The regulation of lung homeostasis and influenza-associated inflammation

Thesis submitted for the degree of Doctor of Philosophy in the Department of Medicine, Imperial College London

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Statement of originality

All work presented in this thesis was performed by me, except where indicated in the text. Specifically, data for Figure 3.5C was provided by Dr. John Goulding and data for Figure 3.15E was provided by Dr. Robert Snelgrove. Experiments were carried out at the Kennedy Institute of Rheumatology from January to July 2008 and at the National Heart and Lung Institute from July 2008 to June 2011.
Abstract

Alveolar macrophages are the main cell population in the naïve airway and are held in a state of tight regulation by several suppressive mechanisms. One would expect them to display a regulatory phenotype but here we show that at homeostasis they express markers of alternative activation such as YM1 and mannose receptor (MR) but not resistin-like molecule (RELM)-α. We show also that these markers are differentially regulated during influenza infection on macrophage populations in the lungs and airways.

We hypothesised that removing the suppressive effects of IL-10 using an IL-10R blocking antibody would alter alveolar macrophage phenotype and the immune response to influenza infection. We now demonstrate that IL-10R blockade does not significantly alter the phenotype of alveolar macrophages at homeostasis but does increase in their numbers and infiltrate of monocyte/macrophages and T cells into the airways during a subsequent influenza infection.

Blockade of the interaction between the co-stimulatory molecule GITR and its ligand GITRL is beneficial for disease outcome in mouse models of chronic lung inflammation; therefore we hypothesised that it may also abrogate influenza-associated immune pathology. We now show that GITR and GITRL are differentially expressed in the lungs and airways during influenza infection; however contrary to expectations, blockade of the interaction between the two accelerated influenza-induced weight loss and lung cellularity. This may indicate a novel regulatory role for GITRL in influenza-induced inflammation.

This thesis shows that alveolar macrophages represent an atypical alternatively activated macrophage population, whose phenotype is not altered by IL-10R blockade. However, we show that prior IL-10R blockade can alter the immune response to subsequent influenza infection, and blockade of GITRL during influenza infection may be detrimental for the outcome of influenza infection.
Dedication

I dedicate this thesis to my parents, who have always supported and inspired me.
Acknowledgements

Firstly, I would like to thank my supervisor, Professor Tracy Hussell, for her unwavering support and guidance and for giving me this fantastic opportunity. My co-supervisor, Dr. Erika Wissinger also deserves many thanks for her patience and support throughout my PhD. I am extremely grateful to Dr. Robert Snelgrove who trained me when I first joined the lab as a technician (sometimes at the expense of his own experiments). Drs. Samira Salek-Ardakani and Jodie Madden deserve special thanks for proof-reading thesis chapters – I hope it wasn’t too painful. My PhD experience would not have been nearly as memorable or enjoyable without the other amazing past and present members of Team Hussell: Lorna, Emily, Jose, Seema, Bobby, Alex Ramirez, Mary, Alex Godlee, Maryam, Gang, Tosh and Rob Sanders. Thank you all for your help with various experiments and for making the lab a fun and productive working environment.

Lastly, I would like to thank my friends, family and my fiancé Trey, without whose love and encouragement this thesis might not have been completed.

Daphne
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<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AITR</td>
<td>Activation induced tumour necrosis factor receptor</td>
</tr>
<tr>
<td>AITRL</td>
<td>Activation induced tumour necrosis factor receptor ligand</td>
</tr>
<tr>
<td>AMCase</td>
<td>Acidic mammalian chitinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C Chemokine ligand 2</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C Chemokine receptor 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CR3/4</td>
<td>Complement receptor 3/4</td>
</tr>
<tr>
<td>CSIF</td>
<td>Cytokine synthesis inhibitory factor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DS</td>
<td>Double stranded</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extra-cellular signal related kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallisable</td>
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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FcεR</td>
<td>Fc epsilon receptor</td>
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<td>FITC</td>
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<td>GATA-3</td>
<td>GATA binding protein 3</td>
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<td>GITR</td>
<td>Glucocorticoid-inducible tumour necrosis factor receptor super family related protein</td>
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<td>GITRL</td>
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<td>GMCSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
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<td>HA, H</td>
<td>Haemagglutinin</td>
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<td>HIV</td>
<td>Human immune deficiency virus</td>
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<td>High mobility group B</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Inter-cellular adhesion molecule</td>
</tr>
<tr>
<td>ICOS</td>
<td>Inducible co-stimulator</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>Inducible nitric oxide synthase</td>
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<tr>
<td>JAK</td>
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<tr>
<td>JNK</td>
<td>C-jun n-terminal kinase</td>
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<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
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<td>LPS</td>
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<td>Molar</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>MARCO</td>
<td>Macrophage receptor with collagenous structure</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MCSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine darby canine kidney</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallo-protease</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
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<td>Neuraminidase</td>
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<tr>
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<td>Nuclear factor kappa B</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<td>NLR</td>
<td>Nucleotide oligomerisation domain-like receptor</td>
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<td>Nitric oxide</td>
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<td>Natural T regulatory cell</td>
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<td>Ovalbumin</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
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<tr>
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<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCp</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>Polyl:C</td>
<td>Polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
</tbody>
</table>
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T cell expresses and secreted</td>
</tr>
<tr>
<td>RELM-α</td>
<td>Resistin-like molecule-alpha</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic acid inducible gene-I like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RORγt</td>
<td>Retinoid orphan receptor gamma t</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIRP</td>
<td>Signal inhibitory regulatory protein</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Sp</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>SS</td>
<td>Single stranded</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIP DC</td>
<td>Tumour necrosis factor-α producing inducible nitric oxide synthase producing dendritic cells</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzimidine</td>
</tr>
<tr>
<td>TNFRSF</td>
<td>Tumour necrosis factor receptor super family</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/interleukin-1 receptor inducing interferon-γ</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
</tbody>
</table>
1.0 Introduction

With a surface area of 70m$^2$, the human lungs provide a huge interface which is exposed to the outside environment. Approximately 500cm$^3$ of air is taken in with each breath, which is loaded with antigen from pathogenic micro-organisms, allergens, pollutants, viruses and harmless plant and animal proteins, and the lungs must distinguish between what is harmful and what must be ignored. Immune responses to harmless inhaled antigen would mean that the average human being would die of chronic lung disease in childhood. However, the lungs must also recognise and kill invading pathogens, otherwise we would die of infections at a very early age. The lungs have developed several immune mechanisms to expel pathogens and tolerate harmless inhaled antigens which do not merit an immune response.

1.1 Innate immune mechanisms of the lung

The conducting airways provide a significant barrier against the entry of pathogens into the lungs. Inhaled particles are trapped in a mucous layer overlying the epithelium which itself is ciliated and functions to waft trapped particles up the airways to be expelled into the oropharynx. This prevents inhaled particulate antigen from entering further into the airways and also prevents it from coming into contact with the underlying epithelium. The mucin macromolecules that constitute the mucous layer are highly adapted to their function and due to the diversity in their carbohydrate side chains, can bind and trap a multitude of particles $^1$.

The mucous layer additionally contains secretory Immunoglobulin (Ig) A, the predominant antibody class found along mucous membranes $^2$. IgA has high valency for antigens and cross links large antigens, preventing their attachment to the epithelium. Complexes of secretory IgA-bound antigen are then trapped in the mucous layer and are eliminated by ciliary beat of the airway epithelium.
As well as expelling particles and microbes trapped in mucous from the airways, epithelial tight junctions further provide a physical barrier to prevent microbes gaining access to the lung parenchyma. The epithelium is coated in a glycocalyx layer which is negatively charged and is therefore a further deterrent for pathogens.

A periciliary liquid layer lies between the epithelium and mucous layer and contains anti-microbial compounds such as lactoferrin, lysozyme and anti-microbial defensins which halt microbial growth.

1.2 Cells of the innate immune system
Not all microbes are excluded by mucociliary export and therefore, cells of the immune system must recognise pathogenic microbes so that they can be cleared and if necessary an immune response can be mounted. Innate immune cells have evolved pattern recognition receptors (PRRs) to recognise pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components. PRRs expressed by cells of the innate immune system include Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and the nucleotide-binding oligomerisation domain- (NOD)-like receptors (NLRs). These PRRs recognise a range of microbial products, such as viral single stranded (ss) and double stranded (ds) RNA, bacterial cell wall components such as lipopolysaccharide (LPS) and lipotechoic acid (LTA), flagellin and viral proteins. Some of these pathogen receptors are summarised in Table 1.1. PRRs can also be activated by host proteins released under conditions of stress by dying or activated cells, known as damage associated molecular pattern proteins (DAMPs). DAMPs include host DNA, heat shock proteins, uric acid, high mobility group box 1, adenosine and the S100 family of proteins, all of which are capable of activating PRR responses.
<table>
<thead>
<tr>
<th>Pathogen receptor</th>
<th>Location</th>
<th>Pamp Sensed</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-1</td>
<td>Cell membrane</td>
<td>Bacterial lipoproteins</td>
</tr>
<tr>
<td>TLR-2</td>
<td>Cell membrane</td>
<td>Lipotechoic acid</td>
</tr>
<tr>
<td>TLR-3</td>
<td>Endosome</td>
<td>dsRNA</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Cell membrane</td>
<td>Certain viral proteins LPS</td>
</tr>
<tr>
<td>TLR-5</td>
<td>Cell membrane</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR-6</td>
<td>Cell membrane</td>
<td>Bacterial lipoproteins</td>
</tr>
<tr>
<td>TLR-7</td>
<td>Endosome</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR-8</td>
<td>Endosome</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR-9</td>
<td>Endosome</td>
<td>CpG DNA</td>
</tr>
<tr>
<td><strong>RLRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIG-I</td>
<td>Cytoplasm</td>
<td>Short dsRNA, ssRNA</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Cytoplasm</td>
<td>dsRNA</td>
</tr>
<tr>
<td><strong>NLRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD1</td>
<td>Cytoplasm</td>
<td>Peptidoglycan from gram−ve bacteria</td>
</tr>
<tr>
<td>NOD2</td>
<td>Cytoplasm</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>NLRC4</td>
<td>Cytoplasm</td>
<td>Flagellin</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Cytoplasm</td>
<td>Bacterial RNA, viral RNA, bacterial cell wall components</td>
</tr>
</tbody>
</table>

**Table 1.1 Pathogen recognition receptors and their ligands.** Some of the pathogen recognition receptors expressed by cells of the immune system. TLR – toll-like receptor. RLR – retinoid acid inducible gene (RIG)-I like receptor. NLR – nucleotide oligomerisation domain (NOD)-like receptor. Ds RNA (double stranded RNA), ssRNA (single stranded RNA).
This thesis will mainly focus on the phenotype of alveolar macrophages but other cell types important for defence of the lung will also be briefly discussed.

1.2.1 Epithelial cells

Epithelial cells represent the first line of defence against invading micro-organisms. As well as providing a physical barrier to the penetration of microbes into the lung parenchyma, mouse and human epithelial cells express TLRs 1-9 and NOD-1 and NOD-2 and can recognise and respond to a range of pathogens.

In response to the presence of a TLR or NLR stimulus, an inflammatory cascade is induced with the activation of nuclear factor kappa B (NFkB) and mitogen activated protein (MAP) kinases. This leads to the production of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8 (or the mouse ortholog macrophage inflammatory peptide (MIP)-2), granulocyte macrophage stimulating factor (GM-CSF) and a host of other cytokines and chemokines which activate resident alveolar macrophages and recruit other leukocytes to the airways. After stimulation, epithelial cells up-regulate the expression of cell adhesion molecules, enhancing the recruitment of cells to the airways and lungs. Furthermore, in response to invading microbes epithelial cells produce anti-microbial compounds such as β-defensins, cathelicidins, lactoferrin, lysozyme and lipocalin which directly kill a range of bacterial species.

1.2.2 Macrophages

Alveolar macrophages compose 90% of the cells in broncho-alveolar lavage (BAL) and are the main cell type in the alveolar space. It is thought that at homeostasis their numbers are renewed by local proliferation, demonstrated by mouse studies in which blood monocytes are depleted without affecting alveolar macrophage numbers; incidentally, peritoneal macrophage numbers are also unaffected, indicating that local proliferation is a means of maintaining tissue-resident macrophage populations.
Similarly, when the lungs are protected from whole body irradiation, alveolar macrophages are still of host origin 45 weeks later \(^{24}\). They are, therefore a long-lived population. It is known that alveolar macrophages proliferate in response to macrophage colony stimulating factor (MCSF) and GMCSF which are produced by epithelium at homeostasis and during inflammation \(^{25}\). When mice are subjected to whole body irradiation, however, the majority of macrophages in the airways are of donor origin 60 days after bone marrow transfer, indicating that alveolar macrophage numbers can also be replenished from the bone marrow \(^{26}\). Human studies, where patients have received allogenic bone marrow transfers, show similar repopulation of alveolar macrophages 81 days after bone marrow transplant \(^{27,28}\). Other studies show that depletion of alveolar macrophages results in their re-population by donor blood monocytes \(^{29}\). During inflammation, monocytes are recruited into the airways via the CC chemokine receptor 2 (CCR2)/CC chemokine (CCL2) axis \(^{30}\).

The main function of alveolar macrophages is to phagocytose invading pathogens and particulate matter, whilst preventing the activation of a systemic immune response. It is thought that a systemic immune response to microbes in the airways is only achieved once the phagocytic capabilities of alveolar macrophages are overcome \(^{31}\). Alveolar macrophages express various scavenger receptors such as macrophage receptor with collagenous structure (MARCO), the mannose receptor (MR) and Fcy receptors, which aid the phagocytosis of a range of bacterial species \(^{32}\). Table 1.2 summarises the main macrophage phagocytic receptor families. Alveolar macrophages further express extracellular and intracellular TLRs, NLRs and RIG-I receptors to recognise PAMPs on microbial species \(^{3}\). Activation through PRRs enhances the phagocytic capacities of alveolar macrophages, demonstrated after stimulation of TLR-4 with LPS \(^{33}\).
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<table>
<thead>
<tr>
<th>Receptor family</th>
<th>Example</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin</td>
<td>CD11c (CR4), CD11b (CR3)</td>
<td>Binds C3b and mediates complement-mediated phagocytosis Adhesion to epithelium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig Superfamily</td>
<td>Fc receptors</td>
<td>Antibody-mediated phagocytosis</td>
</tr>
<tr>
<td>Scavenger receptors</td>
<td>Scavenger receptor A</td>
<td>Bacterial phagocytosis</td>
</tr>
<tr>
<td>C-type lectin receptors</td>
<td>Mannose receptor</td>
<td>Recognise microbial ligands and mediate phagocytosis of certain microbes and endogenous glycoproteins</td>
</tr>
</tbody>
</table>

Table 1.2 Macrophage receptors involved in immune recognition and phagocytosis. Adapted from 32. CR: complement receptor.
Alveolar macrophages are relatively poor antigen presenters compared to other tissue-resident macrophages \(^{34,35}\), and are not efficient at inducing a T cell response to whole antigen \(^{36-38}\). However, alveolar macrophages can induce a robust immune response on activation of PRRs, with production of IL-12, TNF-\(\alpha\), interferon-gamma (IFN-\(\gamma\)) and monocyte chemotactic protein (MCP)-1 \(^{39,40}\). Monocytes recruited into the airways are inflammatory and contribute to the phagocytosis of dead and dying cells as well as invading pathogens \(^{41,42}\).

The lung parenchyma contains another macrophage population termed interstitial macrophages. These macrophages are morphologically similar to alveolar macrophages but are slightly smaller, resembling blood monocytes \(^{29,43}\). Interstitial macrophages are equally as phagocytic as alveolar macrophages, but have a better antigen presenting capacity, with higher expression of major histocompatibility complex (MHC) class II \(^{44}\). Presumably, if microbes enter the lung parenchyma it is vital for interstitial macrophages to be able to instigate a robust immune response and contain the infection.

Depending on the inflammatory stimulus or cytokine milieu they are subject to, macrophages can adopt different activation states with distinct functionality. The factors that affect macrophage phenotype can be derived from other immune cells, such as natural killer (NK) cells or T cells, or they can themselves produce factors that affect their own phenotype and function in an autocrine manner. The different macrophage subsets are discussed in the following sections.

1.2.2.1 Classically activated macrophages

Classically activated macrophages are the best studied macrophage phenotype, resulting from stimulation of macrophages with IFN-\(\gamma\) and/or TNF-\(\alpha\) in addition to a TLR stimulus \(^{45,46}\). They are vital for the control of intracellular pathogens, and mice lacking
IFN-γ are highly susceptible to multiple bacterial, protozoal and viral infections, as are humans with disrupted IFN-γ signalling \(^\text{47}\).

The main source of IFN-γ during early innate immunity is NK cells that produce it in response to stress and infection \(^\text{48, 49}\). As the immune response progresses, however, IFN-γ and TNF-α are likely to be produced by T cells, and therefore the adaptive immune response perpetuates the classical activation of macrophages. IFN-γ-activated macrophages produce large amounts of anti-microbial reactive oxygen species (ROS) and reactive nitrogen species (RNS) and IL-12 which activates T cells and NK cells \(^\text{49, 50}\).

Classically activated macrophages also produce IL-6, IL-23 and IL-1, which contribute to the generation of T helper (Th) 17 cells \(^\text{51}\), whose production of IL-17 in turn recruits neutrophils to the site of infection \(^\text{52}\). Whilst important for the clearance of invading pathogens, these macrophages mediate tissue pathology if unrestrained, exemplified by their role in tissue pathology in rheumatoid arthritis \(^\text{53}\).

### 1.2.2.2 Alternatively activated macrophages

IL-4 and IL-13 are the main factors responsible for inducing alternatively activated macrophages, which are also known as wound healing macrophages \(^\text{45}\). Alternatively activated macrophages are thought to be involved in tissue repair after inflammation. IL-4 appears to be one of the main cytokines produced on tissue damage \(^\text{54}\). Alternatively activated macrophages are usually observed in Th2 inflammatory conditions such as asthma, allergy and parasite infections \(^\text{55-58}\).

They are characterised by high expression of the scavenger receptor MR, the chitinase-like molecule YM1 \(^\text{59-61}\), acidic mammalian chitinase (AMCase) and resistin-like molecule alpha (RELM-α) \(^\text{59, 60, 62}\). Alternatively activated macrophages also express arginase, which competes with the classically activated macrophage-associated inducible nitric oxide synthase (iNOS) for arginine metabolism \(^\text{61, 63, 64}\).
Arginase catalyses the conversion of arginine to ornithine, which is a precursor of polyamines and collagen, and contributes to the synthesis of the extra cellular matrix. RELM-α stimulates collagen 1 and alpha smooth muscle actin expression by lung fibroblasts that contribute to airway remodelling and fibrosis. YM1 is proposed to have carbohydrate and matrix binding activity and to mask lectin binding sites, preventing entry of cells into inflamed tissues.

Alternatively activated macrophages clearly have roles in tissue repair, but are also known to contribute to the clearance of certain pathogens. For example, they are integral for the clearance of the gut nematode *Heligmosomoides plesyurus* and *Schistosoma mansoni*. Additionally, they inhibit T cell proliferation by cell-cell contact mechanisms including the expression of programmed death ligand 1 (PDL-1) and the production of RELM-α, which negatively regulates Th2 cytokine production.

Evidence that alveolar macrophages express YM1 and MR indicates that they may be intrinsically alternatively activated at homeostasis, an idea that will be further explored in this thesis.

### 1.2.2.3 Regulatory macrophages

Regulatory macrophages are characterised by the production of IL-10 and differentiate in response to different conditions. Ligation of macrophage Fc receptors reverses TLR-induced IL-12 production and stimulates IL-10 production. Furthermore, macrophage phagocytosis of IgG- or C3bi-opsonised erythrocytes or apoptotic neutrophils enhances IL-10 and reduces IL-12 production. Regulatory macrophages are necessary to limit TLR-induced inflammation, in order to avoid excess immune pathology. However, they can also be detrimental to the outcome of infection, as in the case of *Leishmania major* infection, where opsonised amastigotes stimulate Fc receptor-mediated production of IL-10, which delays clearance of the pathogen. Similarly, intra-tracheal administration of apoptotic cells prior to intra-nasal
infection with *Streptococcus pneumoniae* leads to reduced clearance of the bacteria accompanied by IL-10 production by macrophages, which is dependent on prostaglandin (PG) E$_2$. Others show that phagocytosis of apoptotic cells enhances the production transforming growth factor (TGF)-β by macrophages and reduces LPS-induced TNF-α production, which is also dependent on PGE$_2$. The role of opsonised antigen and apoptotic cells in deactivating macrophages has been widely studied, but other factors are capable of inducing macrophages with a regulatory phenotype. Adenosine stimulation reduces IL-12 and enhances IL-10 production by macrophages. Despite generally described as being immune suppressive, regulatory macrophages retain the expression of the co-accessory molecules MHC class II and CD86, and are able to activate T cell responses *in vitro*.

The different macrophage activation states and the signals that drive their differentiation are summarised in Figure 1.1.

Macrophages differentiated under a particular condition are not terminally differentiated. Many studies show that alternatively activated macrophages can adopt a classically activated phenotype with the appropriate stimulus. For example, alternatively activated peritoneal macrophages become classically activated upon stimulation with IFN-γ and LPS.

### 1.2.2.4 Macrophage subset nomenclature

In human studies, classically activated macrophages are referred to as M1 macrophages and alternatively activated (wound healing) macrophages as M2a. Some human studies distinguish between IL-10-producing macrophages based on the stimulus used: TLR/immune complex-stimulated macrophages are referred to as M2b and macrophages stimulated with glucocorticoids referred to as M2c.
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Figure 1.1 Macrophage subsets. Macrophages can differentiate into different subsets with distinct cytokine profiles depending on the cytokine and TLR stimulus provided. Adapted from 91.
However this thesis will continue to refer to the three groups of macrophages as classically activated, alternatively activated and regulatory macrophages.

1.2.3 Dendritic cells

Dendritic cells (DCs) are the main antigen presenting cell (APC) population in the lungs and there are several different DC populations identified in the lungs, each with distinct functions. They loosely fall into two categories: conventional DCs and plasmacytoid DCs. Plasmacytoid DCs (pDCs) make up a small population in the lung interstitium and large conducting airways. Different subsets of conventional DCs are distinguished by the expression of the integrins CD11c and CD11b. Intra-epithelial DCs express CD11c and the integrin CD103 but not CD11b. These cells lie on the baso-lateral side of the airways just beneath the epithelium, and extend their dendritic processes into the airway lumen to sample antigen in the air space. The lung interstitium contains another DC subset, resident CD11b$^+$ CD11c$^+$ CD103$^-$ DCs. Conventional DCs migrate to the lymph nodes during infection and inflammation via the CCR5/CCL5 axis and are capable of inducing a T cell response. During influenza infection, these cells also produce TNF-α and iNOS and in the spleen are called TNF-α-producing iNOS-producing DCs (TIP Dcs). pDCs express TLRs 3 and 7 and produce vast amounts of IFN-α during viral infections, playing a vital role in the clearance of respiratory viruses.

Another population of DCs has recently been described in tumours which secrete IFN-γ and are called IFN-γ producing killer DCs; this population is also observed in the lungs following inflammatory stimuli. IFN-γ-producing killer DCs express markers of NK cells but have the ability to present antigen to T cells and activate a T cell response.

1.2.4 NK cells

NK cells are innate lymphocytes which make up 10% of all the lymphocytes in the uninfected lung \(^{100}\), but are also recruited to the lungs from the blood during infection \(^{101}\). NK cells express activating receptors which recognise pathogen glycoproteins \(^{102}\) and molecules expressed on the surface of infected cells \(^{103, 104}\), and inhibitory receptors which recognise MHC class I molecules; the loss of inhibitory molecules leads to NK cell activation \(^{105, 106}\). Inflammatory cytokines such as type 1 IFNs and IL-12 activate NK cells, whereupon they release cytolytic granules to kill infected cells and secrete IFN-γ which activates T cells \(^{107}\). Secretion of IL-2 and/or IFN-γ by NK cells is necessary for robust CD8\(^+\) T cell responses \(^{48}\). NK cells can also produce IL-10 to suppress inflammation \(^{108}\).

1.2.5 Granulocytes

Neutrophils, eosinophils and basophils make up the granulocytes, and are characterised by cytoplasmic granules containing lytic enzymes and bactericidal substances. Neutrophils form an integral part of the host’s defence against invading micro-organisms. They are highly phagocytic and recognise a variety of micro-organisms via the expression of TLRs. Neutrophils have highly microbicidal activity due to the production of reactive oxygen species following the uptake of pathogens, and the release of degradative enzymes such as serine proteases, metalloproteases and lysozyme. Neutrophils also release pro-inflammatory cytokines such as TNF-α and IL-12, which prime the macrophage response \(^{109}\). Eosinophils are important for the immune response against parasites and also mediate allergy. When activated, they degranulate to release their granule contents such as histamine, eosinophil peroxidase and plasminogen, which are toxic to parasites. Basophils are involved in the immune response against parasites and can degranulate to release histamine and proteolytic enzymes \(^{110}\). They can also instruct the T cell response by the production of IL-4 \(^{111}\).
1.3 Cells of the adaptive immune system

The adaptive immune system consists of the humoral arm and the cellular arm. The humoral arm is mediated by antibody produced by B cells. This thesis will focus on the cellular arm of the adaptive immune system.

1.3.1 T cells

T cells mature in the thymus and consist of two populations, the CD4 positive and the CD8 positive populations, which co-express CD3. CD4+ T cells are T helper cells (Th) whose main function is to secrete cytokines that educate other immune cells to respond to infection, and provide the signals to B cells to enhance antibody production. CD4+ T cells recognise antigen loaded onto MHC class II on APCs via specific interactions with their surface-expressed T cell receptor (TCR), which enables them to respond to infection.

CD8+ T cells interact with antigen-MHC class 1 complexes on the surface of virally infected cells or tumour cells, whereupon they differentiate into cytotoxic effector cells. CD8+ effector T cells kill target cells via the release of cytolytic granules containing perforin monomers that polymerise to form cylindrical pores on the target cell membrane. Granzyme is then released into the target cell, which activates apoptotic pathways in the target cell. Another mechanism by which CD8+ T cells kill target cell is via the Fas/Fas ligand (FasL) pathway. FasL on the surface of CD8+ T cells binds to Fas expressed on target cells, leading to the association of Fas with Fas-associated protein with death domain (FADD), which activates a caspase cascade that ultimately leads to apoptosis of the target cells. MHC class II restricted CD4+ T cells are also reported to have cytolytic activity \textit{in vitro} and \textit{in vivo} during viral infections.
1.3.2 T cell activation

T cell activation requires two distinct signals; firstly TCR recognition of antigen-MHC complexes, and a co-stimulatory signal, which is provided by interaction between molecules on the surface of the APC and the T cell.

The TCR consists of αβ or γδ heterodimers. αβ T cells are much more common than γδ T cells, although the latter are usually found at mucosal surfaces. The TCR closely associates with the CD3 complex, which is necessary for the recognition of antigen by the TCR. CD4 and CD8 act as co-receptors, interacting with MHC molecules, and participating in signal transduction. The TCR complex, consisting of CD3 and CD4/CD8, recognises antigen in complex with MHC molecules on the surface of APCs. However, this interaction is insufficient to activate naïve T cells and TCR/MHC activation in the absence of co-stimulation results in T cell anergy.

Co-stimulatory signals are provided by several molecules, the most studied of which are CD28 and cytotoxic T lymphocyte antigen 4 (CTLA4) on the surface of T cells that interact with the B7 molecules on the surface of the APC. Naïve T cells express CD28, and binding of B7 results in proliferation and activation of the effector functions of T cells when the MHC:peptide complex is also recognised. CTLA4 is up-regulated by activated T cells, and its interaction with B7 is inhibitory and down-regulates the activity of the T cell. CD28 co-stimulation is vital for T cell activation; however there exist several other co-stimulatory molecules which contribute to T cell activation. It is proposed that certain co-stimulatory molecules might be required for T cell activation and/or survival at different time points after TCR activation.

Two main classes of co-stimulatory molecules have been studied; the immunoglobulin super family, for example, CD28 and the TNF receptor super family (TNFRSF). Members of the TNFRSF include 4-1BB, CD27 and OX40, all of which have roles in the survival of T cells. CD27 is constitutively expressed by T cells, with increased
expression upon activation \(^{119}\) and blockade of this molecule inhibits proliferation and cytokine secretion by activated T cells \(^{120}\). Additionally, CD27 deficient mice have impaired primary and secondary T cell responses to influenza infection \(^{121}\). OX40 is expressed by T cells 1 to 2 days after antigen encounter \(^{122}\), and signalling through OX40 enhances survival of T cells and boosts cytokine production \(^{123}\). This indicates that OX40 plays a major role in the survival of T cells. Similarly, 4-1BB ligand deficient mice have fewer antigen-specific T cells and fewer memory T cells upon infection \textit{in vivo} \(^{124}\).

1.3.3 T cell subsets

Depending on the antigenic stimulus and resulting cytokine milieu CD4 T cells can differentiate into a number of T cell subsets with distinct cytokine profiles and effector functions.

1.3.3.1 Th1 cells

Th1 cells are induced in response to viral and bacterial infections and autoimmune disease and are characterised by the production of large amounts of IFN-γ \(^{125}\). Differentiation of T cells into Th1 cells is dependent upon DC and NK cell production of IL-12 in response to the invading micro-organism \(^{126}\). Th1 cell differentiation is controlled by the transcription factor Tbet, which induces the production of IFN-γ via activation of signal transducer and activator of transcription 4 (STAT4) \(^{127, 128}\). IFN-γ produced by Th1 cells activates innate immune cells such as macrophages and enhances CD8\(^+\) T cell cytotoxicity \(^{129-131}\). IFN-γ polarises the classical activation of macrophages, inducing their production of inflammatory cytokines such as TNF-α, enhances iNOS activity \(^{130}\) and directly inhibits the differentiation of other T helper subsets \(^{132}\).

Th1 responses are vital for the clearance of several respiratory pathogens, such as influenza. Treatment of influenza-infected mice with IFN-γ rescues mice from lethal
influenza infection by enhancing the NK cell response \(^{133}\). Respiratory syncytial virus (RSV) infection in humans skews the immune response away from a protective Th1 response to a Th2 response that impedes viral clearance \(^{134}\).

### 1.3.3.2 Th2 cells

Th2 cells are induced *in vivo* during allergy or parasitic infections. They are characterised by the production of IL-4, IL-5, IL-13 and IL-25. IL-4 induces B cells class switching to IgE production \(^{135}\). IL-5 recruits eosinophils \(^{136}\) and IL-13, along with IL-4, induce the production of mucous by goblet cells and broncho-constriction of airway smooth muscle cells \(^{137}\). Furthermore IL-4 and IL-13 induce the alternative activation of macrophages \(^45\). Th2 cells are induced by IL-4 activation of STAT6 and subsequently the transcription factor GATA-binding protein 3 (GATA3), which controls the transcription of Th2-associated cytokines \(^{138}-^{140}\). As with Th1 cells, Th2 cells can negatively regulate the development of other T cell subsets. Negative regulation of Th17 responses by Th2 cells is thought to be via the up-regulation of growth factor independent 1 (GFI1), a transcriptional repressor of Th17 generation \(^{141}\).

IL-4, required for the induction of Th2 responses, is thought to originate from basophils, which produce IL-4 upon FcεR crosslinking \(^{142}\), NK T cells, which can produce it upon TCR engagement \(^{143}\), or activated Th2 cells themselves \(^{144}\). Interestingly, DCs are capable of inducing Th2 responses, despite the fact that they are not known to produce significant amounts of IL-4 \(^{145}\). Aside from IL-4, a few other cytokines are known to promote Th2 responses. Thymic stromal lymphopoietin (TSLP) produced by epithelial cells, mast cells and basophils induces Th2 cytokines production, suppresses IL-12 production and enhances the ability of DCs to prime a Th2 response \(^{146},^{147}\). IL-25 induces IL-4, IL-5 and IL-13 production by naive T cells \(^{148}\) and IL-33 induces IL-5 and IL-13 production by T cells and the polarisation of alternatively activated macrophages \(^{149}\).
Th2 cells dominate in asthma, secreting cytokines to recruit eosinophils, basophils and mast cells into the airway and amplifying the amount of pro-inflammatory mediators in the airways. Furthermore, the Th2 cytokine IL-13 is thought to be responsible for airway remodelling during allergic airways disease.

1.3.3.3 Th17 cells

Th17 cells produce IL-17, and are controlled by the orphan retinoid receptor RORγt transcription factor, the expression of which is under the control of STAT3. The transcription factors Runx and interferon regulatory factor 4 (IRF4) are important for the expression of RORγt in CD4+ T cells. The up-regulation of RORγt and thus the differentiation of murine Th17 cells is induced by IL-6 and TGF-β, although it is now known that IL-21 can substitute for IL-6 in inducing Th17 differentiation. Additionally IL-23, while unnecessary for the initial differentiation of Th17 cells, is necessary for their sustained differentiation and proliferation. However, some studies show that human Th17 cells require IL-23 and IL-1β but not TGF-β for their differentiation. Furthermore, TGF-β receptor knockout mice have Rorγt positive lamina propria CD4+ T cells, and naïve T cells differentiate into Th17 cells under the influence of IL-1β, IL-6 and IL-23 in the absence of TGF-β. Others propose a role for IL-18 in the induction of Th17 cells along with IL-23 and IL-1β. It is suggested that Th17 cells generated in the absence of TGF-β are more pathogenic in vivo compared to those generated in the presence of TGF-β.

As well as IL-17, Th17 cells secrete IL-21, which furthers their own differentiation and IL-22, which is involved in the innate immune response of non-haematopoietic cells. Th17 cells can also secrete TNF-α, express the chemokine receptor CCR6 and migrate to its ligand, CCL20 in mouse models of rheumatoid arthritis.

IL-17 recruits neutrophils and macrophages to the site of inflammation and is critical for the clearance of certain intra-cellular pathogens, such as pulmonary infections with...
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*Klebsiella pneumoniae*[^163] and *Pseudomonas aeruginosa* in mice[^164]. IL-17 reportedly plays a pathogenic role in various auto-immune diseases; for example, it is directly involved in bone and cartilage erosion in arthritis[^165] and is thought to play a role in the pathogenesis of experimentally-induced encephalitis (EAE)[^166]. CD8+ T cells can also produce IL-17 and represent a T cytotoxic (Tc) 17 population[^167].

DCs stimulated *in vitro* with a combination of apoptotic cells and TLR agonists produce IL-6, IL-23 and TGF-β, thereby inducing a Th17 response[^168]. This is one of the mechanisms thought to induce Th17 cells during infections with certain pathogens such as *Bacteroides fragilis* and *Citrobacter rodentium*, which induce apoptosis in infected cells[^169]. Additionally, *Mycobacterium tuberculosis* can induce Th17 responses *in vitro* and *in vivo*[^170].

A subset of human skin-homing T cells which secretes IL-22 but not Th17 has recently been described, and denoted Th22 cells[^171, 172]. IL-22 production by these cells is independent of RORγt[^171, 172]. It is not yet known if this subset of T cells is distinct from Th17 cells[^173].

### 1.3.3.4 Regulatory T cells (Tregs)

Tregs are a distinct T cell subset whose main function is to negatively regulate inflammation by the production of immune modulatory cytokines. Two main subsets of Tregs exist, depending on the way in which they are differentiated. Natural Tregs (nTregs) differentiate in the thymus, and inducible Tregs (iTregs) are found in secondary lymphoid organs and non-lymphoid tissue[^174]. Both iTregs and nTregs are characterised by the expression of the transcription factor forkhead box protein 3 (Foxp3). nTregs are generated in the thymus via mechanisms that are dependent upon interactions with MHC class II on the cortical epithelium of the thymus[^175]. The generation of iTregs is dependent on IL-2, TGF-β and TCR stimulation[^176, 177]. iTregs

[^163]: Page 38
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and nTregs suppress effector T cells by the production of IL-10 and TGF-β as well as cell contact mediated suppression by CTLA-4 expression \(^{178}\).

IL-10 and TCR stimulation drives the generation of Foxp3 negative regulatory T cells which produce IL-10 (Tr1 cells) \(^{179}\) or TGF-β (Th3 cells) \(^{180}\) that function to regulate infection-induced inflammation \(^{181}\).

Tregs are vital for regulating inflammation in respiratory syncytial virus (RSV) infections, and their depletion increases morbidity in murine infections \(^{182}\); transfer of Tregs into mice suffering from allergic airway inflammation reverses inflammation and remodelling \(^{183}\).

The generation of the different T cell subsets is depicted in Figure 1.2. Th1, Th2 and Th17 cells can also produce IL-10 and this represents a mechanism by which effector T cells can switch off their own activation.

1.3.3.5 Plasticity of T helper cell subsets

It has become clear that T helper cells that have differentiated into a certain T helper cell subset retain some plasticity with regards to cytokine production and the expression of subset-specific transcription factors. For example, Th17 cells generated in the absence of TGF-β express T-bet \(^{158}\), and highly purified Th17 cells transferred into non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice became IFN-γ-producing Th1 cells \textit{in vivo}, causing diabetes \(^{184}\). Another recent study describes Th17 cells \textit{in vivo} that convert to Th1 cells during the progression of experimental autoimmune encephalitis (EAE) \(^{185}\) and Foxp3\(^+\) CD4\(^+\) T cells that produce IL-17 have been described in the lamina propria of Crohn’s disease patients \(^{186}\). Furthermore, Th1 cells stimulated with ovalbumin and IL-18 have been shown to produce the Th2-associated cytokine IL-13 alongside IFN-γ \(^{187}\). These studies show that T cells already differentiated to become a particular T helper subset are able to switch phenotype depending on the cytokine milieu.
Figure 1.2 T cell subsets. T cells differentiate into distinct subsets with different cytokine profiles depending on the cytokine milieu. The different T cell subsets are reviewed in $^{173}$. 
1.4 Homeostatic regulation of inflammation in the lungs and airways

Epithelial cells represent the first line of defence against pathogens which infiltrate the airways. Additionally they are crucial for maintaining airway cells in a quiescent state so as to avoid unnecessary inflammation. Epithelial cells are therefore a key regulator of immune homeostasis in the airways and lungs.

Alveolar and bronchial epithelial cells produce surfactant proteins (SP) A, B, C and D which comprise 10% of pulmonary surfactant that is vital for maintaining appropriate surface tension in the airways, an absence of which leads to the collapse of the lung. Surfactant proteins also have immune modulatory properties. SP-A and SP-D bind to signal inhibitory regulatory protein (SIRP)-α on the surface of alveolar macrophages, reducing Fcγ receptor-mediated phagocytosis, and inhibiting TNF-α production. However, SP-A and SP-D can also enhance phagocytosis by opsonising apoptotic cells. In this way surfactant proteins prevent inflammation during phagocytosis of apoptotic cells by alveolar macrophages. Interestingly, mice lacking SP-A develop low-grade lung inflammation and age-dependent emphysema.

Epithelial cells produce IL-10 which regulates the activity of alveolar macrophages and DCs by diminishing MHC class II expression, therefore lowering their activation status and ability to present antigen. They express the integrin αvβ6 which catalyses the conversion of locally produced latent TGF-β into its active form. Alveolar macrophages are in close proximity to the epithelium and are therefore subject to the anti-inflammatory effects of TGF-β. Furthermore, mice lacking the β6 integrin have enhanced expression of matrix metal protease (MMP) 12 in alveolar macrophages and develop age-dependent emphysema.

The epithelium exerts a further suppressive mechanism in the airways via the myeloid inhibitory molecule CD200, which is expressed on the luminal surface of the
epithelium. The ligand for CD200, CD200 receptor (CD200R) is highly expressed by alveolar macrophages and when bound to CD200, imparts a negative signal to alveolar macrophages, resulting in the down-regulation of inflammatory cytokines. Alveolar macrophages from CD200R knockout mice have a lower threshold of activation than wild type mice and produce inflammatory cytokines to relatively low levels of inflammatory stimulus.

Alveolar macrophages, as well as being highly regulated at homeostasis, can also regulate adaptive immunity in the lungs. For example, depletion of alveolar macrophages is associated with enhanced inflammation in the lungs and airways and enhanced T cell responses to inhaled antigens. Additionally, alveolar macrophages sequester particulate inhaled antigen by phagocytosis. This prevents DCs gaining access to antigen and therefore prevents the induction of a T cell response. DCs induce a T cell response only when the phagocytic capabilities of alveolar macrophages have been compromised. Like the epithelium, alveolar and lung interstitial macrophages secrete IL-10 at homeostasis, which inhibits the maturation and migration of antigen-loaded DCs.

Treg-derived IL-10 is also important for regulation of airway homeostasis. Mice with IL-10 deficient Treg cells display increased cellular infiltrate in the lungs and airways at homeostasis compared to wild type littermate controls. Additionally, these mice have more severe inflammation in models ovalbumin-induced allergy.
1.5 Influenza virus

1.5.1 Epidemiology

Influenza is a major cause of morbidity and mortality worldwide, with approximately 20% of children and 5% of adults affected each year. Despite developments in vaccines and anti-viral drugs, seasonal influenza epidemics still occur each year, resulting in approximately 12000 fatalities in England and Wales, and affecting 5 – 10% of the population. In the last century there were three influenza pandemics, from influenza viruses of different haemagglutinin (H) and neuraminidase (N) subtypes. Pandemics arose in 1968 (H3N2), 1957 (H2N2) and the 1918 (H1N1) pandemic (or Spanish flu) which was the most devastating, and killed approximately 50 million people worldwide. This century has so far seen the 2009 “swine flu” pandemic (H1N1), which was first documented in Mexico in April 2009 and was thought to have infected over 50 million people worldwide by December 2009. Swine flu cases were still being reported for autumn/winter 2010 although the full epidemiological data for this season is not yet available.

The elderly and immune compromised are usually the most susceptible to seasonal influenza epidemics and pandemics; however, the 2009 and 1918 pandemics disproportionately affected children and young adults compared to the older age groups. The resistance of the older population to the 2009 pandemic swine flu is hypothesised to be due to partial immunity in older people. Indeed, studies show that 33% of adults over the age of 60 have cross-reactive antibodies to H1N1.

Influenza infection can lead to symptoms ranging from mild to severe with myalgia, malaise, headaches, cough, sore throat and fever to viral pneumonia and acute respiratory distress. Certain pandemic influenza strains such as the pandemic 2009 swine flu can induce gastro-intestinal symptoms such as diarrhoea and vomiting.
1.5.2 Classification and structure

Influenza is an enveloped negative sense single stranded RNA virus of the Orthomyxoviridae family, with a segmented genome consisting of 8 genes encoding 10 proteins. There are 3 types, influenza A, B and C, though only influenza A and B have been implicated in seasonal epidemics. Influenza virus has two major surface glycoproteins, neuraminidase (NA) and haemagglutinin (HA), which form the basis for the classification of different virus strains and are the most immunogenic of the viral proteins. Influenza A viruses are sub-divided into 16HA subtypes (H1-16) and 9 NA subtypes (N1-9). All 16 HA and 9 NA subtypes are found in birds but so far only H1, 2, 3 and 5, and N1 and 2 are known to infect humans.  

The segmented nature of the influenza genome allows for reassortment between different virus strains which infect the same cell. For example, the pandemic swine flu is thought to have arisen after reassortment of swine, human and avian viruses. The process of reassortment of different virus strains to create a new strain is called antigenic shift and accounts for the annual nature of influenza epidemics. Antigenic drift can also occur, which is due to the error prone viral RNA polymerase that induces point mutations in viral proteins. Antigenic shift and drift in the HA and NA proteins leads to viruses which are not recognised by neutralising antibody from a previous infection, and can select for viruses which are resistant to anti-viral drugs.

1.5.3 Influenza replication cycle

HA and NA are important virulence factors. HA is necessary for attachment of the virus to sialic acid receptors on epithelial cells, and NA catalyses the cleavage of sialic acid residues, allowing virion progeny to be released from infected cells. Another important virulence factor is the M2 protein, which is an ion channel and acts to regulate the pH of endosomes in infected cells early on in infection. The NS1 proteins of certain influenza viruses such as H5N1 antagonise anti-viral interferons, contributing to their pathogenicity. Three polymerase subunits PB1, PB2 and PA carry out replication
and transcription of influenza virus RNA, which is encapsulated by viral nucleoprotein (NP).

The infective process begins with influenza virus binding to epithelial cells. HA preferentially binds to sialic acid linked to galactose. Human influenza viruses bind to sialic acid linked to galactose via an α2,6 linkage while avian influenza viruses bind to sialic acid linked to galactose via an α2,3 linkage. These specificities match the sialic acid residues on the respiratory epithelium of humans and birds; however, recent data suggests that α2,3 linked sialic acid receptors can be found deeper in the lung tissue of humans.

HA exists as a precursor form which must be cleaved by host proteases into HA1 and HA2. Once the virus has been taken up by the host cell via endocytosis, the vesicle fuses with endosomes and becomes progressively acidified by the action of M2 protein. HA1 and HA2 mediate the fusion of the virus with the endosome and consequently the release of viral nucleoprotein out of the endosome into the nucleus. Seasonal influenza virus HA molecules are cleaved at a conserved arginine, which restricts the proteases that can cleave it, and thus the tissues and cells the virus can infect. Highly pathogenic influenza viruses have a multi-basic cleavage site which is cleaved by a wide range of host proteases and this renders these viruses able to infect multiple cells and tissues outside of the respiratory tract.

Once in the nucleus, viral polymerases replicate viral RNA and synthesise mRNA. The mRNA is exported into the cytoplasm, where it is translated by host ribosomes. Early phase proteins such as the polymerase proteins, are transported back to the nucleus, where they function to amplify the replication of viral RNA. The rest of the viral proteins move to the golgi apparatus where they are glycosylated before assembly near the host cell membrane. The viral nucleoproteins assemble in the nucleus and aggregate with the structural proteins at the cell membrane, where they bud off the cell.
membrane. NA catalyses the cleavage of terminal sialic acid residues on the cell membrane and facilitates virus release. The replication of influenza virus is summarised in Figure 1.3.

1.5.4 Host response

Influenza virus is recognised by PRRs expressed by the innate immune system including TLRs 3, 7, 8 and 9, RLRs and NLRs, which sense viral RNA and proteins \(^{219, 220}\). Influenza-infected epithelial cells produce a range of chemokines, including regulated on activation, normal T cell expressed and secreted (RANTES), MCP-1, IL-8 and type 1 interferons such as IFN-α and IFN-β. IFN-α and β enhance antigen presentation by APCs, and cause up-regulation of MCP-1 and MCP-3, leading to recruitment of monocyte/macrophages \(^{49, 221}\). Macrophages produce RANTES, MCP-1, MCP-3, MIP-1α, and MIP-1β. Pro-inflammatory cytokines such as IFN-γ and TNF-α are produced by NK cells, macrophages and activated T cells \(^{222}\).

The extent of these early responses determines the extent of bystander tissue damage. Excess innate stimulation leads to an excess infiltrate into the lungs of T cells and other immune cells causing occlusion of the airways, in addition to the cell death caused by the virus itself. As vaccine strategies and antiviral drugs may have limited efficacy, attention has turned to the immune response itself, in an effort to reduce the lung pathology associated with influenza infections. Previous work in the laboratory has focused on regulating the inflammatory response to influenza infection, whilst maintaining viral clearance. For example, the blockade of TNF-α reduces pulmonary injury during influenza infection and ameliorates weight loss \(^{223}\).

Manipulation of OX40, an inducible late co-stimulatory molecule has shown promising results. It is up-regulated 1-2 days after antigen encounter and provides a positive signal to activated T cells, preventing activation-induced cell death (AICD) \(^{123}\).
Figure 1.3. The influenza virus replication cycle. 1. The virus attaches to host cells via HA binding to sialic acid residues on respiratory epithelium. 2. The virus is endocytosed and endocytic vesicles fuse with endosomes and M2 mediates the acidification of the endosome, which leads to the release of viral nucleoprotein. 3. HA fuses with the endosome and viral nucleoprotein is released into the cytoplasm and transported to the nucleus. 4. Viral polymerases transcribe mRNA and replicate viral RNA. 5. mRNA is translated in the cytoplasm by host ribosomes. 6. Early phase proteins are transported back to the nucleus and further amplify viral RNA. 7. Late phase proteins are glycosylated in the endoplasmic reticulum and golgi apparatus. 8 and 9. Late phase proteins aggregate with nucleoproteins at the cell membrane. 10. Assembled viral particles bud off the cell membrane.
Blockade of OX40 eliminates influenza-induced weight loss and cachexia and reduces cellular infiltrate into the lungs but importantly, does not impair viral clearance\textsuperscript{224}.

Clearly, it is possible to manipulate respiratory immunity to alleviate symptoms without compromising pathogen clearance. However, to date the laboratory has only tested OX40 and inducible co-stimulator (ICOS) manipulations, both co-stimulatory molecules of the TNF receptor super family. One was beneficial, the other was not. It is postulated that ICOS was less beneficial because it reduced the T cell response too far and impaired viral clearance\textsuperscript{225}. Similarly, others show that 4-1BB is vital for protection against influenza, as its absence leads to an inefficient T cell response and virus outgrowth\textsuperscript{124}. These data highlight the variable outcomes of manipulating co-stimulatory molecules in influenza infection, and to understand this better, interruption of other receptor/ligand pairs are required and will be discussed in this thesis.

1.5.5 Treatment and prevention

Two main classes of anti-viral drugs exist against influenza – the NA inhibitors and the ion channel inhibitors. NA inhibitors, such as zanamavir and oseltamivir, block the enzymatic activity of NA, preventing the release of progeny virion particles from infected cells. However, in recent years high rates of oseltamivir-resistant viruses are reported\textsuperscript{226, 227}. Rimantidine and amantadine block the activity of the M2 ion channel and thus the release of viral nucleoprotein into the cytoplasm, which is a vital part of the virus replication\textsuperscript{226}. However, influenza virus strains with resistance to these drugs are emerging\textsuperscript{226}. Other anti-viral drugs under development include a nucleoside analogue which inhibits the polymerase activity of influenza viruses\textsuperscript{228} and monoclonal antibodies against HA\textsuperscript{229}.

Existing vaccines are grown in fertilised hens’ eggs and inactivated by formaldehyde or β-propiolactone. Vaccines consist of whole inactivated forms of type A and B influenza virus, detergent treated split products or purified HA and NA antigen formulations of the
three virus strains recommended by the WHO. Protection is provided by the production of haemagglutinin specific antibodies, and efficacy rates vary but are usually between 50 and 90%. However, due to antigenic variation of influenza virus, it is difficult to anticipate what strains will emerge in future epidemics. New vaccines in development include live attenuated viruses, virosomes, which consist of the virus surface proteins on liposomes, and DNA vaccines. It is hoped that these new vaccines will be more immunogenic, with quicker production times.

1.6 IL-10 and IL-10 receptor

1.6.1 IL-10

IL-10 is a 178aa cytokine, which was first discovered as a cytokine produced by Th2 cells that was capable of inhibiting cytokine production by Th1 cells, hence it was originally called cytokine synthesis inhibitory factor (CSIF). Although IL-10 was discovered as a Th2-associated cytokine, it is now clear that multiple T cell subsets can produce it. IL-10 is produced by Foxp3 positive Treg cells in vivo, and mediates the immune suppressive effects of Tregs. IL-10 is also produced by Th1 cells which co-express IFN-γ. This is observed in vivo in mouse models of Toxoplasma gondii, RSV and influenza infections, where most of the IL-10 that is vital for limiting immune pathology is produced by Th1 cells which do not express Foxp3. Human studies also show IFN-γ and IL-10-producing CD4+ T cells in the BAL fluid of patients suffering from active pulmonary tuberculosis. Equally, a population of IL-12-dependent IL-10 and IFN-γ co-producing CD4+ T cells is reported in the blood of patients suffering from Lyme disease.

Macrophages and DCs produce IL-10 upon microbial stimulation or after ligation of CD40. Lung and alveolar macrophages produce IL-10 at homeostasis that alters
the ability of DC to induce allergic responses in mice. B cells, mast cells and eosinophils are also known to produce IL-10.

1.6.2 Regulation of IL-10 expression

IL-10 production by macrophages and myeloid DCs in response to TLR stimulation is dependent on the Toll/IL-1 receptor (TIR)-domain-containing adaptor molecule myeloid differentiation primary response protein 88 (MYD88) and TIR-domain-containing adaptor protein inducing IFN-β (TRIF). Deletion of TRIF and MYD88 abrogates TLR stimulation-induced production of IL-10 by murine macrophages in vitro. Similarly, deletion or blockade of MAPK attenuates IL-10 production upon stimulation of macrophages with TLR-4 and TLR-2 agonists. Similar results are reported for the production of IL-10 by macrophages upon stimulation with TLR-9 agonists.

Strong TCR stimulus and high IL-12 induce the production of IL-10 by Th1 cells in vitro. IL-10 production is dependent on STAT4 and extra-cellular signal-related kinases (ERK) 1 and 2 activation, while production of IL-10 by Th2 cells is thought to be under the control of the Th2-associated transcription factor GATA-3. However, the production of IL-10 by Th2 cells also requires ERK activation. IL-10 production by naïve T cells after TCR stimulation can also be induced by IL-21 and IL-27 via STAT1 and STAT3 activation.

1.6.3 Functions of IL-10

IL-10 has broadly immune suppressive effects on a range of cell types. For example, IL-10 suppresses IFN-γ, IL-2, TNF-α and GM-CSF production by Th1 cells and maintains expression of Foxp3 on Tregs. IL-10 inhibits LPS-induction of NFκB activation, the degradation of IkBα, the inhibitory subunit of NFκB, and DNA binding by NFκB. Others show that IL-10 negatively regulates the production of IL-17 by T cells and macrophages and suppresses expression of the Th17 transcription factor RORγt.
IL-10 decreases IL-6 and TNF-α production by activated macrophages \(^{256}\), inhibits the ability of monocytes to induce a T cell response \(^{257}\) by down-regulating their expression of MHC class II expression \(^{258, 259}\) and modulates the expression of the co-stimulatory molecule B7 on macrophages \(^{260-262}\). It decreases TLR-4-induced production of MIP-1α by human peripheral blood monocytes and alveolar macrophages \(^{263}\) and MIP-3α, MIP-3β \(^{264}\) and RANTES \(^{265}\) production by human monocytes. LPS-induced production of MIP-2, MCP-1 and MCP-5 by murine macrophages is reduced in the presence of IL-10 \(^{266}\), as is antigen presentation and subsequent T cell priming by bone marrow-derived macrophages \(^{267}\).

Addition of IL-10 to murine DC/T cell co-cultures partially inhibits the production of IFN-γ by Th1 cells \(^{268}\). It inhibits the expression of the co-stimulatory molecules CD80 and CD86 by human dermal dendritic cells and also reduces their ability to stimulate naïve T cells \(^{269}\). Additionally, IL-10 promotes the apoptosis of human epidermal DCs in culture \(^{270}\) and inhibits the maturation of human DCs \(^{271}\). It decreases IFN-γ-induced TNF-α and IL-12 production by DCs and attenuates the increase in CD80 and CD86 expression induced by IFN-γ \(^{272}\). Therefore, overall IL-10 reduces the ability of DCs to activate and prime a T cell response.

The inhibitory effects of IL-10 extend to mast cells, where Fc epsilon receptor (FceR) expression is reduced \(^{273, 274}\) and apoptosis in developing mast cells increased \(^{275}\). However, others show that IL-10 also stimulates the proliferation of differentiated mast cells, which implies that the effects of IL-10 may be dependent on the stage of their differentiation \(^{276}\). LPS-induced production of IL-8, TNF-α and IL-1β by human neutrophils is reduced by IL-10 \(^{277, 278}\) as is production of the chemokines MIP-1α and MIP-1β \(^{279}\). IL-10 also down-regulates the production of IFN-γ inducible protein 10 (IP-10) on stimulation of neutrophils with IFN-γ and LPS \(^{280}\).
In contrast to its effects on other immune cells, IL-10 promotes B cell activity by increasing MHC class II expression, viability \(^{281}\) and antibody production \(^{282-284}\). Therefore, for B cells IL-10 can act in a stimulatory manner to increase B cell activation.

The effect of IL-10 on different immune cell subsets is displayed in Figure 1.4.

Figure 1.4. Effects of IL-10 on immune cell subsets. IL-10 has broadly immune suppressive effects on immune cells, however, it enhances B cell responses and antibody production. Reviewed in \(^{285, 286}\).
1.6.4 IL-10 receptor

IL-10 receptor (IL-10R) is composed of two subunits and is a member of the class II cytokine receptor super family. It exists as a tetramer comprising 2 molecules each of IL-10R1 and IL-10R2. IL-10R1 constitutively interacts with Jak1 and IL-10R2 is associated with the JAK kinase Tyk2. Binding of IL-10 to its receptor activates Jak1 and Tyk2, which phosphorylate tyrosine residues in the extracellular domain of IL-10R1. These tyrosine residues serve as docking sites for STAT molecules (Figure 1.5).

IL-10R signalling leads to activation of STAT1 and STAT3 in T cells and monocytes. In B cells IL-10 signalling activates DNA binding activity of STAT5 as well as STAT1 and STAT3.

STAT3 is necessary for the anti-proliferative effects of IL-10 on murine macrophages as well as the inhibition of the production of inflammatory cytokines. The importance of STAT3 in mediating the suppressive effects of IL-10 is observed in mice which have macrophages and neutrophils deficient of STAT3; in these mice, macrophages have enhanced MHC class II expression and the mice develop colitis in response to commensal gut flora, as do IL-10 knockout mice which are also highly susceptible to endotoxic shock.

Murine IL-10R can bind both murine and human IL-10, however, human IL-10R is specific for human IL-10. IL-10R is expressed by mouse and human mast cells, B cells, macrophages and epithelial and epidermal cells.
Figure 1.5 The IL-10/IL-10R signalling complex. Binding of IL-10 to IL-10R activates the Jak kinases Jak1 and Tyk2, initiating a chain of events which leads to STAT molecules docking onto IL-10R1. These STAT molecules then become phosphorylated and translocate to the nucleus where they regulate gene expression. Adapted from 290.

IL-10R expression is down-regulated by IFN-γ-activated human macrophages upon Fcy receptor ligation 303, whereas signalling in murine alveolar macrophages is inhibited by TLR stimulation 197. Stimulation of macrophages and DCs with agonists to TLRs 2, 4 and 9 inhibits IL-10-induced STAT-3 activation, inhibiting the ability of IL-10 to suppress macrophage activation 197, 304.
1.6.5 IL-10 and IL-10R in disease

The IL-10/IL-10R axis has been extensively studied in different disease scenarios. The importance of IL-10 is evident by the phenotype of IL-10 and IL-10R knockout mice, which develop spontaneous colitis in response to commensal gut flora \textsuperscript{297}. Generally, a lack of IL-10 renders mice more resistant to certain pathogens, with the drawback that these mice usually develop severe immune pathology. However, the addition of IL-10 can reduce immune pathology, at the risk of mediating a chronic infection.

IL-10 produced by lung interstitial macrophages inhibits DC maturation and migration, preventing induction of allergic airways disease by adoptive transfer of bone marrow-derived DCs stimulated with ovalbumin antigen (OVA) \textsuperscript{43, 305}.

IL-10 in influenza infections is produced by effector CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells which co-produce IFN-γ \textsuperscript{233, 306}. Blockade of IL-10 during influenza infection has however yielded conflicting results. A study by Sun \textit{et al} shows that blockade of IL-10R during influenza infection increases mortality and infiltrating monocyte/macrophages into the lungs, with increased IL-12, and IFN-γ positive virus-specific T cells in BAL fluid \textsuperscript{233}. These authors therefore suggest that T cell-derived IL-10 is vital for ameliorating influenza-induced inflammation. Conversely McKinstry \textit{et al} show that IL-10 knockout mice and mice treated with an IL-10R blocking antibody have enhanced survival during lethal influenza infection due to the presence of a protective Th17 population in the lungs \textsuperscript{306}.

McKinstry \textit{et al} suggest that IL-10 inhibits the formation of a protective Th17 response during influenza infection. A further study from the Metzger group also shows that IL-10 deficient mice have enhanced survival to a high dose influenza infection and display accelerated viral clearance with increased IFN-γ levels in BAL fluid \textsuperscript{307}. The authors attribute the enhanced clearance of influenza virus by IL-10 deficient mice in this study to increased anti-viral antibody production \textsuperscript{307}. 
These discrepancies in the effect of IL-10 on influenza infections might be due in part to different doses of virus used for infections, different strains of mice and different amounts of IL-10R blocking antibody used. For example, the McKinstry and Metzger studies used a high dose of influenza virus, while the Sun study used a lower dose of the virus

Recent reports show that IL-10 can drive the alternative activation of macrophages. IL-10 deficient mice infected with *Schistosoma mansoni* have lower expression of RELM-α and YM1 in lung tissue compared to wild type mice and blockade of IL-10R during *Schistosoma mansoni* infection reduces MR and YM1 expression by peritoneal macrophages.

IL-10 is beneficial for disease outcome in mouse models of experimental autoimmune encephalitis (EAE) and *Toxoplasma gondii*, *Trypanosoma cruzi*, *Plasmodium yoelii* and *Schistosoma mansoni* infections, where it controls immune pathology and enhances the resolution of inflammation. On the other hand, persistence of certain infections is associated with IL-10 production, such as *Leishmania major*, *Leishmania donovani*, *Listeria monocytogenes*, and lymphocytic choriomeningitis virus (LCMV) infections in mice. In human disease, human immune deficiency virus (HIV)-infected individuals with progressive disease have a higher frequency of IL-10-producing CD4+ T cells compared to individuals with non-progressive disease and disease severity in systemic lupus erythematosus (SLE) patients correlates with serum levels of IL-10. Blockade of IL-10 in SLE patients decreases disease severity.

IL-10 therefore plays a major role in the balance between efficient clearance of pathogens and immune pathology and affects macrophage phenotype. IL-10 also has a major role in the regulation of immune homeostasis in the gut, as is evident in IL-10 and IL-10R deficient mice that develop colitis. Like the gut, the lungs are exposed
to myriad antigenic stimuli, including microbial stimuli, and it would be detrimental to
mount a systemic immune response to all antigenic stimuli. This thesis will therefore
investigate the effect of IL-10R blockade on innate immune homeostasis in the lungs
and how this is altered during a subsequent influenza infection, once IL-10R blockade
has ceased. I will explore the concept that a prior blockade of a negative regulator such
as IL-10 can alter the lung micro-environment and the immune response to a
subsequent influenza infection.

1.7 GITR and GITRL

Glucocorticoid-induced TNF receptor-related protein (GITR; also known as TNFRSF18)
and its ligand GITR ligand (GITRL; also known as TNFSF18) belong to the TNF
receptor super family and TNF super family respectively.

1.7.1 Structure and expression profile

GITR was discovered in 1997 as the result of a screen of molecules which were up-
regulated when a murine T cell line was stimulated with dexamethasone. GITR is
induced by T cell activation treatments such as anti-CD3, Concanavalin A or phorbol 12-
myristate 13-acetate (PMA) and ionomycin stimulation. However, others have since
shown that GITR is expressed on resting murine effector T cells, with high expression
on Tregs. Shimizu et al. also show that GITR is expressed on murine NK cells
and splenic macrophages and B cells.

To date, 4 isoforms of mouse GITR have been described, including a splice variant
which lacks the trans-membrane and intra-cellular domains of GITR and therefore is a
secreted protein which could act as a decoy receptor.

Mouse GITRL is expressed by splenic macrophages and B cells, DCs, endothelial cells
and peritoneal macrophages. Expression of mouse GITRL on
splenic macrophages increases transiently upon activation, peaking four hours after stimulation with LPS \(^{326}\).

Human GITR and GITRL were reported soon after mouse GITR, and originally called activation induced TNF receptor family member (AITR) and AITRL \(^{332,333}\). Human GITR bears 55\% identity with mouse GITR and is highly expressed on CD4\(^{+}\)CD25\(^{+}\) Tregs, as is the case in mice, but is weakly expressed on CD4\(^{+}\)CD25\(^{-}\) T cells \(^{332,334,335}\). As well as constitutive expression by T cells, GITR is also expressed by human NK cells \(^{336}\). Human GITRL is expressed by activated endothelial cells \(^{332,333}\), NK cells, activated pDCs \(^{337}\) and human retinal epithelium, on which it is up-regulated by inflammatory cytokines \(^{338}\). A summary of the expression of mouse and human GITRL on various immune cell subsets is shown in Table 1.3.

Mouse GITR is a 228 aa type 1 trans-membrane protein with 3 cysteine pseudorepeats in the extracellular domain \(^{324,329}\) and bears significant homology to other TNF receptor super family members OX40, 4-1BB and CD27 \(^{324}\). The structure of mouse GITR is shown in Fig. 1.6. Human GITR is a 234 aa type 1 trans-membrane protein \(^{332}\) and like mouse GITR, human GITR displays 3 cysteine rich pseudorepeats in its extracellular domain, and bears significant homology with 4-1BB and CD27 \(^{332,333}\).

Mouse and human GITRL exist as a dimer in solution, although human GITRL is also stable as a trimer \(^{339-341}\). Both human and mouse GITRL are type II transmembrane proteins \(^{339}\).
<table>
<thead>
<tr>
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</tr>
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</tr>
<tr>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>• CD4⁺ CD25⁺ (activated)</td>
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<td>+ ? *</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>• Unstimulated</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>• Activated</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NK CELLS</td>
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<td>-</td>
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Table 1.3 Summary of the expression of GITR and GITRL. * Some studies show GITRL expression by T cells after TCR stimulation but not others. GITRL is not reported on activated human T cells.
Figure 1.6 The structure of mouse GITRL. Mouse GITRL is composed of 2 protomers, and exists as a dimer in solution. Protomers A and B are yellow and pink, respectively.

1.7.2 Outcome of GITR signalling

GITR is co-stimulatory for T cells and signalling through GITR leads to their survival and proliferation, enhances production of IL-2 and IFN-γ and renders them less susceptible to the suppressive effects of regulatory T cells. Although GITR triggering leads to the proliferation of CD4+CD25- cells, other studies show that GITR stimulation can lead to the expansion of both CD4+CD25+ Tregs and CD4+CD25- T cells. The abrogation of responsiveness of effector T cells to suppression by Tregs is not observed with human T cells, although GITR is co-stimulatory for human effector T cells.

Ligating GITR on T cells leads to the activation of NFkB and the MAPK family members JNK, ERK and p38. GITR, like other TNFR family members interacts with TNF receptor-associated factor (TRAF) proteins to mediate signalling; GITR interacts with TRAFs 1, 2, 3, 4 and 5. Ligating GITR ultimately leads to survival and proliferation of T cells but others show that GITR also interacts with the pro-apoptotic...
protein Siva, indicating that ligation of GITR could also lead to apoptosis\textsuperscript{352}. Fig 1.7 summarises GITR signalling in mouse T cells.

Blockade of GITR on T cells in \textit{vitro} reduces their proliferative activity in response to TCR stimulus\textsuperscript{326}. It was originally thought that splenocytes and lymph node cells from GITR knockout mice have higher proliferative capacity than wild type T cells, and are more sensitive to AICD\textsuperscript{353}. However other studies since show that CD8\textsuperscript{+} T cells from GITR knockout mice have impaired responses to TCR stimulus while CD4\textsuperscript{+} T cell responses are normal\textsuperscript{354}. GITR is up-regulated by T cells upon TCR stimulation, but if TCR stimulation is sub-optimal, CD28 stimulation is sufficient to up-regulate GITR expression\textsuperscript{326}.

The effects of signalling through GITR on non-T cells appear to be cell-specific. Human and mouse macrophages up-regulate cell adhesion molecules such as ICAM-1 and pro-inflammatory factors such as MCP-1, IL-8, TNF-\alpha and IL-6 and MMP-9\textsuperscript{355, 356}, that are dependent on NFkB\textsuperscript{356}.

NK cells constitutively express low levels of GITR, which is enhanced on activation\textsuperscript{336, 337}. The effect of GITR triggering on NK cells is controversial; certain studies report that it activates NK cell cytotoxicity with increased IFN-\gamma production\textsuperscript{337, 357}, while others show that tumour-expressed human GITRL imparts a negative signal to NK cells via GITR and down-regulates cytotoxicity, including IFN-\gamma production\textsuperscript{336}. The discrepancy between studies is interesting and may indicate that GITR signalling on NK cells has different effects in mice and humans. However, it is possible that in the \textit{in vivo} mouse models the clearance of tumours after treatment with anti-GITRL antibody is due to GITR-mediated T cell activation and not directly due to effects on NK cells.
Figure 1.7 Signalling pathways induced by GITR ligation. GITR interacts with TRAFs 2, 4 and 5, which activates p38 mitogen activated protein kinase (MAPK) and c-jun terminal protein kinases (JNK) via MAPK phosphorylating proteins MAPK kinase (MKK) and MKK kinase (MKKK). GITR triggering also activates NFkB signalling by a sequence of events beginning with the activation of NFkB-inducing kinase (NIK), which activates the downstream kinase IkB kinase (IKK) that phosphorylates the inhibitory IkB complex, leading to its degradation and release of the NFkB subunits from suppression. The result of NFkB, JNK and p38 MAPK activation is survival and proliferation via activation of the pro-survival proteins Bcl-XL and Bfl-1. GITR signalling can also activate the pro-apoptotic protein SIVA, which directly inhibits Bcl-XL and leads to apoptosis via the caspase pathway. Signalling via TRAFs 1 and 3 negatively regulates NFkB activation. Adapted from 118, 358.
Although GITR is expressed by splenic B cells, the effect of ligating GITR on these cells has not yet been elucidated.

1.7.3 Outcome of GITRL signalling

Like GITR, GITRL signalling is cell-specific. Signalling through GITRL on human macrophages induces the up-regulation of pro-inflammatory factors such as IL-6, IL-1β, TNF-α, and IL-8 that is dependent on the phosphorylation of ERK1/2, and inhibited by p38 and JNK1/2. Treatment of murine macrophages with recombinant soluble GITR induces the production of inflammatory mediators such as COX-2, MMP-9, and iNOS. GITRL is expressed by murine pDCs and expression is up-regulated by dexamethasone. Grohmann et al. further show that reverse signalling through GITRL induces the non-canonical pathway of NFkB activation and leads to the production of IDO by pDCs. Therefore, for pDCs, the effect of signalling through GITRL appears to be anti-inflammatory.

Over-expression of GITRL on human myeloid DCs enhances the ability of DCs to stimulate CD8+ T cells, leading to enhanced cytotoxic activity and cytokine production.

1.7.4 GITR and GITRL in human disease and mouse models of human disease

The involvement of GITR and GITRL has been investigated in a range of mouse models of human disease. For example, treatment of mice with an agonistic anti-GITR antibody exacerbates the auto-immune diseases EAE and collagen-induced arthritis, and GITR and GITRL are expressed in synovial fluid from human rheumatoid arthritis patients. However, anti-GITR agonists enhance the immune response to persistent pathogens such as ocular HSV, Leishmania donovani, and Trichinella spiralis. Similarly, GITR-mediated co-stimulation of T cells results in clearance of murine tumours and melanoma, and enhances vaccine efficacy.
Blockade of the interaction between GITR and GITRL has proven beneficial for several mouse models of pulmonary inflammatory disease. During bleomycin-induced lung inflammation, which is an acute model of lung inflammation, GITR knockout mice have improved disease outcome compared to wild type mice, with reduced lung pathology\textsuperscript{379}. Similarly, GITR knockout mice have better disease outcome during carrageenan-induced lung inflammation, which is a model of chronic lung inflammation\textsuperscript{380}. Wild type mice treated with a GITR fusion protein also have improved disease outcome, similar to GITR knockout mice, and the authors attribute this to a blockade of the pro-inflammatory effects of GITR signalling. Potentiating signalling through GITR during ovalbumin-induced airway allergy in mice leads to enhanced production of Th2 cytokines and airways allergic disease; this implies GITR blockade could represent a therapeutic target\textsuperscript{365}.

This thesis will focus on the role of GITR in the context of acute infection-induced inflammation caused by influenza.
1.9 Thesis objectives

The activity of resident innate immune cells in the lung and airway clearly rests on a delicate balance. We hypothesise that slight alterations of this balance will adjust innate immunity and other subsequent responses to lung viral infection. Lung viral infection causes prolonged alterations. We further hypothesise that these alterations are also dictated by the initial starting innate phenotype in the lung.

In this thesis I seek to address the following aims:

1. Determine the precise phenotype of alveolar and lung macrophages at rest and following influenza infection.
2. Determine the effect of transient IL-10R blockade on alveolar macrophage phenotype and the immune response to subsequent influenza infection.
3. Determine the importance of GITR and its ligand in lung homeostasis and during influenza infection.
2.0 Materials and Methods

2.1 Laboratory animals

2.1.1 Mice

Six to eight week old female Balb/c and C57BL/6 mice (Harlan Olac Ltd, Bicester UK) were maintained in specific pathogen-free conditions at BioSafety Level 2. All mice were kept in accordance with institutional and UK Home Office guidelines.

2.2 Mouse infections

On day 0, C57BL/6 or Balb/c mice were anaesthetised with isofluorane and infected with $1.4 \times 10^5$ pfu recombinant influenza virus strain A/HK/X31 (H3N2), a kind gift from Professor Wendy Barclay, Imperial College London. The virus was titrated by specific influenza virus plaque assay (see section 2.8). In some cases, mice were infected again six weeks later with 100 plaque forming units (pfu) recombinant influenza virus strain A/PR/8/1934/H1N1, which had been titrated by influenza-specific plaque assay. Influenza stocks were diluted in 50μl sterile endotoxin-free phosphate-buffered saline (PBS) and administered intra-nasally (i.n.) to mice held in an upright position. Mice were weighed daily and weight recorded as a percentage of original weight. Mice were then culled at various time points after infection by intra-peritoneal (i.p.) injection of pentobarbital and exsanguination via the femoral artery.

2.3 Reagents for in vivo studies

2.3.1 GITRL reagents

Anti-GITRL antibody (Ab) was a kind gift from Professor Steve Cobbold (Oxford University, UK) and supplied as an ammonium sulphate cut. The antibody was then further purified by dialysis with PBS (dialysis cassettes were purchased from Thermo Scientific Inc.) on a protein G column (GE Healthcare), after which it was concentrated
by centrifugation using protein concentration tubes (GE Healthcare). The antibody concentration was then determined using a Bradford assay kit (Thermo Scientific Inc) and tested for endotoxin contamination by limulus amebocyte lysate (LAL) (Kate Tomlinson, UCB).

Balb/c mice were administered 200μg anti-GITRL Ab or rat IgG (BioXcell) i.p. on day 0 (at the same time as infection), and on day 2, day 4 and day 6 post infection. Antibodies were diluted in 300μl sterile endotoxin-free PBS.

Recombinant GITR:Fc fusion protein, composed of the cysteine-rich region of mouse GITR fused to human IgG1 was purchased from Axxorra (Enzo Life Sciences, UK). Mice were given 1μg i.p every from the day of infection. GITR:Fc was diluted in 300μl sterile endotoxin-free PBS.

2.3.2 IL-10R reagents

Anti-IL-10R antibody (IB1.3A) was purchased from Bioxcell (West Lebanon, NH, USA). C57BL/6 mice were injected i.p. with 200μg anti-IL-10R Ab or rat IgG on days -8, -6, -4 and -2. The antibody was diluted in 200μl sterile endotoxin-free PBS.

2.4 Cell recovery and isolation

2.4.1 Airway cells, tissue and serum recovery

Blood from the femoral artery was centrifuged at 8000rpm for 8 minutes and the serum removed and stored at -70°C for analysis of cytokines. Bronchoalveolar lavage (BAL) was performed by inflating the lungs six times with 1.5ml 1mM EDTA in Hanks Balanced Salt Solution (HBSS) [both from Gibco]. BAL fluid was then centrifuged for 5 minutes at 1200rpm, the supernatants frozen at -20°C and the pellet resuspended in R10F (RPMI medium [PAA Laboratories] containing 10% (vol/vol) foetal calf serum (FCS) [Sigma Aldrich, MO, USA], 50U/ml penicillin and 50μg/ml streptomycin [both Sigma Aldrich, MO, USA]). Lungs, spleen and mediastinal lymph node were collected.
and stored in R10F. Lymph nodes were disrupted into single cell suspension by passage through a 100μm sieve (BD Labware, NJ, USA). Lung and spleen tissue was disrupted by digestion with Liberase (Roche, Switzerland) and DNase grade II (Sigma Aldrich MO, USA). Briefly, lung and spleen tissue was finely chopped and incubated for 30 minutes with 2.5mg/ml units of Liberase (Roche, Switzerland) and 50μg/ml DNase grade II (Sigma Aldrich MO, USA) in R10F, after which EDTA was added to a final concentration of 5mM. Tissue was then passed through 100μm sieve (BD Labware, NJ, USA) and washed in R10F. Cell suspensions were spun for 5 minutes at 240 x g, MLN cells were resuspended in R10F and red blood cell lysis was performed for lung and spleen tissue by resuspension of the pellet in 3ml ACK buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate and 0.01 mM EDTA, pH 7.2) before they were spun (5 min at 240 x g) and washed with R10F. Cell viability was assessed by Trypan blue exclusion and all cells were resuspended in R10F and 2 x 10^5 used per stain.

2.4.2 Isolation of alveolar macrophages for in vitro studies

BAL was performed on C57BL/6 or Balb/c mice as described before and cells in BAL fluid enumerated and plated out at 7 x 10^4 per well. Alveolar macrophages were isolated by adherence to plastic for 1 hour at 37°C in 5% CO₂ in DMEM, after which non-adherent cells were washed off. In some cases, LPS (10ng/ml) (Sigma Aldrich, MO, USA), IFN-γ (100ng/ml) (Sigma Aldrich, MO, USA), PolyI:C (20ng/ml) (Sigma Aldrich, MO, USA) or R10F were added in triplicate, otherwise Imiquimod (Invivogen) was added to the cells at concentrations ranging from 1 to 5μg/ml. Cells were then incubated at 37°C in 5% CO₂ for various time points. Cell supernatants were removed at various time points and stored at -70°C for cytokine analysis. In some cases macrophages were harvested by incubation with Versene EDTA (GIBCO) for 10 minutes, after which they were scraped off and after being washed with R10F, were stained with specific antibodies.
2.4.3 Isolation of bone marrow-derived macrophages

Femurs were removed from Balb/c or C57BL/6 mice and bone marrow was extracted by flushing through with RPMI medium. Red blood cell lysis was performed as described above. Bone marrow cells (3x10^5 per ml) were incubated with 20ng/ml MCSF in 10ml RPMI plus 20% (vol/vol) FCS and penicillin (50U/ml), streptomycin 50µg/ml and 10% HEPES (wt/vol) pH 7 for 72 hours at 37°C and 5% CO₂ (all from Sigma Aldrich, MO, USA). Another 5ml of medium containing MCSF were then added to the 10ml already in culture, followed by incubation for another 72hrs at 37°C and 5% CO₂. The cells were then harvested by incubation with EDTA followed by scraping; cells were then enumerated by trypan blue exclusion. Bone marrow-derived macrophages (7x10^4 per well) were then stimulated with LPS (10ng/ml) (Sigma Aldrich, MO, USA), IFN-γ (100ng/ml) (Sigma Aldrich, MO, USA), and incubated at 37°C and 5% CO₂. At various time points, cell supernatants were removed for cytokine analysis. Cells were harvested by incubation with Versene EDTA for 10 minutes followed by scraping. After being washed with R10F the cells were stained with specific antibodies and analysed by flow cytometry.

2.5 Flow cytometry

2.5.1 Cell surface antigens

All antibodies were purchased from eBiosciences, unless otherwise stated. A minimum of 2 x 10^5 or maximum of 1 x 10^6 cells were stained using combinations of FITC, PE, PerCp or PeCy5.5, PE, PECy7, APC, purified or biotin-conjugated antibodies, followed by labelled secondary antibodies. Cells were stained for 30 minutes on ice, with antibodies dissolved in PBA [PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (Sigma Aldrich, MO USA)].

The cells were then washed twice with PBA, in some cases stained with a secondary antibody and then fixed with 2% formaldehyde for 20 minutes at room temperature,
after which the cells were washed twice with PBA and resuspended in PBA and stored at 4°C. The flow cytometry antibodies used are listed in Table 2.1.

2.5.2 Intracellular cytokine staining
For the detection of T cell-derived intracellular cytokines 1 x 10^6 cells were incubated for 3 hours at 37°C and 5% CO₂ with R10F containing phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (500 ng/ml; Calbiochem) and brefeldin A (10 mg/ml). Cells were then stained with anti-CD4 and anti-CD8 antibodies and fixed as above. After permeabilisation for 10 minutes with PBA containing 1% (wt/vol) saponin, cells were stained with anti-TNFα, anti-IFN-γ or anti-IL-17 antibodies diluted in saponin buffer. 30 minutes later cells were washed twice with saponin buffer and once more with PBA. Samples were run on the cytometer within 4 hours of intracellular staining.

2.5.3 RELM-α, YM1 and MR intra-cellular staining
For the detection of intra-cellular RELM-α, YM1 and MR, 1 x 10^6 cells were incubated for 4 hours with R10F containing brefeldin A (10 mg/ml). Cells were then stained with anti-CD11c, anti-CD11b and anti-F480 antibodies and fixed as previously described. After permeabilisation for 10 minutes with PBA containing 1% saponin, cells were stained with anti-RELM-α, anti-YM1 or anti MR antibodies diluted in saponin buffer for 20 minutes. In the case of RELM-α and YM1 after 2 washes in PBA containing 1% saponin buffer, cells were further stained with a fluorophore-conjugated anti-rabbit or anti-rat antibody respectively. Then 30 minutes later stained cells were washed once with saponin buffer and twice more with PBA. Samples were run on the cytometer within 4 hours of intracellular staining. All samples were run on a FACS Canto flow cytometer and results were analysed using FlowJo software.
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Table 2.1 Antibody list
Chapter 2: Materials And Methods

2.5.4 Foxp3 staining

Foxp3 staining was performed according to manufacturer’s instructions (eBiosciences). Briefly, cells were stained with anti-CD4 and and anti-CD25 antibodies as before, and fixed for 30 minutes in Fixation/Permeabilisation buffer supplied by the manufacturers. After washing with Permeabilisation buffer cells were next stained with anti-Foxp3 PE in Permeabilisation buffer for 30 minutes. Cells were then washed again with Permeabilisation buffer and resuspended in PBA. All cells stained with Foxp3 were run on the flow cytometer within 4 hours of staining.

2.6 Tissue imaging

2.6.1 Haematoxylin and Eosin staining of lung tissue

The superior right lobe was inflated with 100μl PBS and fixed with 2% formalin in PBS at room temperature overnight. The tissue was then transferred to 70% ethanol and later embedded in paraffin wax. 4μM sections were stained with haematoxylin and eosin (Lorraine Lawrence, Imperial College, UK).

2.7 Influenza-specific viral plaque assay

Lung lobes were homogenised in DMEM media and the homogenates were freeze-thawed three times and spun at 2100rpm for 5 minutes. Supernatants were then plated out in duplicate doubling dilutions onto a monolayer of 5 x10^4 Madine Darby canine kidney (MDCK) cells per well. After a 3 hour incubation at room temperature, a 1% methyl cellulose overlay was placed on the samples, and the samples were incubated for 72 hours at 37°C and 5% CO₂. Cell monolayers were then washed with PBS and permeabilised with 0.5% Triton-X-100 (Sigma) after which they were washed and incubated with anti-influenza antibody (Serotec) for 90 minutes, followed by anti-mouse horseradish peroxidase (HRP) (Dako) for 90 minutes. Infected cells were then visualised using 3-amino-9-ethylcarbazole substrate and plaque forming units were
enumerated by light microscopy; total plaque forming units were calculated by number of plaques x dilution factor x volume of lung homogenate.

2.8 Detection of soluble factors in lung homogenate and BAL fluid

2.8.1 Detection of soluble factors in lung homogenate and BAL fluid by ELISA

ELISA kits were used to measure soluble levels of IL-6, (Ebioscience) TNF-α (BDBiosciences), and YM1 (R&D) and individual manufacturer’s instructions were followed. Soluble RELM-α was measured using optimised antibody pairs purchased from Peprotech and the manufacturer’s instructions were followed. Briefly, lungs were homogenised in DMEM and centrifuged for 5 minutes at 2100rpm. Supernatants were then used for the ELISA and diluted as appropriate. BAL fluid was used neat or diluted as appropriate. Microtitre plates were coated with 100μl of capture antibody dissolved in PBS or coating buffer specified by the manufacturers; capture antibody was incubated at room temperature or 4°C according to manufacturer’s instructions. After 5 washes with PBS containing 0.05% Tween 20 (Sigma Aldrich, MO USA) plates were blocked with 10% FCS in PBS or 1% BSA in PBS, depending on the manufacturer’s instructions, for 1 hour at room temperature. After a further 5 washes as before, 100μl of each sample was applied to the plate in duplicate and incubated at room temperature for 2 hours. Various dilutions of standards were also plated out according to the manufacturer’s instructions. Plates were washed again and samples were incubated with a biotinylated detection antibody for 1 hour at room temperature, washed as before and incubated with avidin-HRP for 30 minutes at room temperature in the dark. Samples were then washed and incubated with tetramethylbenzidine (TMB) substrate solution. The reaction was stopped with H₂SO₄ and optical densities read at 450nm using 570nm as a reference. The concentration of cytokine in each sample was determined using a standard curve.
2.8.2 Detection of soluble factors in lung homogenate and BAL fluid by Luminex multi-plex assays

Luminex multi-plex assays were performed according to the manufacturer’s (Biolegend) instructions and using reagents provided by the manufacturer. Briefly, filter plates were washed with wash buffer (BioLegend, CA) and samples and standards were added diluted 1:1 with assay buffer (BioLegend, CA) and incubated at room temperature with shaking for 2 hours with pre-mixed beads coated with antibodies against the analytes of interest. Wells were then washed 3 times with assay buffer. The detection antibody cocktail mix (BioLegend, CA) was then added to the wells containing the pre-mixed beads and incubated with shaking for 1 hour at room temperature. Steptavidin-PE (BioLegend, CA) was then added to the pre-mixed bead/detection antibody mix and incubated for 30 minutes with shaking at room temperature. Sample and standard wells were then washed twice and beads resuspended in PBA and read on Luminex machines. Data was analysed using StarStation software.

2.9 Statistics

All mouse experiments were performed with groups of 4/5 mice per group, unless otherwise stated. In vitro stimulations of cells were performed with 3/4 wells per condition unless otherwise stated. GraphPad Prism was used for all statistical calculations. Different statistical tests were used depending on the type of data sets to be analysed. They include: non-parametric Mann Whitney t test, not assuming Gaussian distribution and Kruskal Wallis one-way analysis of variance (ANOVA) with Dunns post test. Data are presented as mean +/- standard error of the mean (SEM). p values were considered significant as follows * = p<0.05 ** =, p<0.01, *** = p<0.001.
3.0 The phenotype of alveolar macrophages

3.1 Introduction

3.1.1 Alveolar macrophages at homeostasis

Alveolar macrophages constitute 90% of the cells in a naïve airway and due to the constant antigenic onslaught experienced by the airways they must perform a balancing act between ignorance of innocuous inhaled antigen and mounting a robust immune response to pathogens. To a large extent the lung micro-environment provides the specific cues that allow alveolar macrophages to respond or not.

Alveolar macrophages are highly phagocytic and one of their main functions is to sequester antigen from dendritic cells in the airway and interstitium in order to avoid an immune response being mounted against harmless antigen. Intra-tracheal administration of antigen activates a T cell response via dendritic cell migration to the lymph node only when the phagocytic abilities of alveolar macrophages have been overwhelmed. This mechanism ensures that particulate antigen can be cleared without inducing inflammation unless it exceeds a certain threshold.

Alveolar macrophages express high levels of scavenger receptors, which facilitate the phagocytosis of un-opsonised antigen. The mannose receptor (MR) is a C-type lectin involved in phagocytosis, which is constitutively expressed by alveolar macrophages, but is inducible on other macrophages after stimulation with IL-4 and IL-13, and therefore is considered a marker for alternatively activated macrophages. MR mediates the endocytosis and phagocytosis of microbes expressing mannose-rich glycoproteins for MHC class II presentation by macrophages. Ligands recognised by MR are found on bacterial species such as Klebsiella pneumoniae, Streptococcus pneumoniae, Leishmania donovani and Trypanosoma cruzi.
Chapter 3: The Phenotype Of Alveolar Macrophages

MR expression is reduced 10-fold by IFN-γ, which also decreases MR-mediated endocytosis. However, others show that the combined actions of IL-4 and IFN-γ can actually enhance MR-mediated phagocytosis. Interestingly, MR expression on human alveolar macrophages is enhanced by SP-A, which is abundant in the airways, and could explain its constitutive expression on alveolar macrophages. Additionally, alveolar macrophages from SP-A deficient mice display lower expression of MR, which supports the findings in human macrophages.

A key mechanism that restricts the activation of alveolar macrophages is the high expression of CD200R that transmits a negative signal to alveolar macrophages upon ligation by CD200. CD200 itself is highly expressed on endothelium and the luminal aspect of epithelium in the un-inflamed lung. CD200R is up-regulated by human macrophages upon treatment with IL-4 and IL-13, and is thought to be a marker of alternative activation on human macrophages. However it is also up-regulated by macrophages on a per cell basis during mouse models of influenza infection where IFN-γ and TNF-α dominate and may represent a strategy to terminate inflammation.

Due to their presence at an environmentally exposed site one might expect alveolar macrophages to display an alternatively activated or regulatory phenotype. Alternatively activated macrophages are usually associated with allergy, worm infections or other Th2 inflammatory diseases, where they play a role in wound healing and regulation of nutrient homeostasis and glucose tolerance. They are highly phagocytic, but have lower killing ability than classically activated macrophages. Alveolar macrophages constitutively express two markers of alternative activation, YM1 and MR – others however are yet to be determined. A number of markers of alternative activation are investigated in this chapter and these include the following:
3.1.2 YM1

YM1 is a secreted protein originally discovered as crystals in the lungs of mice with immune defects, such as mice deficient in NADPH oxidase, CD40L deficient mice and the moth-eaten mouse, which has a severe autoimmune and immune deficiency syndrome. Subsequent studies show that YM1 is a novel mammalian lectin which recognises N-acetyl-D-glucosamine (GlcNac) oligosaccharides and heparan, and has significant homology to known mammalian chitinases, including chitotriosidase a human chitinase. YM1 and its close homologue YM2 (91% identical) are thought to have been formed from a gene duplication event with another mouse chitinase, acidic mammalian chitinase. The two isomers are only distinguishable by gene sequence analysis and therefore protein analysis does not allow a distinction between the two. However, it is generally agreed that YM1 does not have chitinase activity and is now generally regarded as a marker for alternatively activated macrophages.

YM1 mRNA is abundant in the lungs and spleen of mice, and YM2 is abundant in the kidneys, stomach and thymus. YM1 is expressed by alveolar macrophages in the un-inflamed airway and is also produced by epithelial cells, alternatively activated macrophages and neutrophils. It is reportedly the most highly inducible molecule by macrophages upon IL-4 and IL-13 stimulation, which occurs in a STAT-6 dependent manner and is down-regulated by IFN-γ.

YM1 crystals are found in Th2-associated chronic lung inflammatory disease models such as cryptococcosis and YM1 is highly produced in the lungs in mouse models of allergy and asthma. Studies of YM1 in infectious disease models have mainly focused on nematode infections of the gut, where alternatively activated macrophages are induced and YM1 is highly expressed.
The function of YM1 is currently unknown. It is postulated to be an eosinophil chemoattractant; indeed it is often known as eosinophil chemoattractant factor L (ECF-L) but this action of YM1 is contested by others who claim it is not actually chemotactic for eosinophils. YM1 is also proposed to be involved in the resolution of inflammation, by masking lectin binding sites and preventing entry of inflammatory cells to the inflamed tissues. Others propose that YM1 promotes Th2 cytokine production during allergic inflammation in the lung by inhibiting the production of 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) by the enzyme 12/15-lipoxygenase (12/15-LOX); 12(S)-HETE negatively regulates the ability of DCs to stimulate Th2 cytokine production by CD4+ T cells. It is suggested that 12(S)-HETE is a ligand for the myeloid regulator peroxisome proliferator-activated receptor gamma (PPAR-γ), which when ligated decreases lung inflammation and remodelling during allergic inflammatory lung disease. Therefore, YM1 promotes Th2 inflammation in an indirect manner.

To date, YM1 has not been investigated in the context of Th1 cytokine-dominated inflammation in the lung.

3.1.3 Resistin-like molecule alpha

Resistin-like molecule alpha (RELM-α), also associated with the alternatively activated macrophage phenotype, is a secreted protein which belongs to a family of resistin-like molecules including RELM-β and RELM-γ. RELM-α mRNA is present at highest concentrations in adipose tissue but is also found in mammary tissue, the lungs, tongue and heart. In contrast, RELM-β is mainly produced by colonic epithelium. RELM-α is found at high levels in the lungs during allergy and asthma, where it is associated with chronic Th2 pulmonary inflammation, and in the gut during nematode worm infections. RELM-α is produced by epithelial cells, alternatively...
activated macrophages and eosinophils and is highly induced by stimulation of macrophages in vitro with IL-4 and IL-13. Many roles are proposed for RELM-α, some of them conflicting. RELM-α stimulates the production of collagen and smooth muscle actin by fibroblasts, therefore promoting fibrosis during bleomycin-induced lung inflammation and these results have been replicated by others upon over-expression of RELM-α in fibroblasts. It is thought that these effects of RELM-α signalling on fibroblasts imply a role in wound healing and tissue repair, especially during nematode infections. On the other hand Munitz et al show that RELM-α promotes inflammation in a mouse model of colitis, inducing the recruitment of neutrophils, lymphocytes and eosinophils and enhancing the production of TNF-α and IL-6 by macrophages upon stimulation with LPS. Furthermore, Nair et al demonstrate that RELM-α down-regulates the production of Th2 cytokines in the lung, and RELM-α deficient mice have significantly worse disease severity during Th2-dependent Schistosoma mansoni egg-induced lung inflammation. Others propose a role for RELM-α in glucose tolerance during intestinal inflammation and the regulation of eosinophil chemoattractants. RELM-α expression has not been fully mapped on macrophage populations in the un-inflamed lung, nor has it been investigated in the context of a Th1-biased acute inflammatory infection.
3.1.4 Hypothesis

The goal of this chapter is to determine the phenotype of alveolar macrophages at homeostasis. I will test the hypothesis that alveolar macrophages are intrinsically alternatively activated due to the environment they reside in. I will attempt to answer the following questions:

- Do alveolar macrophages express markers of alternative activation at homeostasis and how does this compare to other macrophage populations in the lung?
- How does the expression of the above markers change during influenza-induced inflammation?
- What factors control the expression of markers of alternatively activation on alveolar macrophages?
- Do macrophages co-express co-stimulatory molecules with markers of alternative activation?
3.2 Results

3.2.1 Lung and airway myeloid populations at homeostasis

Broncho-alveolar lavage (BAL) was performed to isolate alveolar macrophages and the cells recovered from naïve C57BL/6 mice were stained with anti-CD11c, anti-CD11b and anti-F4/80 antibodies. CD11c is the \( \alpha \times \) subunit of the integrin \( \alpha \times \beta_2 \), also known as complement receptor 4 (CR4) and was originally identified as a marker of dendritic cells \(^{419} \). However, it is now also widely used as a marker for alveolar macrophages \(^{420} \). CD11b is the \( \alpha \text{M} \) subunit of the \( \alpha \text{M} \beta_2 \) integrin, also known as CR3, which is a receptor for C3b \(^{421} \). CD11b (also known as mac-1 or CD18) is expressed by dendritic cells, monocytes, macrophages, neutrophils, eosinophils and sometimes by T cells \(^{421} \). Both CR3 and CR4 enhance cell adhesion and phagocytosis activity of macrophages \(^{422, 423} \).

Myeloid cells were first selected on the basis of size and granularity by flow cytometry (Fig. 3.1A) and then analysed for the expression of CD11c and CD11b. Naïve alveolar macrophages expressed high levels of CD11c and low to intermediate levels of CD11b (Fig. 3.1B). They were also positive for F4/80 (Fig. 3.1C), which is a monocyte and macrophage-specific marker \(^{424} \), and this was consistent with current protocols for identifying alveolar macrophages \(^{43, 425} \). Airway lavage produces a virtually pure population of cells, as 90 to 95% of the cells recovered from the airways of a naïve mouse are alveolar macrophages \(^{426} \).

Lung homogenates were also stained with the same markers to provide a tissue-resident comparison. After selection of myeloid cells on the basis of size and granularity, these cells were analysed for expression of CD11b and CD11c, which revealed 4 main populations; (Fig. 3.2A). The CD11c\(^+\) CD11b\(^{\text{low}}\) cells were separated into 2 populations; expression of the integrin CD103 was used to identify CD11c\(^+\) CD103\(^+\) intra-epithelial dendritic cells \(^{92} \) (Fig. 3.2B; R2)
Figure 3.1 Alveolar macrophages are the main population in the naïve airway. Broncho-alveolar lavage (BAL) cells derived from C57BL/6 mice were stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7. Viable cells (A) were gated based on forward and side scatter and alveolar macrophages were selected based on co-expression of CD11b and CD11c (B) and these cells were also positive for F4/80 (C). Data represent 5 mice and 3 independent experiments.
Figure 3.2 Myeloid populations in the naïve lung. The lungs were extracted from uninfected C57BL/6 mice, digested and single cell suspensions stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PE-Cy7 and anti-CD103 PE. (A) Viable cells were selected and gated based on CD11b and CD11c expression. (B) CD11c<sup>+</sup> CD11b<sup>low</sup> cells were analysed for CD103 expression. R2: CD11b<sup>low</sup>CD103<sup>+</sup> DCs. (C) Lung DCs (R3) were defined as CD11b<sup>+</sup>CD11c<sup>+</sup> cells (R1; A), which were F4/80<sup>-</sup>. (D) Lung CD11c<sup>+</sup> macrophages, R4 were defined as CD11c<sup>high</sup>F4/80<sup>-</sup>CD11b<sup>low</sup> (E) Lung CD11b<sup>+</sup> monocyte macrophages were defined as CD11b<sup>high</sup>CD11c<sup>low</sup>F4/80<sup>-</sup>. R5. Data are representative of 5 mice per experiment and 3 independent experiments.
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Lung myeloid dendritic cells were CD11b⁺ CD11c⁺ cells (R1; 3.2A), which were F4/80 negative (Fig 3.2C; R3). CD11c⁺ lung macrophages were identified by the expression of F4/80 (Fig. 3.2D; R4). These CD11c⁺ macrophages were consistently seen in the lungs and were similar to alveolar macrophages in their size and granularity, and expression of CD11c, CD11b and F4/80. Others have indeed defined them as alveolar macrophages. It is likely that airway lavage does not flush all the cells out of the airways and so CD11c⁺ macrophages in the lung probably represent this population of residual alveolar macrophages. It is also possible that the CD11c⁺ macrophage population observed in the lungs represents lung macrophages in an intermediate stage of differentiation on the way to becoming alveolar macrophages. R5 (Fig. 3.2E) shows a second population of lung monocyte/macrophages, which were CD11c<sup>low</sup> CD11b<sup>high</sup> F4/80⁺, and these have been shown by others to be found in the lung interstitium.

3.2.2 Comparing the expression of different markers of alternative activation

We hypothesised that due to the regulated environment of the lungs and airways perhaps alveolar macrophages were alternatively activated or regulatory. Others report that mouse and human alveolar macrophages constitutively express MR and YM1, and therefore we commenced to confirm MR and YM1 protein expression on macrophage populations in the un-inflamed lung and airways. We also examined the expression of another marker of alternative activation, RELM-α.

C57BL/6 mice were culled, BAL performed and the lungs removed. BAL cells and lung homogenates were stained for intra-cellular expression of YM1, RELM-α and MR, which is only weakly expressed on the cell surface.
Alveolar macrophages were almost all YM1 positive (97 +/- 1.2%), as were lung CD11c⁺ macrophages (97% +/- 0.7%), which we hypothesise to be residual alveolar macrophages not washed out by BAL (Fig. 3.3A). The similar YM1 expression displayed by these two populations lends further support to the theory that they may be the same population. There was a lower percentage of YM1 positive lung monocyte/macrophages with 86.7 +/- 3.9% positive for YM1 (Fig. 3.3A).

Next, RELM-α expression was examined on lung and airway macrophages. Unlike YM1, there was a very low percentage of RELM-α positive alveolar and lung CD11c⁺ macrophages; however, there was a significant proportion of RELM-α positive lung monocyte/macrophages (62 +/- 6.6% positive) (Fig. 3.3B). Interestingly, alveolar and lung CD11c⁺ macrophages showed similar expression of RELM-α, which was dissimilar to expression by lung monocyte/macrophages.

Most alveolar macrophages were positive for MR (98 +/- 0.5% positive), as previously published and there was a similarly high percentage of MR positive lung CD11c⁺ macrophages (67 +/- 12%) (Fig. 3.3C). In contrast, there was a very low percentage of MR positive lung monocyte/macrophages (Fig. 3.3C). Surfactant protein A is known to up-regulate expression of MR on human macrophages. It is possible to imagine a similar scenario in the mouse as alveolar macrophages, which associate with alveolar epithelium and the surfactant monolayer, have high expression of MR compared to lung CD11b⁺ monocyte/macrophages which reside in the interstitial lung tissue. Airway macrophages are therefore YM1⁺, RELM-α⁻, MR⁺ and CD200R⁺.
Figure 3.3 Expression of YM1, RELM-α and MR by macrophage populations in the lungs and airways. C57BL/6 mice were culled, BAL performed and the lungs extracted. BAL cells and lung homogenates were stained with anti-F4/80 PE-Cy7, anti-CD11c APC and anti-CD11b PerCp to isolate lung and airway macrophage populations: alveolar macrophages (CD11c<sup>high</sup> CD11b<sup>low</sup> F4/80<sup>+</sup> - left hand column), lung CD11c<sup>+</sup> macrophages (CD11c<sup>high</sup> CD11b<sup>low</sup> F4/80<sup>+</sup> - middle column), lung CD11b<sup>+</sup> macrophages (CD11c<sup>low</sup> CD11b<sup>high</sup> F4/80<sup>+</sup> - right hand column). After fixing, cell were permeabilised and then stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC (A), or rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC (B) or anti-mouse MR FITC (C). Histograms are representative of n=5; data from 3 experiments. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
3.2.3 Soluble YM1 and RELM-α in the lungs and airways

Intra-cellular staining of lung and airway macrophages revealed different expression profiles of YM1 and RELM-α, but it was important to assess whether the amount of protein secreted into either compartment differed. Therefore, the levels of soluble RELM-α and YM1 were compared in lavage alone and in lung homogenate that would also contain some residual airway sample.

C57BL/6 mice were culled, BAL performed and the lungs extracted and snap-frozen. Supernatants of lung homogenate, together with BAL fluid, were analysed by ELISA for soluble YM1 and RELM-α. Levels of YM1 in the BAL fluid were on average 2.6 times higher than levels in the lung homogenate (Fig. 3.4A), and correlates with intra-cellular staining of YM1 on airway and lung macrophages, which showed that there was a higher percentage of YM1 positive macrophages in the airways compared to the lungs. This suggests some element of site specific production, and as mentioned before we cannot rule out that levels present in lung homogenates do not actually represent residual airway protein. However, regardless of contamination of lung specimens with airway proteins, the lavage specimen still shows higher levels and we therefore believe that it represents a valid comparison.

Based on flow cytometry data which indicated that there was a low percentage of RELM-α positive alveolar macrophages and lung residual CD11c+ macrophages, it was expected that there would be little soluble RELM-α in the airways. Conversely we expected moderate amounts in the lungs as lung monocyte/macrophages stained positive by flow cytometry (Fig. 3.3B).

On the contrary, there was some RELM-α in the BAL fluid – at 5.1 +/- 3.9 ng/ml this was approximately 193 times less than the amount of YM1 (Fig. 3.4B). Levels of RELM-α in the lung were similarly low, at an average of 3.9 +/- 1.7ng/ml in the lung homogenates and approximately 94 times less than YM1 levels (Fig. 3.4B).
Figure 3.4 Levels of soluble YM1 and RELM-α in the naïve lung and airways. Naïve C57BL/6 mice were culled and BAL performed and the lungs extracted. BAL fluid and lung homogenate were then tested for soluble YM1 (A) and RELM-α (B) levels by ELISA as described in the Methods section. n=5 +/- SEM, data are representative of 2 experiments; * = p<0.05.
RELMA is produced by epithelial cells and perhaps this is the source of the soluble RELMA observed in the BAL fluid.

Overall, there was significantly more YM1 in the lungs and airways compared to RELMA. This correlated with flow cytometry data which indicated that there was a higher percentage of YM1 positive cells in the lungs and airways than there were RELMA positive cells.

### 3.2.4 Macrophages during inflammation

RSV is a common respiratory infection which induces a mixed Th1 and Th2 response. RSV infection induces the production of IL-4 and IL-13 by alveolar macrophages, which polarises their transformation into alternatively activated macrophages with elevated expression of arginase 1, YM1 and RELMA. Shirey et al further show that IL-4R null mice, which fail to produce alternatively activated macrophages during RSV infection, suffer worse pulmonary inflammation and lung pathology compared to wild type mice. The authors suggest that this induction of alternatively activated macrophages provides a mechanism by which the lung can repair inflammation-induced pathology.

Like RSV, influenza infection causes severe lung pathology, and we hypothesised that during influenza infection alveolar macrophages might change their phenotype to become more alternatively activated in order to resolve the excessive lung pathology.

To analyse alveolar macrophage phenotype during influenza infection, a time course experiment was performed. C57BL/6 mice were infected intra-nasally (i.n.) with 1.4x10⁵ plaque-forming units (pfu) of influenza strain X31 on day 0 and sacrificed 1, 3, 6 or 14 days after infection, at which time the airways were sampled by BAL and the lungs were removed. Day 1 represents the induction phase of the immune response, day 3 the peak of innate immunity, day 6 the peak of adaptive immunity and day 14 represents the resolution phase of infection.
As expected, influenza infection led to weight loss, which peaked at day 6 of the infection (Fig. 3.5A). The increase in weight loss corresponded with an increase in inflammatory infiltrate into the lungs and airways, which was reflected in the total viable cell counts in BAL fluid and lung homogenate (Fig. 3.5B) and could be seen in haematoxylin and eosin (H and E) stained lung sections (Fig. 3.5C). Peak viral load occurred between days 1 and 3 of infection and influenza virus was undetectable in the lungs by day 14 of infection (Fig. 3.5D).

During influenza infection there is a substantial inflammatory infiltrate into the airways. We show this in our influenza model in the airways, represented by the forward and side scatter plot of cells from BAL fluid at day 3 of infection (Fig. 3.6A). Alveolar macrophages up-regulated the expression of CD11b, but retained high CD11c expression (Fig 3.6B; R1) and remained F4/80 positive (Fig. 3.6C; R3). This up-regulation of CD11b by alveolar macrophages during pulmonary inflammation is reported by others. Although alveolar macrophages can renew by self-proliferation, during inflammation monocytes are also recruited into the lungs and airways. These cells were distinguishable by high CD11b expression, medium expression of CD11c and expression of F4/80 (Fig. 3.6B; R2 and Fig. 3.6D; R4). These monocyte/macrophages are described as inflammatory, induce apoptosis of epithelial cells during influenza infection, and contribute to influenza-induced pathology. The number of alveolar macrophages and airway monocyte/macrophages was calculated by multiplying the percent of cells positive for the various markers by the percentage of cells in the myeloid gate and then by the total viable cell count (Fig. 3.6E).
Figure 3.5 Influenza infection induces weight loss and infiltration of cells into the airways. C57BL/6 mice were infected intra-nasally (i.n.) on day 0 with 1.4x10^5 plaque-forming units (pfu) of influenza X31 on day 0 and weight loss monitored daily and expressed as a percentage of original weight (A). Mice were sacrificed on days 1, 3, 6 and 14 after infection, BAL performed and the lungs extracted. Total viable cell counts of airway cells in BAL fluid (blue line) and lung homogenates (red line) were determined by Trypan blue exclusion (B). (C) Haematoxylin and eosin staining of histology sections of naïve and influenza-infected mouse lungs (day 6 of infection) was carried out as described in the Methods section. (D) Snap-frozen lung lobes were assessed for influenza virus by plaque assay as described in the Methods section. Data shown are representative of 3 experiments; n=5 +/- SEM. ND = not detected. Data for Figure 3.5C was provided by Dr. John Goulding.
Figure 3.6 Macrophages in the airway at day 3 of influenza infection. C57BL/6 mice were infected i.n. with 1.4x10^5 pfu influenza X31; at day 3 of infection, BAL was performed and lungs extracted. Single cell suspensions of lung homogenate and BAL cells were stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy7. Myeloid cells were selected on the basis of size and granularity (A) and then by CD11c and CD11b expression (B). Alveolar macrophages were identified as being CD11c+ cells (R1; B) which were F4/80+ (R3; C) and airway monocyte macrophages were classed as CD11b+ cells (R2; B) which were F4/80+ (R4; D). (E) The number of alveolar macrophages (blue line) and monocyte/macrophages (red line) was calculated by multiplying the percentage positive cells x percent of myeloid cells x viable cell count. (F) The numbers of lung CD11c+ macrophages (blue line) and lung CD11b+ monocyte/macrophages (red line) were determined as for the airways. Data are representative of 3 experiments; n=5 mice per group +/- SEM.
Like the airways (Fig. 3.6E), the lungs contained a significant macrophage infiltrate during influenza-induced inflammation that consisted of the two lung-resident macrophage populations, the CD11c⁺ macrophages and the CD11b⁺ macrophages (Fig. 3.6F), the numbers of which were calculated as described above for airway cells.

### 3.2.5 YM1 and RELM-α expression on airway macrophages during influenza infection

The expression of YM1 was then determined on airway macrophage populations at days 1, 3, 6 and 14 of influenza infection.

Most naïve alveolar macrophages were positive for YM1 (97 +/- 1.2%) and at day 1 this had not changed significantly (Fig. 3.7A and C). However, at day 3 and 6 of the infection, there was a reduction in YM1 positive cells to approximately 32 +/- 2% (Fig. 3.7A and C). At day 14 of the infection, the percentage of YM1 positive cells was once again high and most alveolar macrophages were YM1 positive (91 +/- 2.12%) (Fig. 3.7A and C), as observed for naïve alveolar macrophages. The reduction in the percentage of YM1 positive alveolar macrophages during influenza correlates with reports that suggest that YM1 mRNA expression is down-regulated by IFN-γ, which is highly produced during influenza infection. Alternatively, the reduction in the percentage of YM1-expressing alveolar macrophages could indicate an increase in the secretion of this soluble protein. Importantly, the percentage of YM1 positive alveolar macrophages was restored to levels seen in naïve mice after resolution of influenza infection.
Figure 3.7 YM1 expression on airway macrophages during influenza infection. C57BL/6 mice were infected intra-nasally on day 0 with 1.4x10^5 pfu influenza X31 and culled 1, 3, 6 and 14 days after infection. BAL was performed and BAL cells stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy7, fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat FITC. Representative histograms show YM1 expression by alveolar macrophages (CD11c^{high} CD11b^{medium} F4/80^{+}) (A) and airway monocyte/macrophages (CD11c^{medium} CD11b^{high} F4/80^{+}) (B). The percentage of YM1 positive alveolar macrophages (C) and monocyte/macrophages (D) was determined by flow cytometry. Data are representative of 2 independent experiments; n=5 +/- SEM. * = p<0.05, ** p<0.01 compared to day 0 for alveolar macrophages and day 1 for monocyte/macrophages. ND = not detected. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
At homeostasis, alveolar macrophages constitute 90% of the cells in the airways, however during inflammation, monocyte/macrophages are recruited into the airways; this population is only observed in the airways during inflammation and so it was not possible to analyse them for expression of YM1 during influenza infection. Like alveolar macrophages, most airway monocyte/macrophages expressed YM1 (97 +/- 8.27%) at day 1 of infection and there was a slight decrease of around 10% in the percentage positive at day 3 and 6 post infection (Fig. 3.7B and D). However, at day 14, unlike the alveolar macrophages, there was a further reduction in the percent of YM1 positive monocyte/macrophages in the airways, with 24 +/- 3.06% YM1 positive (Fig. 3.7B and D).

Compared to the decrease in the percentage of YM1 positive alveolar macrophages, the percentage of YM1 positive airway monocyte/macrophages did not significantly decrease until day 14 of infection. At day 14 of influenza infection, the number of airway monocyte/macrophages is relatively low, as infection is resolving and therefore the percentage of YM1 positive cells is a fraction of a relatively small number of cells and may not be physiologically relevant.

In contrast to YM1 expression, naïve alveolar macrophages did not express RELM-α – less than 1% positive – but the percentage positive increased from day 1 of infection, with 9 +/- 3.09% positive at day 1 and 68 +/- 2.85% RELM-α positive at day 3 (Fig. 3.8A and C). At day 6 of infection, the percentage of RELM-α positive alveolar macrophages decreased to 48.72 +/- 4.92% and at day 14 of the infection, the resolution phase of infection, the percentage of RELM-α positive alveolar macrophages was once again reduced with only 14 +/- 7% positive (Fig. 3.8A and C). It was interesting that the percentage of RELM-α positive alveolar macrophages increased during a classic Th1 infection such as influenza, as the accepted dogma dictates that it is induced by IL-4 and IL-13 \(^{46}\). This indicates that factors produced in the airways during influenza infection are capable of inducing RELM-α expression.
Figure 3.8 RELM-α expression on airway macrophages during influenza infection. C57BL/6 mice were infected i.n. with 1.4x10^5 pfu influenza X31 and culled 1, 3, 6 and 14 days after infection. BAL was performed and BAL cells stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy7, fixed, permeabilised and stained with rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC. Representative histograms show RELM-α expression by alveolar macrophages (CD11c<sup>high</sup>CD11b<sup>medium</sup>F4/80<sup>-</sup>) (A) and airway monocyte/macrophages (CD11c<sup>medium</sup>CD11b<sup>high</sup>F4/80<sup>-</sup>) (B). The percentage of RELM-α positive alveolar macrophages (C) and monocyte/macrophages (D) was determined by flow cytometry. Data represent 2 independent experiments; n=5 +/- SEM. * = p<0.05, ** p<0.01 compared to day 0 for alveolar macrophages and day 1 for monocyte/macrophages. ND = not detected. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
It would be of interest to test the effect of TNF-α, IFN-γ and type 1 interferons on RELM-α expression by alveolar macrophages in vitro, as these cytokines are highly produced during influenza infection\(^{221}\). Again, importantly, we observed that although the percentage of RELM-α positive alveolar macrophages increased during influenza infection, it was low again at resolution, as observed for naïve mice.

Monocyte/macrophages could not be analysed in the airways at day 0 because they are not present in the airways of naïve mice, so analysis of this population began at day 1 of influenza infection. At day 1, the percentage of RELM-α positive monocyte/macrophages was higher than alveolar macrophages at the same time point, with 75% +/- 4.6% positive (Fig. 3.8B and D). The percentage of RELM-α positive monocyte/macrophages increased at day 3, with 88 +/- 2.3% positive and this fell to 63 +/- 6.88% at day 6 and by day 14 only 22 +/- 5.49% were RELM-α positive (Fig. 3.8B and D).

The increase in the percentage of RELM-α positive airway monocyte/macrophages was not as dramatic as observed for alveolar macrophages. However, at day 1, the majority of airway monocyte/macrophages already expressed RELM-α. This would indicate that either these monocytes come into the airways already expressing RELM-α, or they up-regulate it upon entry into the airways. Again, we see unexpected expression of RELM-α during influenza infection. This might indicate that cytokines produced early during influenza infection might induce and maintain RELM-α expression at a high level. Like YM1, there was a low percentage of RELM-α positive airway monocyte/macrophages at day 14 of influenza infection. This could indicate that these cells change their phenotype after the inflammatory insult is gone or that they are being cleared after the infection.
3.2.6 YM1 and RELM-α expression on lung macrophage populations during influenza infection

Like alveolar macrophages, most naïve lung CD11c⁺ macrophages were YM1 positive (97 +/- 0.73%) and this did not significantly change at day 1 of the influenza infection (Fig. 3.9A and C). However at day 3, there was a 13% reduction in the percentage of YM1 positive lung CD11c⁺ macrophages (85 +/- 1.63%) and this was further reduced at day 6 to 61.8 +/- 1.87% (Fig. 3.9A and C). At day 14, the percentage of YM1 positive lung CD11c⁺ cells had increased to 89 +/- 2.9% (Fig. 3.9A and C). The trend in YM1 expression by these lung-associated CD11c⁺ macrophages is similar to CD11c⁺ macrophages found in the airways and lends further support to the idea that these might represent residual alveolar macrophages not washed out by lavage.

The decrease in the percentage of YM1 positive lung CD11c⁺ macrophages during inflammation might indicate that secretion of YM1 was enhanced or factors in the influenza-infected lung micro-environment might down-regulate YM1 expression. As we observed for alveolar macrophages, the percentage of YM1 positive alveolar macrophages returned to levels seen for naïve mice after resolution of influenza.

The percentage of YM1 positive CD11b⁺ monocyte/macrophages in the lungs generally decreased during the influenza time course. At day 0 87 +/- 3.94% of monocyte/macrophages were positive for YM1 and this was reduced to 71 +/- 7.96% at day 1 and by day 3 only 25 +/- 6.22% were YM1 positive; this did not significantly change for the rest of the time points (Fig. 3.9B and D). The decrease in YM1 expression by lung CD11b⁺ monocyte/macrophages was interesting as the percentage of YM1 positive cells never returned to levels seen for naïve mice. It would be interesting to analyse a later time point to determine if the majority of cells again expressed YM1.
Figure 3.9 YM1 expression on lung macrophages during influenza infection.
C57BL/6 mice were infected with 1.4x10^5 pfu influenza X31 i.n. and culled 1, 3, 6 and 14 days after infection. Lungs were removed, digested and single cell suspensions stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy7, fixed permeabilised and stained with rat anti- mouse YM1 followed by goat anti-rat IgG FITC. Representative histograms show YM1 expression by lung CD11c^+ macrophages (CD11c^high CD11b^medium F4/80^+ ) (A) and CD11b^+ monocyte/macrophages (CD11c^medium CD11b^high F4/80^+ ) (B). The percentage of YM1 positive lung CD11c^+ macrophages (C) and CD11b^+ monocyte/macrophages (D) was determined by flow cytometry. Data represent 2 experiments. n=5 +/- SEM. * = p<0.05, ** p<0.01, *** p<0.001 compared to day 0. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
The expression of RELM-α by lung CD11c⁺ macrophages during influenza infection was similar in trend to the expression observed on alveolar macrophages, with the percentage positive starting off low and peaking at day 3 of infection, and back to pre-infection levels by day 14 (Fig. 3.10A and C). There was a general reduction in the percentage of RELM-α positive lung CD11b⁺ monocyte/macrophages throughout influenza infection (Fig. 3.10B and D). At day 0, 62 +/- 6.63% of CD11b⁺ macrophages were RELM-α positive and this decreased throughout the influenza infection up to day 14 (Fig. 3.10B and D).

The reduction of RELM-α positive lung CD11b⁺ monocyte/macrophages was similar to the reduction of RELM-α positive airway monocyte/macrophages and in contrast to the increase in RELM-α expression by alveolar macrophages.

3.2.7 Soluble YM1 and RELM-α in the lungs and BAL fluid during influenza infection

Having observed decreased intra-cellular YM1 and enhanced RELM-α expression by macrophage subsets in the lungs and airways during influenza infection, it was of interest to determine if soluble levels were also altered during influenza infection.

C57BL/6 mice were infected i.n. with 1.4 x 10⁵ pfu influenza X31 and culled 1, 3, 6 and 14 days post infection, BAL performed and the lungs removed. Lung homogenates and BAL supernatants were assessed for RELM-α and YM1 by ELISA as described in the Methods section.
Figure 3.10 RELM-α expression on lung macrophages during influenza infection. C57BL/6 mice were infected i.n. with 1.4x10^5 pfu influenza X31 and culled 1, 3, 6 and 14 days after infection. Lungs were extracted, digested and single cell suspensions stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy7 fixed, permeabilised and stained with rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC. Representative histograms show RELM-α expression by lung CD11c⁺ macrophages (CD11c<sup>high</sup> CD11b<sup>medium</sup> F4/80⁺) (A) and airway monocyte/macrophages (CD11c<sup>medium</sup> CD11b<sup>high</sup> F4/80⁺) (B). The percentage of RELM-α positive lung CD11c⁺ macrophages (C) and CD11b⁺ monocyte/macrophages (D) was determined by flow cytometry. * = p<0.05, ** p<0.01, *** p<0.001 compared to day 0. Data are representative of 2 independent experiments. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
Soluble YM1 increased in lung homogenates during influenza infection, with levels increasing 2 fold from day 0 to day 1, over 5 fold at day 3 and up to 10 fold at 6 (Fig. 3.11A). Levels were still 5 fold higher at day 14 compared to day 0 but this difference was not statistically significant (Fig. 3.11A). Soluble YM1 levels dramatically increased in the BAL fluid during influenza infection, increasing two fold from day 0 to day 1, four fold up to day 3 and up to ten fold at day 6 and day 14 (Fig. 3.11B). This similar increase in levels of soluble YM1 in BAL fluid and lung homogenates was in contrast to the trend seen for intra-cellular levels of YM1 on airway and lung macrophage populations, which decreased during influenza infection. This decrease in intra-cellular YM1 was therefore presumably due to increased secretion of YM1 during influenza infection, which is reflected in higher levels of YM1 observed in BAL fluid and lung homogenates. However, it is possible that other cell types apart from macrophages can secrete YM1, including neutrophils which could partly be responsible for the increase in soluble YM1 observed. It is interesting to note that soluble YM1 had not returned to pre-infection levels at day 14 despite the virus being eliminated by days 7 to 10 (Fig. 3.5E).

Soluble RELM-α in lung homogenates increased around a hundred fold from day 0 to days 1, 3, 6 and 14 of influenza infection (Fig. 3.11C). Similarly, soluble RELM-α in BAL fluid increased significantly up to days 3, 6 and 14 of influenza infection (Fig. 3.11D). The increase in soluble RELM-α in BAL fluid and lung homogenates during influenza infection correlates with an increase in the level of intra-cellular RELM-α expression by lung and airway macrophages. It is possible that macrophages are only partially responsible for the soluble RELM-α observed, as it is known that epithelial cells can also produce it. However it would be interesting to determine what factors induced during influenza up-regulate the production of RELM-α, a protein usually associated with chronic Th2-associated inflammation.
Figure 3.11 Soluble levels of YM1 and RELM-α in the lungs and airways during influenza infection. C57BL/6 mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 and culled 1, 3, 6 and 14 days post infection. BAL was performed and the lungs removed. ELISA was used to measure soluble YM1 in lung homogenate (A) and BAL fluid (B) and RELM-α in lung homogenates (C) and BAL fluid (D). n=5 +/- SEM * = p<0.05, ** = p<0.01 using one way ANOVA. Data are representative of 2 independent experiments.
3.2.8 MR expression throughout influenza infection

The expression of MR by alveolar macrophages was next examined during influenza infection. In contrast to expression of YM1 and RELM-α, MR expression was only analysed at day 0, 7 and day 21 in this experiment. This represents the un-inflamed airway, the peak of inflammation and the resolved airway.

The majority of alveolar macrophages were MR positive at day 0, but the percentage positive was decreased at day 7, with only 51 +/- 6.2% remaining positive for MR (Fig. 3.12A and B). By day 21, when influenza infection is resolved and the virus is cleared, the percentage of MR positive alveolar macrophages had not returned to naïve levels, with only 68 +/- 7% MR positive cells (Fig. 3.12A and B). The reduction in the percentage of MR positive alveolar macrophages is not surprising in light of the fact that it is known that IFN-γ negatively regulates its production. However, even at day 14 when levels of IFN-γ are reduced, the percentage of MR positive alveolar macrophages had not returned to naïve levels (Fig. 3.12A and B). This indicates that influenza infection could permanently alter the expression of MR on alveolar macrophages. It would be of interest to investigate a later time point after influenza infection.

Like alveolar macrophages, the majority of lung CD11c+ macrophages expressed MR, and the percentage positive decreased at day 7 of influenza infection (Fig. 3.12C and D). Again IFN-γ produced during influenza infection is likely to be responsible for the down-regulation of MR, but levels of IFN-γ decrease by day 10 of influenza infection and it is interesting that levels of MR are not back to naïve levels at day 21.
Figure 3.12 MR expression on lung and airway macrophages during influenza infection. C57BL/6 mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 on day 0 and culled at day 0, 7 or 21 days after infection. Lungs were extracted, digested and single cell suspensions stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy7, fixed permeabilised and stained with anti-MR FITC. The percentage of MR-expressing alveolar macrophages (A and B), lung CD11c+ macrophages (C and D) and lung monocyte/macrophages (E and F) was determined by flow cytometry. n=5 +/- SEM. Data are representative 2 independent experiments. Shaded histograms represent isotype control and clear histograms represent specific antibody staining.
Next lung CD11b+ monocyte/macrophages were analysed for MR expression. At day 0 very few lung CD11b+ monocyte/macrophages expressed MR and contrary to what we observed for alveolar and lung CD11c+ macrophages, the percentage positive actually increased up to day 7 and 21 of influenza infection (Fig. 3.12D and E). This is interesting and indicates that MR might be differentially regulated on lung CD11b+ monocyte/macrophages during influenza infection. If alveolar macrophages and lung CD11c+ macrophages are in fact the same population that resides in the airways then it is possible that the cytokine milieu might differ between the interstitial lung CD11b+ macrophages and the airway-resident alveolar macrophages during influenza infection, and this might account for the different expression of MR observed during influenza infection on these two populations.

Airway CD11b+ monocyte/macrophages were not shown as they are not present in the airways at day 0 or day 21, only at day 7. Therefore a comparison could not be made with other time points.

A summary of MR expression by airway and lung macrophages during influenza infection is shown on Table 3.1 and 3.2 respectively, including RELM-α and YM1 expression.
### Table 3.1 Expression of RELM-α, YM1 and MR by airway macrophage populations during influenza infection

The percentage of alveolar and airway monocyte/macrophages expressing the different markers at day 0, day 6 and day 14. Percentages for MR expression are from day 0, day 7 and day 21. Values are percentage +/- SD. ND = not detected as monocyte/macrophages are not present in the airways at day 0. Data are representative of 2 independent experiments.

<table>
<thead>
<tr>
<th>Influenza time point</th>
<th>ALVEOLAR MACROPHAGES</th>
<th>AIRWAY MONOCYTE/MACROPHAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 6</td>
</tr>
<tr>
<td>RELM-α</td>
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<td>48 +/- 5</td>
</tr>
<tr>
<td>YM1</td>
<td>97 +/- 1</td>
<td>32 +/- 2</td>
</tr>
<tr>
<td>MR</td>
<td>99 +/- 0.3</td>
<td>51 +/- 7</td>
</tr>
<tr>
<td></td>
<td>(day 7)</td>
<td>(day 21)</td>
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</tbody>
</table>
Table 3.2 Expression of RELM-α, YM1 and MR by airway macrophage populations during influenza infection. The percentage of alveolar and airway monocyte/macrophages expressing the different markers at day 0, day 6 and day 14. Percentages for MR expression are from day 0, day 7 and day 21. Values are percentages +/- SD. Data are representative of 2 independent experiments.
3.2.9 Influenza-induced factors up-regulate RELM-α expression by alveolar macrophages

After observing an increase in the percentage of RELM-α and a decrease in the percentage of YM1 positive alveolar and lung CD11c+ macrophages, it was of interest to determine if we could recreate this effect in vitro using factors that are present in the influenza-infected lung and airway. IFN-γ is highly induced during influenza infection and is known to negatively regulate MR expression. 389 It also reportedly negatively regulates YM1 up-regulation by IL-4. 406 Polyinosinic:polycytidylic acid (PolyI:C) is a TLR3 agonist and mimics double stranded RNA, which is associated with viral infections. 437 It was of interest to stimulate TLR3 in vitro to determine if this induced changes in YM1 and RELM-α expression by alveolar macrophages.

Alveolar macrophages were isolated from C57BL/6 mice as described in the Methods section and incubated with media, BAL supernatant from an influenza-infected mouse, 100ng/ml IFN-γ or 20ng/ml PolyI:C and incubated for 24 or 48 hours at 37°C and 5% CO₂. We assume that factors present during influenza infection were responsible for the up-regulation of RELM-α and down-regulation of intra-cellular YM1 observed during influenza infection. Therefore BAL supernatants from influenza-infected mice at day 6 were tested for effects on RELM-α and YM1 levels.

None of the conditions used altered the percentage of YM1 positive cells; the majority of alveolar macrophages remained YM1 positive 24 hours (Fig. 3.13A) and 48 hours (Fig. 3.13B) post stimulation. However, at 48 hours there was a slight decrease in the percentage of YM1 positive cells compared to 24 hours for all conditions used. This might indicate that factors present in the naïve airway are required for the expression of YM1; however this could be an artefact and due to the fact that the cells had been in culture for 48 hours.
Figure 3.13 RELM-α is induced by BAL fluid from influenza-infected mice. C57BL/6 mice were culled, BAL performed and alveolar macrophages isolated as described in the Methods section and plated out at 7x10⁴ cells per well of a 96 well plate. Cells were stimulated with media (clear bars), BAL supernatant from a day 6 influenza-infected mouse (orange bars), 100ng/ml IFN-γ (red bars) or 20ng/ml Poly I:C (blue bars) and incubated for 24 or 48 hours at 37°C and 5% CO₂. The cells were then harvested as described in the Methods section and stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy7, fixed, permeabilised and stained with rabbit anti-mouse RELM-α and rat anti-mouse YM1 followed by goat anti-rat IgG FITC or goat anti-rabbit IgG PE. The percentage of YM1 positive alveolar macrophages was determined at 24 (A) and 48 hours (B) post stimulation and the percentage of RELM-α positive alveolar macrophages was determined by flow cytometry 24 (C) and 48 (D) hours after stimulation. n=3 +/- SEM. Data are representative of 2 independent experiments.
Incubation of alveolar macrophages with BAL fluid taken from an influenza-infected mouse at day 6 of infection induced a consistent increase in the percentage of RELM-α positive cells 24 hours (Fig. 3.13C) and 48 hours (Fig. 3.13D) post stimulation. This difference was statistically insignificant but the trend was consistent and indicates that factors present in the influenza-infected airway are capable of inducing RELM-α expression in alveolar macrophages. A caveat of this experiment is that RELM-α is present in BAL fluid from influenza-infected mice and this could be the source of the intra-cellular RELM-α expression observed for macrophages stimulated with BAL fluid.

Next, alveolar macrophages were extracted as before and stimulated with TLR agonists against TLRs 3 and 7 along with IFN-γ. This was to more efficiently mimic the situation in vivo during influenza infection, when TLRs are stimulated in an environment rich in inflammatory cytokines. However, the combination of TLR stimulation with IFN-γ had no effect on YM1 expression and did not induce the expression of RELM-α (data not shown).

3.2.10 Characterising the expression of RELM-α by lung and airway macrophages in a BALB/c model of influenza infection

Having observed that RELM-α is highly up-regulated by macrophages during influenza infection in our C57BL/6 model, it was of interest to confirm these results in a BALB/c model of the disease.

Therefore BALB/c mice were infected i.n. as before with $1.4 \times 10^5$ pfu influenza X31 on day 0 and 1, 3, 6 or 14 days post infection. BAL was performed and the lungs extracted and homogenised. Lung and airway macrophage populations were analysed for RELM-α expression as before.

There was a low percentage of RELM-α positive CD11c+ alveolar macrophages at day 0, and the percentage positive increased from day 1 up to day 6 of infection, falling again at day 14 (Fig. 3.14A).
Figure 3.14 RELM-α positive alveolar macrophages express GITRL during influenza infection. BALB/c mice were infected i.n. with $1.4 \times 10^4$ pfu influenza X31 on day 0 and culled at day 0, 1, 3, 6, or 14 post infection. BAL was performed and the lungs extracted. BAL fluid was stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy-7 and anti-GITRL PE. Cells were then fixed, permeabilised and stained with rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC. The percentage of RELM-α positive alveolar macrophages (A) and airway monocyte/macrophages (B) was determined by flow cytometry. The percentage of RELM-α positive cells expressing GITRL was next determined for alveolar macrophages (C) and airway monocyte/macrophages (D). n=5 +/- SEM. * = p < 0.05 ** = p < 0.01. ND = not detected. Data represent one experiment.
This trend was consistent with that seen for C57BL/6 mice during influenza infection (Fig 3.8A). Similarly, as we observed for CD11b+ airway monocyte/macrophages in C57BL/6 mice (Fig. 3.8B), there was a general reduction in the percentage of RELM-α positive cells throughout influenza infection. These cells could not be analysed at day 0 as they are absent from the airways of naïve mice but at day 1 the majority of CD11b+ airway monocyte/macrophages expressed RELM-α and the percentage positive decreased throughout influenza infection (Fig. 3.14B).

It was of interest to further characterise this population of RELM-α positive macrophages which appeared during influenza infection. Of particular interest was their expression of co-stimulatory molecules. Macrophages constitutively express co-stimulatory molecules such as CD80 and CD86, which are up-regulated on activation. Other co-stimulatory molecules are absent but induced upon activation. For example, OX40L, whose receptor is the co-stimulatory molecule OX40 which is expressed by late activated T cells, is not expressed by macrophages in the naïve state, but is up-regulated on activation. We were interested in analysing the expression of a related molecule, GITRL on RELM-α positive macrophages in the lungs and airways.

GITRL is reportedly expressed by splenic and bone marrow-derived macrophages but so far the expression of GITRL has not been determined in the naïve lung and airways. GITRL is up-regulated by murine peritoneal and bone marrow-derived macrophages upon stimulation with LPS and signalling through GITRL on macrophages leads to the production of inflammatory factors.

Due to very low numbers of RELM-α positive alveolar macrophages at day 0 it was not possible to analyse GITRL expression on these cells. However at day 1 of influenza infection a small percentage of RELM-α positive alveolar macrophages was also GITRL positive and this increased up to day 6 of influenza infection (Fig. 3.14C).
At day 14 it was impossible to analyse GITRL expression on RELM-α positive alveolar macrophages as this comprised a very small cell population. GITRL is up-regulated by macrophages upon stimulation with LPS \(^{330}\). RELM-α positive alveolar macrophages also up-regulated it during influenza infection, which is consistent with its up-regulation as a result of TLR stimulation.

It was not possible to analyse airway monocyte/macrophages at day 0, and at day 1 of influenza infection RELM-α positive airway monocyte/macrophages did not express GITRL (Fig. 3.14D). However at day 3 of infection there was a small percentage of RELM-α positive airway monocyte/macrophages expressing GITRL (6 +/- 2%) which increased slightly to 13 +/- 4% GITRL positive cells at day 6 of influenza infection. The difference in GITRL expression between alveolar macrophages and airway monocyte/macrophages was interesting and the expression of GITRL by these two populations will be further characterised in Chapter 5.

Lung CD11c\(^+\) macrophages were next analysed for RELM-α expression in the BALB/c model of influenza infection. As in the C57BL/6 model (Fig. 3.10A), at day 0 there was a low percentage of RELM-α positive lung CD11c\(^+\) macrophages and this increased throughout influenza infection, peaking at day 3 of infection (Fig. 3.15A). The increase in RELM-α positive cells was however not as dramatic as observed for alveolar macrophages (Fig. 3.14). As in the C57BL/6 influenza infection model (Fig. 3.10B), we observed that the majority of lung CD11b\(^+\) monocyte/macrophages expressed RELM-α at day 0 and this percentage decreased throughout influenza infection (Fig. 3.15B). However, unlike the case in C57BL/6 mice (Fig. 3.10B), where the percentage of RELM-α positive cells was lowest at day 14, in the BALB/c model the percentage of RELM-α positive lung CD11b\(^+\) monocyte/macrophages was high at day 14, as observed at day 0 (Fig. 3.15B).
Figure 3.15 RELM-α positive lung macrophages express GITRL during influenza infection. BALB/c mice were infected i.n. with 1.4 x 10⁴ pfu influenza X31 on day 0 and culled at day 0, 1, 3, 6, and 14 post infection and the lungs extracted. Lung homogenates were stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy-7 and anti-GITRL PE, stained fixed, permeabilised and stained with rabbit-anti mouse RELM-α followed by goat anti-rabbit IgG FITC. The percentage of RELM-α positive lung CD11c⁺ macrophages (A) and lung CD11b⁺ monocyte/macrophages (B) was determined by flow cytometry. The percentage of RELM-α positive cells expressing GITRL was next determined for lung CD11c⁺ macrophages (C) and lung CD11b⁺ monocyte/macrophages (D). (E) Lung lobes were tested for influenza virus via plaque assay as described in the Methods section. n=5 +/- SEM. * = p < 0.05 ** = p < 0.01. ND = not detected. Data represent one experiment. Data for Figure 3.15E was obtained from Dr. Robert Snelgrove.
At day 14, like C57BL/6 mice (Fig. 3.5D), BALB/c mice have also cleared the influenza virus from the lungs (Fig. 3.15E) and inflammation has resolved, therefore this apparent strain difference is not due to impaired viral clearance in the BALB/c mice.

Next, it was again of interest to analyse the expression of the co-stimulatory molecule GITRL on RELM-α positive cells in the lungs. At day 0 and day 1 the percentage of RELM-α positive lung CD11c+ macrophages expressing GITRL was low, but significantly increased up to day 3 and 6 of influenza infection (Fig. 3.15C). By day 14 the percentage of GITRL positive cells was again low as at day 0 (Fig. 3.15C). This pattern resembled that observed for GITRL positive RELM-α positive alveolar macrophages.

The expression of GITRL was next analysed on RELM-α positive lung CD11b+ monocyte/macrophages. There was a very low percentage of GITRL-expressing RELM-α positive lung monocyte/macrophages at days 0 and 1 of influenza infection but this increased significantly up to day 3 of influenza infection, falling again at days 6 and 14 (Fig. 3.15D).

3.2.11 YM1 expression by macrophage populations in a BALB/c model of influenza infection

The expression of YM1 during influenza infection was next analysed on airway and lung macrophage populations in BALB/c mice. As before BAL and lung lobes were isolated from influenza-infected mice on days 0, 1, 3, 6 and 14 after influenza infection and analysed by flow cytometry for YM1.

YM1 was expressed by the majority of alveolar macrophages, as observed before for C57BL/6 mice (Fig. 3.7A), and was similarly lost at days 3 and 6 post influenza infection (Fig. 3.16A). By day 14 however, the majority of alveolar macrophages again expressed YM1 (Fig 3.16A).
Figure 3.16 YM1 positive airway macrophages co-express RELM-α during influenza infection. BALB/c mice were infected i.n. with 1.4 x 10⁴ pfu influenza X31 on day 0 and culled at days 0, 1, 3, 6, or 14 post infection. BAL was performed and BAL cells stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy-7. Cells were then fixed, permeabilised and stained with rat-anti mouse YM1 followed by goat anti-rat IgG FITC, and rabbit-anti mouse RELM-α followed by donkey-anti-rabbit IgG PE. The percentage of YM1 positive alveolar macrophages (A) and airway CD11b⁺ monocyte/macrophages (B) was determined by flow cytometry. The percentage of YM1 positive macrophages co-expressing RELM-α was determined for alveolar macrophages (C) and airway CD11b⁺ monocyte/macrophages (D). n=5 +/- SEM. ND = not detected. ND = not detected. * = p<0.05 compared to day 0 for alveolar macrophages and day 1 for airway monocyte/macrophages. Data represent one experiment.
Airway CD11b⁺ monocyte/macrophages could not be analysed at day 0 as they are only recruited into the airways during inflammation. However, at day 1 of influenza infection 79 +/- 7% of airway monocyte/macrophages expressed YM1 and the percentage of YM1 positive cells decreased during influenza infection up to day 14 (Fig. 3.16B). This pattern was similar to that previously seen for C57BL/6 mice (Fig. 3.17B).

Next it was of interest to determine whether YM1 and RELM-α were co-expressed at any time during influenza infection. At day 0, there were no YM1⁺RELM-α⁺ double positive alveolar macrophages, as alveolar macrophages did not express RELM-α at day 0. However, from day 1 of influenza infection there was an insignificant increase in the percentage of YM1⁺RELM-α⁺ double positive alveolar macrophages, with a reduction at day 14 (Fig. 3.16C). Airway monocyte/macrophages could not be analysed at day 0 but the percentage of YM1⁺RELM-α⁺ double positive cells increased from day 1 to day 3, and decreased up to day 14 (Fig. 3.16D).

Representative flow plots showing co-expression of YM1 and RELM-α by alveolar macrophages (left hand side panels) and airway CD11b⁺ monocyte/macrophages (right hand side panels) is displayed in Fig. 3.17.

Next, lung macrophage populations were analysed for expression of YM1 in BALB/c mice. As observed for C57BL/6 mice, the expression of YM1 by lung CD11c⁺ macrophages during influenza closely resembled the pattern seen for alveolar macrophages, with the majority of cells expressing it at day 0 and a reduction of YM1 positive cells at days 3 and 6 of infection, but at day 14 the majority of cells were once again YM1 positive (Fig. 3.18A). In contrast to what was seen previously for C57BL/6 mice, YM1 was expressed by few CD11b⁺ lung monocyte/macrophages at day 0, but the percentage positive increased up to days 3 and 6 of influenza infection, decreasing again at day 14 (Fig. 3.18B).
Figure 3.17 YM1 and RELM-α co-expression by airway macrophages in BALB/c mice during influenza infection. BALB/c mice were infected i.n. with $1.4 \times 10^4$ pfu influenza X31 on day 0 and culled at day 0, 1, 3, 6, and 14 post infection. BAL was performed and BAL cells were stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy-7. Cells were then fixed, permeabilised and stained with rat-anti mouse YM1 followed by goat anti-rat IgG FITC, and rabbit-anti-mouse RELM-α followed by donkey-anti-rabbit IgG PE. Alveolar macrophages (left hand side panels) and airway monocyte/macrophages (right hand side panels) co-expressing RELM-α and YM1 were determined by flow cytometry. Data represent 5 animals. ND = not detected. Data represent one experiment.
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Figure 3.18 YM1 positive lung macrophages co-express RELM-α during influenza infection. BALB/c mice were infected i.n. with $1.4 \times 10^4$ pfu influenza X31 on day 0 and culled at day 0, 1, 3, 6, and 14 post infection. BAL was performed and the lungs extracted and homogenised. Lung homogenates were stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy-7. Cells were then fixed, permeabilised and stained with rat-anti mouse YM1 followed by goat anti-rat IgG FITC, and rabbit-anti-mouse RELM-α followed by donkey-anti-rabbit IgG PE. The percentage of YM1 positive lung CD11c$^+$ macrophages (A) and lung CD11b$^+$ monocyte/macrophages (B) was determined by flow cytometry. The percentage of YM1 positive macrophages co-expressing RELM-α was determined for lung CD11c macrophages (C) and lung CD11b$^+$ monocyte/macrophages (D). n=5 +/- SEM. ND = not detected. * = p<0.05, ** = p<0.01 compared to day 0. Data represent one experiment.
Next, as with alveolar macrophages, the co-expression of YM1 and RELM-α was determined during influenza infection. There was a very low percentage of YM1⁺RELM-α double positive cell lung CD11c⁺ macrophages at day 0, with a slight increase at day 1 of infection, but no significant changes at any of the time points analysed (Fig. 3.18C). There was a consistently low percentage of YM1⁺RELM-α⁺ double positive CD11b⁺ lung monocyte/macrophages at all the time points analysed, with a slight increase at day 14 of influenza infection (Fig. 3.18D).
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3.3 Discussion

Findings from this chapter

- At homeostasis alveolar macrophages express high levels of YM1 and MR
- RELM-α is expressed by lung monocyte/macrophages but not alveolar macrophages at homeostasis
- Intracellular YM1 decreases during influenza infection, with an increase in secreted YM1
- MR expression decreases during influenza infection
- RELM-α is highly inducible by lung and airway macrophages during influenza infection
- YM1 and MR show similar expression profiles during influenza infection, while RELM-α appears to be differentially regulated.
- RELM-α positive macrophages co-express GITRL at the peak of influenza infection

3.3.1 Alveolar macrophages express high levels of YM1 and RELM-α at homeostasis

YM1 and RELM-α are perceived as markers of alternatively activated macrophages and are usually associated with Th2-associated inflammation and helminth infections. However, it is known that alveolar macrophages express MR, and human studies implicate another molecule constitutively expressed by alveolar macrophages, CD200R, as a marker of alternative activation. We set out to investigate the phenotype of alveolar macrophages in the un-inflamed lung. As well as high expression of MR, alveolar macrophages also expressed high levels of YM1, but not RELM-α. Interestingly, like alveolar macrophages, lung CD11c+ macrophages expressed high levels of MR and YM1 but not RELM-α. In contrast, lung CD11b+ monocyte/macrophages expressed fairly high levels of RELM-α and YM1 but very little MR.
It is interesting that i) some markers of alternative activation, which are usually associated with Th2 inflammation were expressed by alveolar macrophages in the uninflamed airway and ii) the markers of alternative activation were not always co-expressed; we observed RELM-α and YM1 expression by lung monocyte/macrophages but not MR expression, and YM1 and MR but not RELM-α expression by alveolar macrophages and lung CD11c⁺ macrophages. This indicates a measure of site specific regulation of these proteins.

Others show that surfactant protein A up-regulates MR expression by alveolar macrophages, and alveolar macrophages from surfactant protein A knockout mice have reduced MR expression. Alveolar macrophages associate closely with airway epithelial cells and alveolar epithelial cells are known to produce surfactant proteins. It is possible that airway epithelial cell-derived surfactant protein A is responsible for the high expression of MR on alveolar macrophages. Lung monocyte/macrophages, which presumably are found in the lung interstitium and do not associate closely with alveolar epithelial cells would not be subject to alveolar epithelium-derived surfactant proteins in the same manner as alveolar macrophages.

High YM1 expression by alveolar macrophages may also be due to alveolar epithelium-derived surfactant proteins. Additionally, others show that YM1 and MR can be expressed independently of IL-4. For example, IL-10 induces MR expression and IL-10 knockout mice have reduced MR expression by macrophages. Another study shows that IL-10 drives MR and YM1 expression by peritoneal macrophages in IL-4R knockout mice during Schistosoma mansoni infection and IL-10R blockade in IL-4 knockout mice abolishes the expression of YM1 and MR by these cells. It is thought that IL-10 is crucial for maintaining lung homeostasis, and others show significant IL-10 levels in the un-inflamed lung. It is known that IL-10 is produced by epithelial cells and alveolar and lung macrophages at homeostasis. It is possible that YM1 and MR are maintained at high levels on alveolar macrophages by locally produced IL-10.
Interestingly, unlike MR, YM1 is also expressed by lung monocyte/macrophages; this might also be due to the influence of IL-10. It is known that lung monocyte/macrophages themselves produce IL-10 which could act in an autocrine manner to induce YM1 expression.

The presence of markers of alternative activation on alveolar macrophages in the un-inflamed airway begs the question: what is their function at this site? Many studies show that MR is a scavenger receptor and is highly expressed by alveolar macrophages. The function of YM1 is less certain. There are many proposed functions for this chitinase-like molecule, but the one that stands out in the context of the un-inflamed lung is the suggestion that it YM1 could be involved in the resolution of inflammation, by masking lectin binding sites and preventing entry of inflammatory cells to the inflamed tissues. One of the main functions of alveolar macrophages is to sequester antigen and prevent it from encountering DCs to induce an inflammatory response. It is possible that YM1 is also involved in maintaining immunological tolerance of inhaled antigen in the lung and airway. To address this hypothesis it would be of interest to determine if blockade of YM1 leads to the enhanced recruitment of immune cells into the lungs and airways in naïve mice.

Intra-cellular production of RELM-α by lung monocyte/macrophages, but not alveolar macrophages, alludes to another mechanism by which these markers of alternative activation are differentially regulated. To date RELM-α expression has only been shown to be up-regulated under strongly Th2 polarised conditions or in the gut after chemically-induced colitis and in vitro after IL-4/IL-13 stimulation. IL-4 and IL-13 are not thought to be prevalent in the lungs of naïve mice, and therefore an alternate mechanism of RELM-α regulation may exist. The lack of RELM-α expression by alveolar macrophages and its expression by lung monocyte/macrophages further indicates that it is negatively regulated by factors present in the alveolar space or alternatively, the factors required to induce its expression may be absent from the
airways. The presence of soluble RELM-α in BAL fluid from naïve mice is probably due to production by epithelial cells, which are known to produce it.

3.3.2 YM1 and RELM-α are differentially regulated during influenza infection

Once the phenotype of alveolar macrophages had been established in the un-inflamed lung and airway, we mapped the markers of alternative activation YM1, RELM-α and MR during influenza infection. Alveolar macrophage expression of intra-cellular YM1 decreased throughout influenza infection and was least at day 6 of infection, the peak of infection. Lung and airway monocyte/macrophage expression of YM1 was similar, with a general loss of intra-cellular YM1 throughout influenza infection. Similarly, MR also appeared to be lost during influenza infection.

The loss of MR and YM1 is perhaps not surprising as others show that IFN-γ antagonises their up-regulation by IL-4. Treatment of alveolar macrophages in vitro did not alter the expression of YM1 in our system; however it is possible that other pro-inflammatory factors produced during influenza infection such as type 1 interferons, TNF-α and IL-6 might be responsible for the observed loss or release of intra-cellular YM1. Additionally, the loss of the epithelium during influenza infection might result in the loss of epithelium-derived surfactant proteins and this could also account for the loss of MR, and release of YM1.

The observation that RELM-α was produced by alveolar macrophages during influenza infection was surprising; the accepted dogma is that RELM-α is induced by allergic Th2-biased inflammatory diseases and worm infections. Influenza infection leads to highly Th1-polarised inflammation and IL-4 is not present at high levels compared to IFN-γ and IL-12. However, Munitz et al demonstrate the presence of RELM-α in acute chemically-induced colitis, which is characterised by high levels of TNF-α, IL-6 and IL-17, and slightly less IFN-γ; IL-4 is not present at high levels in the serum in the acute colitis model, which is more biased towards Th1 inflammation. This indicates...
that RELM-α can be induced under Th1 biased conditions. The fact that the expression patterns of RELM-α and YM1 during influenza are reciprocal alludes to the fact that they might be reciprocally regulated; perhaps RELM-α is induced by TNF-α or IL-6 binding to their receptors on alveolar macrophages, which could down-regulate YM1 production.

We observed that *in vitro* IFN-γ or PolyI:C did not affect alveolar macrophage expression of RELM-α or YM1. Similarly, stimulation with TLR agonists and an inflammatory cytokine such as IFN-γ did not affect RELM-α or YM1 expression. Future experiments will test the involvement of TNF-α, IL-6 and type 1 interferons, all of which are present at high levels during influenza infection. IL-33, which is induced during influenza infection (E. L. Wissinger and T. Hussell; unpublished observations), is known to amplify the polarisation of alternatively activated macrophages and the effect of this cytokine on alveolar macrophage expression of RELM-α will also be tested in the future.

### 3.3.3 RELM-α positive lung and airway macrophages express GITRL during influenza infection

After establishing differential expression of RELM-α and YM1 in a C57BL/6 model of influenza infection, we also showed similar kinetics of expression in influenza-infected BALB/c mice. Additionally, RELM-α positive lung and airway macrophages expressed GITRL, a co-stimulatory molecule induced on macrophages upon activation.

GITRL expression is not associated with a Th1 or Th2 bias, but merely functions to provide a survival and proliferation signal to T cells.

RELM-α positive macrophages up-regulated GITRL at the peak of influenza infection, which was expected, as GITRL is up-regulated upon stimulation of macrophages with TLR agonists \(^330\). A full characterisation of GITRL expression by lung and airway macrophages will be discussed in Chapter 5.
3.3.4 Conclusion

This chapter shows that alveolar macrophages express markers of alternative activation YM1 and MR, unlike lung monocyte/macrophages, which express RELM-α and YM1 but not MR. Furthermore we show that during influenza infection these markers of alternative activation are differentially regulated with a loss of intra-cellular YM1 accompanied by an increase in secreted YM1. We also show an increase in the percentage of RELM-α positive macrophages during influenza infection, which challenges the dogma that this protein is only inducible by IL-4 and IL-13. We propose that alveolar macrophages represent an intermediate phenotype of macrophages which expresses some but not all markers of alternative activation. Their production of IL-10 indicates that they may be a hybrid of a regulatory and alternatively activated macrophage.

It has not been previously shown that alveolar and lung macrophages represent a unique atypical alternatively activated macrophage population. YM1 and MR might perform homeostatic lung functions, such as regulating inflammation and phagocytosis of inhaled particulate matter, respectively. It is interesting that YM1 and RELM-α production in the BAL and lung homogenate is enhanced during a Th1-skewed infection such as influenza. Although these molecules are usually associated with Th2-skewed inflammation, it is possible that it is not the Th2 cytokine milieu but the tissue damage, for example in worm infections, which induces their expression. It is possible that damage-associated signals induce the production of YM1 and RELM-α, or perhaps factors in the Th1 cytokine milieu are also capable of up-regulating these proteins.
Chapter 4: The impact of IL-10R blockade on lung homeostasis

4.0 The impact of IL-10R blockade on lung homeostasis

4.1 Introduction

4.1.1 Control of airway homeostasis

In the previous chapter we showed that airway macrophages are phenotypically different from those that reside in the deeper lung tissues in the absence of inflammation. This may be regulated by a number of site-specific factors. TGF-β is present in the lungs at homeostasis, but is secreted as a latent form in a complex of three proteins. The integrin αvβ6, which is expressed at high levels on airway epithelial cells, catalyses the cleavage of the latent form of TGF-β into the active form. Because of the proximity of alveolar macrophages to the epithelium, they are then subject to the anti-inflammatory effects of TGF-β. Expression of αvβ6 is up-regulated on the epithelium in response to inflammation, making more TGF-β available; this serves as a mechanism to control inflammation as well as maintaining lung homeostasis.

The epithelium is involved in another myeloid regulatory mechanism, involving CD200 and its receptor CD200R, which is highly expressed on alveolar macrophages. CD200 is expressed on the lumen of airway epithelial cells and binding to its receptor CD200R on alveolar macrophages, imparts a negative signal to the alveolar macrophage and results in down-regulation of inflammatory cytokines. The role of the CD200/CD200R interaction is evident in the phenotype of mice lacking either molecule. Influenza-infected CD200 null mice display excessive uncontrolled lung inflammation during influenza infection and die from the infection. Even un-infected CD200 null mice have more alveolar macrophages compared to wild type mice, suggesting an important role for this pathway in suppressing inflammation in general. Alveolar macrophages from CD200R knockout mice have a lower threshold of
activation than wild type controls and produce higher amounts of inflammatory cytokines to relatively low levels of inflammatory stimuli.

IL-10 is proposed to play a central role in immune homeostasis in the lung and the effect of neutralising IL-10/IL-10R interactions will be the focus of this chapter.

4.1.2 IL-10 and lung homeostasis

IL-10 is a potent immuno-suppressive cytokine and important for the maintenance of immune homeostasis in mucosal areas such as the lungs, where an inappropriate immune response might result in lung pathology, which would be detrimental to the host. The importance of IL-10 in controlling the inflammatory response can be observed in IL-10 knockout mice, which develop spontaneous enterocolitis in response to normal gut flora; IL-10R knockout mice similarly develop colitis and splenomegaly by 12 weeks of age.

In the lungs and airways IL-10 is constitutively produced by a range of cells; epithelial cells, Tregs, lung myeloid and plasmacytoid DCs and alveolar and lung interstitial macrophages.

IL-10 is abundant in the lungs at homeostasis and suppresses inflammatory cytokine production by T cells and macrophages and decreases the expression of co-stimulatory molecules and MHC class II on monocytes and macrophages, thereby lowering their activation status and their ability to present antigen. IL-10 directly acts on Tregs, maintaining their expression of Foxp3. As well as negatively regulating inflammatory processes, IL-10 can also act in a stimulatory manner, for example by promoting MHC class II expression on B cells and enhancing their ability to produce antibodies. DCs pre-treated with IL-10 lose the ability to induce allergy in mouse models of OVA-induced allergic airways disease. IL-10 production by DCs in the lungs is an important mechanism for the tolerance induced by exposure to inhaled antigen. IL-10 acts directly on T cells, macrophages and DCs to limit inflammation.
but also affects other myeloid regulators. For example, IL-10 up-regulates the expression of the myeloid regulator, CD200R on macrophages, which makes them more susceptible to suppression by CD200 ligation.

Furthermore, IL-10 can affect the alternative activation of macrophages. IL-10 knockout mice have lower basal mRNA levels of YM1 and RELM-α in lung tissue during *Schistosoma mansoni* infection. Additionally, others show that IL-10 can be as potent at inducing MR expression by macrophages as IL-4. In the absence of IL-4, IL-10 can drive the expression of YM1 and MR on peritoneal macrophages. Dewals et al further show that MR and YM1 expression by peritoneal macrophages is abolished in IL-4R knockout mice by blockade of IL-10R.

IL-10R is a member of the class II IFN receptor-like cytokine receptor family and signalling occurs via the JAK/STAT pathway and is highly dependent on STAT-3. IL-10R is widely expressed by most mouse and human haematopoietic cell types such as DCs, macrophages, monocytes, B cells, NK cells, mast cells and T cells, which is demonstrated by their responsiveness IL-10. TLR stimulation inhibits IL-10R function on DCs and macrophages in a MyD88-dependent manner and decreases the ability of these cells to respond to IL-10 once activated by TLRs.

### 4.1.3 Manipulation of IL-10/IL-10R interactions in disease

Due to the immune suppression associated with this pathway, IL-10 and IL-10R have been manipulated in a variety of human diseases and mouse models of human diseases. In general, blockade of IL-10R enhances the immune response and addition of IL-10 dampens inflammation. For example, IL-10R blockade enhances the clearance of persistent pathogens in chronic infectious disease models such as LCMV, Hepatitis C, and *Leishmania major* infections.

Clinical trials using IL-10 to treat psoriasis result in decreased local and peripheral inflammation. Additionally, mice lacking IL-10 do not recover from EAE, and
local administration of IL-10 renders mice completely resistant to EAE. IL-10 is also thought to play a major role in the regulation of asthma-associated inflammation; there are lower levels of IL-10 in the lungs of asthmatic patients and they have fewer IL-10-producing T cells in peripheral blood compared to healthy controls.

Mice deficient in IL-10 have high immune-pathology and therefore worse disease outcome upon infection with certain pathogens such as *Toxoplasma gondii*, *Pseudomonas aeruginosa* and *Trypanosoma cruzi*. However, IL-10 knockout mice are resistant to other pathogens associated with persistence such as *Leishmania major*, and *Mycobacterium bovis* Bacillus Calmette-Guérin. The immuno-stimulatory properties of IL-10 towards certain immune subsets must also be considered in any therapy. For example, IL-10 has been implicated to have a role in the cause and/or exacerbation of the B cell-associated disease SLE. In Crohn’s disease IL-10 administration was only effective at reducing inflammation at intermediate doses, but slightly higher doses resulted in enhanced inflammation. Whether this is due to enhanced B cell proliferation or an outgrowth of commensal organisms is currently unknown.

IL-10 is important for disease outcome in murine models of influenza infection and IL-10 deficient mice are protected against a high dose influenza infection and mount a more efficient immune response to infection. Similar results are seen upon IL-10R blockade during a high dose influenza challenge. Conversely, another group shows that IL-10 produced by CD8 effector T cells during influenza infection is essential for controlling inflammation and bystander lung pathology and that blockade of IL-10R during influenza infection leads to increased mortality and immune-pathology. A third study shows that IL-10 deficient mice have improved viral clearance and survival after influenza infection, with enhanced specific anti-viral antibody response, probably due to heightened APC activity.
Clearly IL-10 is important for the outcome of influenza infection, but its role in the maintenance of lung homeostasis is not known.

### 4.1.4 Hypothesis

The altered homeostasis and response to infection observed in IL-10, CD200 and CD200R knockout mice indicate that a lack of these myeloid regulators alters the activation status of immune cells in the absence of inflammation. Additionally, IL-10 clearly has an impact on the phenotype of macrophages, as it is involved in the regulation of YM1, MR and RELM-α. It is interesting to consider whether blockade of such regulation is sufficient to alter lung homeostasis and the immune response to a subsequent infection.

This chapter will examine the effects of blocking IL-10R using an anti-IL-10R blocking antibody, which has previously been shown by others to block the effects of IL-10 in vivo. We hypothesise that blockade of IL-10R will alter lung homeostasis and macrophage phenotype, and that the immune response to a subsequent infection will be more robust after IL-10R blockade. To address these hypotheses I will aim to answer the following questions:

- Does IL-10R blockade result in enhanced inflammation at homeostasis?
- Is alveolar and lung macrophage phenotype altered by IL-10R blockade?
- What is the effect of prior IL-10R blockade on a subsequent influenza infection?
4.2 Results

4.2.1 The effect of IL-10R blockade on cell numbers in the lungs and airways at homeostasis

In order to examine the effects of IL-10 on lung homeostasis, an IL-10R blocking antibody was used. C57BL/6 mice were injected intra-peritoneally (i.p.) with 200μg anti-IL-10R antibody (Ab) or rat IgG1 isotype control on days -8, -6, -4 and -2 (Fig. 4.1). At day 0 mice were culled, BAL performed to isolate airway cells and the lungs were extracted; analysis was performed as detailed in Fig. 4.1.

As IL-10 is known to suppress inflammation and the proliferation of immune cells, it was of interest to analyse the effect of IL-10R blockade on the numbers of the different lung and airway cell populations.

IL-10R blockade resulted in increased numbers of cells in the BAL fluid compared to IgG-treated controls (Fig. 4.2A). As alveolar macrophages are the main population in the naïve airway, there was also an increase in alveolar macrophage numbers in the anti-IL-10R Ab-treated mice compared to IgG-treated controls (Fig. 4.2B). Interestingly, IL-10R blockade had no effect on the total number of cells in the lungs or the number of lung CD11c+ macrophages or CD11b+ monocyte/macrophages (data not shown).

Next it was of interest to investigate the effect of IL-10R blockade on DC populations in the lungs. Sub mucosal DCs were defined as CD11c+CD11b+CD103- and intra-epithelial DCs were identified by CD11c and lack of CD11b expression as well as expression of the integrin CD103. However, IL-10R blockade did not alter the number of sub mucosal (Fig. 4.2C) or intra-epithelial DCs in the lungs (4.2D).

There are very few T cells in the naïve airway but the effect of IL-10R blockade on the number of T cells in the lungs was examined. IL-10R blockade had no effect on the number of CD4+ or CD8+ T cells in the lungs (data not shown), nor was there any effect on the number of Tregs (Fig. 4.2E).
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Figure 4.1 Experimental set up. C57BL/6 mice were injected intra-peritoneally (i.p.) with 200 μg anti-IL-10R Ab or rat IgG1 every other day for 8 days, after which bronchoalveolar lavage (BAL) was performed and the lungs extracted; BAL fluid and lung homogenates were analysed as shown above.
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Figure 4.2 The effect of IL-10R blockade on cell populations in the lungs and airways. C57BL/6 mice were injected i.p. with 200μg anti-IL-10R Ab (squares) or rat IgG1 every other day for 8 days, after which the mice were sacrificed, BAL performed and the lungs extracted. Numbers of airway cells in BAL fluid (A) were determined by trypan blue exclusion and the cells stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7 to identify alveolar macrophages (B). Lungs were digested and single cell homogenates stained for intra-epithelial DCs (C; CD11c+ CD11b+ F4/80), sub mucosal DCs (D; CD11c+ CD11b- CD103+) and Tregs (E; CD4+ CD25+ FoxP3+). Numbers of each cell type were determined by multiplying the percentage positive x number of cells in the myeloid/lymphocyte gate x viable airway or lung cell count. (A and B) n=15 +/- SEM and represents data from 3 experiments. n=5 per group (C-E), +/- SEM. * = p<0.05
4.2.2 Effect of IL-10R blockade on macrophage phenotype

The absence of myeloid regulators at homeostasis can lead to altered macrophage phenotype. For example, alveolar macrophages lacking the myeloid regulator PPAR-γ switch to a Th1 phenotype and the mice develop pulmonary Th1 inflammation in the absence of any external antigenic stimulation. IL-10 knockout mice have lower basal expression of YM1 and RELM-α in lung tissue. Alveolar macrophages from mice lacking the myeloid regulator CD200R also have a heightened state of activation and produce higher levels of inflammatory cytokines in response to TLR stimulation.

In the absence of IL-4, IL-10 can drive the expression of YM1 and MR by macrophages, and therefore it was of interest to determine if alveolar macrophage phenotype was altered after IL-10R blockade. Geometric mean (GM) was used as a crude measure of the intensity of expression of MR and YM1. IL-10R blockade did not significantly alter the percentage or GM of MR positive alveolar macrophages (Fig. 4.3A), nor was the percentage or GM of YM1 positive alveolar macrophages altered (Fig. 4.3B). There appeared to be an increase in the GM of both YM1 and MR but this difference was statistically insignificant. At homeostasis alveolar macrophages did not express RELM-α, and this was not altered by IL-10R blockade (data not shown).

Neither YM1 nor RELM-α expression at the intra-cellular level was affected by IL-10R blockade; however both proteins are secreted and therefore it was of interest to assess the amount of secreted protein. BAL fluid was therefore analysed by ELISA for soluble YM1 and RELM-α. There appeared to be slightly higher levels of soluble YM1 in BAL fluid of IL-10R Ab-treated mice but this did not reach statistical significance and there was similarly no significant difference in RELM-α levels (Fig. 4.3C).
Figure 4.3 Alveolar macrophage phenotype is unaffected by IL-10R blockade. C57BL/6 mice were injected i.p. with 200μg anti-IL-10R Ab (squares or clear bars) or rat IgG1 (circles or filled bars) every other day for 8 days, after which the mice were culled and BAL was performed; BAL fluid was stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7. The cells were then fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC or anti-MR FITC. The percentage (closed symbols) and geometric mean (open symbols) of MR (A) and YM1 (B) positive alveolar macrophages (CD11c<sup>high</sup>CD11b<sup>low</sup>F4/80<sup>+</sup>) were determined by flow cytometry. Levels of soluble YM1 (C) and RELM-α (D) in the BAL fluid were determined by ELISA. Data shown are representative of 3 experiments; n=5 +/- SEM.
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Lung macrophage populations were next examined for the expression of YM1, RELM-\(\alpha\) and MR. The percentage and GM of MR (Fig. 4.4A) and YM1 (Fig. 4.4B) positive lung CD11c\(^+\) macrophages was unchanged by IL-10R blockade. The percentage of RELM-\(\alpha\) positive lung CD11c\(^+\) macrophages was negligible and this was not altered by IL-10R blockade (data not shown). Neither the percentage nor GM of MR or YM1 positive lung CD11b\(^+\) monocyte/macrophages was altered by IL-10R blockade (Fig. 4.5A and B respectively). Although the GM of YM1 on the lung monocyte/macrophages from the anti-IL-10R Ab-treated mice appeared to be lower than that of the controls, this difference was not statistically significant. However, the percentage and GM of RELM-\(\alpha\) positive lung monocyte/macrophages was significantly higher after IL-10R blockade (Fig. 4.5C). This would indicate that these monocyte/macrophages were more alternatively activated, but this was not consistent with the fact that there was no significant difference in the expression of YM1 or MR. Therefore this could be an indication that YM1 and RELM-\(\alpha\) are differentially regulated at homeostasis.

Again, it was of interest to measure the amounts of secreted protein in the lungs by measuring levels of soluble YM1 and RELM-\(\alpha\) in lung homogenates. Snap-frozen lung lobes were homogenised and the supernatants tested for YM1 and RELM-\(\alpha\) by ELISA. IL-10R blockade did not alter the amount of secreted YM1 or RELM-\(\alpha\) (Fig. 4.6A and B respectively).

Next, the activation status of alveolar and lung macrophages was examined; CD200R and MHC class II were used as activation markers. CD200R is highly expressed by alveolar macrophages \(^{200}\) and expression is diminished upon activation, while MHC class II expression is enhanced on macrophages upon activation \(^{389}\). However, IL-10R blockade did not alter the expression of CD200R or MHC class II by alveolar or lung macrophages (data not shown).
Figure 4.4 Lung CD11c\(^+\) macrophage phenotype is unaffected by IL-10R blockade. C57BL/6 mice were injected i.p. with 200\(\mu\)g anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days, after which the mice were culled and the lungs extracted, digested and lung homogenates stained with anti-CD11c APC, anti-CD11b PerCp, and anti-F4/80 PE-Cy7; the cells were then fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC or anti-MR FITC. The percentage (closed symbols) and geometric mean (open symbols) of MR (A) and YM1 (B) positive cells were determined by flow cytometry. Data shown are representative of 3 experiments; n=5 +/- SEM.
C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days, after which the lungs were extracted, digested and lung homogenates stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7; the cells were then fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC, or anti-MR FITC or rabbit anti-mouse RELM-α followed by donkey anti-rabbit IgG PE. The percentage (closed symbols) and geometric mean (open symbols) of MR (A), YM1 (B) and RELM-α (C) positive lung CD11b+ monocyte/macrophages were determined by flow cytometry. Data shown are representative of 3 experiments; n=5 +/- SEM. * = p<0.05
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**Figure 4.6** Blockade of IL-10R does not impact on the production of soluble YM1 or RELM-α in the lungs. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (open bars) or rat IgG1 (grey bars) every other day for 8 days, after which the lungs were extracted and snap-frozen. Snap-frozen lungs were homogenised and supernatants assayed for soluble YM1 (A) and RELM-α (B) by ELISA as described in the Methods section. Data represent 2 experiments, n=5 +/- SEM.
4.2.3 Prior IL-10R blockade does not alter the expression of CCR2

Alveolar macrophages renew their numbers by self-proliferation, but during inflammation monocytes are recruited into the airways from the bone marrow via a series of chemokine gradients; MCP-1 and MIP-1α are highly induced during lung inflammatory disease. IL-10 inhibits the production of mouse, rat and human MCP-1 and MIP-1α. We hypothesised that a lack of the suppressive effects of IL-10 might release the inhibition of MCP-1 or MIP-1α and could result in the increased number of alveolar macrophages observed on IL-10R blockade.

Alveolar and lung macrophages were examined for the expression of CCR2, which is the ligand for the monocyte chemo-attractant MCP-1. Despite the difference seen in alveolar macrophage numbers upon IL-10R blockade, there was no significant difference in the percentage or GM of CCR2 positive alveolar macrophages (Fig. 4.7A and B respectively).

4.2.4 Prior IL-10R blockade does not affect weight loss or viral load during a subsequent influenza infection

Although the lung micro-environment did not appear to have been dramatically altered by IL-10R blockade, it was important to investigate whether the temporary absence of IL-10 would alter the immune response to a subsequent influenza infection.

As before, C57BL/6 mice were injected i.p. with 200μg anti-IL-10R Ab or rat IgG1 isotype control on days -8, -6, -4 and -2; on day 0 the mice were then intra-nasally infected with $1.4 \times 10^5$ pfu influenza strain X31 and culled at various time points after infection (Fig. 4.8). Mice were weighed daily, as influenza-induced weight loss is a key indication of disease severity. As expected, the mice lost up to 20% of their original body weight and the peak weight loss occurred at day 6 of infection; however, there was no difference in weight loss between the anti-IL-10R Ab-treated mice compared to control mice (Fig. 4.9A).
Figure 4.7 IL-10R blockade does not alter CCR2 expression by alveolar macrophages. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days. BAL was performed and BAL fluid stained with anti-CD11c APC, anti-CD11b PerCP, anti-F4/80 PE-Cy7 and purified anti-CCR2 followed by goat anti-rabbit PE. The percentage and geometric mean of CCR2 positive alveolar macrophages was determined by flow cytometry. n=5 +/- SEM. Data are representative of 2 experiments.
Figure 4.8 Experimental set up. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab or rat IgG1 every other day for 8 days, after which the mice were infected intranasally (i.n.) with 1.4x10^5 pfu influenza strain X31. At the time points indicated BAL was performed and the lungs extracted and analysed as displayed above.
Figure 4.9 Prior IL-10R does not impact on weight loss during subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (blue line, A; clear bars, B) or rat IgG1 (red line, A; grey bars, B) every other day for 8 days, after which the mice were infected i.n. with 1.4x10^5 pfu influenza strain X31. Mice were weighed daily and weight loss was expressed as a percentage of original weight (A). Mice were culled and lungs extracted at the time points shown; viral load was determined by plaque assay as described in the Methods section (B). Data shown represent results from 2 experiments; n=5 +/- SEM.
Mice were culled 1, 3, 6 and 14 days after influenza infection, and lung lobes extracted. Lung lobes were tested for viral load by influenza plaque assay, as described in the Methods section. However, prior IL-10R blockade did not alter the ability of mice to clear influenza virus, as viral load was comparable between the two groups at the time points tested (Fig. 4.9B). Virus particles were not detectable at day 14 of influenza infection in either group.

4.2.5 Prior IL-10R blockade impacts on total cellular infiltrate into the airways during influenza infection

Cellular infiltrate into the lungs and airways during influenza infection correlates closely with weight loss in mouse models of influenza and the peak cellular infiltrate coincides with the peak of weight loss at day 6 of infection. Mice were culled 1, 3, 6 and 14 days after influenza infection, airway cells washed out by BAL and the lungs extracted. As expected during influenza infection there was a steady increase in cell numbers in the airways which peaked at day 6 of infection; at day 3 of infection, however, there were significantly more cells in the airways of mice previously treated with anti-IL-10R Ab (Fig. 4.10A). At day 6 there again appeared to be increased numbers of cells in the airways of the anti-IL-10R Ab-treated mice, but this difference did not gain statistical significance.

The numbers of cells in the lungs increased steadily throughout influenza infection, again peaking at day 6 of infection (Fig. 4.10B). At all the time points analysed there were comparable numbers of cells in the lungs of anti-IL-10R Ab-treated mice compared to control mice (Fig. 4.10B).
Figure 4.10 Prior IL-10R blockade increases total cell numbers in the airways at day 3 of a subsequent influenza infection but lung numbers are unaffected. C57BL/6 mice were injected i.p. with 200μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which the mice were infected i.n. with 1.4x10^5 pfu influenza strain X31. Mice were culled at the time points shown, BAL performed to isolate airway cells and lungs extracted. Lungs were digested and made into single cell homogenate and total cell numbers in BAL fluid (A) and lungs (B) were determined by Trypan blue exclusion. Data are representative of 2 experiments; n=5 +/- SEM. *=p<0.05
4.2.6 Prior IL-10R blockade increases T cell infiltrate into the lungs and airways during a subsequent influenza infection

T cell infiltrate into the lungs and airways is a key feature of influenza infections and the peak in T cell infiltrate directly correlates with disease severity in mouse models of influenza infection \(^{470}\). Therefore, it was of interest to examine the T cell infiltrate into the influenza-infected lungs and airways in mice previously treated with anti-IL-10R Ab.

Mice were pre-treated with \(200\mu\text{g}\) anti-IL-10R Ab as before on days -8, -6, -4 and -2, and on day 0 they were infected i.n. with \(1.4\times10^5\) pfu influenza X31 and culled 1, 3, 6 and 14 days afterwards; BAL was then performed, the lungs extracted and numbers of T cells determined by flow cytometry analysis.

As expected, the numbers of both CD4\(^+\) and CD8\(^+\) T cells in the lungs peaked at day 6 of influenza infection (Fig. 4.11A and B) and this coincided with the peak of weight loss at day 6. There were comparable numbers of CD4\(^+\) T cells in lungs of the IL-10R Ab-treated mice and IgG-treated controls, except at day 14, when there were significantly more CD4\(^+\) T cells in the lungs of the IL-10R Ab-treated mice (Fig. 4.11A). Similarly, there was no difference in lung CD8\(^+\) T cell numbers between the anti-IL-10R Ab-treated mice and control mice, except at day 14, when there appeared to be more CD8\(^+\) T cells in the lungs of the anti-iL-10R Ab-treated mice (Fig. 4.11B). However, this difference stopped short of reaching statistical significance (\(p=0.056\)).

T cells in the airways were not analysed at early time points, as there are few T cells in the airways of naïve mice and the peak of T cell infiltrate into the airways is day 6 of influenza infection \(^{223}\). T cells were therefore analysed at day 6 of infection and at day 14, the resolution phase of infection. At day 6 there were significantly more CD4\(^+\) T cells in the airways of the IL-10R Ab-treated mice compared to IgG1-treated control mice (Fig. 4.11C).
Figure 4.11 T cell numbers in the lungs and airways during influenza infection following IL-10R blockade. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which the mice were infected i.n. with 1.4x10⁵ pfu influenza strain X31. Mice were culled at the time points shown, BAL performed and the lungs extracted. BAL cells and single cell suspensions of lung homogenate were stained with anti-CD4 APC and anti-CD8 PerCp. The number of CD4⁺ (A) and CD8⁺ (B) T cells in the lungs was calculated by multiplying the percentage CD4 or CD8 positive cells x the percentage of cells in the lymphocyte gate x viable cell count. Airway cells were only analysed for CD4⁺ (C) and CD8⁺ (D) T cells at day 6; numbers of T cells were calculated as previously specified for the lungs. Data represent 2 experiments; n=5 +/- SEM. *=p<0.05, ** = p<0.01.
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However, numbers of CD8\(^+\) T cells were comparable between the two groups (Fig. 4.11D). There was no difference in T cell numbers in the airways of either anti-IL-10R Ab- or IgG1-treated mice at day 14 of influenza infection (data not shown).

4.2.7 Prior IL-10R blockade augments numbers of macrophages in the airways during a subsequent influenza infection

During influenza infection, there is a vast infiltrate of macrophages into the lungs and airways, recruited as a result of the secretion of chemokines by resident immune cells. Total cell numbers in the airways were increased at day 3 of influenza infection after IL-10R blockade (Fig. 4.12A), and this time point is the peak of innate immunity during influenza infection. Hence it was of interest to analyse macrophage populations in the airways at day 3.

Anti-IL-10R Ab-treated mice had significantly higher numbers of monocyte/macrophages in the airways compared to IgG1-treated controls (Fig. 4.12B) and slightly higher numbers of alveolar macrophages at day 3 but this difference did not reach statistical significance (Fig. 4.12C). The increase in numbers of macrophages in the airways correlated with the increase in total number of cells in the airways observed at day 3 of influenza infection.

Numbers of monocyte/macrophages and CD11c\(^+\) macrophages were also analysed in the lungs, however there were comparable numbers of the two macrophage populations between the anti-IL-10R Ab-treated mice and IgG1-treated controls (data not shown).
Figure 4.12 Prior IL-10R blockade increases total cells and macrophages in the airways at day 3 of a subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days, after which the mice were infected i.n. with 1.4x10^5 pfu influenza strain X31. Mice were culled at day 3 and BAL performed. BAL fluid was stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7. The total number of cells in the airways was determined by trypan blue exclusion (A) and the number of monocyte/macrophages (B) and alveolar macrophages (C) was calculated by multiplying the percentage positive x percentage of cells in the myeloid gate x viable cell count in the airways. * = p<0.05, ** = p<0.01 n=10+/− SEM. Data represent 3 experiments.
4.2.8 Prior IL-10R blockade results in airway macrophages with a more alternatively activated phenotype during a subsequent influenza infection

A lack of myeloid regulators in the airways can lead to altered homeostasis and change the phenotype of macrophages; for example, alveolar macrophages lacking PPAR-γ become spontaneously activated with a Th1 bias. Although only lung monocyte/macrophage expression of RELM-α was altered in naïve mice after IL-10R blockade, it was important to investigate if there was a change in macrophage phenotype during influenza-induced inflammation.

We had previously seen, surprisingly, that YM1 and RELM-α were differentially regulated during influenza infection (see Chapter 3). The expression of YM1 and RELM-α was therefore compared between the anti-IL-10R Ab-treated mice and IgG1-treated controls. At day 6 of influenza infection, prior IL-10R blockade resulted in a significantly higher percentage of YM1 positive alveolar macrophages compared to IgG1-treated controls, however there was no difference in the GM of YM1 between the two groups (Fig. 4.13A). The increase in intra-cellular YM1 expression by macrophages during influenza infection subsequent to IL-10R blockade might imply decreased secretion and/or increased intra-cellular production.

Similarly, after IL-10R blockade, there was a higher percentage of RELM-α positive alveolar macrophages at day 6 of a subsequent influenza infection, but again, no increase in GM of RELM-α (Fig. 4.13B). Prior IL-10R blockade did not alter YM1 or RELM-α expression at any other time points during influenza infection.
Figure 4.13 Prior IL-10R blockade augments the proportion of YM1 and RELM-α positive airway macrophages at day 6 of a subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days, after which mice were infected i.n. with 1.4x10⁵ pfu influenza strain X31. Mice were sacrificed at day 6, BAL performed and BAL fluid stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7; the cells were then fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC or rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC. The percentage (closed symbols) and geometric mean (open symbols) of YM1 (A) and RELM-α (B) positive alveolar macrophages and YM1 (C) and RELM-α (D) positive airway monocyte/macrophages were determined by flow cytometry. Data shown are representative of 2 experiments; n=5 +/- SEM. * = <0.05
Next, airway recruited monocyte/macrophages were analysed for expression of YM1 and RELM-α. In a similar trend to that displayed by alveolar macrophages, there was a higher percentage of YM1 positive monocyte/macrophages at day 6 of a subsequent influenza infection (Fig. 4.13C). There was also a slight increase in the GM of YM1 positive cells but this difference did not reach statistical significance (Fig. 4.13C). Surprisingly, there was no difference in the percentage or GM of RELM-α positive monocyte/macrophages between the anti-IL-10R Ab-treated group and IgG1-treated controls (Fig. 4.13D). There was no difference in the percentage of YM1 or RELM-α positive airway monocyte/macrophages between the two groups at any other time point analysed (data not shown).

4.2.9 Prior IL-10R blockade enhances the alternative activation of lung macrophages during subsequent influenza infection

The expression of YM1 and RELM-α was next analysed on lung macrophage populations during influenza infection following IL-10R blockade. In a similar result to that seen in the airways, the percentage of YM1 positive lung CD11c⁺ macrophages was higher at day 6 of influenza infection after IL-10R blockade, but there was no change in the GM of YM1 (Fig. 4.14A). Similarly, there was a higher percentage of RELM-α positive lung CD11c⁺ macrophages at day 6 in the anti-IL-10R Ab-treated mice, but there was no difference in the GM of RELM-α (Fig. 4.14B). As discussed in the previous chapter these may represent residual airway cells not removed by BAL. There was no difference in the percentage or GM of YM1 or RELM-α positive lung CD11c⁺ macrophages at any other time point analysed (data not shown).

Interestingly, there was no difference in YM1 expression on lung monocyte/macrophages at day 6 of influenza infection, contrary to the result seen for the other lung and airway macrophage populations (Fig. 4.14C). However, after IL-10R blockade there was a higher percentage of RELM-α positive lung monocyte/macrophages at day 6 of a subsequent influenza infection (Fig. 4.14D).
Figure 4.14 Prior IL-10R blockade increases the proportion of YM1 and RELM-α positive lung CD11c+ macrophages at day 6 of a subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (squares) or rat IgG1 (circles) every other day for 8 days, after which mice were infected i.n. with 1.4x10⁵ pfu influenza strain X31. Mice were sacrificed at day 6, BAL performed and BAL fluid stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PE-Cy7; the cells were then fixed, permeabilised and stained with rat anti-mouse YM1 followed by goat anti-rat IgG FITC or rabbit anti-mouse RELM-α followed by goat anti-rabbit IgG FITC. The percentage (open symbols) and geometric mean (closed symbols) of YM1 (A) and RELM-α (B) positive lung CD11c+ macrophages and YM1 (C) and RELM-α (D) positive lung monocyte/macrophages were determined by flow cytometry. Data shown are representative of 2 experiments; n=5 +/- SEM. ** = p<0.01
There was no difference in YM1 or RELM-α expression at any other time points analysed.

4.2.10 The effect of prior IL-10R blockade on levels of soluble YM1 and RELM-α during subsequent influenza infection

As there was increased intra-cellular expression of YM1 and RELM-α by macrophages, it was of interest to also analyse the levels of soluble protein. Therefore BAL fluid and lung homogenates were analysed for soluble YM1 and RELM-α by ELISA. Consistent with a higher proportion of RELM-α positive airway macrophages in the anti-IL-10R Ab-treated animals at day 6 of influenza infection (Fig. 4.13), levels of soluble RELM-α in the BAL fluid were also higher in anti-IL-10R Ab-treated mice compared to IgG-treated controls at this time point (Fig. 4.15A). Conversely, although there was a higher proportion of YM1 positive alveolar macrophages and airway monocyte/macrophages in the BAL fluid of anti-IL-10R Ab-treated mice at day 6 of influenza infection (Fig. 4.13), this did not correlate with a difference in the levels of soluble YM1 in the BAL fluid at this time point (Fig. 4.15B). However, there were significantly higher levels of YM1 in the BAL fluid of anti-IL-10R Ab-treated mice at day 3 of influenza infection (Fig. 4.15B).

Next, lung homogenates were tested for levels of YM1 and RELM-α. Intra-cellular levels of YM1 and RELM-α were higher at day 6 of influenza infection in lung CD11c+ of mice which had been previously treated with anti-IL-10R Ab (Fig. 4.14), therefore we hypothesised that secreted levels of both proteins might also be different. However, there was no significant difference in the levels of RELM-α (Fig. 4.15C) or YM1 (Fig. 4.15D) in lung homogenates at any of the time points analysed during influenza infection.
Figure 4.15 The effect of prior IL-10R blockade on levels of soluble YM1 and RELM-α during influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which mice were infected i.n. with 1.4x10^5 pfu. Mice were sacrificed at day 6, BAL performed and the lungs extracted. BAL fluid was then assessed for soluble RELM-α (A) and YM1 (B) and snap-frozen lung lobes were homogenised and similarly analysed by ELISA for soluble RELM-α (C) and YM1 (D). Data are representative of 2 experiments.
4.2.11 Prior IL-10R blockade enhances production of inflammatory cytokines by T cells in the airway during a subsequent influenza infection

During influenza infection, activated T cells infiltrate the lungs and airways and secrete vast amounts of inflammatory cytokines which, whilst importance for viral clearance also cause a significant amount of bystander damage to the lungs and can impact on weight loss. It was therefore important to establish whether prior IL-10R blockade had affected the ability of T cells to secrete inflammatory cytokines during subsequent influenza infection.

T cell production of inflammatory cytokines TNF-α and IFN-γ was assessed by intracellular cytokine staining of lung homogenate and BAL fluid. Prior IL-10R blockade resulted in a higher number of IFN-γ- and TNF-α-producing CD4+ (Fig. 4.16A) and CD8+ (Fig. 4.16B) T cells at day 6 of influenza infection compared to IgG1 treatment. However, this enhancement of TNF-α- and IFN-γ-producing T cells was not seen in the lungs (data not shown).

Previous studies show that IL-10R blockade or a lack of IL-10 during a high dose influenza infection results in enhanced numbers of Th17 cells in the lungs and airways. Although by comparison infection with 1.4x10^5 pfu of influenza virus is not a high dose, it was important to establish if the formation of Th17 cells had been altered by prior blockade of IL-10R. IL-17 production by T cells was therefore determined by intracellular cytokine staining. At day 6 of influenza infection after IL-10R blockade, there was a higher number of IL-17+ CD4+ T cells in the airways compared to IgG1-treated controls (Fig. 4.16C). There was no difference in the number of IL-17 positive CD8+ T cells between the anti-IL-10R Ab-treated groups and IgG1-treated controls (Fig. 4.16D).
Figure 4.16 Prior IL-10R blockade increases the number of IFN-γ-, TNF-α-, and IL-17- producing T cells at day 6 of a subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which mice were infected i.n. with 1.4x10^5 pfu. Mice were sacrificed at day 6, BAL performed and airway cells were stained with anti-CD4 PerCp and anti-CD8 FITC after which the cells were fixed and permeabilised and stained with anti-TNF-α PE, anti-IFN-γ PE-Cy7 and anti-IL-17 APC. The number of IFN-γ and TNF-α positive CD4⁺ (A) and CD8⁺ T cells (B) and the number of IL-17 positive CD4⁺ (C) and CD8⁺ (D) T cells was calculated by multiplying the percentage positive x the number of cells in the lymphoid gate x viable cell count. Data are representative of 2 experiments; n=5 +/- SEM. *= p<0.05, ** = p<0.01.
The differentiation of Th17 cells is driven by TGF-β, IL-6 and IL-21 and IL-23 is required for the maintenance of this population. As mice previously treated with anti-IL-10R Ab had higher numbers of Th17 cells during influenza infection (Fig. 4.16), it was of interest to analyse the production of these factors. Lung homogenate from IgG1- and anti-IL-10R Ab-treated mice was then analysed for IL-6 and IL-23 levels.

At day 0 and day 3 of influenza infection there were significantly higher levels of IL-6 in the lung homogenates of mice previously treated with anti-IL-10R (Fig. 4.17A). This was consistent with the hypothesis that blockade of IL-10R might result in lung inflammation in the un-infected state and might enhance inflammation during influenza infection. On the other hand, there was no significant difference in the levels of IL-23 in the lungs of either group of mice during influenza infection (Fig. 4.17B).

Figure 4.17 IL-6 but not IL-23 levels are enhanced during influenza infection in the lungs of mice previously treated with anti-IL-10R Ab. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which mice were infected i.n. with 1.4x10⁵ pfu influenza X31 on day 0. Mice were sacrificed at the time points shown and the lungs extracted. Lung lobes were homogenised and supernatants assessed for IL-6 (A) and IL-23 (B) by ELISA. n=5 +/- SEM * = p<0.05, ** = p<0.01. Data are representative of 2 experiments.
4.2.12 Prior IL-10R blockade does not significantly alter chemokine production during influenza infection

Due to the increased numbers of monocyte/macrophages seen in the airway during influenza infection following IL-10R blockade (Fig.4.12B) it was of interest to analyse the production of macrophage chemoattractants in the lungs and airways. Macrophage chemoattractants MCP-1 and MIP-1α were of particular interest as they are highly induced during influenza infection.\textsuperscript{41, 94}

C57BL/6 mice were pre-treated on days -8, -6, -4 and -2 with 200μg anti-IL-10R Ab or rat IgG, after which they were infected i.n. at day 0 with $1.4 \times 10^5$ pfu influenza X31. Mice were culled 1, 3, 6 or 14 days post infection and the lungs extracted. Lung homogenates were then analysed for the production of MCP-1 and MIP-1α. Levels of MCP-1 (Fig. 4.18A) and MIP-1α (Fig. 4.18B) were comparable between anti-IL-10R Ab-treated mice and rat IgG1-treated mice at all time points analysed except day 6, when levels of both chemokines were lower in the lungs of mice which had been previously treated with anti-IL-10R Ab. This is interesting as numbers of macrophages in the lungs of anti-IL-10R Ab-treated mice were comparable with control mice at day 6 of influenza infection.

4.2.13 The effect of IL-10R blockade on macrophage responses to TLR stimulus

During \textit{Pneumocystis carinii} infection, IL-10 knockout mice display heightened clearance of bacteria, and have increased levels of IFN-γ in BAL fluid.\textsuperscript{472} The authors suggest that alveolar macrophages from these mice have a lower threshold of activation than wild type mice. Additionally, IL-10-treated \textit{Mycobacterium bovis}-infected macrophages have defective IFN-γ-mediated killing, which suggests that IL-10 can alter macrophage function.\textsuperscript{473}
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Figure 4.18 Prior IL-10R blockade reduces chemokine levels in the lungs at day 6 of subsequent influenza infection. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days after which mice were infected i.n. with 1.4x10^5 pfu on day 0. Mice were sacrificed at the time points shown and the lungs extracted. Lung lobes were homogenised and supernatants assessed for MCP-1 (A) and MIP-1α (B). n=5 +/- SEM. * = p<0.05. Data are representative of 2 experiments.

In order to assess if IL-10R blockade had altered the ability of alveolar macrophages to respond to microbial stimulus, it was of interest to stimulate alveolar macrophages ex vivo with TLR agonists. C57BL/6 mice were injected i.p. with 200μg anti-IL-10R ab or rat IgG1 isotype control every other day for 8 days, after which the mice were sacrificed and BAL performed. Alveolar macrophages were then isolated from BAL fluid as described in the Methods section, and stimulated with an agonist to TLR-7, Imiquimod, at concentrations of 5μg/ml, 4μg/ml, 3μg/ml, 2μg/ml and 1μg/ml. Cell supernatants were collected 6 hours and 24 hours post stimulation. Imiquimod mimics the effects of viral TLR-7 agonists and induces the activation of NFκB \(^{474}\). IL-6 and TNF-α production were used a read-out of responsiveness to TLR stimulation.

Contrary to expectations, alveolar macrophages from mice which had been previously treated with anti-IL-10R ab had markedly reduced levels of IL-6 and TNF-α at 6 hours.
post stimulation compared to rat IgG1-treated controls (Fig 4.19A and B respectively). At 24 hours production of IL-6 and TNF-α was similar between the anti-IL-10R Ab-treated alveolar macrophages and IgG-treated controls (Fig.4.19C and D respectively).

**Figure 4.19** Prior IL-10R blockade impairs TLR responsiveness at early time points. C57BL/6 mice were injected i.p. with 200 μg anti-IL-10R Ab (clear bars) or rat IgG1 (grey bars) every other day for 8 days, after which the mice were culled and BAL performed. Alveolar macrophages were isolated as described in the Methods and stimulated with concentrations of Imiquimod (R387) as displayed above. Supernatants were harvested and IL-6 and TNF-α levels at 6 hours (A and B respectively) and 24 hours (C and D respectively) by ELISA. n=3 +/- SEM. Data represent one experiment.
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4.3 Discussion

4.3.1. IL-10R blockade does not dramatically alter lung and airway homeostasis

It was hypothesised that a lack of regulation by IL-10 in the lung at homeostasis would alter the lung micro-environment and result in low grade pulmonary inflammation, which would have been evident in increased lung and airway cellularity, as well as enhanced macrophage activation. However, the differences observed were fairly subtle. The main difference observed on IL-10R blockade was enhanced numbers of airway cells and alveolar macrophages. This result is consistent with reports that a lack of the myeloid inhibitory receptor CD200R leads to enhanced numbers of alveolar macrophages; similar results were observed by our group upon TGF-β blockade (J. Goulding and T. Hussell; unpublished observations). The increase in alveolar macrophage number indicates a dysregulation of immune homeostasis in the airways and could be due to enhanced proliferation; this will be tested in future. Cell numbers in the lung were unaffected by IL-10R blockade, with T cell, DC and macrophage numbers unaffected by IL-10R blockade.

It is not altogether surprising that IL-10R blockade did not lead to more striking airway inflammation, as several mechanisms exist in the lungs and airways to regulate inflammation. For example, TGF-β is present and is constantly being activated from its latent form by the integrin αvβ6 on the epithelium. TGF-β is a potent anti-inflammatory cytokine and has a major role in the maintenance of lung homeostasis. In the liver IL-10 and TGF-β redundantly protect against liver injury and mortality during acute schistosomiasis. It is possible that a similar compensatory mechanism exists in the lungs and airways, which would explain the partial effect on alveolar macrophage numbers and no other evidence of altered homeostasis. Additionally, although IL-10 enhances the expression of the regulatory molecule CD200R on macrophages, blockade of IL-10R did not alter CD200R expression (data not shown) and it is possible that other factors present in the airways such as TGF-β, GMCSF or surfactant proteins...
have roles in keeping the expression of CD200R at normal levels, and therefore allowing macrophages to be regulated.

Interestingly, neither TGF-β nor CD200R knockout mice display overt lung inflammation until a much later age and only have dysregulated responses to infection. Although IL-10 is clearly vital for maintaining homeostasis in the gut (as IL-10 knockout mice develop spontaneous colitis), it is possible that several compensatory mechanisms exist in the lungs and airways. It would be interesting to investigate the combined effect of IL-10R and TGF-β blockade, or the effects of blocking IL-10R in a CD200R knockout mouse. If there is redundancy between these regulatory pathways then it would be expected that blockade of another regulatory pathway in addition to the IL-10/R pathway would have an additive and therefore more pronounced effect on the dysregulation of homeostasis.

4.3.2 Prior IL-10R blockade enhances the alternative activation of macrophages during subsequent influenza infection

A lack of the myeloid regulator PPAR-γ in alveolar macrophages results in spontaneous pulmonary Th1 inflammation, and IL-10R blockade in IL-4R knockout mice decreases YM1 and MR expression. Therefore it was of interest to determine if IL-10R blockade affects macrophage phenotype at homeostasis. Interestingly, IL-10R blockade did not alter alveolar macrophage expression of markers of alternative activation such as MR, YM1 or RELM-α in the absence of inflammation; consistently, there was no change in the amount of soluble YM1 or RELM-α in the BAL fluid. Lung CD11c+ macrophage expression of MR, YM1 and RELM-α was similarly unchanged after IL-10R blockade, which is consistent with the idea that they may be residual alveolar macrophages not washed out by lavage. Interestingly, although lung monocyte/macrophage expression of MR and YM1 was unchanged, expression of RELM-α was enhanced upon IL-10R blockade.
Recent reports indicate that IL-10 and IL-4 redundantly drive the expression of YM1 and MR \(^{308, 439}\) and this might account for the lack of a change in YM1 and MR expression upon IL-10R blockade. The increase in the percentage of RELM-\(\alpha\) positive lung monocyte/macrophages is interesting and suggests that RELM-\(\alpha\) is regulated by additional factors distinct from those that regulate YM1 and MR.

Interestingly, at day 6 of influenza infection, mice which had been previously treated with anti-IL-10R Ab had a significantly higher percentage of RELM-\(\alpha\) positive alveolar, lung CD11c\(^+\) and lung monocyte/macrophages, and a higher percentage of YM1 positive alveolar macrophages, airway monocyte/macrophages and lung CD11c\(^+\) macrophages. Soluble RELM-\(\alpha\) was also increased in BAL fluid at day 6, and soluble YM1 was increased at day 3 during influenza infection subsequent to IL-10R blockade.

Alternatively activated macrophages are conventionally induced by IL-4/IL-13 stimulation \(^{45, 475}\), and have been shown to be vital for the resolution of RSV infection in mice \(^{431}\). However during influenza infection, the increase in the proportion of RELM-\(\alpha\) and YM1 positive macrophages in the anti-IL-10R-treated group was not associated with faster resolution of weight loss, nor was inflammatory infiltrate lessened in these mice. It is unclear what role alternatively activated macrophages play in influenza infection and indeed what factors up-regulate their expression (this is discussed more in the previous chapter) as there is not a significant amount of IL-4 produced during influenza infection; studies report as little as 10pg/ml in lung homogenate at day 7 of influenza infection \(^{442}\) and fewer than 1% of lung and airway T cells producing IL-4 at day 7 of influenza infection \(^{441}\).

It is known that IL-33 amplifies the polarisation of alternative activation of alveolar macrophages \(^{149}\) and studies in the laboratory have shown that IL-33 is present in the lungs and airways at homeostasis and during influenza infection (E.L Wissinger and T. Hussell; manuscript in preparation). We hypothesised that IL-10R blockade may have
enhanced the production of IL-33, which drives the polarisation of alternatively activated macrophages in the lung. However, IL-10R blockade had no effect on IL-33 production (data not shown). This indicates that other factors may be driving the increased expression of markers of alternative activation by lung and airway macrophages seen upon IL-10R blockade.

The half-life of the anti-IL-10R antibody is not known, however, treatment regimens in other studies have ranged from daily administration to every four days. Therefore it is possible that the effects of the antibody might last into the influenza infection, although treatment was stopped two days prior to infection. It is also possible that, as the antibody was administered intra-peritoneally, it has systemic effects on sites other than the lung, and might impact on the phenotype of cells in other organs, such as the gut.

4.3.3 IL-10R blockade leads to increased cellular infiltrate into the lungs and airways during a subsequent influenza infection

At day 3 of influenza infection, mice which had been treated with anti-IL-10R Ab had increased total cell numbers and numbers of monocyte/macrophages in the airways; there was also a non-significant trend towards higher numbers of alveolar macrophages at this time point. MCP-1 and MIP-1α are chemoattractants for macrophage recruitment into the lungs and airways during inflammation; we hypothesised that prior IL-10R blockade led to production of more of these chemokines during influenza infection, and therefore increased recruitment of macrophages. It is also possible that the partial increase in macrophage numbers seen at homeostasis was an indication that the recruitment of macrophages had been altered. Although there was no effect on CCR2 expression by alveolar macrophages at homeostasis it is possible that other receptors for macrophage chemoattractants and their ligands were up-regulated. Additionally, MCP-1 and MIP-1α levels were not altered during influenza infection except at day 6, when they were actually reduced in IL-10R Ab-treated mice.
It is possible that the increase in monocyte/macrophage numbers seen at day 3 of influenza infection in the anti-IL-10R-treated animals is due to proliferation of cells *in situ* or a lack of cell death. This will be tested in future by inclusion of markers for proliferation such as BRDU, Ki67 or markers of apoptosis such as Annexin V.

4.3.4 Prior IL-10R blockade augments T cell numbers in the airways during subsequent influenza infection

T cells are primed by DCs in the lymph node and migrate to sites of infection via homing signals, where they then proliferate under the influence of cytokines like IL-2 and co-stimulatory molecules. The increase in T cell numbers in the airways at day 6, which is the peak of T cell infiltrate during influenza infection, might result from the increased monocyte/macrophage numbers earlier in the infection. Monocyte/macrophages secrete myriad cytokines and chemokines such as TNF-α and IFN-γ which activate T cells and the endothelium. Activated monocyte/macrophages also express co-stimulatory molecules such as GITR ligand and OX40 ligand, which when bound to their receptors on T cells result in T cell proliferation and survival. Higher monocyte/macrophage expression of these co-stimulatory molecules would result in prolonged T cell survival and T cell proliferation.

The increase in total T cell number in the airways of the IL-10R Ab-treated mice correlates with a higher number of TNF-α-, IFN-γ- and IL-17-producing T cells, which contribute to a highly inflammatory environment. Higher influenza virus load could lead to increased T cell infiltrate into the lungs and airways, but as viral load was comparable between anti-IL-10R Ab-treated and IgG1-treated mice, this was not the reason for the enhanced T cell numbers. Interestingly, the increase in T cell number and consequently IFN-γ and TNF-α did not result in faster clearance of the virus, nor did it result in increased weight loss.
4.3.5 Prior IL-10R blockade alters macrophage responses to TLR stimulus

There is evidence that IL-10 can alter the function of macrophages; for example IL-10 inhibits IFN-γ-mediated killing of *Mycobacterium bovis*[^473] and *Paracoccidioides brasiliensis*[^476]. Additionally, IL-10 knockout mice have enhanced IFN-γ production at early time points during *Bordetella pertussis* infection, indicating that they are intrinsically more activated[^477]. Furthermore, IL-10 decreases the production of TNF-α and IL-6 by macrophages[^257, 258].

It was therefore hypothesised that a lack of the suppressive effects of IL-10 at homeostasis would enhance the response of alveolar macrophages to antigenic stimuli. However when alveolar macrophages were treated with various dilutions of the TLR7 agonist Imiquimod, the alveolar macrophages from mice pre-treated with anti-IL-10R Ab produced less IL-6 and TNF-α at early time points than those from mice treated with rat IgG1 (Fig. 4.18), indicating that perhaps they are actually less activated. Therefore, the absence of the regulatory effects of IL-10, instead of making alveolar macrophages inherently more responsive to antigenic stimuli, might just change the nature of the response. It would be informative to analyse the expression of co-stimulatory molecules on these macrophages; it is possible that instead of producing vast amounts of cytokines, these macrophages act in a more “professional” manner, up-regulating the expression of co-stimulatory molecules such as GITR ligand or OX40 ligand.

4.4 Conclusion

It is known that IL-10 and IL-10R knockout mice have altered immune homeostasis and depending on the dose of influenza used, better or worse outcome upon influenza infection. The aim of this study was to determine if a transient blockade of IL-10R would significantly alter lung homeostasis and/or alter the subsequent immune response to influenza infection. While there were no major alterations in lung

[^473]: Chapter 4: The impact of IL-10R blockade on lung homeostasis
[^476]: Chapter 4: The impact of IL-10R blockade on lung homeostasis
[^257]: Chapter 4: The impact of IL-10R blockade on lung homeostasis
[^258]: Chapter 4: The impact of IL-10R blockade on lung homeostasis
homeostasis, prior IL-10R blockade resulted in increased cellularity at key time points during influenza infection, as well as altering the expression profile of the markers of alternative activation YM1 and RELM-α. This indicates that although there is redundancy in myeloid regulators such that the transient blockade of IL-10R did not result in an overtly altered homeostasis, transient blockade of this one regulatory pathway was enough to alter the immune response to a subsequent influenza infection. Therefore, although the different myeloid regulators may be redundant in maintaining lung homeostasis, once an infection is added then the true magnitude of an individual component can be seen, such as in the case of pulmonary infections in CD200, CD200R, IL-10 and IL-10R knockout mice.
5.0 The role of GITR and GITRL in influenza-induced inflammation

5.1 Introduction

The last chapter revealed that blockade of IL-10R in the un-inflamed lung did not have a major impact on lung homeostasis; however, during a subsequent influenza infection mice had greater airway inflammatory infiltrate and higher production of inflammatory cytokines by T cells. This indicates that a transient blockade of the regulatory effect of the IL-10/IL-10R interaction in the un-inflamed lung is sufficient to alter the immune response to a subsequent influenza infection. While this is useful for understanding the relative contribution of the IL-10/IL-10R axis in maintaining lung and airway homeostasis, physiologically it is more relevant to explore a pathway that could be manipulated therapeutically to improve disease outcome during influenza infection.

5.1.1 Ameliorating influenza-associated immune pathology

Influenza-associated lung pathology directly correlates with the pathogenicity of the viral strain and in turn the inflammatory infiltrate into the lungs and airways \(^{223, 369, 478}\). Infected epithelial cells produce RANTES, MCP-1 and IL-8, which recruit T cells, monocytes and neutrophils into the lungs and airways \(^{49}\). Infected alveolar macrophages and monocyte/macrophages secrete pro-inflammatory cytokines such as MIP-1α, MCP-1, TNF-α, IL-1β, IL-6 and IFN-α and IFN-β \(^{467}\). This in turn leads to induction of other cytokines and chemokines such as MCP-1, MCP-3 and IP-10, which recruit more inflammatory cells into the lungs and airways \(^{49}\). T cells are also recruited, and produce IFN-γ and TNF-α, which, while important for viral clearance, in excess are highly pro-inflammatory and contribute to lung pathology \(^{223, 479, 480}\). The mere presence of the vast number of inflammatory cells recruited into the airways during influenza infection causes acute respiratory distress syndrome in humans and mice and occludes gaseous exchange in the airways \(^{481, 482}\). Therefore, recent studies in mouse
models have focused on reducing the influx of cells into the airways in an effort to reduce bystander damage to the lungs.

Therapeutic neutralisation of pro-inflammatory cytokines during influenza infection is beneficial for disease outcome. For example neutralisation of TNF-α reduces weight loss and lung pathology during influenza infection and mice lacking MIP-1α also have reduced lung immune pathology during influenza but suffer from delayed viral clearance. Conversely CCR2 null mice have better disease outcome during influenza infection, and the same result is seen with blockade of CCR2. Neutralisation of IFN-γ during influenza infection decreases lung cellular infiltrate, and depletion of T cells enhances survival to lethal influenza infection.

Previous efforts in the laboratory have sought to reduce the influenza-associated lung pathology and improve disease outcome by targeting co-stimulatory molecules during infection. One such co-stimulatory molecule is OX40, which is expressed on late activated T cells and when bound by its ligand, OX40L, promotes T cell survival and cytokine production. OX40 and OX40L are highly up-regulated in the lungs during influenza infection. Blockade of OX40 ameliorates influenza-induced weight loss and lung and airway inflammatory infiltrate leading to significantly reduced immune pathology without affecting viral clearance. This reduction in immune pathology is attributed to diminished proliferation and increased apoptosis of T cells.

All of these studies suggest that neutralisation of one component of immunity, especially therapeutically, may benefit immune pathology during lung viral infection. This chapter will explore the role of another co-stimulatory pair which, like OX40 belongs to the TNF receptor super family: glucocorticoid-induced TNF receptor-related protein (GITR) and its ligand, GITRL.
5.1.2 GITR and GITRL in inflammatory lung disease

GITR expression is enhanced on lung and pleural macrophages during carrageenan-induced pleurisy and GITR⁺ mice display significantly reduced cell numbers in the pleural cavity compared to wild type mice, which is associated with a reduction in the number of neutrophils and macrophages. Additionally GITR⁻/⁻ mice display lower levels of pro-inflammatory factors such as COX-2, PGE₂ and iNOS in lung homogenates. Treatment of wild type mice with GITR:Fc fusion protein, which binds GITRL and blocks signalling to GITR, equally leads to improved disease outcome during carrageenan-induced pleurisy. The authors attribute this diminished inflammation to a reduction in NFκB signalling and decreased expression of cell adhesion molecules on endothelium in the absence of GITR. Indeed, other groups show that GITR signalling leads to NFκB activation and that signalling through GITR on macrophages leads to the production of inflammatory mediators such as COX-2 and cell adhesion molecules such as ICAM-1.

A lack of GITR is also beneficial for bleomycin-induced chronic lung inflammation; GITR⁻/⁻ mice and wild type mice treated with GITR:Fc have reduced immune pathology and inflammation in the lungs and airways. Again, the authors attribute this improvement in disease outcome to an absence of GITR. However, GITR/GITRL signalling is bi-directional, and therefore a GITR⁻/⁻ mouse will lack the effects of both GITR and GITRL signalling whereas GITR:Fc binds GITRL and prevents signalling through GITR but signalling through GITRL may remain intact. Because the phenotype of the GITR⁻/⁻ mice and the GITR:Fc-treated mice was similar, the authors suggest that perhaps GITR signalling is more important for the activation of macrophages.

The above studies propose a role for GITR in the activation of macrophages and NFκB signalling, and further studies suggest that interactions between GITR on leukocytes and GITRL on the endothelium could aid extravasation, thereby implicating this co-stimulatory pair in the progression of inflammation.
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While many show that GITRL signalling is inflammatory \(^{359, 485}\) it appears that its effects may be cell-specific. For example, signalling through GITRL on plasmacytoid (p) DCs leads to induction of IDO and is therefore anti-inflammatory \(^{363}\). Furthermore, Grohmann *et al* show that dexamethasone up-regulates GITRL on pDCs and dexamethasone-dependent induction of IDO occurs via the interaction between GITR and GITRL \(^{363}\). They subsequently show that improved outcome of allergic bronchopulmonary aspergillosis after treatment with dexamethasone is via a GITRL-dependent up-regulation of IDO production \(^{363}\).

While blockade of GITR signalling is clearly beneficial for disease outcome in chronic lung inflammation, potentiating the signal is detrimental. For example in mouse models of asthma, treating mice with a GITR agonist exacerbates disease severity, with enhanced T cell activity and concomitant production of Th2 cytokines and airway hyper-responsiveness \(^{365}\).

GITR and GITRL are clearly important for the development of pulmonary inflammation but their role in acute, viral-induced pulmonary inflammation has not yet been determined.

### 5.1.3 Hypothesis

This chapter will explore the hypothesis that GITR and GITR ligand play a role in influenza-induced inflammation and will answer the following questions:

- Are GITR and GITRL expressed in the lungs and airways during influenza infection?
- What is the effect of blocking the interaction between this co-stimulatory pair?
- What factors up-regulate GITR and GITRL expression?
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5.2 Results

5.2.1 GITR and GITR ligand expression on lung and airway cells

The expression of GITR and GITRL on immune cell subsets in the lungs and airways has not been definitively characterised, therefore it was of interest to ascertain expression of this co-stimulatory pair on lung and airway immune cell subsets. Single cell suspensions of lung homogenates and BAL cells from un-infected BALB/c mice were stained with specific antibodies against GITR and GITRL.

As previously published 325, 326 GITR is constitutively expressed by T cells, and CD4+ and CD8+ T cells in the lungs were also GITR positive (Fig. 5.1A and C). B cells were next examined in the lung, and appeared to have modest expression of GITR (43+/−4% were GITR positive) compared to CD4+ and CD8+ T cells (Fig. 5.1A and 5.1C). T cells and B cells were also analysed for expression of GITRL. The percentage of GITRL positive CD4+ and CD8+ T cells was very low (Fig. 5.1B and D). On the other hand, approximately 21 +/-2.9% of B cells were positive for GITRL (Fig. 5.1B and D). At present it is unclear why only a proportion of B cells express GITR and GITRL. It would be interesting to sub-divide these B cells into those expressing specific antibody isotypes to determine if GITR or GITRL expression correlates with a switch from IgM positive to B cells double positive for IgD, IgG, or IgA. IgA would be particularly interesting in this mucosal site. The same is true for the proportions of CD4+ and CD8+ T cells expressing GITRL. It is possible that low level antigen stimulation occurs in small numbers of T cells in mucosal tissues. A comparison with cells in the spleen should also be performed in the future. It was not possible to analyse lymphoid cells in the air spaces due to their very low numbers in healthy mice.
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Figure 5.1 GITR and GITRL ligand expression on lymphocytes in un-infected lungs and airways. BALB/c mice were culled and the lungs extracted, homogenised into single cell suspension and stained with anti-CD4 PerCp and anti-CD8 APC or anti-B220 PerCp and anti-CD3 APC, and anti-GITR or anti-GITRL PE. The proportion of CD4+ (circles) and CD8+ (squares) T cells and B cells (triangles) expressing GITR (A) and GITRL (B) was determined by flow cytometry. Representative histograms are shown of GITR (C) and GITRL (D) expression on CD4+ and CD8+ T cells and B cells. Data are representative of 3 independent experiments, n=4/5 ±/− SEM. Shaded histograms represent isotype control staining, and clear histograms represent specific antibody staining.
Next, macrophage populations were examined for GITR and GITRL expression. Neither alveolar macrophages nor lung CD11c+ macrophages expressed significant levels of GITR (Fig. 2A and C). We believe that the CD11c+ macrophages found in lung homogenates may be residual alveolar macrophages that are not washed out of the airways during lavage and the similar lack of GITR expression observed on these two macrophage populations further supports this idea. The percentage of GITR positive lung monocyte/macrophages was low, with just approximately 8.8 +/- 1% GITR positive cells (Fig. 2A and C).

Like GITR, GITRL was not expressed by alveolar and lung CD11c+ macrophages (Fig. 2B and D). However, GITRL was expressed on approximately 18 +/- 4.9% of lung monocyte/macrophages (Fig. 2B and D). The low expression of GITRL on monocyte/macrophages and the absence of GITRL on alveolar macrophages is in contrast with reports that GITRL mRNA and protein is expressed by splenic, peritoneal and bone marrow-derived macrophages and may suggest site-specific regulation of GITRL. Low expression of GITR and GITRL is also reported in ocular tissues and may represent regulation of this co-stimulatory pair at sites where expression of this inflammatory pair would be highly deleterious.

5.2.2 GITR and GITRL are differentially up-regulated on lymphocytes in the lungs and airways during influenza infection

GITR and GITRL were differentially expressed in the un-inflamed lung, but in order to analyse the role this co-stimulatory pair play during influenza infection, it was of interest to follow their expression during influenza infection. Therefore a time course experiment was performed, and BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 at day 0. Mice were culled 4, 7 or 10 days after influenza infection, BAL performed and the lungs removed and homogenised.
Figure 5.2 GITR and GITR ligand expression on myeloid cells in un-infected lungs and airways. BALB/c mice were culled, BAL performed, and the lungs removed. BAL fluid and lung homogenates were stained with anti-CD11c APC, anti-CD11b PerCp, anti-F4/80 PECy-7 and anti-GITR or anti-GITRL PE. The percentage of alveolar macrophages (circles), lung monocyte/macrophages (squares) and lung CD11c+ macrophages (triangles) expressing GITR (A) and GITRL (B) were determined by flow cytometry. (C) and (D) display representative histograms of GITR and GITRL expression respectively on the above cell populations. Data are representative of 3 independent experiments, n=4/5 +/-SEM. Shaded histograms represent isotype control staining, and clear histograms represent specific antibody staining.
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T cells in the airways were only analysed at day 7 and 10 due to limiting cell numbers in BAL at early time points during influenza infection. As observed in the un-inflamed lung, the majority of CD4+ and CD8+ T cells in the airways expressed GITR at day 7 and this remained unchanged at day 10 (Fig. 5.3A). The GM was used as a crude method to determine up-regulation of GITR on T cells. Like the percentage positive, the GM of GITR on T cells in the airways did not significantly change from day 7 to 10 of influenza infection (Fig. 5.3B).

Figure 5.3 GITR expression is unchanged on T cells in the airways during influenza infection. BALB/c mice were infected with 1.4x10^5 pfu influenza X31 on day 0 and the mice culled at the time points indicated. BAL was then performed and the lungs extracted. BAL cells were stained with anti-CD4 PerCp, anti-CD8 FITC, and anti-GITR or anti-GitRL PE. The percentage (A) and GM (B) of airway CD4+ (clear bars) and CD8+ (grey bars) T cells expressing GITR were determined by flow cytometry. n=5 +/- SEM; data represent 2 independent experiments

CD4+ and CD8+ T cells in the un-inflamed lung were almost all positive for GITR and this did not change at any of the time points examined during influenza infection (Fig. 5.4A). However, the percentage of GITR positive B cells in the lungs increased during influenza infection from approximately 43 +/- 4% at day 0, to 66 +/- 2% positive at day 7 (Fig. 5.4A). Like the expression of GITR on a subset of B cells in the un-inflamed lung, the increase in the percentage positive during influenza infection might reflect cells that have undergone an isotype switch. This will be analysed in the future.
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Figure 5.4 The intensity of GITR and GITRL on lymphocytes is differentially regulated during influenza infection. BALB/c mice were infected with 1.4x10⁵ pfu influenza X31 on day 0 and the mice culled at the time points indicated. BAL was then performed and the lungs extracted. Lung homogenates and BAL cells were stained with anti-CD4 PerCp, anti-CD8 FITC, anti B220 PerCp and anti-GITR or anti-GITRL PE. The percentage (A) and GM (B) of GITR on CD4⁺ (clear bars) and CD8⁺ (grey bars) T cells and B cells (striped bars) in the lungs; and the percentage (C) and GM (D) of GITRL on CD4⁺ (clear bars) and CD8⁺ (grey bars) T cells and B cells (striped bars) in the lungs were determined by flow cytometry. * = p<0.05, ** = p<0.01, *** = p<0.001 compared to day 0. n=5 +/- SEM; data represent 2 independent experiments.
Previous studies report that GITR is up-regulated by T cells upon activation \(^{324}\). Therefore, although the percentage of GITR positive T cells in the lung did not significantly change during influenza infection, it was of interest to examine the intensity of GITR on these cells, by analysing the GM. As expected, the GM of GITR on CD4\(^+\) and CD8\(^+\) T cells increased during influenza infection, peaking at day 7 of infection, a time point when T cell numbers are maximal and levels of inflammatory cytokines in the lungs are high and therefore T cells are activated \(^{224}\) (Fig. 5.4B). At day 10 of influenza infection the GM of GITR on T cells remained high (Fig. 5.4B). Therefore, GITR expression was increased on a per cell basis during influenza infection. Although the percentage of GITR positive B cells increased during influenza infection, the intensity of GITR on these cells did not change, as indicated an absence of change in GM values (Fig. 5.4B).

Some studies report that GITRL is up-regulated on T cells upon TCR stimulation \(^{326, 354}\), while others report no up-regulation of GITRL \(^{330}\). As activated T cells infiltrate the lungs during influenza infection it was important to assess whether T cells in the highly inflammatory environment of the influenza-infected lung expressed GITRL. There was very low expression of GITRL on T cells in the un-inflamed lung and for CD4\(^+\) T cells there was no significant change in the percentage positive at the time points examined during influenza infection (Fig. 5.4C). However, the percentage of GITRL positive CD8\(^+\) T cells increased significantly up to day 7 of infection (Fig. 5.4C). Unlike GITR expression on B cells, there was no significant change in the percentage of GITRL positive B cells in the lungs at any of the time points investigated (Fig. 5.4C). Once again, the question arises: why do only a subset of CD8\(^+\) T cells express GITRL and why do only a subset up-regulate it? The up-regulation is by approximately 10%. Perhaps this proportion are specific for influenza MHC class I epitopes, a hypothesis that could be tested by staining with MHC class I pentamers loaded with immune dominant peptides specific for BALB/c mice.
In accordance with flow cytometry data showing a low percentage of GITRL positive CD4+ T cells and no significant change during influenza infection, there was no change in the GM of GITRL on these cells (Fig. 5.4D). There was also no change in the GM of GITRL on CD8+ T cells and a small but significant increase in GM of GITRL on B cells at day 10 of influenza infection compared to day 0 (Fig. 5.4D).

5.2.3 GITR and GITRL are up-regulated on myeloid cells in the lungs and airways during influenza infection

Myeloid cells in the lungs were next examined for GITR and GITRL expression during a time course experiment of influenza infection. Mice were infected as before on day 0 with 1.4x10^4 pfu influenza strain X31 and culled 4, 7 or 10 days afterwards.

The up-regulation of GITR and GITRL on macrophage populations was the most dramatic effect of any cell population during influenza infection. From a low starting percentage, GITR positive CD11c+ alveolar macrophages increased significantly during influenza infection, peaking at day 7 (Fig. 5.5A). Interestingly, at day 10 the percentage of GITR positive cells was still high compared to day 0 (Fig. 5.5A). It would be of interest to determine whether GITR ever returned to base line by examining later time points. Airway monocyte/macrophages expressing CD11b but low levels of CD11c infiltrate the airways during influenza infection and these were examined at days 4, 7 and 10 post infection but are not present at day 0 so could not be analysed at that time point. There was a slight but insignificant increase in the percentage of GITR positive monocyte/macrophages in the airways throughout influenza infection (Fig. 5.5A). However, this was not as dramatic as the increase in GITR expression by CD11c+ alveolar macrophages.
Figure 5.5 GITR and GITRL expression on airway macrophages during influenza infection. BALB/c mice were infected with $1.4 \times 10^5$ pfu influenza X31 on day 0, the mice culled and BAL performed at the time points indicated. BAL cells were stained with anti-CD11c APC, anti-CD11b PerCp, anti F4/80 PECy7 and anti-GITR or anti-GITRL PE. The percentage and GM of of alveolar macrophages (clear bars) and airway monocyte/macrophages (grey bars) expressing GITR (A and B, respectively) and GITRL (C and D respectively) were determined by flow cytometry. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ compared to day 0. n=5 +/- SEM; data represent 2 independent experiments.
The intensity of GITR expression on macrophage populations in the airways was next measured by analysing GM. The GM of GITR on CD11c+ alveolar macrophages increased significantly from day 0 to day 4 of influenza infection, but there was no significant increase in GM at any other time points compared to day 0 (Fig. 5.5B). On the other hand, the intensity of GITR on infiltrating CD11b+ airway monocyte/macrophages decreased from day 4 to day 7 and 10, contrary to the trend seen for the percentage of positive cells (Fig. 5.5B).

Like GITR, there was a low percentage of GITRL positive alveolar macrophages at day 0, but the percentage positive increased significantly during influenza infection up to days 7 and 10 of infection (Fig. 5.5C). GITRL expression on infiltrating monocyte/macrophages could not be examined at day 0 but there was no change in the percentage positive between days 4, 7 and 10 post influenza infection (Fig. 5.5C). GITRL intensity on alveolar macrophages followed a similar pattern to the percentage positive, with a significant increase up to day 7; however, there was no change in GITRL intensity on airway monocyte/macrophages at any of the time points analysed (Fig. 5.5D). In the airways the up-regulation of GITR and GITRL therefore seems to occur predominantly on CD11c+ alveolar macrophages.

GITR and GITRL expression was next examined on myeloid cells in the lungs. Lung CD11c+ macrophages, which we believe may be residual alveolar macrophages not washed out by lavage, had little expression of GITR at day 0 but this increased significantly from day 4 of the infection, peaking at day 7; at day 10 the percentage of GITR positive CD11c+ macrophages decreased but was still slightly higher than that observed at day 0 (Fig. 5.6A).
Figure 5.6 The regulation of GITR and GITRL expression on myeloid cells in the lungs during influenza infection. BALB/c mice were infected with 1.4x10^5 pfu influenza X31 on day 0, mice culled and the lungs extracted at the time points indicated. Lung homogenates were stained with anti-CD11c APC, anti-CD11b PerCp, anti F4/80 PECy-7 and anti-GITR or anti-GITRL PE. The percentage and GM of lung CD11c^+ macrophages (clear bars) and lung monocyte/macrophages (grey bars) expressing GITR (A and B, respectively) and GITRL (C and D respectively) were determined by flow cytometry. * = p<0.05, ** = p<0.01, *** = p<0.001 compared to day 0. n=5 +/- SEM; data represent 2 independent experiments.
Lung monocyte/macrophages similarly up-regulated expression of GITR, with the peak of percentage positive cells at day 7; there was still a significantly higher percentage of GITR positive monocyte/macrophages at day 10, compared to day 0 (Fig. 5.6A). The intensity of GITR on lung CD11c+ macrophages peaked at day 4 of influenza infection, unlike the percentage positive, which was highest at day 7, while the intensity of GITR on lung monocyte/macrophages was highest at day 4 and day 7 (Fig. 5.6B).

The percentage of GITRL positive lung CD11c+ macrophages and lung monocyte/macrophages increased during influenza infection, peaking at day 7 of infection in a similar trend to GITR expression on these cell types (Fig. 5.6C). GITRL intensity similarly increased on lung monocyte/macrophages during influenza infection, peaking at day 7 for both lung CD11c+ macrophages and lung monocyte/macrophages (Fig. 5.6D).

The expression of GITR and GITRL on macrophage and lymphocyte populations during influenza infection is summarised in Table 5.1. The percentage of GITR and GITR ligand positive alveolar and lung CD11c+ macrophages significantly increased during influenza infection, however the percentage of GITR and GITRL positive airway monocyte/macrophages did not significantly change during influenza infection. On the other hand, influenza infection led to an increase in the percentage of GITR and GITRL positive lung CD11b+ monocyte/macrophages.
### Table 5.1 Summary of GITR and GITRL expression on lymphocyte and macrophage populations during influenza infection.

The percentage of GITR and GITRL positive cells is shown at day 0 (naïve mice) and at day 7, the peak of influenza infection. Values shown are averages +/- SD. GITR expression is high on naïve T cells and remains high during influenza infection while GITRL is only significantly up-regulated by CD8^+^ T cells. Roughly half of B cells in the lungs express GITR at homeostasis and this percentage significantly increases during influenza infection. All macrophage populations analysed up-regulated GITR and GITR ligand during influenza infection. ND= not detected. Data represent 2 independent experiments.

<table>
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<th>Cell type</th>
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<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
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<td>Lung CD4^+^ T cells</td>
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<td>99 ( +/- 0.2)</td>
<td>3.6 ( +/- 0.3)</td>
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<td>Lung CD8^+^ T cells</td>
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<td>98 ( +/- 0.3)</td>
<td>8 ( +/- 3)</td>
<td>21 ( +/- 1)</td>
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<tr>
<td>Lung B cells</td>
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<td>21 ( +/- 3)</td>
<td>13 ( +/- 3)</td>
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<td>31 ( +/- 7)</td>
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<tr>
<td>CD11b^+^ lung macrophages</td>
<td>8.8 ( +/- 1)</td>
<td>75 ( +/- 3)</td>
<td>18 ( +/- 4.9)</td>
<td>62 ( +/- 8)</td>
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5.2.4 Blockade of GITRL during influenza infection leads to increased weight loss and lung cellular infiltrate at early time points during influenza infection

As GITR and GITRL were up-regulated on immune cell subsets in the lungs and airways during influenza infection, it was of interest to determine the importance of the interaction between the two during infection. Signalling through GITRL on macrophages leads to the production of pro-inflammatory cytokines \(^{359}\) and signalling through GITR on T cells leads to survival and proliferation of T cells \(^{325}\). Additionally, signalling through GITR on macrophages is also pro-inflammatory, leading to the production of TNF-\(\alpha\) and IL-8 \(^{355}\). Therefore we hypothesised that blockade of GITRL might lead to a reduction in inflammation in the lungs during influenza infection and therefore decreased immune pathology.

BALB/c mice were infected i.n. with \(1.4 \times 10^5\) pfu influenza virus, strain X31 on day 0 and injected with 200\(\mu\)g anti-GITRL Ab or rat IgG1 i.p. on days 0, 2, 4 and 6. Mice were culled 4 or 7 days after infection and tissues were harvested as shown in Fig. 5.7A. Anti-GITRL binds to GITRL and blocks bi-directional signalling between GITR and GITRL (Fig. 5.7B). At present studies assume that the anti-GITRL antibody does not signal to GITRL.

Mice were weighed daily and weight loss measured as a percentage of original body weight; mice were then culled and BAL performed and the lungs and mediastinal lymph nodes extracted. Viable cell counts in the BAL fluid, mediastinal lymph node and lung homogenates were enumerated by Trypan blue exclusion.
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Figure 5.7 Experimental protocol. (A) BALB/c mice were infected i.n with 1.4x10^5 pfu influenza X31 on day 0; mice were then injected i.p. with 200μg anti-GITRL Ab or rat IgG1 i.p. on day 0, 2, 4 and 6. 4 mice per group were then culled at day 4 or 7 post influenza infection and the rest of the mice rested for four weeks, after which they were infected i.n. with 100 pfu influenza A/PR/8 (H1N1) and culled 4 days later. BAL was performed on sacrificed mice and the lungs and mediastinal lymph nodes extracted. (B) Anti-GITRL Ab binds GITRL and blocks bi-directional signalling between GITR and GITRL. GITR is shown on T cells and GITRL on APCs for simplicity but during influenza macrophages could also be GITR positive and T cells GITRL positive.
Mice treated with anti-GITRL Ab displayed transient enhanced weight loss at days 4 and 5 post infection compared to IgG1-treated controls, but there was no difference at any other time points, indicating that anti-GITRL treatment may have accelerated influenza-induced weight loss during innate immune activation (Fig. 5.8A). There was a slight but insignificant increase in the viable cell count in the BAL fluid of anti-GITRL Ab-treated mice compared to IgG1-treated controls at day 4 of influenza infection, but no difference at day 7 (Fig. 5.8B). Anti-GITRL Ab treatment resulted in a significant increase in total cell numbers in the lungs at day 4 of influenza infection, but like in the airways, cell numbers were similar between the two groups at day 7 (Fig. 5.8C). Weight loss during influenza infection is associated with excess inflammatory infiltrate into the lungs; therefore the increase in cell numbers in the lungs was consistent with increased weight loss observed at day 4 in anti-GITRL Ab-treated mice.

Total viable cell numbers in the mediastinal lymph nodes, which drain the lungs were unaffected by GITRL blockade (Fig. 5.8D).

5.2.5 GITRL blockade enhances T and myeloid cell numbers in the lungs during influenza infection

Influenza infection is associated with recruitment of T cells and macrophages into the lungs and airways, and this inflammatory infiltrate is associated with disease severity in influenza-infected mice. It is reported that signalling through GITR on T cells leads to T cell proliferation and signalling through GITRL on macrophages causes production of inflammatory factors, which could lead to recruitment of more cells into the airways. Therefore, we hypothesised that blocking the interaction between the two would lead to a reduction in T and myeloid cell numbers in the lungs and therefore a reduction in immune pathology.
Figure 5.8 The impact of GITRL blockade on influenza-associated weight loss and cellular infiltrate. BALB/c mice were infected with 1.4x10^5 pfu influenza X31 on day 0 and injected i.p. with 200 µg anti-GITRL Ab (closed symbols/grey bars) or rat IgG1 (open symbols/clear bars) on day 0, 2, 4 and 6; mice were then culled 4 or 7 days after infection, BAL performed and the lungs and mediastinal lymph nodes extracted. Weight was measured daily and expressed as a percentage of original weight (A). Total number of viable cells in the BAL fluid (B), and homogenates of lungs (C) and mediastinal lymph nodes (D) were determined using Trypan blue exclusion. * = p<0.05 n=4 +/- SEM. Data represent one experiment.
However, at day 4 of infection, there was a higher number of both CD4$^+$ and CD8$^+$ T cells in the lungs of anti-GITRL Ab-treated mice compared to IgG1-treated controls (Fig. 5.9A). In contrast, GITRL blockade did not alter the number of T cells in the lungs at day 7 (5.9B) nor was there a difference in T cell numbers in the airways at day 7 of infection (Fig. 5.9C). Airway T cells were not analysed at day 4 as the low number of T cells present at this time point make the results hard to interpret.

There was a slight but insignificant increase in the number of lung CD11c$^+$ macrophages, and a significant increase in the number of lung monocyte/macrophages at day 4 of influenza infection upon GITRL blockade (Fig. 5.10A). In contrast, there was no difference in numbers of either macrophage population in the lung at day 7 of influenza infection (Fig. 5.10B). Anti-GITRL Ab-treated mice had significantly higher numbers of CD11c$^+$ alveolar macrophages in the airways at day 4 compared to IgG1-treated controls; there were also slightly more CD11b$^+$ airway monocyte/macrophages in anti-GITRL Ab-treated mice at day 4, but this difference did not achieve statistical significance (Fig. 5.10C). At day 7, in contrast to day 4, IgG-1 treated mice had higher numbers of alveolar macrophages in the BAL fluid compared to anti-GITRL Ab-treated mice (Fig. 5.10D).

The increase in numbers of T cells and macrophages observed upon GITRL blockade reflected the increase in total viable cell count in the lungs observed at day 4 post infection.
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**Figure 5.9** GITRL blockade increases T cell numbers in the lungs at day 4 but not day 7 of influenza infection. BALB/c mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 on day 0, and injected with 200 ug anti-GITRL Ab (grey bars) or rat IgG1 (clear bars) on day 0, 2, 4 and 6. Mice were then culled 4 or 7 days after infection, BAL performed and the lungs removed. Lung homogenates and BAL cells were stained with anti-CD4 APC and anti-CD8 PerCp and the number of CD4+ and CD8+ T cells in the lungs at day 4 (A) and day 7 (B) and airways at day 7 (C) was determined by multiplying the percentage positive x number of cells in the lymphocyte gate x total number of viable cells. * p<0.05 n=4 +/- SEM. Data represent one experiment.
Figure 5.10 The effect of GITRL blockade on macrophage populations in the lungs and airways during influenza infection. BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 on day 0 and injected i.p. with 200 μg anti-GITRL Ab (grey bars) or rat IgG1 (clear bars) on days 0, 2, 4 and 6; mice were then culled 4 or 7 days after influenza infection and BAL performed and lungs removed. Single cell suspensions of lung homogenate and BAL cells were stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy-7 to identify lung CD11c macrophages and lung monocyte/macrophages at day 4 (A) and day 7 (B), and alveolar macrophages and airway monocyte/macrophages at day 4 (C) and day 7 (D). * p<0.05 n=4 +/- SEM. Data represent one experiment.
5.2.6 The impact of GITRL blockade on intra-cellular cytokine production by T cells

During influenza infection, T cells are recruited into the lungs and airways and produce high amounts of IFN-γ and TNF-α, which, while important for their anti-viral effects and activation of macrophages, also contribute to immune pathology. Production of the above two intra-cellular cytokines are an indicator of the activation state of T cells, and therefore it was important to assess the effect of anti-GITRL treatment on intracellular expression of these cytokines by T cells.

Anti-GITRL treatment did not alter the proportion of IFN-γ positive CD4+ T cells in the lungs at day 4 post influenza infection; but there was a slight but insignificant increase in the percentage of IFN-γ positive CD8+ T cells (Fig. 5.11A). When taking total recruited cells into account, anti-GITRL Ab-treated mice had significantly higher numbers of IFN-γ positive CD4+ and CD8+ T cells in the lungs compared to IgG-treated controls at day 4 (Fig. 5.11B). At day 7 of influenza infection anti-GITRL treatment induced an increase in the percentage of IFN-γ positive CD8+ T cells but had no effect on the percentage of IFN-γ positive CD4+ T cells (Fig. 5.11C); there was, however a slight but insignificant increase in the total number of IFN-γ positive CD4+ and CD8+ T cells (Fig. 5.11D).

Intra-cellular production of TNF-α by T cells was also assessed. Like IFN-γ, TNF-α is associated with influenza-induced immune pathology. Unlike IFN-γ, anti-GITRL Ab treatment did not alter the percentage of TNF-α-producing CD4+ or CD8+ T cells in the lungs at day 4 (Fig. 5.12A), however, the total number of TNF-α positive CD4+ and CD8+ T cells was enhanced (Fig. 5.12B). In contrast, anti-GITRL Ab treatment had no effect on the percentage or total numbers of CD4+ or CD8+ T cells producing TNF-α at day 7 (Fig. 5.12C and D respectively).
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Figure 5.11 The effect of GITRL blockade on intracellular IFN-γ production by T cells in the lungs. BALB/c mice were infected i.n. with $1.4\times10^5$ pfu influenza X31 on day 0 and injected i.p. with 200 μg anti-GITRL Ab (circles) or rat IgG1 (squares) on days 0, 2, 4 and 6. Mice were culled 4 or 7 days after influenza infection and the lungs removed. Single cell suspensions of lung homogenate were stained with anti-CD4 APC and anti-CD8 PerCp, fixed, permeabilised and stained with anti-IFN-γ FITC. The percentage of CD4+ (closed symbols) and CD8+ (open symbols) IFN-γ-producing T cells at day 4 (A) and day 7 (C) was determined by flow cytometry; the number of IFN-γ positive CD4+ (closed symbols) and CD8+ (open symbols) at day 4 (B) and day 7 (D) was determined by multiplying the percentage positive x percentage of CD4/CD8 T cells x percent of lymphocytes x total viable lung cell count. n=4 +/- SEM * = p<0.05. Data represent one experiment.
Figure 5.12 The effect of GITRL blockade on intracellular TNF-α production by T cells in the lungs. BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 on day 0 and injected i.p. with 200 μg anti-GITRL Ab (circles) or rat IgG1 (squares) on day 0, 2, 4 and 6. Mice were culled 4 or 7 days after influenza infection and the lungs removed. Single cell suspensions of lung homogenate were stained with anti-CD4 APC, anti-CD8 PerCp, fixed, permeabilised and stained with anti-TNF-α PE. The percentage of CD4+ (closed symbols) and CD8+ (open symbols) TNF-α positive T cells at day 4 (A) and day 7 (C) was determined by flow cytometry; the number of TNF-α positive CD4+ (closed symbols) and CD8+ (open symbols) at day 4 (B) and day 7 (D) was determined by multiplying the percentage positive x percentage of CD4/CD8 T cells x percentage of lymphocytes x total viable lung cell count. * p<0.05 n=4 +/- SEM. Data represent one experiment.
Intra-cellular cytokine production by T cells in the BAL fluid was also assessed on day 4 and day 7 of influenza infection post anti-GITRL treatment. However, GITRL blockade did not impact on either the proportion or number of IFN-γ- or TNF-α-producing T cells (data not shown).

Next, the total amount of inflammatory cytokines in the BAL fluid and lung homogenate was determined. IL-6 is produced predominantly by macrophages in the early phase of influenza infection and is highly pro-inflammatory; hence it was of interest to assess IL-6 levels as a reflection of lung inflammation. IL-6 was present in BAL fluid at day 4 and 7 in both anti-GITRL Ab- and IgG1-treated mice; however, at day 7 there were significantly higher levels of IL-6 in BAL fluid of anti-GITRL Ab-treated mice (Fig. 5.13A). Furthermore, anti-GITRL Ab-treated mice had slightly higher levels of IL-6 in lung homogenates at day 4 and day 7 but this difference did not reach statistical significance (Fig. 5.13B). The increase in IL-6 levels further indicates that anti-GITRL Ab treatment enhanced influenza-associated inflammation during innate immune activation.

TNF-α, though mainly produced by T cells in the later stages of infection, is also produced by monocytes and macrophages in the early stages of influenza infection. We therefore measured TNF-α in BAL fluid and lung homogenate. Unlike IL-6, GITRL blockade did not alter TNF-α levels in BAL fluid (Fig. 5.13C) or lung homogenate (Fig. 5.13D).
Figure 5.13 The impact of GITRL blockade on cytokine production during influenza infection. BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 on day 0 and injected i.p with 200μg anti-GITRL Ab (grey bars) or rat IgG1 (clear bars) on day 0, 2, 4 and 6. Mice were culled 4 or 7 days after influenza infection and BAL performed and lungs removed. IL-6 was then quantified in the BAL fluid (A) and lung homogenate (B), and TNF-α levels were determined in the BAL fluid (C) and lung homogenate (D) at day 4 and 7 post influenza infection by ELISA, as described in the Methods section. n=4 +/- SEM. * p<0.05. Data represent one experiment.
5.2.7 GITRL blockade does not affect viral clearance during influenza infection

We hypothesised that though anti-GITRL treatment may have caused increased lung inflammation, this may have enhanced viral clearance, or perhaps inflammation was increased due to increased viral load.

Lung lobes were therefore tested for influenza virus via an influenza plaque assay as described in the Methods section. Anti-GITRL Ab treatment did not affect viral clearance; at day 4 anti-GITRL Ab- and IgG1-treated mice had comparable viral load in the lungs, and by day 7 neither group had detectable levels of influenza virus (Fig. 5.14).

**Figure 5.14** GITRL blockade during influenza infection does not affect viral clearance. BALB/c mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 on day 0 and injected i.p. with 200 μg anti-GITRL Ab (grey bars) or rat IgG1 (clear bars) on day 0, 2, 4 and 6. Mice were culled 4 or 7 days after influenza infection and the lungs removed. Snap-frozen lung lobes were homogenised and viral plaque assay performed to detect viral particles. n=4 +/- SEM. (ND: not detected). Data represent one experiment.
5.2.8 GITRL blockade does not impact on the immune response to subsequent infection with a heterologous strain of influenza virus

To test if GITRL blockade had an impact on the immune response to a re-challenge infection, mice previously treated with anti-GITRL Ab or rat IgG1 during a primary influenza X31 infection (H3N2) were infected with influenza A/PR/8 (H1N1) as displayed in the experimental protocol in Fig. 5.7. These viruses share the same NP protein, which is one of the dominant intracellular proteins against which T cells are raised. There is no antibody cross-reactivity between the two viruses, as the surface HA and NA proteins are different, but as the NP proteins are the same, there is T cell cross-reactivity. Mice previously infected with influenza X31 mount a swift T cell response to infection with an influenza PR/8 virus which results in rapid clearance of the virus.

As expected, mice previously infected with influenza X31 did not lose a significant amount of weight after infection with influenza PR/8 (Fig. 5.15A). BAL was performed and the lungs and mediastinal lymph nodes extracted. Prior GITRL blockade during the primary influenza infection had no effect on cellular infiltrate into the airways during subsequent PR/8 infection (Fig. 5.15B) but induced a slight but insignificant reduction in cell numbers in the lungs compared to IgG1-treated controls (Fig. 5.15C), and a significant reduction in cell numbers in the lymph node (Fig. 5.15D). This suggests reduced inflammation in mice previously treated with anti-GITRL during the last influenza infection.

During secondary influenza infection, there is a rapid expansion of T cells in the lungs and therefore numbers of T cells were assessed after secondary influenza infection. Anti-GITRL treatment during a primary infection led to a slight decrease in the number of CD4+ T cells and a significant decrease in the number of CD8+ T cells in the lungs during secondary influenza infection with influenza A/PR/8 (Fig. 5.16A and B respectively).
Figure 5.15 The impact of prior treatment with anti-GITRL during primary influenza infection on re-infection with a subsequent heterologous influenza virus. BALB/c mice were infected i.n. with 1.4 \times 10^5 pfu influenza X31 and injected i.p. with 200 \mu g anti-GITRL Ab (circles) or rat IgG1 (squares) on day 0, 2, 4 and 6. Four weeks after primary X31 infection, mice were then infected with 100 pfu influenza PR/8 and weighed daily, with weight loss expressed as a percentage of original weight (A). Mice were culled 4 days after PR/8 infection, BAL performed and the lungs and mediastinal lymph nodes removed. Total viable cell count in BAL fluid (B) and single cell suspensions of lungs (C) and lymph node (D) were determined by Trypan blue exclusion. * = p<0.05, n=4 +/- SEM. Data represent one experiment.
Figure 5.16 Prior treatment with anti-GITRL Ab during primary influenza infection leads to decreased cellular infiltrate into the lungs during subsequent heterologous influenza infection. BALB/c mice were infected i.n. on day 0 with 1.4 x10^5 pfu influenza X31 and injected i.p. with 200 μg anti-GITRL Ab (circles) or rat IgG1 (squares) on day 0, 2, 4 and 6. Four weeks after primary X31 infection, mice were then infected with 100 pfu influenza PR/8. Mice were culled 4 days after PR/8 infection and the lungs removed. Single cell suspensions of lung homogenate were then stained with anti-CD4 APC and anti-CD8 PerCp or anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy7. The number of CD4^+ (A) and CD8^+ (B) T cells and lung CD11c^+ macrophages (C) and lung monocyte/macrophages (D) was determined by multiplying the percent positive for the various markers x percentage of cells in the lymphocyte/myeloid gate x total viable cell count in the lungs. * = p<0.05, n=4 +/- SEM. Data represent one experiment.
T cell production of intra-cellular IFN-γ and TNF-α was also analysed, but prior GITRL blockade did not have an effect on IFN-γ or TNF-α production by lung T cells (data not shown). T cells were not analysed in the airways due to limiting cell numbers. Next the number of macrophages in the lungs was examined. Anti-GITRL-treated mice had fewer CD11c+ macrophages in the lungs (Fig. 5.16C) but numbers of monocyte/macrophages were unaffected (Fig. 5.16D). Likewise, there was no difference in macrophage populations in the airways (data not shown).

Next, in order to assess the level of inflammation in the lungs and airways, the amount of TNF-α in BAL fluid and lung homogenate was measured by ELISA. Mice which had been treated with anti-GITRL Ab during primary influenza infection had slightly lower levels of TNF-α in BAL fluid (Fig. 5.17A) and lung homogenates (Fig. 5.17B) which did not gain statistical significance. The reduction in TNF-α levels correlated with lower numbers of macrophages and T cells observed in anti-GITRL Ab-treated mice.

Neither anti-GITRL- nor IgG1-treated mice had detectable influenza virus in the lungs at day 4 post PR/8 infection (data not shown).
Figure 5.17 GITRL blockade during primary influenza infection does not impact on cytokine production during a secondary infection. BALB/c mice were infected i.n. on day 0 with 1.4x10^5 pfu influenza X31 on day 0 and injected i.p. with 200μg anti-GITRL Ab or rat IgG1 on days 0, 2, 4 and 6. Mice were then rested for 3 weeks and infected i.n. with 100 pfu influenza PR/8 and culled 4 days after. BAL was performed and the lungs removed. TNF-α levels were determined in BAL fluid (A) and lung homogenate (B) by ELISA as described in the Methods section. n=4 +/- SEM. Data represent one experiment.
5.2.9 Blockade of the GITR:GITRL interaction during influenza infection using GITR:Fc

The results above suggest that blockade of the GITR:GITRL interaction actually enhanced influenza-induced inflammation. GITR:Fc contains the cysteine-rich region of mouse GITR fused to the Fc portion of human IgG1. Some studies report that GITR:Fc not only binds to GITRL and prevents GITRL engaging GITR, but that GITR:Fc signals through GITRL. In contrast, others show that GITR:Fc simply binds to GITRL and blocks GITR binding. Whatever the mode of action of GITR:Fc, however, all studies report that treatment with this protein ameliorates inflammation in different mouse models of inflammatory disease. Therefore, it was of interest to determine if this would also be the case in influenza-induced inflammation.

Mice were infected i.n. on day 0 as before with 1.4x10⁵ pfu influenza X31 and injected i.p. with 1μg of GITR:Fc or control Fc daily (Fig. 5.18A and B). The dose of 1μg/mouse/day was inferred from studies where mice were given 6.25μg GITR:Fc via mini-osmotic pump over 7 days, for treatment of bleomycin-induced lung inflammation, which was successful. Treatment was given i.p. so a comparison could be made with the previous study with anti-GITRL Ab treatment. Mice were culled at day 7 of influenza infection, BAL performed and the lungs and lymph nodes removed and homogenised.

Unlike treatment with anti-GITRL, GITR:Fc had no effect on weight loss during influenza infection (Fig. 5.19A). Similarly, GITR:Fc treatment did not alter cellular infiltrate into the airways (Fig. 5.19B), lungs (Fig. 5.19C) or mediastinal lymph nodes (Fig. 5.19D) at day 7 of influenza infection. The absence of an effect of GITR:Fc on weight loss correlated with the lack of an impact on cellular infiltrate.
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Figure 5.18 Experimental set up. (A) BALB/c mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 on day 0; mice were then injected daily i.p. with 1 μg GITR:Fc or control Fc daily. Mice were culled 7 days later and BAL performed and lungs and mediastinal lymph nodes removed. (B) GITR:Fc binds to GITRL and it is not known if it signals through GITRL or simply binds and blocks bi-directional signalling between GITR and GITRL. GITRL can also be expressed by T cells and GITR by APCs.
Figure 5.19 Treatment with GITR:Fc does not alter weight loss or cell recruitment into the airways, lungs and lymph node. BALB/c mice were infected i.n. with $1.4 \times 10^5$ pfu influenza X31 on day 0 and injected daily with 1μg GITR:Fc (circles) or control Fc (squares). Mice were weighed daily and weight loss expressed as a percentage of original body weight (A). Mice were culled 7 days after infection and BAL performed and lungs and lymph node extracted. Total viable cell counts in BAL fluid (B), lung homogenate (C) and mediastinal lymph nodes (D) were enumerated by Trypan blue exclusion. n=5 +/- SEM. Data represent one experiment.
T cell numbers were next determined in the BAL fluid and lung homogenate at day 7 of influenza infection. Unlike GITRL blockade, GITR:Fc treatment did not affect the influx of CD4$^+$ (Fig. 5.20A) or CD8$^+$ T cells (Fig. 5.20B) into the airways. Similarly, numbers of CD4$^+$ (Fig. 5.20C) and CD8$^+$ (Fig. 5.20D) T cells in the lungs were not affected by GITR:Fc treatment.

Next, macrophage numbers were analysed at day 7 of influenza infection post GITR:Fc treatment. GITR:Fc- and control Fc-treated mice had similar numbers of alveolar macrophages (Fig. 5.21A) and airway monocyte/macrophages (Fig. 5.21B) in the BAL fluid. GITR:Fc treatment equally did not affect numbers of lung CD11c$^+$ macrophages (Fig. 5.21C) and lung monocyte/macrophages (Fig. 5.21D).

T cell activation was then examined, and production of intra-cellular IFN-γ was used as a indicator of T cell activation. GITR:Fc treatment had no impact on intra-cellular IFN-γ production by CD4$^+$ T cells in the airways (Fig. 5.22A) or lungs (Fig. 5.22C) or CD8$^+$ T cells in the airways (Fig. 5.22B) or lungs (Fig. 5.22D).

There was also no difference in the numbers of IFN-γ-producing CD4$^+$ or CD8$^+$ T cells in the lungs and airways (data not shown).

In our model of influenza infection, the dose of GITR-Fc used did not result in less severe inflammation, as observed in chemically-induced lung inflammation models$^{379,380}$. It is possible that this is due to insufficient dose of the protein or the route of administration was sub-optimal. It was not possible to test other doses/administration routes due to the cost of this reagent.
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Figure 5.20 Treatment with GITR:Fc does not alter T cell recruitment into the airways or lungs. BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 on day 0 and injected daily with 1μg GITR:Fc (circles) or control Fc (squares) i.p. Mice were culled 7 days after infection and BAL performed and lungs extracted. Lung homogenates and BAL fluid were stained with anti-CD4 APC and anti-CD8 PerCp. The number of CD4+ (A) and CD8+ (B) T cells in the airways and the number of CD4+ (C) and CD8+ (D) T cells in the lungs was determined by multiplying the percentage positive by flow cytometry x percent of cells in the lymphocyte gate x total viable cell count in the airways or lungs. n=5 +/- SEM. Data represent one experiment.
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Figure 5.21 Treatment with GITR:Fc does not affect macrophage infiltrate into the airways or lungs. BALB/c mice were infected i.n. with 1.4x10^5 pfu influenza X31 on day 0 and injected daily with 1 μg GITR:Fc (circles) or control Fc (squares) i.p. Mice were culled 7 days after infection and BAL performed and lungs extracted. Lung homogenates and BAL cells were stained with anti-CD11c APC, anti-CD11b PerCp and anti-F4/80 PECy7 to identify alveolar macrophages (A), airway lung monocyte/macrophages (B), lung CD11c+ macrophages (C) and lung monocyte/macrophages (D). n=5 +/- SEM. Data represent one experiment.
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Figure 5.22 Treatment with GITR:Fc does not affect intra-cellular cytokine production by T cells in the lungs. BALB/c mice were infected i.n. with 1.4x10⁵ pfu influenza X31 on day 0 and injected daily i.p. with 1μg GITR:Fc (circles) or control Fc (squares) i.p. Mice were culled 7 days after infection and BAL performed and the lungs extracted. BAL cells and lung homogenates were stained with anti-CD4 APC, anti-CD8 PerCp, fixed and then permeabilised and stained with anti-IFN-γ FITC. The percentage of IFN-γ-producing CD4⁺ T cells (A;C) and CD8⁺ T cells (B;D) in the airways and lungs respectively was determined by flow cytometry. n=5 +/- SEM. Data represent one experiment.
5.2.10 Regulation of GITRL on alveolar macrophages

Previous studies show an up-regulation of GITRL mRNA transcripts on splenic B cells and peritoneal and bone marrow-derived macrophages in response LPS stimulation\(^\text{330}\) and GITR and GITRL mRNA is induced during carrageenan-induced lung inflammation\(^\text{380}\). GITR and GITRL expression was up-regulated on macrophage populations in the lungs and airways and was sustained during influenza infection. It was therefore of interest to determine what factors up-regulate GITR and GITRL on alveolar macrophages during influenza infection.

GITRL expression was high in the lungs and airways at day 7 of influenza infection, when the lung micro-environment is highly inflammatory, with pro-inflammatory cytokines such as IFN-γ and TNF-α present at high levels\(^\text{48, 479}\). Therefore we hypothesised that IFN-γ might have a role in the up-regulation of GITRL observed during influenza infection. Alveolar macrophages were isolated from un-infected BALB/c mice as described in the Methods section and stimulated with 10ng/ml IFN-γ or a media only control. Other studies report that GITRL expression upon stimulation \textit{in vitro} is transient and peaks at 4 hours\(^\text{330}\). However, no expression of GITRL was observed at 4 hours (Fig. 5.23A) or 24 hours (Fig. 5.23B) post stimulation.

LPS treatment up-regulates GITRL expression on peritoneal and bone marrow-derived macrophages\(^\text{330}\), therefore alveolar macrophages were isolated as before and stimulated with LPS (10ng/ml). Another TLR agonist, lipotechoic acid (LTA) was also used, along with 20ng/ml IL-1α, which is extremely pro-inflammatory for many cell types including macrophages\(^\text{493}\). Because GITRL expression on alveolar macrophages was maximal at day 7 of influenza infection, which is relatively late in infection, we hypothesised that up-regulation \textit{in vitro} might also be at a later time point. Therefore, in the next experiment, alveolar macrophages were harvested 24 or 48 hours post stimulation. However, no GITRL expression was detected on alveolar macrophages 24 hours (Fig. 5.23C) or 48 hours (Fig. 5.23D) post stimulation.
Figure 5.23 GITRL is not up-regulated after in vitro stimulation of alveolar macrophages. BALB/c mice were culled and BAL performed to isolate alveolar macrophages as described in the Methods section. 7 x 10⁴ alveolar macrophages were stimulated with 10ng/ml IFN-γ (grey bars) or incubated with media (clear bars) and the cells harvested 4 (A) or 24 hours (B) later and stained with anti-CD11c APC, anti-CD11b PerCp and anti-GITRL PE. In another experiment, alveolar macrophages were isolated as previously described and incubated with media (clear bars) or media containing 10μg/ml lipotechoic acid (LTA) (grey bars), 10ng/ml LPS (striped bars) or 20ng/ml IL-1α (chequered bars) and harvested after 24 hours (C) or 48 hours (D). (E) Alveolar macrophages were next incubated overnight with media (clear bars), LPS (10ng/ml) (grey bars), or PolyI:C (20 ng/ml) and harvested after 24 hours. n=3 +/- SEM. Data represent two experiments.
PolyI:C (polynosinic-polycytidylic acid) is an agonist for TLR-3 and mimics dsRNA, which is associated with virus infections. As GITRL was up-regulated on macrophages during influenza infection, we hypothesised that the use of a viral TLR agonist might induce GITRL \textit{in vitro} on macrophages, as it might better replicate the situation seen \textit{in vivo}. Therefore alveolar macrophages were isolated as described previously and stimulated with 20ng/ml PolyI:C or incubated with media for 24 hours; cells were also stimulated with LPS (10ng/ml). As before, GITRL was not up-regulated by alveolar macrophages after stimulation with either LPS or PolyI:C (Fig. 5.23E).

Lastly, it was of interest to determine the factors that up-regulate GITR expression. Alveolar macrophages were isolated as previously described and stimulated with 10ng/ml IFN-γ, 10ng/ml LPS or a media control and harvested 24 hours later and stained for GITR expression. Like GITRL, GITR was not up-regulated by alveolar macrophages after stimulation \textit{in vitro} (Fig. 5.24).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5.24}
\caption{GITR is not up-regulated on alveolar macrophages upon activation \textit{in vitro}. BALB/c mice were culled and BAL performed and alveolar macrophages were isolated as described in the Methods section. 7 x 10^6 alveolar macrophages were then incubated for 24 hours with media (clear bar), LPS (10ng/ml) (grey bar), IFN-γ (10ng/ml) (striped bar) or LPS and IFN-γ (chequered bar). Macrophages were harvested as described in the Methods section and stained with anti-CD11c APC, anti-CD11b PerCp and anti-GITR PE. Data represent 2 experiments; n=3 +/- SEM. Data represent two experiments.}
\end{figure}
This result was interesting as GITR and GITRL were up-regulated by alveolar macrophages during influenza infection. However, it is possible that TLR stimulus is not sufficient to up-regulate GITR and GITRL, and this may require a combination of factors including the inflammatory cytokine milieu associated with the influenza-infected airway. Furthermore, previous studies only show GITRL mRNA up-regulation after stimulation *in vitro* so it is possible that the products are post-transcriptionally regulated, which would correspond with our data showing no expression at the protein level upon stimulation *in vitro*. 
5.3 Discussion

5.3.1 GITR and GITRL are differentially regulated on T cells during influenza infection

GITR is reportedly constitutively expressed by mouse splenic T cells \(^{330}\) and the same was true for T cells in the lung. GITRL was not expressed by T cells in the un-inflamed lung. Consistent with reports that GITR is up-regulated on activated T cells \(^{324, 326, 367}\), we observed an increase in GM of GITR on T cells during influenza infection, peaking at day 7 of infection, a time point which correlates with a multitude of inflammatory cytokines in the lungs and airways, and when T cell activation is maximal \(^{223}\). Some studies report GITRL up-regulation on CD4\(^+\) and CD8\(^+\) T cells after TCR stimulation \textit{in vitro} \(^{326, 354}\) while others report no up-regulation of GITRL \(^{330}\). This discrepancy may be due to the experimental conditions used. However, during influenza infection, GITRL was only up-regulated by CD8\(^+\) T cells, and there was no significant increase in GITRL expression on CD4\(^+\) T cells. The peak of GITRL expression on CD8\(^+\) T cells was at day 7 of infection, which coincided with the peak of GITR intensity on these cells.

It is not currently known if GITRL is preferentially up-regulated on CD8\(^+\) T cells. However, GITR co-stimulation is reportedly more important for CD8\(^+\) T cells, as GITR null CD8\(^+\) T cells have lower NFkB activation and produce lower amounts of inflammatory cytokines upon co-stimulation with CD28, while co-stimulation of CD4\(^+\) T cells is unaffected \(^{354}\). Other TNFRSF molecules are known to have different effects on CD4\(^+\) versus CD8\(^+\) T cell co-stimulation; for example, 4-1BB co-stimulation enhances anti-CD3-induced CD8\(^+\) T cell proliferation 10 to 100 fold compared to a 2 to 3 fold induction of CD4\(^+\) T cell proliferation \(^{495}\). The function of GITRL on T cells has not yet been elucidated; it is possible that as well as APC-T cell interactions between GITRL and GITR, T-T cell interactions might occur between GITR and GITRL. Future studies to elucidate the effect of GITRL signalling on T cells would involve using TCR stimulation to up-regulate GITRL in the first instance, and then the cells would be
treated with an anti-GITRL agonistic antibody. Changes in proliferation of cells and production of IL-2 could be used to measure the effect of GITRL signalling.

It was interesting that only a subset of CD8$^+$ T cells in the lungs expressed GITRL during influenza infection. This may denote antigen-specific cells that should be examined using MHC class I pentamers loaded with influenza-specific CD8 peptides. Preferential up-regulation of co-stimulatory molecules on antigen-specific CD8$^+$ T cells is reported for OX40, ICOS and 4-1BB. In this case the result of ligation would be enhanced survival of antigen-specific T cells.

5.3.2 GITRL is up-regulated by lung and airway macrophages in vivo during influenza infection

In the absence of inflammation GITR and GITRL were not expressed by alveolar and lung CD11c$^+$ macrophages. The lack of GITR and GITRL expression on alveolar or lung CD11c$^+$ macrophages compared to splenic, peritoneal and bone marrow-derived macrophages indicates that there may be some site specific regulation involved in the control of GITR and GITRL expression. There was a small but consistent proportion of GITR and GITRL positive monocyte/macrophages in the un-inflamed lung, however, and the relevance of this population would be of interest to investigate. The low expression of GITR and GITRL seen in the lungs and airways is consistent with data showing similar low expression of this pair on APCs in ocular tissue, which implies that in areas where even low level inflammation is detrimental, this pair is tightly regulated. It would also of interest to isolate alveolar macrophages from their environment or neutralise some of the anti-inflammatory mediators known to be expressed in the un-inflamed lung to see if GITR/L expression appears.

GITR and GITRL were up-regulated by alveolar and lung CD11c$^+$ macrophages during influenza infection. Lung monocyte/macrophages also up-regulated GITR and GITRL; however, there was no significant up-regulation of GITR or GITRL airway...
monocyte/macrophages. The up-regulation of GITR and GITRL correlates with reports that this pair is up-regulated on macrophages in the lungs during chemically-induced inflammation and in ocular tissue during HSV infection of the eyes^{367, 379, 380}. The lack of significant up-regulation of GITR and GITRL on airway monocyte/macrophages is interesting and warrants further investigation. It is possible that these cells lose GITR and GITRL upon entry into the airways or perhaps they represent an immature monocyte population that is yet to acquire expression.

5.3.3 GITRL up-regulation requires complex factors

Attempts to induce GITRL expression in vitro by stimulation of alveolar macrophages with LPS were unsuccessful, though other studies show GITRL mRNA up-regulation by splenic and bone marrow-derived macrophages in vitro, with peak expression 4 hours after stimulation with LPS^{330}. As GITRL was maximally up-regulated at day 7 of influenza infection, we next attempted to up-regulate GITRL on alveolar macrophages by stimulation with IFN-γ, which is abundant in the lungs and airways at that time point^{221}. However, IFN-γ treatment did not alter GITRL expression. Stimulation of alveolar macrophages with the TLR agonists LTA and PolyI:C also proved unsuccessful in up-regulating GITRL in vitro. It is possible that for alveolar macrophages GITRL up-regulation requires several pro-inflammatory factors. Perhaps a combination of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6 is required to up-regulate GITRL on alveolar macrophages, in addition to TLR stimulation. Additionally cell-cell contact may be required between macrophages and T cells for GITRL up-regulation. It would be interesting to analyse the effect of several pro-inflammatory factors on GITRL expression in vitro. A positive control would be to stimulate cells with BAL fluid from influenza-infected mice at the peak of infection. Presumably all the factors required to up-regulate GITRL would be present in the supernatant. However, the BAL procedure causes extensive dilution of mediators. A concentration of BAL fluid may therefore be required.
The need for multiple signals to induce expression of GITR and GITRL on alveolar macrophages implies tight control of this pair at homeostasis, which is probably due to the reportedly pro-inflammatory outcome of signalling through this pair.\textsuperscript{359, 361, 362}

5.3.4 GITRL blockade enhances influenza-associated inflammation

Blockade of the GITR-GITRL interaction during influenza infection resulted in accelerated weight loss and increased T cell and macrophage numbers early in influenza infection, as well as increased levels of IL-6 at day 7 of influenza infection. The increase in cellular infiltrate into the lungs indicates that GITRL blockade may have induced an increase in the production of chemokines such as MCP-1, MIP-1α and cell adhesion molecules which facilitate extravasation of cells into the lungs. Overall, it appeared that GITRL blockade increased influenza-induced inflammation in the lungs. This result was surprising as other groups have shown that a lack of GITR:GITRL signalling is beneficial for mouse models of inflammatory disease.\textsuperscript{379, 380} These studies have employed both GITR null mice and wild type mice treated with GITR:Fc fusion protein, with similar results. Our studies, however, indicated that GITRL could have an anti-inflammatory role in influenza-induced inflammation. Others have indeed shown that GITRL signalling on pDCs is anti-inflammatory via production of IDO.\textsuperscript{363}

As this thesis was written, a study was published highlighting the role of GITR on CD8\textsuperscript{+} T cells during influenza infection. Snell \textit{et al} show impaired survival of donor GITR\textsuperscript{-/-} antigen-specific CD8\textsuperscript{+} T cells during primary and secondary influenza infection compared to wild type CD8\textsuperscript{+} T cells.\textsuperscript{500} This is attributed to a lack of GITR-activated Bcl-xL, a pro-survival molecule induced upon GITR signalling in T cells.\textsuperscript{500} These data are in contrast with the increased T cell numbers observed upon GITRL blockade in our model of influenza infection. It would be of interest to analyse antigen-specific T cell numbers. The discrepancy between the results observed by Snell \textit{et al} might have implications for the mode of action of the anti-GITRL blocking antibody used in this study – see section 5.3.6. Differences in results might also be due to the fact that most
of the studies carried out by Snell et al were performed with GITR\textsuperscript{−−} transferred to influenza-infected recipient mice, and not in mice which had the GITR/GITRL interaction knocked out globally, as in our model.

On re-challenge with a heterologous influenza virus, mice previously treated with anti-GITRL Ab during primary influenza infection had reduced T cell numbers in the lungs. This data is consistent with reports from the above study that GITR\textsuperscript{−−} CD8\textsuperscript{+} T cells have impaired survival during secondary influenza infection\(^500\). Furthermore, mice lacking 4-1BBL, a related molecule of the TNF super family, have reduced expansion of memory T cells during secondary infection\(^498\). As observed during GITRL blockade, Bertram et al show that 4-1BBL knockout mice did not have impaired T cell responses during primary influenza infection but responses to secondary infection were impaired\(^498\). Similarly, Dawicki et al show that OX40 is necessary for recall responses during secondary infection with influenza virus\(^501\). The authors suggest that 4-1BBL and OX40L are responsible for providing survival signals to T cells which ensure their entry into the memory pool\(^498,501\). It is known that signalling through GITR on T cells induces their expansion\(^344\). Therefore a lack of the proliferation signals from GITR could account for the reduced T cell infiltrate upon secondary influenza infection. Although anti-GITRL Ab-treated mice had a bigger T cell infiltrate into the lungs in the primary infection, perhaps GITR signalling is required to ensure the survival of these cells.

An alternative explanation is that the enhanced T cell infiltrate upon GITRL treatment during the first influenza infection actually enhanced the memory T cell pool and that on re-challenge resolution of infection and inflammation is faster, leading to an apparent reduced response at day 4. Though anti-GITRL and IgG-treated groups of mice cleared culturable virus by day 4, the viral plaque assay is relatively insensitive and so RT PCR at earlier time points should be employed together with antigen-specific ELISPOT or MHC class I pentamer staining to rule out this possibility.
5.3.5 Treatment with GITR:Fc does not impact on influenza-induced inflammation

To confirm the results using anti-GITRL blocking antibody, we treated mice with GITR:Fc during influenza infection in order to replicate previous results in other inflammatory disease models. Treatment with GITR:Fc fusion protein ameliorates disease severity in mouse models of chronic and acute chemically-induced lung inflammation. Additionally, GITR null mice also have reduced disease severity. Other studies report improved outcome of spinal cord injury upon treatment with GITR:Fc and treatment with GITR:Fc reduces inflammation and disease severity in mouse models of acute pancreatitis.

In contrast to other studies where GITR:Fc proved beneficial for reduction of inflammation, GITR:Fc treatment did not alter any of the phenotypic markers of influenza-induced inflammation; there was no effect on weight loss, cellular infiltrate or cytokine production. This result is difficult to interpret because 1) the dose of GITR:Fc might not have been optimal and further studies testing different doses of GITR:Fc would indicate whether a different result might have been achieved with a higher dose of GITR:Fc. 2) It is not definitively known if GITR:Fc signals through GITRL. To confirm the role of GITRL during influenza-induced lung inflammation, it would be of interest to use an agonistic anti-GITRL antibody treatment during influenza infection.

5.3.6 Confounding factors

It is interesting that in our system blockade of the interaction between GITR and GITRL is not beneficial for resolution of inflammation. It might indicate an anti-inflammatory role for GITRL in our model of influenza, but it might also indicate that the antibody used to block GITRL, although marketed as a blocking antibody, may actually have signalled through GITRL. This would result in a blockade of signal through GITR but would also result in the pro-inflammatory activation of macrophages through GITRL. One of the goals of up-regulating GITRL in vitro was to then ligate it with the anti-GITRL Ab and test the effect of signalling through this antibody on alveolar
macrophages. This was not possible because of the lack of up-regulation of GITRL on alveolar macrophages in vitro.

Without testing the effects of the GITR:Fc and the anti-GITRL antibodies on signalling through GITRL, it is impossible to accurately make an interpretation of the results. The in vivo data from the anti-GITRL antibody experiments indicate that the antibody could be signalling through GITRL, but without further study on the mode of action of this antibody it would be impossible to interpret the results. Similarly, one cannot draw any conclusions from the GITR:Fc experiments because i) the dose and route of administration of the antibody needs to be validated and ii) the mode of action of the action also needs to be determined.

5.4 Conclusion

The aim of this chapter was to determine the role of GITR and GITRL in influenza-induced inflammation. We showed that GITR was constitutively expressed by T cells in the lungs and GITR and GITRL were up-regulated on T and myeloid cells in the lungs and airways during influenza infection. We focused on blocking GITRL because unlike GITR, it was not highly expressed in the un-inflamed lung and was highly inducible during influenza infection. The results indicate that GITRL might have a novel anti-inflammatory role in influenza-induced inflammation. Teasing apart the relative contributions of this co-stimulatory pair in influenza-induced inflammation is difficult because both are inducible on different cell types and signalling through GITR and GITRL on different cells yields different effects.

The following important questions have been raised by the work in this chapter.

1. Does GITRL expression denote antigen-specific CD8+ T cells?

2. Is GITRL expression on CD8+ T cells pro- or anti-inflammatory?
3. Can multiple pro-inflammatory cytokines and TLR agonists as well as cognate interactions with T cells cause an increase in GITRL expression on alveolar macrophages \textit{in vitro}? 

4. What is the impact of anti-GITRL and GITR:Fc on GITRL function – blocking or stimulatory? 

5. Do GITRL null mice display enhanced lung inflammation naturally or during infection?
6.0 Concluding discussion

This thesis has shown several important concepts that warrant further discussion beyond the results presented in each chapter:

1. Alveolar macrophages express a distinct profile of markers of alternative activation compared to interstitial lung macrophages.
2. Markers of alternative activation are differentially regulated during influenza infection.
3. The outcome of influenza infection can be altered by manipulating immune modulatory molecules prior to, and during influenza infection.

6.1 Alveolar macrophages as a unique macrophage subset

The results in this thesis show that at homeostasis alveolar macrophages express YM1 and MR but not RELM-α (Fig. 6.1A), while interstitial lung macrophages express YM1 and RELM-α but not MR (Fig. 6.1B). Alveolar macrophages are distinct from interstitial lung macrophages in many other aspects. For example, interstitial lung macrophages are reportedly better at priming a naïve T cell response, secrete higher levels of IL-6 and IL-1 upon activation and have higher MHC class II expression compared to alveolar macrophages. On the other hand alveolar macrophages have increased Fc-receptor-independent phagocytosis and production of reactive oxygen species compared to interstitial macrophages.

Data from the laboratory shows much higher expression of CD200R on alveolar macrophages compared to interstitial lung macrophages. We further show that CD200R interaction with CD200 on the luminal aspect of respiratory epithelium is a key mechanism of macrophage suppression. Furthermore, alveolar macrophages uniquely express high levels of CD11c (Fig. 6.1A), a complement receptor associated with DCs, and low expression of CD11b compared to tissue macrophages, including interstitial lung macrophages, which express CD11b but not CD11c (Fig. 6.1B).
Figure 6.1. The homeostatic airway. A) CD11c+ alveolar macrophages express YM1 and MR at homeostasis. B) CD11b+ interstitial lung macrophages express RELM-α and YM1 but not MR. C) Surfactant proteins bind to TLRs and SIRP-α on alveolar macrophages and decrease phagocytosis and the release of inflammatory cytokines. D) CD200, IL-10, MUC-1 and TGF-β activated by αvβ6 negatively regulate alveolar macrophages.

These differences are likely to be as a result of the anatomical location of alveolar macrophages, which are found in the alveolar space and are easily isolated by airway lavage, as opposed to lung interstitial macrophages, which are found in the lung tissue and can only be isolated by digestion of lung tissue. Differences in phenotype of lung versus alveolar macrophages could therefore be as a result of:

i) Differences in secretion of soluble factors

Soluble factors which are high in the airways include surfactant proteins that are secreted by alveolar type II epithelial cells, Clara cells and sub mucosal cells and immuno-histochemical staining shows localisation of surfactant proteins on the luminal
aspect of epithelium and on alveolar macrophages. Surfactant proteins have potent immune modulatory effects on alveolar macrophages; SPA and D bind to SIRP-α, a negative regulator on alveolar macrophages, decreasing inflammatory cytokine production in response to LPS stimulation by inhibiting p38 and NFkB activation (Fig. 6.1C). Additionally, SPA binds to TLRs 2 and 4 on alveolar macrophages and interferes with their binding to peptidoglycan, and therefore decreases peptidoglycan-induced TNF-α production by alveolar macrophages.

It is possible that interstitial lung macrophages are not subject to the effects of surfactant proteins, which is supported by reports that they are expressed on the luminal aspect of the epithelium (Fig. 6.1C). As surfactant proteins are known to promote MR expression, this could account for the different expression of MR observed between the two macrophage populations. Interestingly, treating bone marrow-derived macrophages with SPD results in their expression of CD11c. It is not currently known how this occurs but it would be interesting to isolate alveolar or bone marrow-derived macrophages, incubate them with SPA or D and examine their expression of YM1; like MR, expression might be induced by SPA or D.

A key regulator of alveolar macrophage activity is TGF-β, which is activated from its latent form by αvβ6 produced by the epithelium (Fig. 6.1D). TGF-β and IL10 up-regulate CD200R expression by splenic macrophages that do not normally express it. Similarly, differences in TGF-β and IL-10 expression between the lung tissue and alveolar space might account for differences observed in markers of alternative activation between the two compartments. Again, this could be tested as described above. Unpublished observations from our laboratory suggest that alveolar macrophages alter their phenotype once removed from the lung, which may be due to removal of site-specific factors that regulate their phenotype. This would be an interesting experiment to perform in the future to see if MR and YM1 expression decrease.
Chapter 6: Concluding discussion

ii) Oxygen tension

Alveolar macrophages are exposed to high oxygen partial pressures, as they are in direct contact with the outside environment. Presumably, interstitial lung macrophages in the lung tissue are not subject to such high oxygen partial pressures. Culture of bone marrow-derived and peritoneal macrophages in conditions of high oxygen tension results in them behaving more like alveolar macrophages; they become less able to prime a naïve T cell response and produce lower levels of inflammatory cytokines upon stimulation with LPS. This is attributed to altered intracellular redox potential in macrophages cultured at high oxygen tension.

iii) Mucous

Alveolar epithelial cells secrete mucous that traps foreign particles and microbes which are wafted up out of the airways by the muco-ciliary elevator. Muc 1, a mucin-like glycoprotein found in the mucous layer has immune modulatory properties and suppresses alveolar macrophage responses to TLRs 2, 3, 4, 5, 7 and 9 by decreasing NFkB activation in response to TLR stimulation (Fig. 6.1D). Again, unlike alveolar macrophages, interstitial macrophages would not be subject to the immune modulatory properties of mucins and this might account for some of the differences observed between the two populations.

It is possible that differences in the above mentioned parameters could account for the unique expression pattern of YM1, RELM-α and MR by alveolar versus lung macrophages. Additionally their presence in the homeostatic lung implies that factors besides IL-4 and IL-13 could regulate the induction of these proteins. Perhaps they contribute to the maintenance of homeostasis in the lungs and airways. Certainly uptake of microbes mediated by MR would be beneficial for avoiding the induction of an immune response. Studies have not yet interrogated the function of YM1 or RELM-α at homeostasis; only during Th2-biased inflammation. It would be of interest to
determine what effect blockade of either of these proteins has on the lung micro-environment.

6.2 Influenza infection and the effect on macrophage phenotype

During influenza infection, much of the homeostatic regulation of the airways is lost. Influenza virus infects and kills epithelial cells, and this leads to the loss of epithelium-derived regulatory factors such as TGF-β and CD200. Additionally, the presence of the virus activates the immune response leading to production of inflammatory cytokines and chemokines by epithelial cells and resident alveolar macrophages. This leads to a highly inflammatory environment in which TNF-α and IFN-γ dominate.

It is in this inflammatory environment that RELM-α, GITR and GITRL expression is observed by alveolar macrophages and airway recruited monocyte/macrophages (Figure 6.2). GITRL is up-regulated by bone marrow-derived macrophages upon stimulation with LPS, which implies it is up-regulated upon activation. GITR is up-regulated by lung macrophages during bleomycin-induced inflammation. Presumably activation of viral TLRs combined with inflammatory cytokines produced by epithelial cells and infiltrating cells could account for the up-regulation of GITR and GITRL observed during influenza infection.

The expression of RELM-α during influenza infection is less easy to account for. Other studies of RELM-α have focused on models of Th2 inflammation and report that it is highly induced in that scenario. It reportedly negatively regulates Th2 inflammation in Schistosoma mansoni-induced lung inflammation and promotes airway remodelling in mouse models of allergic airway disease. However, RELM-α is up-regulated in DSS-induced colitis and promotes inflammation in this model.
Figure 6.2. Influenza-induced changes in macrophage phenotype. Influenza infection leads to epithelial cell death and epithelium-associated regulatory factors are lost. The airways are highly inflammatory, with inflammatory cytokines produced by macrophages, epithelial cells and other leukocytes. Intra-cellular and secreted RELM-α increase, as does secreted YM1. Intra-cellular YM1 largely decreases. Airway and lung macrophages up-regulate GITR and GITRL expression.

The authors show that treatment of mice suffering from colitis with RELM-α leads to increased eosinophils, neutrophils and lymphocytes \textsuperscript{415}. Additionally, RELM-α augments LPS-induced IL-6 and TNF-α production by bone marrow-derived macrophages \textsuperscript{415}. Blockade of RELM-α during influenza infection would shed light on its role in this infection model. It is possible that it might augment inflammatory cytokine production by activation of viral TLRs during influenza infection, and could therefore promote influenza-induced inflammation.
It must be noted that the apparent induction of RELM-α by alveolar macrophages might indicate that alveolar macrophages are changing their phenotype, but it is possible that it could represent a proportion of RELM-α positive infiltrating monocyte/macrophages which have up-regulated CD11c once in the airways. To rule out this possibility it would be necessary to label the resident airway cells prior to influenza infection in order to differentiate the infiltrating monocyte/macrophages from the resident alveolar macrophages.

Intra-cellular YM1 is reduced during influenza infection while extra-cellular levels increase throughout influenza infection. This might suggest that the reason lower levels are observed intra-cellularly is because secretion is enhanced. YM1 expression has not to date been studied in a classical Th1 inflammation setting. In vitro stimulation of alveolar macrophages with IFN-γ did not affect YM1 expression and therefore it is possible that several factors are necessary for its regulation. Perhaps inflammation/tissue damage induces its secretion. Previous studies suggest that YM1 might be involved in the resolution of inflammation. It binds oligosaccharides with a free amine group, found in many lectin receptors. Therefore, it is proposed that soluble YM1 may compete with inflammatory cells for binding sites to lectin receptors and in this way prevent the entry of inflammatory cells into the site of infection. This correlates with its release in the lungs during influenza infection. However, a blockade of YM1 would be necessary to dissect its role in influenza-induced inflammation.

By days 10 to 14 of influenza infection, inflammation has largely resolved in the lungs and airways and influenza virus is cleared. However, RELM-α is still present in BAL fluid but intra-cellular expression is absent from alveolar macrophages. RELM-α present in BAL fluid might be produced by epithelial cells, which are known to secrete it. Interestingly levels remain high in the resolved lung and airway compared to the naïve state. Similarly extra-cellular YM1 remains high and there remains fairly high expression of GITR and GITRL on alveolar macrophages compared to naïve mice.
Additionally, MR expression remains low. This is potentially due to residual low levels of IFN-γ, TNF-α or other inflammatory cytokines. Epithelium and mucous cell metaplasia are reported at day 21 of influenza infection, when inflammation is largely resolved and this might also impact on the ability of alveolar macrophages to return to their naïve phenotype. The influenza time course should be extended to determine if alveolar macrophage phenotype ever returns to that observed in naïve mice. This altered phenotype may also contribute to the reported desensitisation of alveolar macrophages for prolonged periods of time following influenza infection and the increased susceptibility to subsequent bacterial infection (J. Goulding and T Hussell; in press).

6.3 Manipulation of immunity in the lungs and airways

Previous work from the laboratory suggests the concept of the innate immune rheostat in the airways. This suggests that there is a threshold of antigen below which an inflammatory response will not be mounted. The presence of negative regulators in the airways such as IL-10, TGF-β, SIRP-α and CD200R keeps this threshold fairly high in resting alveolar macrophages to prevent them from responding inappropriately to innocuous antigen. However, this regulation is lost during influenza infection and alveolar macrophages become activated, with increased inflammatory cytokine production and oxidative burst. Once the infection has cleared, in an effort to once more raise the threshold of activation, levels of negative regulators such as CD200R are enhanced during resolution of infection and the airways are once more highly regulated. This highly regulated state can leave the airways susceptible to infection. For example, bacterial super-infection post influenza infection in mice is associated with high levels of IL-10, which leads to bacterial outgrowth and death.

A redundancy in negative regulators in the lung is probably the reason why there was not a more pronounced inflammatory phenotype upon IL-10R blockade, or potentially the blocking antibody regimen was not long enough. However, the increased IL-6
production and higher alveolar macrophage numbers hints at enhanced inflammation. This correlates with previous data from the laboratory which shows increased alveolar macrophage numbers in CD200R knockouts and upon blockade of TGF-β in wild type mice (J Goulding and T Hussell; unpublished observations).

IL-10 promotes the expression of MR and YM1, however the lack of a change in expression of these proteins upon IL-10R blockade correlates with studies showing that surfactant proteins can also drive MR expression and YM1 may similarly be regulated by surfactant proteins or other factors in the airway micro-environment such as GMCSF.

It is interesting to note that though IL-10R blockade had minimal effects in the non-inflamed lung, following influenza infection several parameters were affected including increased cellular infiltrate into the lungs and airways and higher levels of inflammatory cytokines. Unlike previous studies where IL-10R was blocked during influenza infection, this was a blockade of IL-10R prior to influenza infection and caused increased inflammation with enhanced T cell activation. This indicates that merely a transient blockade of an immune modulatory pair such as IL-10 and IL-10R can alter the outcome of a subsequent infection.

It is possible that although IL-10R blockade did not result in an obvious inflammatory phenotype at homeostasis, the threshold of activation had been altered. The increase in macrophage numbers in the airways and increased IL-6 levels would correlate with enhanced innate immunity, which ultimately could have resulted in enhanced T cell activation. Levels of CD200R and MHC class II on macrophages were unchanged, however, future experiments would test SIRP-α and TGF-β expression.

Efforts to reduce influenza-induced immune pathology in the past have focused on blocking activation signals to leukocytes. For example, blockade of OX40 causes apoptosis of activated T cells and reduces inflammatory mediators, with accelerated
recovery from influenza \(^{224}\). Similarly, GITRL blockade was hypothesised to reduce influenza-induced inflammation by blocking the pro-inflammatory signal to macrophages by GITRL signalling \(^{359}\), and the pro-survival signal to T cells upon GITR triggering \(^{344}\). However, this led to increased inflammation and potentially worse disease. This result might imply that GITRL is important for the regulation of influenza-induced inflammation. Indeed, it is known that reverse signalling through GITRL on pDCs leads to IDO production, which protects against Th2 inflammation in the lungs \(^{363}\). This would imply a novel role for GITRL in the regulation of influenza-induced immunity and suggests that potentiating GITRL signalling on pDCs might be beneficial for the outcome of influenza infection. A recent study showed that GITRL expansion of Tregs is necessary for the prevention of corneal allografts \(^{518}\). It is possible that GITRL blockade decreased Treg numbers during influenza infection and led to the increased inflammation observed. Future experiments should be performed to analyse the effect of GITRL blockade on Tregs during influenza infection.

### 6.4 Conclusion

This thesis shows for the first time that alveolar macrophages and lung macrophages have distinct expression profiles with regards to markers of alternative activation, which are differentially regulated during Th1-biased influenza-induced inflammation. I also show that prior blockade of IL-10R is sufficient to alter the immune response to a subsequent influenza infection, and that blocking GITRL during influenza infection is detrimental. These results provide further insight into the phenotype of alveolar macrophages and regulation of lung and airway homeostasis and influenza-induced inflammation.
Publications arising from this work


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