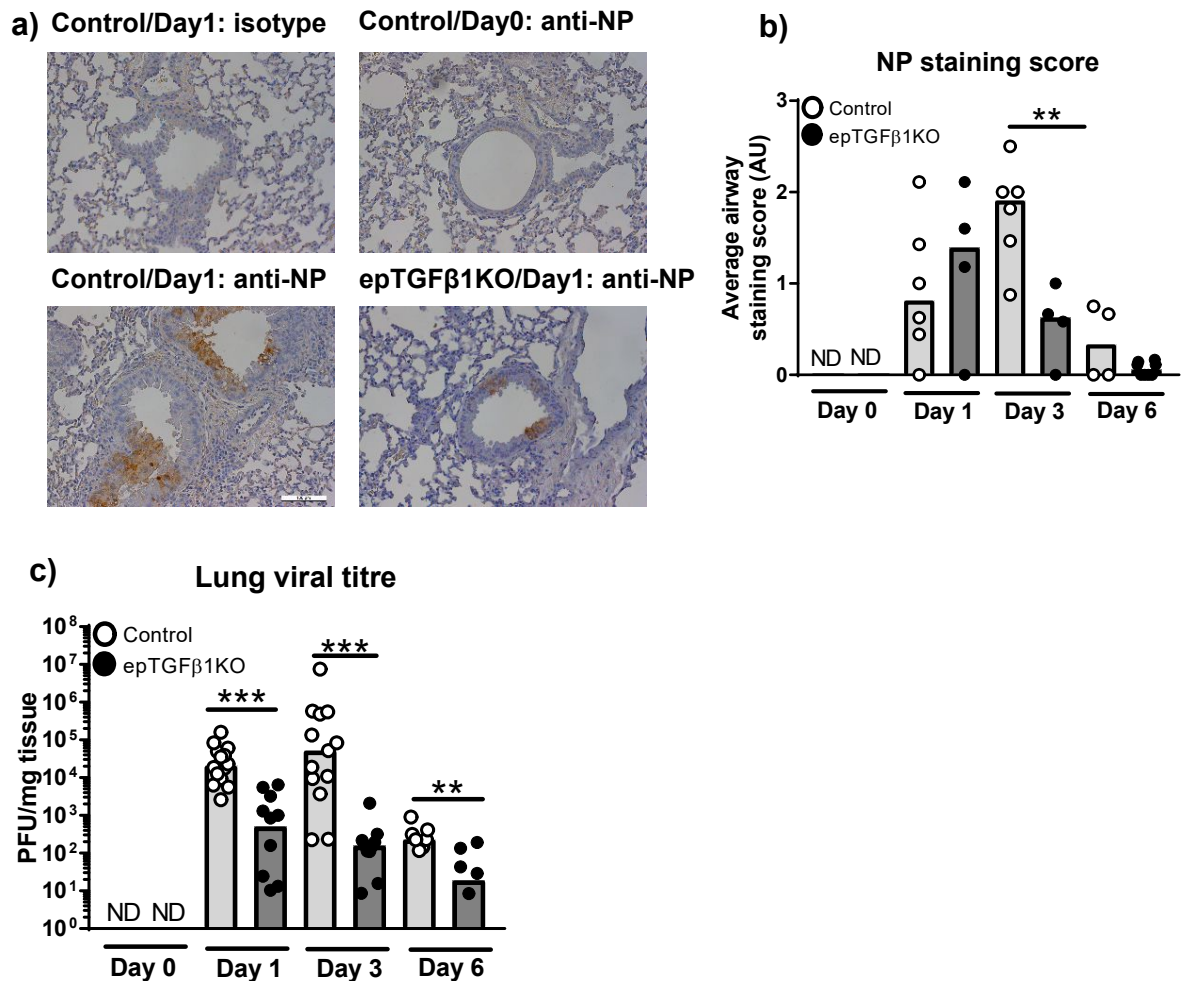


Fig. 7.7. Rapidly reduced pulmonary influenza viral load in epTGFβ1KO mice

a) Representative images at 200x magnification of airways stained for influenza NP. Isotype and uninfected controls are included to show specificity of staining. **b)** Semi-quantitative scoring of NP staining. Data shown are medians of the mean score of all airways in individual mice (4-6 per group). **c)** Numbers of plaque forming viral units (PFU) in lung tissue. Data shown are medians of 7-14 mice per group and are pooled from two (day 0-3) or one (day 6) independent experiment(s). Note that 3 points from the 8 day 6 epTGFβ1KO mice are not visible on the log₁₀ scale as no viral units were detected (i.e. virus had been cleared), but these points contribute to the displayed median value and statistical analysis. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; **, $p \leq 0.01$; ***, $p \leq 0.001$. ND, not detected.

ELISA analysis of BAL fluid showed little detection of IFN β above the assay detection limit in BAL fluid of control mice at 1 dpi, but was significantly increased in epTGF β 1KO mice at this time-point (**Fig. 7.8a**). IFN β concentrations in BAL were comparable between epTGF β 1KO mice and controls at 3 dpi and had decreased to below the detection limit in both groups at 6 dpi (**Fig. 7.8a**). Notably, the concentration of IFN β in BAL of epTGF β 1KO mice at 1 dpi was comparable to that observed at 3 dpi in control mice, suggesting that mice showed more rapid release of IFN β in the absence of club cell-derived TGF- β 1, rather than an increase in maximal production of this cytokine. Enhanced early IFN production appeared to be restricted to IFN β , since the concentration of IFN α in BAL fluid was not elevated in epTGF β 1KO mice compared to controls at 1 dpi and IFN λ was not detectable at this time-point (**Fig. 7.8a**), as reported previously in a murine influenza model⁴⁹⁶. Concentrations of both IFN α and IFN λ in BAL fluid were highest at 3 dpi and both were significantly decreased in epTGF β 1KO mice at this time-point (**Fig. 7.8a**), suggesting that levels of these mediators decreased in line with the reduced viral burden and overall attenuated inflammatory response in the absence of club cell-derived TGF- β 1. Thus, protection of epTGF β 1KO mice from influenza infection was accompanied by a rapid spike in release of IFN β into the airways.

To determine the effects of enhanced early IFN β release on the antiviral response in different cellular compartments of the lung, airway epithelial cells and leukocytes were obtained from tracheal brushings and BAL cell pellets, respectively, and gene expression assessed by qPCR. In an attempt to observe effects preceding the elevated airway IFN β levels and decreased viral load apparent at 1 dpi, cells were analysed 12h after *in vivo* infection. At the 12h time-point, a trend towards increased *Ifnb1* mRNA expression was observed in epTGF β 1KO mouse epithelial cells ($p = 0.09$, **Fig. 7.8b**), suggesting that epithelial cells may contribute to the enhanced airway IFN β levels observed at 1 dpi. There was also a small but significant increase in relative expression of the ISG *Oas2*, which is involved in RNase L-dependent degradation of viral RNA⁴⁹⁷, in epTGF β 1KO epithelial cells compared to controls at 12 hours post-infection (hpi; **Fig. 7.8b**), further supporting an increased early epithelial IFN β response in these mice. Airway leukocytes, which comprised > 90 % of AM at this time-point (**Fig. 7.1f**), had also upregulated *Ifnb1* and *Oas2* by 12 hpi compared to those from uninfected mice, however no differences were observed between epTGF β 1KO mice and controls (**Fig. 7.8b**), suggesting that the antiviral state was not enhanced above control levels in the airway leukocytes of epTGF β 1KO mice at this stage of infection.

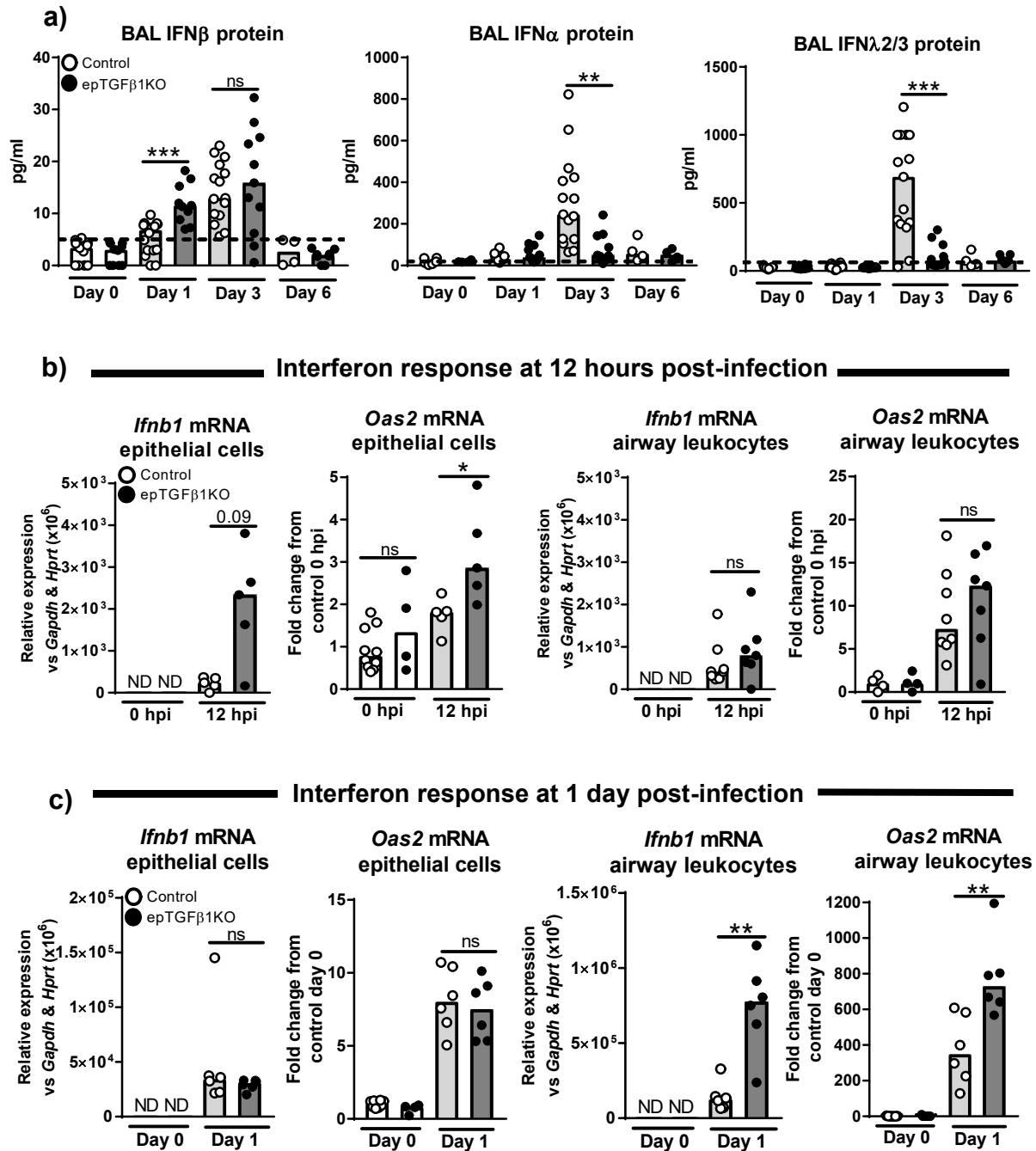


Fig. 7.8. Enhanced early IFN β response to influenza in epTGF β 1KO mice

a) Concentrations of IFN β , IFN α and IFN λ 2/3 protein in BAL fluid. Dashed lines indicate lower limits of detection. Data shown are medians of 4-15 mice per group. Day 0-3 data are pooled from two to three independent experiments. Day 6 data are representative of two independent experiments that were not pooled due to use of different X31 batches. **b-c)** Expression of mRNA for *Ifnb1* and *Oas2* in tracheal epithelial cells and airway (whole BAL) leukocytes relative to housekeeping genes as determined by qPCR at 12h (**b**) and 24h (**c**) post-infection. *Oas2* data are expressed as fold changes from control day 0 samples. Data shown are medians of 4-8 mice per group. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, non-significant. ND, not detected.

Analysis of gene expression at 1 dpi revealed greater induction of both *Ifnb1* and *Oas2* mRNA in epithelial cells than that seen at 12 hpi, but expression of both genes was comparable between epTGFβ1KO and controls (**Fig. 7.8c**). In contrast, expression of both genes was significantly higher in airway leukocytes from epTGFβ1KO mice than controls at this time-point (**Fig. 7.8c**). Leukocytes obtained by BAL at 1 dpi consisted of around 75 % AM and around 10 % neutrophils and did not differ in composition between epTGFβ1KO mice and controls (**Fig. 7.1f**), together suggesting that the antiviral state in these cells was elevated by 24 hpi in the absence of club cell-derived TGF-β1. These data support a model in which enhanced early IFNβ production, resulting from a lack of TGF-β1 release from club cells upon influenza infection, drives rapid adoption of an airway antiviral state.

Having demonstrated enhanced early IFNβ production in epTGFβ1KO mice, it remained unclear how lack of TGF-β1 release from club cells was mechanistically allowing increased IFNβ production. Previous work had shown exogenous and endogenous TGF-β to reduce rhinovirus-induced type I IFN secretion in cultured human bronchial epithelial cells⁴⁹⁸, suggesting a potential common mechanism of TGF-β regulation of IFN responses in human and mouse. In these studies, antibody neutralisation of TGF-β in cultures enhanced type I IFN secretion in response to rhinovirus, accompanied by decreased expression of the negative regulators of type I IFN signalling, *SOCS1* and *SOCS3*⁴⁹⁸, which can be induced by TGF-β⁴⁹⁹. However the murine homologues of these *SOCS* genes, *Socs1* and *Socs3* were not differentially expressed in airway epithelial cells of epTGFβ1KO mice at 12 hpi with influenza (**Fig. 7.9**), suggesting that enhanced early IFNβ production in these mice is unlikely to result from decreased expression of these negative regulators.

If driven by the absence of TGF-β1 release from club cells, rather than an as yet undetermined off-target effect of the epTGFβ1KO genotype, then no enhancement of the airway antiviral response would be expected in epTGFβ1KO mice following infection with RSV, which does not activate TGF-β *in vivo* (**Fig. 7.5b**). Analysis of gene expression in airway cells from RSV-infected mice confirmed this to be the case. *Ifnb1* mRNA expression was detectable in airway leukocytes, but not epithelial cells at 1 dpi, while induction of *Oas2* was apparent in both compartments at this time-point (**Fig. 7.10**). However, no differential expression was observed between cells from epTGFβ1KO and control mice (**Fig. 7.10**), indicating that deletion of TGF-β1 from club cells does not enhance the airway antiviral response to RSV infection.

Collectively, these data suggest that enhanced early adoption of an IFNβ-driven antiviral state occurs as a result of the lack of TGF-β1 release from club cells, consistent with a local pro-viral role for this cytokine.

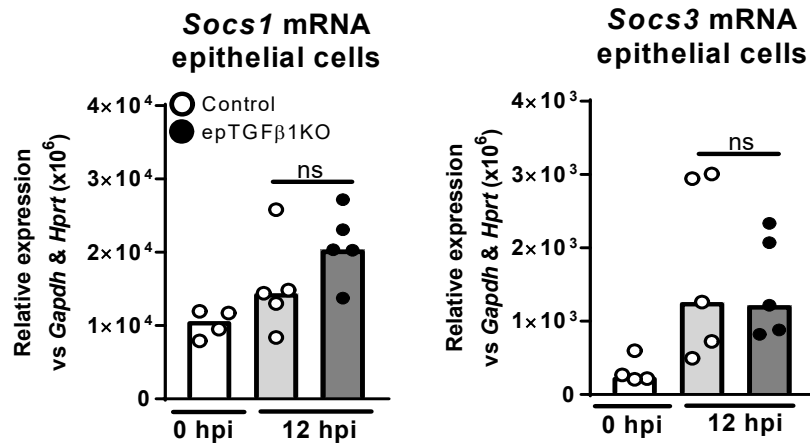


Fig. 7.9. No difference in expression of negative regulators of IFN β expression in epithelial cells of epTGF β 1KO mice early in influenza infection

Expression of *Socs1* and *Socs3* mRNA in tracheal epithelial cell brushings relative to housekeeping genes, as determined by qPCR. Data shown are medians of 4-5 mice per group. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ns, not-significant.

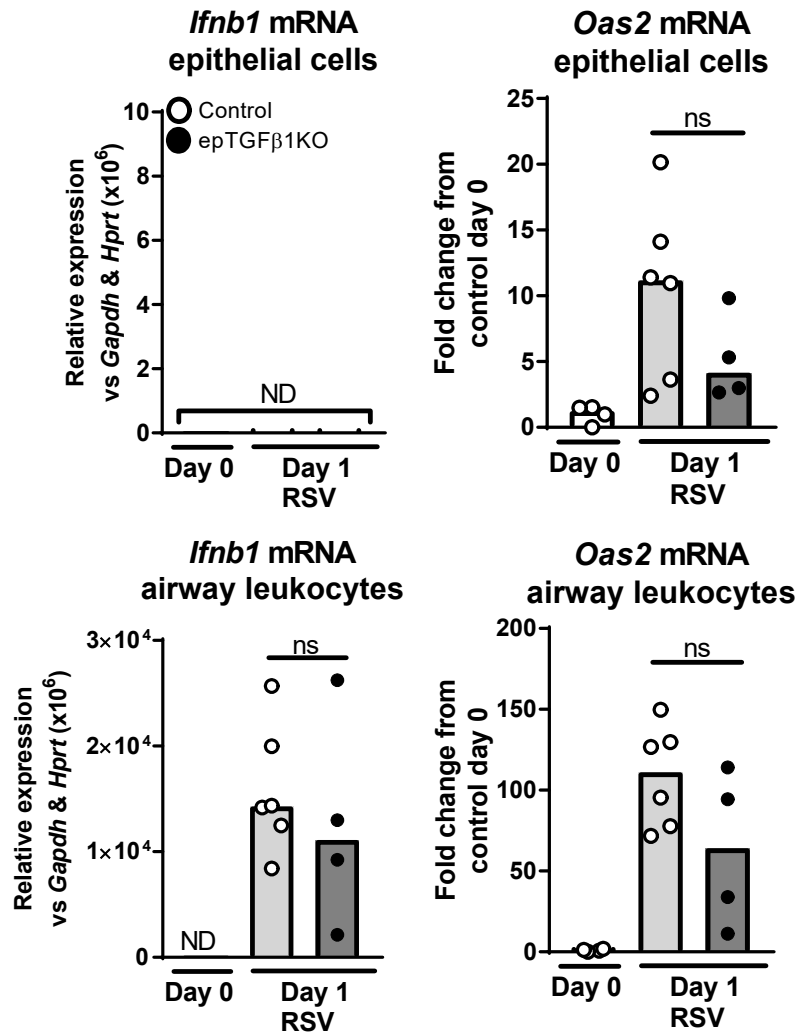


Fig. 7.10. Early interferon response to RSV infection is not enhanced in epTGFβ1KO mice

Expression of *Ifnb1* and *Oas2* mRNA in tracheal epithelial cells and airway (whole BAL) leukocytes relative to housekeeping genes as determined by qPCR at 24h post-infection with RSV. *Oas2* data are expressed as fold changes from control day 0 samples. Data shown are medians of 4-6 mice per group. Statistics: Kruskal-Wallis test followed by Mann-Whitney tests between indicated groups; ns, non-significant. ND, not detected.

7.9. Enhanced early viral clearance in epTGFβ1KO mice is dependent on IFNβ

Finally, to confirm a mechanistic link between enhanced early IFNβ release and reduced pulmonary viral burden in influenza-infected epTGFβ1KO mice, a prophylactic antibody blockade approach was employed. After induction of Cre expression, mice were administered either a monoclonal anti-IFNβ antibody (αIFNβ) or isotype control via the airways 24h prior to influenza infection and lung viral burden assessed at 1 dpi (**Fig. 7.11a**). As shown in earlier experiments, lung viral titre was significantly lower in epTGFβ1KO mice than controls at 1 dpi where both strains received isotype control antibody (**Fig. 7.11b**). However, prophylactic blockade of IFNβ was sufficient to reverse this effect in epTGFβ1KO, resulting in viral titres close to those observed in control mice (**Fig. 7.11b**). Thus, protection of epTGFβ1KO mice from early influenza virus replication was dependent on the action of IFNβ, released rapidly upon infection.

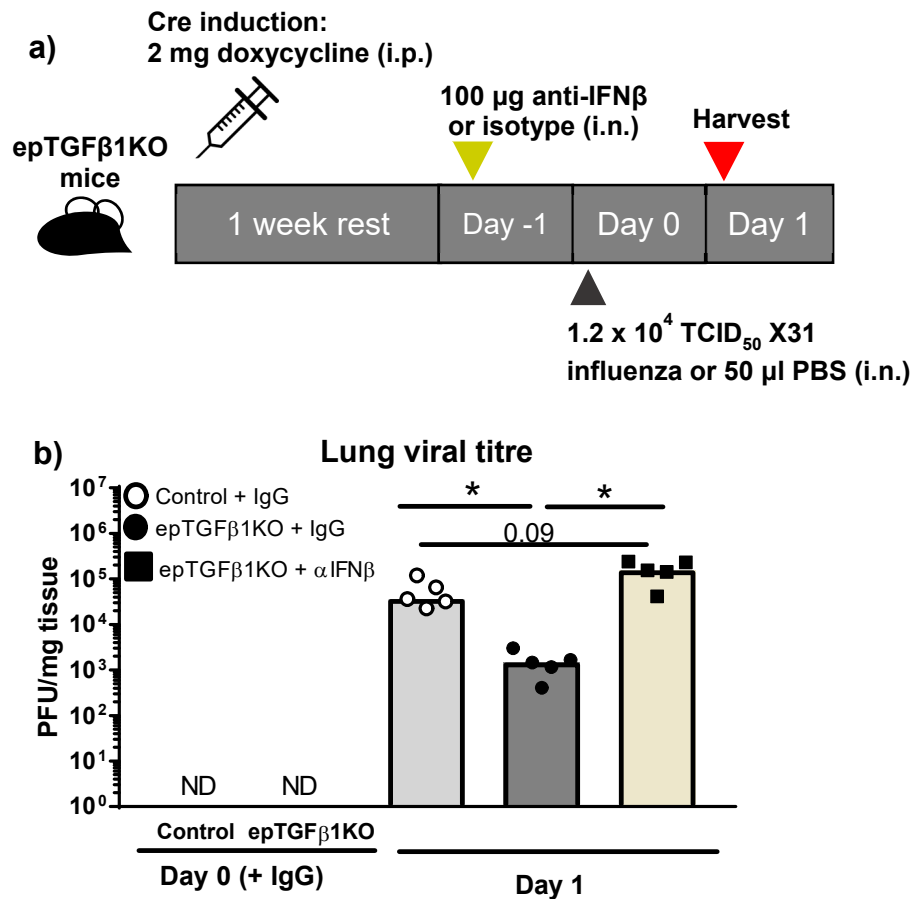


Fig. 7.11. Blockade of airway IFN β reverses the protective effect of club cell-specific TGF- β knockout on early influenza viral load

a) Schematic of blocking experiment. After Cre induction, mice were administered 100 μ g of intranasal anti-IFN β (α IFN β) or isotype control (IgG) in a 100 μ l volume, before infection with X31 influenza. Day 0 control mice received IgG control and a mock infection with intranasal PBS. **b)** Numbers of plaque forming viral units (PFU) in lung tissue. Data shown are medians of 5 mice per group. Statistics: Kruskal-Wallis test followed by Bonferroni-corrected Mann-Whitney tests between indicated groups; *, $p \leq 0.05$; **, $p \leq 0.01$. Where relevant, p values are shown between groups. ND, not detected.

7.10. Discussion

The aim of this chapter was to determine whether bronchiolar epithelial club cells were a source of TGF- β 1 released following influenza infection and to assess the effects of this source of TGF- β 1 on pulmonary immunity to influenza. Based on the impaired ILC2 recruitment observed in epTGF β 1KO mice in AAD models²⁷³ and the apparent regulatory role of TGF- β activated by α v β ₆ integrin in limiting AM activation during infection²⁴⁵, it was hypothesised that club cell-derived TGF- β 1 might regulate recruitment and activation of airway myeloid cells during influenza infection. Instead, club cells appeared to provide a spike in bioactive TGF- β 1 release into the airway lumen at 1 dpi, absence of which resulted in earlier maximal release of IFN β , coincident with a heightened antiviral gene expression response and rapid reduction in lung viral burden.

The findings described here show some parallels with the work of Meliopoulos *et al*²⁴⁵, in that loss of early TGF- β bioactivity in the airways results in protection from the pathogenesis of influenza infection. However, there are several important distinctions. The work of Meliopoulos *et al* demonstrates a homeostatic effect of integrin β ₆ knockout on airway bioactive TGF- β levels and AM activation, which is suggested to prime AM for antiviral activity, resulting in restricted spread of influenza virus²⁴⁵. A general priming effect on AM may explain why β ₆ knockout mice were also protected against the distinct pathogens Sendai virus and *Streptococcus pneumoniae* in these studies²⁴⁵, although these pathogens were not assessed in our epTGF β 1KO system to test this hypothesis. In the present work, TGF- β 1 was deleted from a specific source, as opposed to global knockout of the epithelial integrin α v β ₆, which could impair integrin-dependent activation of TGF- β from several cellular sources. This temporally and spatially restricted ablation of early epithelial TGF- β 1 release caused an enhanced IFN β -driven antiviral response, resulting in decreased lung viral load at 1 dpi, which was not observed in β ₆ knockout mice²⁴⁵. Instead, viral dissemination from conducting airways to alveolar spaces was restricted in the absence of α v β ₆ integrin, consistent with a mechanism dependent on increased AM activation when bioactive TGF- β levels were reduced²⁴⁵. In contrast, data at homeostasis and after influenza infection, as well as previously published work in AAD models²⁷³, showed no evidence of heightened AM activation in epTGF β 1KO mice, indicating that the source of TGF- β required to regulate AM is unlikely to be club cells. Together, the work described here and that published by Meliopoulos and colleagues are complementary studies highlighting the potential for TGF- β to dampen innate immunity to influenza infection in different contexts.

The protective effect of club cell-specific TGF- β 1 knockout seems at first contradictory to the previously reported protective effects of TGF- β signalling on influenza-induced immunopathology^{275,485}. However, TGF- β -dependent protection in these published studies appears to be independent of viral load and was most apparent during the adaptive immune response to influenza infection^{275,485}, suggestive of a mechanism dependent on regulation of T cell-mediated immunity. This was in marked contrast to the early protection observed in epTGF β 1KO mice. Thus, TGF- β from different cellular sources has distinct regulatory effects on multiple stages of the immune response to influenza infection.

An interesting observation from the present work was that enhanced influenza viral clearance in epTGFβ1KO mice appeared to be mediated by increased IFNβ release at 1 dpi, despite the fact that both IFNα and IFNλ were present in the airways at higher maximal concentrations than IFNβ at later time-points. However, it is important to note that neither IFNα nor IFNλ were significantly elevated in BAL fluid of epTGFβ1KO mice at 1 dpi, which appeared to be the decisive time-point for the heightened antiviral state and reduced viral load preceding the subsequently dampened airway inflammatory response. Thus, the timing as well as magnitude of IFN release are critical in controlling early viral replication in the lung. IFNβ is the most rapidly produced type I IFN, since unlike most IFNα genes, it does not require prior IFN-dependent induction of interferon regulatory factor 7 for transcription⁵⁰⁰. Maximal IFNλ release is also partially dependent on type I IFN signalling through IFNAR⁴⁹⁶ and was not detected until 3 dpi in both the present work and a prior study of influenza infection in mice⁴⁹⁶. Thus type I and III IFNs appear to act in a hierarchical manner, with early production of IFNβ likely to be pivotal to the outcomes of influenza infection.

There is some controversy surrounding the importance of IFNβ in clearance of influenza infection, particularly in inbred laboratory mouse strains, the majority of which lack a functional allele for the key ISG *Mx1*⁵⁰¹ and so do not mount optimal IFN-dependent antiviral responses. Accordingly, varying results have been obtained regarding the antiviral role of IFNβ in murine influenza models depending on influenza strain, mouse genetic background and *Mx1* status. IFNAR knockout mice lacking functional *Mx1* displayed impaired viral clearance compared with *Mx1*-deficient mice with intact IFNAR signalling, in some studies⁵⁰² but not others⁴⁹⁶, suggesting that type I IFN signalling can promote clearance of influenza independently of *Mx1* in certain settings. However, the optimal antiviral response to IFNβ during influenza infection occurs in mice expressing both IFNAR and functional *Mx1*⁵⁰³. Although the epTGFβ1KO mice used in the present study are on a mixed genetic background that includes the *Mx1*-deficient 129 and C57BL/6 strains^{288,501}, the enhanced early IFNβ release observed in the absence of club cell-derived TGF-β1 appeared to protect against viral replication, since reduced viral load in these mice was reversed by specific blockade of IFNβ.

Type I IFNs can also promote inflammation during respiratory infection^{60,482,483}, with enhanced signalling via IFNAR in 129 compared with C57BL/6 mice shown to drive immunopathology during acute influenza infection⁴⁸³. Increased IFNβ in epTGFβ1KO mice did not cause enhanced inflammation or immunopathology, instead resulting in a reduction in these features due to rapidly reduced viral load. This is likely due to the enhanced IFNβ response in epTGFβ1KO mice reflecting more rapid kinetics of release (peak at 1 dpi compared with 3 dpi in controls), rather than a greater overall magnitude of IFNβ release, which might be expected to promote excessive inflammation.

In the present study, we took advantage of the mouse RSV model as an infection that causes acute lung injury and recruitment of neutrophils, monocytes and T cells to the airway, without the rapid activation of club cell-derived TGF-β1 as observed in influenza infection. Lack of protection

of epTGF β 1KO mice from RSV infection strongly suggested that observed protection from influenza was dependent on the lack of influenza-driven TGF- β 1 release from club cells. However, this comparison was not perfect, since RSV and influenza are viruses with distinct biology that differ in their epithelial cell tropism in the lungs^{120,121}, with influenza being biased towards bronchiolar epithelium¹²⁰ and therefore more likely to directly interact with club cells. A more suitable comparison experiment would be to determine whether epTGF β 1KO mice are protected from influenza viruses that do not activate latent TGF- β , as has been demonstrated for a number of H5N1 strains²⁷⁵. Delivery of exogenous active TGF- β 1 to the airways of epTGF β 1KO mice shortly after influenza infection would also be a useful experiment to confirm that the observed phenotype is due to the early reduction in bioactive TGF- β in the airway lumen. Such an approach has been shown to reverse the AM activation phenotype observed in $\alpha_v\beta_6$ knockout mice²⁴⁵ and so would also be expected to reverse the protective effects of the club cell-specific TGF- β 1 knockout on IFN β release and viral clearance.

The precise mechanism linking the lack of TGF- β 1 release from club cells upon influenza infection to enhanced early IFN β production will require further elucidation. The trend towards increased *Ifnb1* and *Oas2* expression in airway epithelial cells of epTGF β 1KO mice at 12 hpi, followed by markedly increased expression of these genes in airway leukocytes at 1 dpi, suggest a model in which enhanced IFN β production begins in the TGF- β 1-deficient epithelial cells and is rapidly amplified to induce a pulmonary antiviral state (**Fig. 7.12**). The transience and location-dependency of the enhanced IFN β response proposed in this work makes it a difficult phenomenon to study *in vivo* and *in vitro* systems would likely be required to dissect the mechanism further. Indeed, *in vitro* work using human bronchial epithelial cells demonstrated a suppressive effect of TGF- β on IFN β release induced by rhinovirus and showed some evidence that SOCS1 and SOCS3 are involved in this process⁴⁹⁸. However, expression of neither *Socs1* nor *Socs3* was decreased in airway epithelial cells from epTGF β 1KO mice, suggesting that such a mechanism does not apply in our system. Whole transcriptome analysis of isolated epithelial cells from epTGF β 1KO following influenza infection would likely be of use in identifying differentially regulated signalling processes linking TGF- β 1 knockout and enhanced IFN β production, however this was outside the scope of this project and thesis.

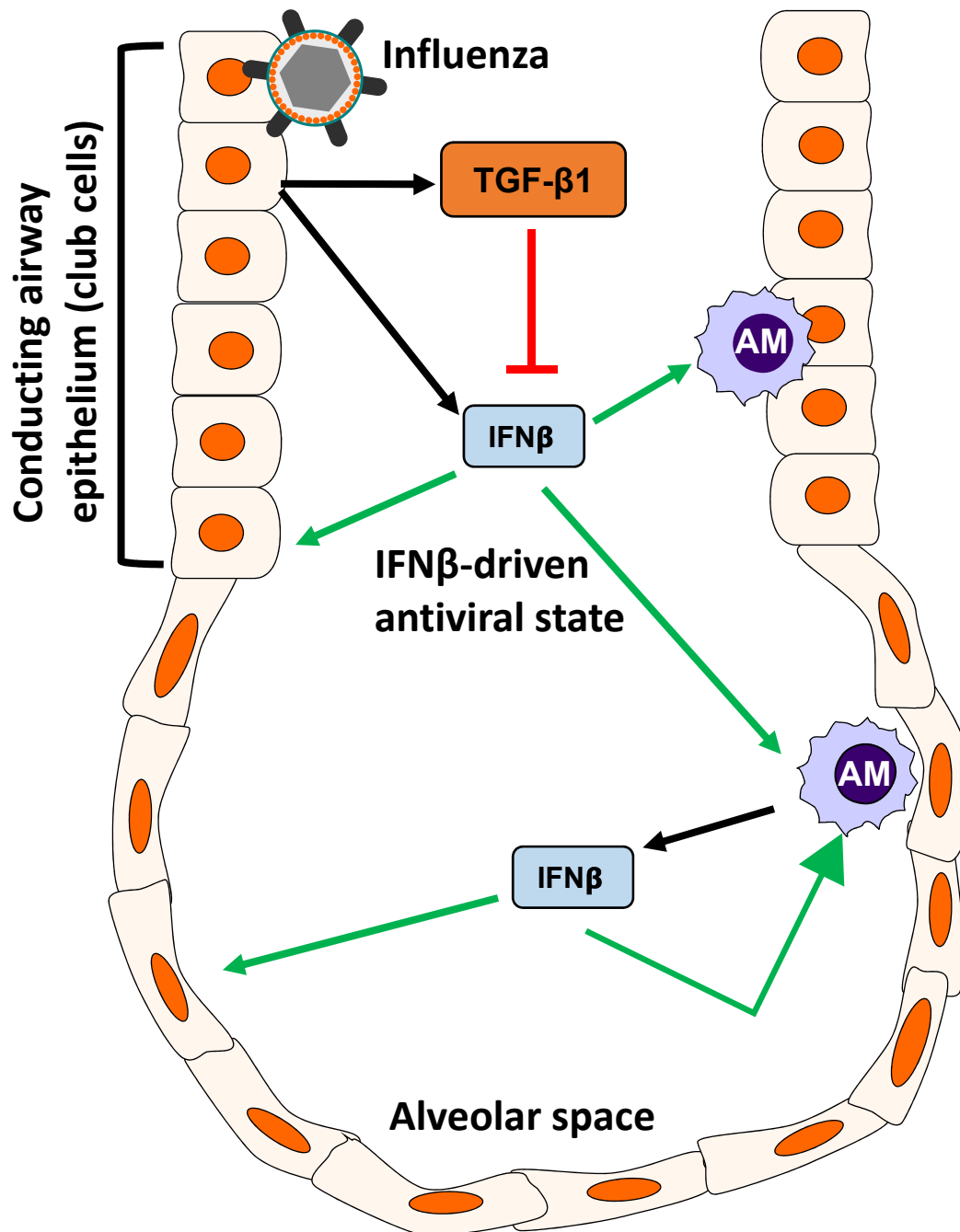


Figure 7.12. Proposed pro-viral function of club cell-derived TGF-β1 during the early stages of influenza infection

Model based on experimental observations described in this chapter. Influenza A virus infection of conducting airway epithelium results in release of TGF-β1 by club cells. TGF-β1 acts to suppress the production of IFNβ from epithelial cells, which would otherwise rapidly promote an antiviral state in airway epithelial cells and leukocytes, principally airway macrophages (AM) to restrict viral replication in the lung. In the absence of suppression by TGF-β1, early action of IFNβ results in further IFNβ production by airway leukocytes, amplifying the pulmonary antiviral response. Black arrows show mediator production; green arrows show activation of the antiviral state and red lines show suppression.

Viral survival in host organisms is dependent on their effective evasion of host immunity. Viruses have therefore evolved a diverse range of mechanisms for dampening or evading the type I and III IFN responses, including interference with ISG transcription and expression of decoy molecules that compete for IFN signalling⁵⁰⁴. It is tempting to speculate from the results of this work that promoting production and activation of club cell-derived TGF- β 1 by influenza represents an evolutionary adaptation of the virus, allowing subversion of a host factor to dampen early antiviral responses and favour its own replication. Notably, suppression of immunity by influenza-induced TGF- β 1 release in this system appeared to be independent of the effects of TGF- β 1 on promoting FoxP3 expression in Tregs⁹⁹ and was instead reliant on rapid restriction of the earliest host immune responses.

7.11. Conclusions

Overall, these studies showed club cell-derived TGF- β 1 to be a key regulator of the innate immune response to influenza infection, with potential to act as a pro-viral factor and facilitate influenza replication in the host. Key differences were observed from published studies in which TGF- β activation in the vicinity of AM is reduced by knockout of an epithelial integrin²⁴⁵ and from global blockade of TGF- β throughout the immune response to influenza²⁷⁵. These findings highlight the temporal and spatial context-specificity with which TGF- β acts to condition the local immune microenvironment.

7.12. Creative commons statement

The main findings from this chapter were included in a joint first author publication, from which a subset of data are reproduced here under a Creative Commons license. The abstract of the published paper is included in Appendix A of this thesis.

Citation: Denney, L*, Branchett, W*, Gregory, L. G., Oliver, R. A. & Lloyd, C. M. Epithelial-derived TGF-beta1 acts as a pro-viral factor in the lung during influenza A infection. *Mucosal Immunol*, doi:10.1038/mi.2017.77 (2017).

*, denotes joint first authors.

8. Final discussion and conclusions

8.1. Final discussion and future work

Constant exposure of the airways to the external environment necessitates a high degree of immune regulation, to allow defence against respiratory pathogens while remaining tolerant to innocuous airborne particles. The cytokines IL-10 and TGF- β 1 have been widely implicated in regulation of immune responses both in lymphoid organs and at mucosal surfaces, including the lung. However, the cellular interactions mediated by these cytokines in pulmonary immune regulation and inhalation tolerance are yet to be fully dissected. Improved knowledge of these basic mechanisms is crucial for better understanding of human diseases in which regulation of pulmonary immunity is insufficient to mitigate immunopathology, including allergic asthma and severe influenza. The strong mechanistic association between augmented early inflammation upon infection and lethality of pandemic influenza⁸⁻¹⁰ illustrates the importance of regulation of the timing and magnitude of immune responses for the outcomes of infection. Furthermore, asthma is increasingly recognised to be a complex syndrome, governed by multiple distinct immunological sub-phenotypes, many of which are not adequately treated with current therapies such as inhaled corticosteroids⁵⁰⁵. Better characterisation of immunoregulatory interactions in the lung are likely to be critical in determining how these phenotypes emerge and how they can be tackled therapeutically. Immune regulation can occur at the level of initiation, maintenance and resolution of immune responses. The focus of this thesis was on airway epithelial cells and macrophages, sentinel cells that are optimally positioned to influence pulmonary immunity at all three of these levels.

Since both IL-10 and TGF- β 1 are pleiotropic cytokines, with the outcomes of signalling differing depending on the receiving cell and cross-talk with other mediators^{181,218}, conditional knockout approaches were employed to study the role of airway sentinel cells as a source of these cytokines. Additionally, flow cytometry, assays of cytokine production and transcriptomic analysis were utilised to more broadly examine the phenotype of airway macrophages (AM) in response to a range of stimuli, with the aim of better understanding their potential for driving inflammation and tolerance in the lung. Through addressing these research aims, insights were gained into a number of central questions and avenues were identified for further investigation. These themes are discussed in the remainder of this chapter.

8.1.1 How does IL-10 regulate immunity in the allergic lung?

Although IL-10 can mediate diverse effects by acting on a wide range of different cell types¹⁸¹, early studies of IL-10 function highlighted its role in suppression of type 1 immune responses¹⁹³, particularly through direct suppressive action on macrophages and dendritic cells (DC)¹⁹⁴⁻¹⁹⁶. In work described in Chapter 6 of this thesis, both global antibody blockade of IL-10R and genetic deletion of *Il10* from T cells (CD4^{Il10} mice) resulted in more severe airway hyperresponsiveness (AHR) and augmentation of the Th1 cell response following repeated HDM inhalation, in a model that otherwise drives a mixed Th2 and Th17 cell response. In the case of T cell-specific IL-10 deletion, but not antibody blockade of IL-10R, increased numbers of Th17 cells and levels of IL-17A were also observed in the lung tissue following HDM exposure. Importantly, this Th1^{hi}

eosinophil^{low} AAD phenotype was accompanied by increased activation of pulmonary macrophages, suggesting that the dysregulated T cell response in the absence of functional IL-10 signalling in the lung may be driven by aberrantly activated macrophages, potentially reflecting a regulatory mechanism similar to the classical model of IL-10 suppression of Th1 responses via antigen-presenting cells (APC)¹⁹⁴⁻¹⁹⁶.

Enhanced pulmonary IFN γ and Th1 cell responses have been demonstrated previously in a 'severe asthma' mouse model of AAD and in human severe asthmatics, compared to mild and moderate asthmatic controls⁴⁷⁴. Although this mouse model is reliant on the use of the bacterial second messenger cyclic di-GMP as a mucosal adjuvant to generate steroid-refractory disease with enhanced AHR, it highlights the potential for the immune response to inhaled HDM to be skewed towards Th1 and for such skewing to result in more severe disease⁴⁷⁴. Ray and colleagues did not report any changes in IL-10 production as causative features or correlates of their severe asthma model⁴⁷⁴, however it remains possible that this model shares common immunological mechanisms with the Th1^{hi} AAD observed in the absence of T cell-derived IL-10 in the present work. Interestingly, further study of the cyclic di-GMP and HDM severe asthma model revealed the observed Th1 skewing to be dependent on expression of IRF5⁵⁰⁶, a key transcriptional regulator in driving a classical activation phenotype in macrophages capable of supporting Th1 and Th17 differentiation¹⁷⁵. Mechanistic experiments in the published work demonstrated a dependency on IRF5 for initiation of Th1 and Th17 responses by migratory DC in the draining lymph nodes during allergic sensitisation in the presence of cyclic di-GMP⁵⁰⁶. However, this does not exclude a role for IRF5 expression in lung macrophages, which would be expected to promote expression of classical M[IFN γ]-like genes and possibly promote local maintenance of Th1 and Th17 responses in the lung, given the induction of these T cell fates by IRF5-expressing macrophages *in vitro*¹⁷⁵. Indeed, IRF5 expression has been shown to be a critical regulator of macrophage function in a type 2-dominant model of HDM-driven AAD, with its absence in AM driving increased type 2 immunity and airway remodelling in response to HDM inhalation¹⁷⁶. Taken together, the work of Ray and colleagues and the studies in this thesis highlight that Th1^{hi} AAD can occur in response to mucosal allergen exposure and is associated with markers of classical macrophage activation, which could conceivably be directly regulated by T cell-derived IL-10.

Additional work is required to better understand these cellular interactions. As discussed in Chapter 6, it will be necessary to determine whether impaired signalling of IL-10 to AM during HDM-driven AAD is indeed a major driver of the overall phenotype arising in the absence of T cell-derived IL-10, or whether the observed increase in AM activation is merely a downstream consequence of the augmented type 1 immune response in these mice. Conditional knockout mice lacking expression of IL-10RA on AM will be used for these experiments. A model in which T cells regulate immunity at the mucosal interface of the airway by direct action on macrophages is comparable to the recently described mechanism by which T cell-derived IL-10 maintains tolerance of resident intestinal macrophages to the commensal microbiota, thus preventing

spontaneous colitis^{205,206}. Future experiments will therefore examine the intriguing possibility that analogous regulatory circuits occur in the steady-state gut and the allergen-exposed airway.

The increased 'classical' activation of AM and interstitial/inflammatory monocytes/macrophages (IMM) observed in the absence of pulmonary IL-10 signalling was inferred from the large proportion of these cells expressing MHC-II. This is a relatively crude definition considering that IL-4 can also induce MHC-II expression on mononuclear phagocytes³²⁶, that macrophages adopt a spectrum of activation states depending on integration of cytokine signals¹⁶⁷ and that RNAseq data in this thesis strongly suggested that AM integrate signals from cytokines such as IFN γ and IL-4 during HDM-driven AAD. In future experiments, AM will be sorted from HDM-treated CD4^{Il10} mice to examine their phenotype and function in more detail. Expression of genes associated with IFN γ - and IL-4-driven macrophage activation will be examined, in particular *Irf5*, which would be expected to be increased in AM with a classically activated, M[IFN γ]-like phenotype in the absence of T cell-derived IL-10 during AAD. If these activated AM are active contributors to the severe Th1^{hi} AAD phenotype observed in the absence of T cell-derived IL-10, then transfer of these cells into the airways of HDM-sensitised IL-10-proficient mice might be expected to promote Th1 skewing in response to subsequent HDM challenges. Conversely, transfer of AM from IL-10-proficient, HDM-sensitised donors to HDM-sensitised CD4^{Il10} recipients could correct the Th1 skewing. Such transfer experiments may be useful in understanding the role of AM in regulation of AAD by IL-10.

Demonstration of a role for AM dysregulation in the absence of pulmonary IL-10 signalling in the pathogenesis of Th1^{hi} AAD would have potential therapeutic implications for severe asthma. Ray and colleagues showed that in patients with severe asthma, which is poorly controlled by inhaled corticosteroids, both IFN γ ⁺ CD4⁺ T cell numbers and *IRF5* mRNA expression were significantly increased in BAL compared to mild to moderate asthmatics^{474,506}. Thus Th1^{hi} AAD is a clinically relevant disease phenotype. In the cyclic di-GMP and HDM severe asthma mouse model, IRF5 deletion rendered the disease sensitive to systemic corticosteroids⁵⁰⁶, suggesting that modification of myeloid cell phenotype can influence type 1 immune responses to inhaled allergen. The authors propose that inhibition of IRF5 activity could be used as an adjunct therapy with corticosteroids in severe asthma⁵⁰⁶. Notably, a polymorphism associated with low IL-10 expression is significantly overrepresented in severe asthmatics⁵⁰⁷ and CD8-depleted peripheral blood mononuclear cells (PBMC) from children with paediatric severe therapy-resistant asthma display diminished IL-10 production compared to non-asthmatic controls⁵⁰⁸. Moreover, CD8-depleted PBMC from adult steroid-resistant asthmatics fail to produce IL-10 in response to corticosteroid treatment *in vitro*⁵⁰⁹, prompting efforts to restore the IL-10 response to corticosteroids with combination therapies such as vitamin D3⁵¹⁰.

The association of severe asthma with reduced IL-10 signalling^{508,509}, increased IRF5 expression⁴⁷⁴ and more abundant Th1 cells^{474,506} suggests potential parallels with the observed HDM-driven AAD phenotype observed in CD4^{Il10} mice in this thesis. Characterisation of an axis

linking impaired IL-10 signalling, altered pulmonary macrophage activation and heightened Th1 immunity to inhaled allergen could therefore provide insights into mechanisms of immunopathogenesis and steroid resistance relevant to severe asthma. Future studies will be conducted with this in mind, such as determining whether HDM-driven Th1^{hi} AAD in CD4^{Il10} mice is resistant to inhaled corticosteroids.

Another key finding from analysis of pulmonary IL-10 expression and function in this thesis was that T cell-restricted IL-10 deletion phenocopied the effects of global IL-10 blockade in HDM-driven AAD and reduced pulmonary IL-10 levels in HDM-treated mice to below those observed in littermate controls prior to allergen exposure. These data suggest that T cells are the major and functionally dominant source of IL-10 during AAD induced by repeated HDM inhalation, despite a proportion of DC and IMM showing IL-10 reporter expression in this model. In contrast, few IL-10-competent T cells were detected in the lungs at steady state and IL-10-competent T cells in HDM-treated mice had an antigen-experienced phenotype, suggesting that these cells are generated in an antigen-specific manner. Taken together with published data demonstrating that interstitial macrophages (IM) are a source of IL-10 in the steady state lung that can be expanded by intranasal TLR9 agonist stimulation to prevent allergic sensitisation^{31,214}, these data support a division of labour between innate and adaptive sources of IL-10 in the lung, with CD4⁺ effector T cells dominating during active immune responses and IM-derived IL-10 being more important at homeostasis. Importantly, IL-10 production was largely undetectable in AM at homeostasis and during inflammation, suggesting that AM and IM employ different immunoregulatory mechanisms in the lung.

The proposed division of labour of pulmonary IL-10 production between IM and T cells has potential implications for therapies aimed at limiting allergic inflammation in the lung by enhancing IL-10 production. Therapies intended to prevent initial allergic sensitisation may be better aimed at innate sources of IL-10, as demonstrated by the efficacy of prophylactic TLR9 agonist administration in preventing HDM-driven allergic inflammation by expanding IL-10⁺ IM²¹⁴, while established allergic inflammation, particularly with a Th1 component, may be better targeted by enhancement of T cell IL-10 production.

8.1.2. TGF-β1 acts locally to perform context-specific immune regulatory functions

Cell-type specific functions of TGF-β1 were examined in two distinct models in the present work, examining AM-derived TGF-β1 in HDM-driven AAD and club cell-derived TGF-β1 in the early stages of primary influenza A virus infection. Deletion of TGF-β1 in AM and DC using CD11c-Cre resulted in enhanced pulmonary type 2 immunity following 3 weeks of exposure to HDM, accompanied by increased numbers of monocyte-derived cells in the lungs and increased cytokine and chemokine production from AM *in vitro*. Although much more work is required to understand the mechanisms underlying these effects, they are suggestive of an autocrine regulatory effect of TGF-β1 on AM function, similar to, but less extreme than, that proposed recently by Yu et al²⁸². In contrast, knockout of TGF-β1 specifically in club cells resulted in

reduced inflammation following influenza infection, due to an enhanced early antiviral response and rapidly diminished viral burden.

Although the outcomes and apparent mechanisms of these two effects differed substantially, with one knockout enhancing and one limiting the overall severity of disease, both appear to reflect local regulatory functions of TGF- β 1 on immune activation. In the case of AM, evidence obtained so far suggests that TGF- β 1 controls inflammatory mediator production in an autocrine manner; a hypothesis supported by the dysregulation of AM observed in $\alpha_V\beta_6$ knockout mice^{244,245}, which lack a major means of TGF- β activation in the airway niche. Similarly, club cell-derived TGF- β 1 appears to act locally on epithelial cells to limit the IFN β response upon influenza infection, since increased interferon-stimulated gene expression was apparent within 12 hours of infection in TGF- β 1-deficient epithelium, suggestive of an effect close to the site of infection and TGF- β 1 release. Such exquisitely local action of TGF- β 1 likely explains why no differential activation of AM was observed in club cell-specific TGF- β 1 knockout mice at homeostasis, during acute influenza infection and in AAD²⁷³, in contrast to $\alpha_V\beta_6$ knockout mice²⁴⁵, as compensatory sources of TGF- β are present in the vicinity of AM.

Further experiments will be required to better elucidate the proposed local regulatory functions of TGF- β 1. In the case of AM, more analysis will be performed on the production of pro-inflammatory mediators by TGF- β 1-deficient AM, both at baseline and in response to pro-inflammatory stimuli, beginning by examining expression of a wider range of chemokines to build upon the CCL2 expression data described in this thesis. The role of TGF- β 1-deficient AM in promoting monocyte recruitment to the lungs will also be analysed further in relevant mouse models of allergic inflammation and respiratory infection. To better understand regulation of epithelial IFN β production by club cell-derived TGF- β , an *in vitro* system is likely to be required to allow superior kinetic and mechanistic analysis of the signalling pathways linking reduced TGF- β 1 and heightened IFN β compared with *in vivo* infections. A system for generating air-liquid interface cultures from mouse airway epithelium has previously been used to study interferon responses to influenza infection and may be of use for this¹²⁵.

Highly localised action is increasingly understood to be a key feature of TGF- β function, particularly at the level of activation of latent TGF- β by spatially-restricted integrins²¹⁸. For example, mice lacking the TGF- β -activating integrin $\alpha_V\beta_8$ on FoxP3⁺ Tregs fail to control colitogenic T cell responses in the intestine²¹⁸, suggesting that co-localisation of effector T cells and Tregs is an important determinant of TGF- β regulation in the gut. Similarly, spatially-restricted activation of TGF- β by integrins expressed on keratinocytes has been shown to maintain the residency of Langerhans cells in the epidermis, with reduced keratinocyte integrin expression in response to exogenous stimuli acting as a signal to allow Langerhans cell migration⁵¹¹. The data described in this thesis, along with work published previously from our group²⁷³, suggest that release of TGF- β 1 from specific cell types is another means of regulating local TGF- β activity to allow context-dependent functions.

Overall, although TGF- β is widely expressed throughout the lung^{272,273} and can mediate diverse and potentially contradictory functions *in vivo*^{218,278}, specific cellular sources and activation mechanisms act to control exposure of target cells to TGF- β signalling. This raises the intriguing possibility that therapeutics targeting specific cellular sources of TGF- β or its activating molecules could be used to selectively enhance its protective functions and/or block pathogenic mechanisms. Indeed, a low dose of blocking antibody to integrin $\alpha_v\beta_6$ effectively prevented bleomycin-induced fibrosis *in vivo*, accompanied by reduced pulmonary Smad signalling but without increasing inflammation, providing preclinical proof-of-concept for such an approach⁵¹². Understanding the cell type-specific roles of TGF- β 1 is therefore exciting in terms of both basic and translational research.

8.1.3. How do airway macrophages promote and regulate the pathogenesis of allergic airway disease?

A central aim of this thesis was to better understand the potentially conflicting roles of AM in pulmonary immune regulation, particularly in the context of AAD. AM depletion in naïve or allergen-sensitised animals results in enhanced inflammatory responses to subsequent allergen challenge^{63,144,145,513,514}, impaired resolution of inflammation¹⁴⁴ and increased T cell stimulation capacity of pulmonary DC¹⁴³, strongly suggesting that AM have an anti-inflammatory function *in vivo*. However, AM also produce pro-inflammatory mediators such as CCL24 and CCL3 in murine AAD models¹⁵⁷ and human asthma¹⁵⁶ and can promote airway remodelling following allergen inhalation¹⁷⁶, illustrating their potential to contribute to the pathogenesis of AAD. It is notable that although endogenous AM appear to retain some protective immunoregulatory function in HDM-allergic mice¹⁴⁴, AM from allergen-sensitised animals lose the ability to ameliorate AAD upon adoptive transfer^{145,146,180} and overproduce pro-inflammatory cytokines such as TNF α ^{145,146}. Taken together, these data suggest that AM have regulatory capacity at homeostasis and during allergic inflammation, but the balance between pro-inflammatory and regulatory functions is dysregulated during AAD. Understanding the balance between pro-inflammatory and regulatory functions of AM was therefore central to this thesis.

In the present work, AM phenotype during AAD was assessed by flow cytometry for surface activation markers and RNAseq analysis of gene expression. Increased expression of CD11b on the surface of AM was determined to be a robust indicator of AM activation in AAD, as well as all other pulmonary inflammation models tested in this thesis, consistent with published reports^{311,323}. CD11b, also known as integrin α_M and complement receptor 3, forms part of the Mac1 complex with CD18 that is constitutively expressed on monocytes and the majority of recruited and tissue-resident macrophages^{515,516}, but not steady-state murine AM^{311,323}. The Mac1 complex is widely implicated in canonical macrophage functions such as complement-dependent opsonophagocytosis and cell adhesion processes, so its upregulation by AM from HDM-exposed mice (AM[HDM]) is suggestive of increased capacity to perform such functions. Consistent with AM adopting features of canonical inflammatory macrophages during AAD, transcriptomic data showed increased expression of gene signatures associated with phagosome maturation and

antigen presentation in AM[HDM] and cell surface MHC-II expression was also robustly increased on AM in both HDM- and *Alternaria alternata*-driven models of AAD. However, gene expression analysis of AM[HDM] in this thesis also revealed downregulation of a number of genes related to actin dynamics, which were suggestive of a potentially decreased capability of AM to perform phagocytosis and other functions dependent on cytoskeletal remodelling. The phagocytic capacity of AM has previously been shown to be impaired in certain rodent models of AAD¹⁴⁶ and bacterial phagocytosis is reported to be reduced in AM of asthma patients with severe or poorly controlled disease^{432,433}. It is therefore possible that while functions related to phagosome and antigen presentation were increased in AM[HDM], phagocytosis may be impaired, although further experiments would be required to confirm this. Nonetheless, dysregulation of AM during AAD was clearly accompanied by increased expression of genes and proteins relating to canonical macrophage and antigen-presenting cell function, with CD11b and MHC-II expression as cell surface markers of this.

Another major effect of repeated *in vivo* HDM exposure observed on AM was the substantial upregulation of genes encoding pro-inflammatory mediators, particularly chemokines such as *Ccl24*, *Ccl2*, *Ccl7* and *Ccl8*. Increased gene expression appeared to be selective to a subset of inflammatory mediators, since *Cx3cl1* and *Tnf* mRNA levels were decreased in AM[HDM]. Increased production of chemokines by AM in AAD is in agreement with previously published work¹⁵⁷ and supports a model in which dysregulated AM promote leukocyte recruitment into the allergic lung. CCL24 was among the most highly induced genes in AM[HDM] and is important for sustained recruitment of eosinophils into the lung⁵¹⁷. Although bronchial epithelial cells are also substantial producers of CCL24⁵¹⁸, it seems likely that AM directly contribute to eosinophil recruitment during AAD. Indeed, a model of AM dysfunction induced by HDM exposure in mice lacking the macrophage scavenger receptor CD163 resulted in heightened CCL24 production and eosinophilic inflammation⁵¹⁹. CCL2, CCL7 and CCL8 are all chemotactic for monocytes⁵⁸, which promote type 2 immune responses during AAD^{40,63}. Although CCL2 in particular is largely produced by bronchial/bronchiolar epithelial cells following allergen exposure^{363,520,521}, these data suggest that AM are also likely to contribute to monocyte recruitment during allergic inflammation. Notably, *Ccl2* expression was shown in the present work to be increased in TGF- β 1-deficient AM from CD11c^{Tgfb1} mice, suggesting that autocrine TGF- β 1 is a mechanism by which AM restrain their potential for monocyte recruitment. In future work, the importance of AM-derived chemokines and their regulation by TGF- β 1 will be assessed using adoptive transfers of TGF- β 1-deficient and control AM.

Given that the gene expression profile of AM during HDM-driven AAD was suggestive of active contribution to pathogenesis, it will be interesting to determine whether pathogenic features of AM are effectively suppressed by inhaled corticosteroids, which remain the major first-line maintenance therapy for asthma in the UK (see guidelines at www.brit-thoracic.org.uk). A placebo-controlled study of inhaled budesonide in mild asthmatics showed *in vivo* corticosteroid exposure to reduce expression of pro-inflammatory cytokines and increase expression of IL-10

in adherent BAL leukocytes, the majority of which were AM, suggesting that corticosteroids can reverse the inflammatory state of AM¹⁵⁶. However, it is unclear whether the observed effect of budesonide in this study was completely attributable to AM and not contaminating lymphocytes or granulocytes in BAL. In murine studies described in Chapter 3 of this thesis, inhaled budesonide was shown to prevent some, but not all, of the changes in surface marker expression on AM, particularly CD71 and CD206 in the highly eosinophilic 129 mouse strain. These findings suggest that AM activation may be incompletely suppressed by corticosteroids and that AM dysfunction may therefore be an important determinant of steroid-resistance in asthma. However, it would be necessary to equate steroid-resistant expression of markers such as CD71 and CD206 observed in HDM-treated 129 mice to production of mediators of asthma pathogenesis to further relate this model to the clinic. Notably, LPS-induced cytokine production from AM isolated from severe, but not mild to moderate, asthmatics is refractory to *in vitro* dexamethasone suppression⁵²², suggesting that steroid-resistant AM activation can occur in asthma patients.

In addition to reversal by corticosteroids, better understanding of the molecular basis of AM dysfunction during AAD could reveal novel therapeutic approaches for reducing pathogenic phenotypes in AM. The observed increase in expression of the majority of genes encoding mitochondrial electron transport chain (ETC) components in AM[HDM], accompanying the pro-inflammatory gene expression changes in these cells, is potentially of interest to this, given the increasingly appreciated role of metabolic reprogramming in macrophage activation and polarisation^{427,428}. Increased mitochondrial oxidative phosphorylation is associated with IL-4- and/or IL-13-induced macrophage polarisation^{427,428} and is required for full responsiveness of macrophages to IL-4 *in vitro*⁴²⁹, suggesting that the predicted metabolic shift in AM[HDM] is intimately associated with induction of M[IL-4] genes. Since heightened M[IL-4]-like AM activation, as observed in IRF5-deficient mice, promotes increased type 2 immunity and airway remodelling following HDM exposure¹⁷⁶, it may be beneficial to limit this activation during AAD. The increased expression of ETC genes in sorted AM[HDM], but not unsorted bronchoalveolar lavage (BAL) cells from HDM-treated mice, suggested that these gene expression changes were largely restricted to AM in the allergic airway. Fine-tuning of AM metabolism to reduce oxidative phosphorylation could therefore potentially allow selective dampening of M[IL-4]-like activation in AM[HDM]. Suppression of the mammalian target of rapamycin (mTOR) signalling pathway has been implicated in mechanisms of IL-4-induced macrophage polarisation, the metabolic shift towards oxidative phosphorylation and suppression of mitochondrial dysfunction by IL-10^{430,523}, suggesting that increased signalling via mTOR could counter the M[IL-4]-like phenotype of AM[HDM]. To further pursue these observations in future work, it would first be necessary to verify the RNAseq findings by using cellular metabolism assays to demonstrate increased oxidative phosphorylation in AM[HDM], before using genetic knockout and/or chemical inhibition strategies to examine the role of metabolic enzymes and pathways in AM[HDM] activation.

Aside from increased production of inflammatory mediators, AM dysfunction in AAD could also depend upon impairment of homeostatic functions. The potential reduction of phagocytic capacity

of AM[HDM] suggested from RNAseq data as discussed above warrants further investigation. More specifically, AM dysfunction in AAD could partially stem from defects in efferocytosis, the process of receptor-mediated apoptotic cell clearance⁵²⁴. Efferocytosis is thought to be performed by AM in the lung and to prevent inflammation by promoting anti-inflammatory gene expression in the efferocytic cell and removing potentially pro-inflammatory cellular debris⁵²⁴. Recent work from Hussell and colleagues has shown Axl to be a major efferocytosis receptor expressed on murine and human AM^{371,372}. Notably, Axl expression was decreased in asthmatics and inversely correlated with levels of potentially pro-inflammatory necrotic cell debris in sputum³⁷², suggesting that defective efferocytosis by AM may contribute to inflammation in asthma. In line with this, *Axl* mRNA expression was shown to be significantly reduced in murine AM[HDM] in the RNAseq study described in this thesis, suggesting that defective Axl-mediated efferocytosis may also contribute to experimental HDM-induced AAD. However, despite decreased mRNA expression, *Axl* was still among the most abundant transcripts in AM[HDM] and these cells also significantly upregulated genes encoding C1q, which promotes efferocytosis in macrophages⁵²⁵. The overall effects of repeated HDM exposure on the capacity of AM to perform efferocytosis are therefore unclear from the data in this thesis but warrant further study, for example using assays for fluorescently labelled apoptotic cell uptake *ex vivo*.

Equally important to understanding AM function during inflammation is characterisation of anti-inflammatory mechanisms employed by these cells. A major finding from this thesis was that AM do not produce functionally important levels of IL-10 at homeostasis, during AAD or after stimulation with TLR agonists *in vitro*. This minimal IL-10 production is supported by work in RSV¹⁰⁸ and *Mycobacterium tuberculosis*¹⁹⁷ infection and highlights the contrast to intestinal macrophages, which are an abundant source of IL-10⁴⁵⁶. Although intestinal macrophage IL-10 production is redundant in the steady state, it was shown to be essential for limiting immunopathology following enteric infection with *Citrobacter rodentium*²⁰⁷, suggesting that intestinal macrophage-derived IL-10 is important for avoiding pathology following certain deviations from immune homeostasis. Thus, tissue-resident macrophages utilise distinct mechanisms of immune regulation in their specific locational niches.

As discussed in section 8.1.2, AM-derived TGF- β 1 appears to have an important autocrine regulatory role in limiting production of pro-inflammatory mediators by AM, which could conceivably restrain potentially pathogenic AM function both at homeostasis and during inflammation. It remains to be seen whether AM-derived TGF- β 1 regulates other cell types in the lung, such as epithelial cells, T cells or DC. Indeed, although TGF- β was shown to be important for induction of FoxP3 expression in naive CD4⁺ T cells *in vitro*¹⁵³, no defect in HDM-elicited FoxP3⁺ Tregs was observed in mice lacking AM-derived TGF- β 1, suggesting that this is not a relevant mechanism of immune regulation during HDM-driven AAD. Further dissection of the mechanisms of enhanced AAD in CD11c^{Tgfb1} mice will allow the role of AM-derived TGF- β 1 on other cell populations to be probed.

Although IL-10 and TGF- β 1 are well characterised and widely studied anti-inflammatory cytokines, it is likely that additional mediators are involved in the regulatory function of AM *in vivo*, particularly given their minimal IL-10 production. Such regulatory molecules could be constitutively expressed or upregulated in response to inflammatory stimuli. Despite the large number of differentially expressed genes observed in AM[HDM] in the RNAseq analysis in this thesis, changes were predominantly associated with pro-inflammatory and tissue repair functions, with no regulatory pathways or gene ontology annotations highly enriched within the differentially expressed genes. A notable exception was *Aldh1a2*, encoding the RALDH2 enzyme required for production of retinoic acid. Since retinoic acid has been implicated in regulatory T cell generation by AM *in vitro*¹⁵³, it would be interesting to determine the *in vivo* importance of AM-derived retinoic acid by conditional knockout of *Aldh1a2* in AM.

Additionally, since AM are important for the resolution of AAD following cessation of allergen exposure¹⁴⁴, it is likely that regulatory mediators will be induced in AM later after HDM challenge than the 24h timepoint used for studies in this thesis. Follow-up RNAseq analysis will therefore be required to determine the molecular changes occurring to AM in the days and weeks following an allergen challenge, to better understand how these cells control homeostasis and resolution of inflammation.

The location of AM in the airway lumen makes them an attractive target for future therapeutic strategies. Proof-of-concept studies showed that macrophages could successfully engraft into the empty airway niches of mice lacking the GM-CSF receptor and correct pulmonary alveolar proteinosis³⁶². Moreover, gene editing on the donor macrophages could be performed prior to transplant³⁶², suggesting that defects in autologous macrophages could theoretically be corrected and the airway repopulated with functional AM. It remains to be seen whether such transplantation will be applicable to recipients that do not have an empty airway niche, or whether it will be applicable in the clinic. However, the recent demonstration that human and mouse induced pluripotent stem cells can adopt a primitive macrophage phenotype capable of adopting tissue-resident macrophage-like fates⁵²⁶ suggests that an autologous transplant approach could eventually be possible. Alternatively, since AM readily phagocytose liposomes, liposomal drug delivery could be developed to allow specific modulation of gene expression, metabolism or signalling pathways in AM. Such an approach has been suggested for targeted delivery of highly toxic or poorly soluble antimicrobial drugs⁵²⁷ and may be of use if molecular pathways were identified that would benefit from specific modulation in AM.

The plasticity of AM might also provide novel therapeutic approaches. RNAseq analysis of AM[HDM] strongly suggested integration of multiple signals in the allergic airway microenvironment, such as IFN γ , type I IFN and IL-4 and IL-13, resulting in expression of clusters of functionally related genes. Dissection of the links between groups of molecules and specific functions of AM could conceivably allow their pro-inflammatory features to be dampened, while preserving their ability to regulate pulmonary immunity. The importance of microenvironmental

signals in driving AM phenotype are well established, as demonstrated by studies in which several distinct precursor cell types could populate the airway niche and differentiate into AM at steady state^{62,362}. Although GM-CSF and TGF- β have been identified as two factors required for local differentiation of AM^{24,25,282}, the combination of microenvironmental signals required for functional maturation and homeostasis of AM is likely to be much more complicated than this. Understanding how to restore such signals of 'health' in the airways, in addition to dampening the activating and polarising signals present in inflammatory states, might allow better therapeutic restoration of normal AM function.

8.2. Concluding remarks

Collectively, the work described in this thesis has helped illuminate context-specific functions of IL-10 and TGF- β 1 in pulmonary inflammation, as well as gain greater understanding of the conflicting pro-inflammatory and immunoregulatory roles of AM. IL-10 was shown to be a crucial regulator of pulmonary immunity to inhaled allergen, independently of its production by AM, and the mechanisms by which T cell-derived IL-10 controls AM activation will be the subject of ongoing research. This work also reinforced the key message that TGF- β 1 operates within temporally- and spatially-restricted niches to mediate distinct physiological and immunological effects. This thesis provides a platform on which to further dissect the basic mechanisms of pulmonary immunoregulation, with the ultimate aim of better understanding how to prevent and treat inflammatory lung disorders.

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Appendix A: publications arising from this thesis

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Denney, L.* , **Branchett, W.***, Gregory, L.G., Oliver, R.A. & Lloyd, C.M. **Epithelial-derived TGF-beta1 acts as a pro-viral factor in the lung during influenza A infection.** *Mucosal Immunology* (2017). DOI: 10.1038/mi.2017.77

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Mucosal surfaces are under constant bombardment from potentially antigenic particles and so must maintain a balance between homeostasis and inappropriate immune activation and consequent pathology. Epithelial cells have a vital role orchestrating pulmonary homeostasis and defense against pathogens. TGF- β regulates an array of immune responses—both inflammatory and regulatory—however, its function is highly location- and context-dependent. We demonstrate that epithelial-derived TGF- β acts as a pro-viral factor suppressing early immune responses during influenza A infection. Mice specifically lacking bronchial epithelial TGF- β 1 (epTGF β KO) displayed marked protection from influenza-induced weight loss, airway inflammation, and pathology. However, protection from influenza-induced pathology was not associated with a heightened lymphocytic immune response. In contrast, the kinetics of interferon beta (IFN β) release into the airways was significantly enhanced in epTGF β KO mice compared with control mice, with elevated IFN β on day 1 in epTGF β KO compared with control mice. This induced a heightened antiviral state resulting in impaired viral replication in epTGF β KO mice. Thus, epithelial-derived TGF- β acts to suppress early IFN β responses leading to increased viral burden and pathology. This study demonstrates the importance of the local epithelial microenvironmental niche in shaping initial immune responses to viral infection and controlling host disease.

Appendix B: conference abstracts arising from this thesis

1) Oral presentation at EAACI Immunology Winter School, Pocol, Italy, 2016

Pulmonary epithelial-derived transforming growth factor β 1 is critical for the pathogenic immune response to influenza infection

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Introduction:

Epithelial cells lining the airways are constantly exposed to the external environment and are therefore critical in directing immune responses to inhaled particles, such as allergens and viruses. Airway epithelial cells are a major source of TGF- β 1 (transforming growth factor β 1), which has a multitude of immune regulatory functions but can also drive tissue remodelling and fibrosis. Given its pleiotropic nature, the role of TGF- β 1 is highly context-dependent, necessitating the use of cell type-specific strategies for TGF- β 1 manipulation *in vivo*. Here, the role of bronchiolar epithelial TGF- β 1 during influenza infection was examined using conditional knockout mice.

Methods:

Inducible bronchiolar club cell TGF- β 1 knockout (epTGF β 1^{-/-}) mice and littermate controls were bred in house and knockout induced by doxycycline administration. Mice were infected by intranasal administration of 1.2×10^4 TCID₅₀ (50 % tissue culture infective dose) units of influenza X31 and disease severity assessed based on weight loss. Influenza NP protein was detected in lung sections by immunohistochemistry. Immune cells were analysed by flow cytometry and inflammatory mediators were quantified by ELISA and/or real-time PCR.

Results:

EpTGF β 1^{-/-} mice were significantly protected from weight loss after influenza infection (mean maximum weight loss 3.5 % vs 15 % in controls). This was accompanied by reduced airway inflammation, including more than two-fold reductions in peak numbers of neutrophils, monocytes and macrophages. Influenza proteins were detectable in the bronchiolar epithelium of both epTGF β 1^{-/-} and control mice at 1 and 3 dpi (days post infection), indicating that viral entry into TGF- β 1-deficient epithelium was not impaired. However, clear reductions in levels of type 1 interferon pathway genes, chemokines CCL2 and MIP2, and inflammatory cytokines IL-6 and TNF α were apparent in the lungs of epTGF β 1^{-/-} mice by 3 dpi, suggestive of a rapidly curtailed immune response in the absence of epithelial TGF- β 1.

Conclusions:

Lack of bronchiolar epithelial cell-derived TGF- β 1 limited the generation of a pathogenic immune response following influenza infection. These data highlight a critical role for local TGF- β 1 production in mediating the pulmonary response to viral infection.

Investigating the role of transforming growth factor β 1 in pulmonary epithelial-myeloid cell interactions

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Interactions between airway macrophages (AMs) and epithelial cells are critical in controlling pulmonary immunity. Both of these cell types produce transforming growth factor 1 (TGF β 1), making it a potential mediator of epithelial-macrophage interactions in the airway. Our laboratory has recently generated bronchiolar club cell-specific TGF β 1 knockout (*Ccsp-Cre Tgfb1^{-/-}*) mice, showing that local TGF β 1 drives innate lymphoid cell recruitment during allergic airways disease¹. Here, we assessed whether epithelial TGF β 1 also controls the recruitment and phenotype of airway myeloid cells, at homeostasis and during influenza A virus (IAV) infection.

At homeostasis, AMs from *Ccsp-Cre Tgfb1^{-/-}* mice and littermate controls were equally abundant and showed equivalent expression of the activation markers CD11b and CD64. Comparable AM activation was also apparent 1 day post infection (dpi) with IAV in *Ccsp-Cre Tgfb1^{-/-}* mice and controls, overall suggesting little regulation of AM activation by bronchiolar epithelial TGF β 1. In contrast, recruitment of neutrophils, monocytes and macrophages to the airways was significantly reduced in *Ccsp-Cre Tgfb1^{-/-}* mice following IAV infection, accompanied by protection from weight loss (mean 3.5 % vs 15 % in controls at 6 dpi). Initial IAV entry to epithelium was not impaired, but plaque-forming viral units were significantly reduced, suggestive of a rapidly attenuated infection in *Ccsp-Cre Tgfb1^{-/-}* mice. Interestingly, despite the reduced inflammatory monocyte and macrophage numbers in *Ccsp-Cre Tgfb1^{-/-}* mice, significantly more of these cells expressed the sialoglycoprotein CD24 at high levels, suggesting an imbalance in recruited myeloid populations in the absence of epithelial TGF β 1.

These data support a critical role for local TGF β 1 in establishing pathogenic IAV infection and dictating the resultant pulmonary myeloid cell infiltrate.

¹Denney et al., 2015. *Immunity* 43, 945-958

3) Poster presentation at British Society for Immunology Congress, Liverpool, United Kingdom, 2016

Understanding the relationship between Interleukin 10 and airway macrophages in regulating allergic airways disease

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Background

Airway macrophages (AMs) contribute to maintenance of immune tolerance in the healthy lung and their dysregulation may facilitate allergic inflammation. Although the factors underlying macrophage tolerance are incompletely understood, they are thought to include the production of immunoregulatory cytokines. Here we investigated the role of the prototypic regulatory cytokine IL-10 in the allergic airway and assessed the potential of AMs as sources of IL-10 both *in vitro* and *in vivo*.

Methods

Allergic airways disease (AAD) was induced in adult mice by intranasal administration of house dust mite extract (HDM) or PBS for controls for 1-3 weeks. In some experiments, mice received either intranasal anti-IL10RB or isotype control twice weekly throughout the model. AMs were obtained by bronchoalveolar lavage of naïve mice, enriched by adhesion and stimulated *in vitro*.

Results

Assessment of the cellular sources of IL-10 during AAD using IL-10 reporter mice revealed less than 2 % of AMs to be IL-10-competent *in vivo*. Instead, inflammatory macrophages and lymphocytes were the most abundant IL-10-producing cells in the allergic lung. Accordingly, purified AMs showed barely detectable levels of IL-10 mRNA when stimulated *in vitro* with LPS and/or IL-4. Furthermore, LPS-induced cytokine synthesis was not exaggerated in IL-10-deficient AMs, consistent with minimal autocrine IL-10 signalling after TLR4 ligation. However, pulmonary IL-10 was shown to be an important component of the immune response to inhaled allergen. Local antibody blockade of IL-10 signalling throughout continuous HDM exposure induced a distinct, severe AAD phenotype marked by enhanced airway hyperresponsiveness, concomitant with decreased eosinophilia, exacerbated Th1 and Th17 responses and a lung-specific deficiency in Tregs.

Conclusions

IL-10 production is unlikely to be a major immunoregulatory mechanism employed by AMs during AAD. However, local IL-10 signalling clearly controls the balance of T cell populations in the allergic lung.

Understanding the relationship between Interleukin-10 and airway macrophages in regulating allergic airways disease

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Introduction

Airway macrophages (AMs) contribute to maintenance of immune tolerance in the healthy lung and their dysregulation may facilitate allergic airway inflammation such as that in asthma. Although the factors underlying AM tolerance are incompletely understood, they are thought to include production of immunoregulatory cytokines. Here we sought to investigate the role of the prototypic regulatory cytokine IL-10 in allergic airways disease (AAD) and assess the potential of AMs as a source of IL-10.

Methods

Allergic airways disease (AAD) was induced in adult mice by intranasal administration of house dust mite extract (HDM) 5 times weekly for 1 or 3 weeks. In some experiments, mice received intranasal anti-IL10RB blocking antibody or isotype control twice weekly throughout the regimen. AMs, peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMDMs) were obtained from naïve mice and stimulated *in vitro* with LPS.

Results

Using 10BIT IL-10 reporter mice we found less than 2 % of AMs to be IL-10-competent *in vivo* during AAD. Instead, inflammatory macrophages/monocytes and lymphocytes were the most abundant IL-10-producing cells in the allergic lung. Accordingly, stimulation of purified AMs with HDM extract or LPS, an immunologically active component of HDM, induced little to no IL-10 mRNA or protein expression. This was in marked contrast to other macrophage populations, PMs and BMDMs, which contained readily detectable levels of IL-10 mRNA at baseline and secreted IL-10 following LPS treatment.

Although pulmonary IL-10 was largely not AM-derived, it was a key regulator of the immune response to inhaled allergen. Local blockade of IL-10 signalling throughout continuous HDM exposure induced a distinct, severe AAD phenotype marked by enhanced airway hyperresponsiveness; concomitant with decreased eosinophilia, exacerbated Th1 and Th17 responses and a lung-specific deficiency in Tregs. This T cell imbalance was also accompanied by elevated pulmonary IFN γ levels and increased MHC-II expression on AMs.

Conclusions

Local pulmonary IL-10 signalling controls the balance of T cell populations in the allergic lung and mitigates the severity of AAD. However, IL-10 production is unlikely to be a major immunoregulatory mechanism employed by AMs during AAD. We are currently exploring further regulatory mechanisms employed by AMs and the role of IL-10 from other cellular sources in regulating airway inflammation.

Cell-specific Sources of Immunoregulatory Cytokines in Allergic Airway Inflammation

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Discrimination between benign and harmful inhaled particles is critical for maintenance of healthy airway function. Regulatory T cells and CD11c⁺ Siglec F⁺ Airway macrophages (AM) are thought to regulate pulmonary immune responses to inhaled allergens. However, the role of the key mucosal regulatory cytokines TGF- β 1 and IL-10 in these processes is unclear.

Murine AM were shown to express high levels of TGF- β 1, both *in vitro* and *in vivo*. In contrast, IL-10 was largely undetectable in AM, at baseline, after *in vitro* LPS stimulation and during house dust mite (HDM)-elicited allergic airways disease (AAD). Instead, FoxP3⁻ CD4⁺ T cells were the major IL-10-producing cells in the allergic lung. Accordingly, CD11c-Cre-driven knockout of IL-10 from AM and dendritic cells (DC) did not affect AAD severity. In contrast, intranasal IL-10 receptor blockade or CD4-Cre-driven IL-10 knockout resulted in an enhanced, Th1-skewed, AAD phenotype. Thus, pulmonary IL-10 from CD4⁺ T cells regulates T cell responses to HDM exposure.

In contrast to IL-10, CD11c-Cre-driven TGF- β 1 knockout in AM and DC resulted in enhanced airway hyperresponsiveness and increased pulmonary Th2 cell numbers following sustained HDM exposure, independently of increased DC numbers in lungs or mediastinal lymph nodes. These findings suggest a role for AM-derived TGF- β 1 in regulating type two immunity to inhaled allergen.

These data suggest that AM-derived TGF- β 1, rather than IL-10, is a key regulator of allergic airway inflammation. However, IL-10, predominantly derived from FoxP3⁻ CD4⁺ T cells, is crucial for control of T cell subsets in the allergic lung. Thus, regulatory cytokines in the lung operate within cell type-specific niches, further understanding of which may provide novel insights into control of pulmonary inflammation.

T cell-derived interleukin-10 regulates airway macrophage activation and T helper cell polarisation to limit the severity of allergic airway disease

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Background:

Interleukin-10 (IL-10) is a key modulator of immunity at mucosal surfaces, including the lung, where IL-10 regulates responses to inhaled allergens and respiratory pathogens. The mechanisms of immunoregulation by IL-10 during allergic airway disease (AAD) are incompletely understood, with T cells, stromal cells and airway macrophages (AM) all suggested to be important cellular sources. In this study, we aimed to dissect the cellular sources and functions of IL-10 in the allergic lung.

Methods:

C57BL/6, IL-10 reporter (10BiT), CD11c-Cre x *Il10*^{flox/flox} or CD4-Cre x *Il10*^{flox/flox} mice were exposed to house dust mite extract (HDM) via daily inhalation for 3 weeks. Intranasally administered anti-IL10RB antibody was used to block pulmonary IL-10 signalling.

Results:

CD4⁺ T lymphocytes were the major IL-10 producing cells in lungs of HDM-treated mice. IL-10⁺ CD4⁺ T cells displayed a highly activated phenotype and consisted of both FoxP3⁺ (20 %) and FoxP3⁻ (80 %) cells. Conversely, CD11c⁺ Siglec F⁺ AM, key regulatory cells in the allergic lung, showed little to no IL-10 reporter signal or gene expression. Accordingly, conditional knockout of IL-10 in CD11c⁺ cells did not affect AAD, suggesting that the regulatory function of AM is independent of IL-10 production. In contrast, local blockade by antibody or CD4⁺ cell-targeted deletion of IL-10 caused a severe Th1^{high} eosinophil^{low} AAD phenotype, featuring reduced pulmonary FoxP3⁺ Treg numbers and enhanced airway hyperresponsiveness. Augmented AAD was also accompanied by marked classical activation of AM, suggesting that aberrant T cell-macrophage interactions occur in the absence of IL-10.

Conclusions:

We have demonstrated a context-specific role for CD4⁺ T cell-derived IL-10 in mitigating the severity of HDM-driven allergic lung inflammation by limiting skewing towards type 1 immunity and promoting pulmonary Tregs. We are now pursuing the mechanisms underlying this regulation, including suppression of airway macrophage activation by CD4⁺ T cell-derived IL-10.