

Gene Modified Macrophages as Therapies for Respiratory Diseases

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Abstract

Gene therapy in the context of pulmonary disease often targets gene transfer to the lung epithelium. Recently there has been a growing interest in alternative macrophage-based cell therapies, where following *ex vivo* modifications, macrophages are transplanted to the lung to target inherent macrophage dysfunction or drive expression of a secreted therapeutic protein *in vivo*.

Here I demonstrated successful *ex vivo* transduction of bone marrow-derived macrophages (BMDMs) by a proprietary fusion (F) and haemagglutinin (HN) pseudotyped simian immune deficiency (SIV) lentiviral vector (rSIV.F/HN) encoding a chimeric hybrid cytomegalovirus enhancer/elongation factor 1 alpha (hCEF) promoter. Following pulmonary transplantation of these modified macrophages, production of secreted transgenic proteins was detected in the lungs, where in wildtype mice, *in vivo* pulmonary expression of a Gaussia luciferase secreted reporter was sustained for up to 16 weeks in bronchoalveolar lavage fluid.

In autoimmune pulmonary alveolar proteinosis (aPAP) anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) antibodies lead to defective surfactant clearance and deposition in alveoli. Ongoing clinical trials suggest the benefit of therapeutic interventions aiming to increase bioavailability of GM-CSF with recombinant protein therapy. Proof-of-concept for this platform macrophage-based technology was therefore performed in a mouse model of aPAP (GM-CSF knockouts) delivering macrophages transduced with a GM-CSF transgene, where four-weeks post-transplantation biomarkers of aPAP disease improved in GM-CSF treated mice in comparison to Gluc controls.

To increase the efficacy of this platform technology, I also investigated gene transfer optimisation strategies. Here, the F/HN pseudotype reported similar transduction efficiencies to the gold-standard vesicular stomatitis virus G glycoprotein (VSV-G) pseudotype. Promoter comparisons between hCEF, elongation factor 1 alpha promoter (EF1a) and a cytomegalovirus enhancer/promoter (CMV), demonstrated that following transduction, more macrophages are positive for transgene expression using an EF1a promoter, while the relative strength of CMV was significantly higher than hCEF and EF1a. Additionally, transducing monocyte precursors during macrophage differentiation significantly increased transduction efficiency across multiplicities of infection.

In cystic fibrosis, patients have mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and research suggests that intrinsic loss-of-function mutations in alveolar macrophages reduce anti-microbial efficacy and promote pro-inflammatory phenotypes. To determine if this platform technology could target these defects, I attempted to characterize the bactericidal activity of macrophages lacking functional CFTR *in vitro*. However, no difference in bacterial phagocytosis or killing of internalized bacteria was observed between BMDMs derived from wildtype and CFTR knockout mice.

This work presents initial work towards the development of an *ex vivo* macrophage-based platform technology for pulmonary disease. I have demonstrated proof-of-concept for pre-clinical efficacy of secreted transgene expression in a model of aPAP and lay the foundation for the treatment of diseases caused by alveolar macrophage dysfunction or a lack of secreted proteins like aPAP and alpha-1 antitrypsin deficiency.

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Declaration of Originality

I hereby declare that the work presented in this thesis was performed by me, or else has been appropriately referenced, in the Gene Therapy Group, National Heart and Lung Institute, Imperial College London.

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Abbreviations

1V	1 Vector system
2V	2 Vector System
A549	Adenocarcinomic human alveolar basal epithelial cells
AaDO₂	Alveolar-arterial oxygen gradient
AAT	Alpha-1 Antitrypsin
AATD	Alpha-1 Antitrypsin Deficiency
AAV	Adeno-Associated Virus
ABC	ATP-binding cassette
AdV	Adenovirus
ALD	Adrenoleukodystrophy
AM	Alveolar Macrophage
ANOVA	Analysis of Variance
aPAP	Autoimmune pulmonary alveolar proteinosis
APC	Antigen Presenting Cells
ASGCT	American society for gene and cell therapy
ASL	Airway surface liquid
AT2	Type II alveolar
<i>B. cepacia</i>	<i>Burkholderia cepacia</i>
B2M	Beta-2 microglobulin
BALF	Bronchoalveolar lavage fluid
BetaC	CSF2R β Null Mice
BHK	Baby Hamster Kidney
BM	Bone marrow
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
BV	Brilliant violet
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CNS	Central nervous system

CO₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disorder
cPPT	Central polypurine tract
cPRMI	Complete RPMI media
CRISPR	Clustered regularly interspaced short palindromic repeats
CSF2R	Colony stimulating factor 2 receptor
CSF2Rα	GM-CSF-binding α -chains
CSF2Rβ	GM-CSF affinity-enhancing β -chains
CSFE	Carboxyfluorescein succinimidyl ester
DAPI	4', 6-Diamidino-2-Phenylindole, Dihydrochloride
DC	Dendritic cell
ddH₂O	Double distilled water
DLCO	Diffusing capacity for carbon monoxide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EF1α	Translation elongation factor 1 alpha
EGFP	Enhanced green fluorescent protein
EIAV	Equine infectious anaemia virus
ELF	Epithelial lining fluid
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
EMP	Erythro-myeloid progenitors
F508del	F508 mutation
FABp	Fatty acid binding promoter
FCS	Forward scatter
FDA	Food and drug administration
FEV1	Forced expiratory volume
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Foetal calf serum
Ftc4	Fusion protein from Sendai virus

FVC	Forced vital capacity
Gag	Gag glycoprotein
Gal4-DBD	Gal4 DNA binding domain
GEV	GALA4-E1b expression vector
GFP	Green fluorescent protein
Glux	Gaussia luciferase
GM	Gene modified
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTC	Gene therapy consortium
hCEF	Human CMV enhancer (hC) and elongation (EF) factor 1a hybrid promoter
HD	High dose
HEK293T	Human embryonic kidney 293T
HIV	Human immunodeficiency virus
HN	Hemagglutinin
hPAP	Hereditary pulmonary alveolar proteinosis
hPR-LBD	Human progesterone receptor ligand binding domain
HRCT	High-resolution computed tomography
HSC	Hematopoietic stem cells
HSV	Herpes simplex virus
ICV	Inducible combination virus
IFN-γ	Interferon gamma
IL	Interleukin
IM	Interstitial macrophage
IP	Intraperitoneal
IPF	Idiopathic pulmonary fibrosis
iPSC	Induced pluripotent stem cells
IR/DR	Inverted repeats in direct orientation
IRES	Internal ribosome entry site
IV	Intravenous
Kan	Kanamycin resistance gene
KGF	Keratinocyte growth factor
KO	Knockout
LB	Lucia broth
LD	Low dose
LH	Lung homogenate

LLD	Lower limit of detection
LMO2	LIM domain only 2
LPS	Lipopolysaccharides
LTR	Long terminal repeats
Lux	Firefly luciferase
M	Molar
M-CSF	Macrophage colony-stimulating factor
MAS	Macrophage activation syndrome
MFD	Maximum feasible dose
mGM-CSF	Murine GM-CSF
MIC	Minimum inhibitory concentration
MLV	Murine leukaemia virus
MOI	Multiplicity of infection
MSC	Mesenchymal stem cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NIR	Near-infrared
NLR	NOD-like receptors
ns	Not significant
OD	Optical density
ORF	Open reading frame
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
p65AD	NF κ B transcription factor p65
PaO₂	Partial pressure of oxygen
PAP	Pulmonary alveolar proteinosis
PAS	Periodic acid–Schiff
PB	Phosphate buffer
PBMC	Peripheral-blood mononuclear cells
PBS	Phosphate buffered Saline
PBST	Phosphate buffered Saline 0.1% Tween
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PFA	Paraformaldehyde
PIC	Pre-integration complex
PLA	Proximity ligation assay

POL	Pol reverse transcriptase
PolyA	Bovine growth hormone poly-adenylation sequence
Ppia	Peptidylpropyl isomerase A
PPR	Pattern recognition receptors
qPCR	Quantitative PCR
<i>rcf</i>	Relative centrifugal force
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRE	Rev response element
rSIV	Recombinant Simian immunodeficiency virus
RT	Room temperate
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1
SB	Sleeping beauty
SCID	Severe combined immunodeficiency
SEM	Standard error of mean
SeV	Sendai virus
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
SOC	Super optimal broth
SP-A	Surfactant protein-A
SP-B	Surfactant protein-B
SP-C	Surfactant protein-C
SP-D	Surfactant protein-D
SSC	Side scatter
TGF-β	Transforming growth factor
TK	Thymidine kinase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TU	Transduction units
UCOE	Ubiquitous chromatin opening element
UT	Untransduced
VCN	Vector copy number
VEGF	Vascular endothelial growth factor

VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus G
WHV	Woodchuck hepatitis virus
WLL	Whole lung lavage
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
WT	Wildtype

Chapter 1 – Introduction

1.1 Macrophages

Macrophages are mononuclear phagocytes with wide-ranging and fundamental functions in host defence, regulation of tissue inflammation, and homeostasis (1). They are ubiquitously represented in all tissues by heterogeneous populations of highly plastic resident cells that exhibit tissue-specific functions and phenotypes as they respond to their local microenvironment (2, 3). Macrophages are integral players in the innate immune system, with direct anti-microbial functions including pathogen phagocytosis, the release of antimicrobial mediators, and the recognition and clearance of apoptotic/diseased cells (4, 5). They actively regulate the adaptive immune system through antigen presentation following fluid phase endocytosis and anti-microbial phagocytosis and the release of immunomodulatory signals (2, 6, 7). Following initial studies where the recognition of macrophage anti-microbial phagocytic function first resulted in the development of cellular immunity as a concept (8, 9), we now understand that macrophages have a wide-ranging repertoire of functions including regulating the initiation and resolution of inflammation, roles in tissue remodelling during ontogenesis, and regulation of metabolic homeostasis (10).

1.1.1 M1/M2 Paradigm

The large repertoire of macrophage function *in vivo* is mediated by their plasticity in response to dynamic stimuli (11, 12). Mature macrophages can be activated to perform polarizing functions for which the nomenclature, M1 ‘classically-activated’ and M2 ‘alternatively-activated’ was developed which presents the two states of activation as diametric opposites (13, 14). Our understanding and characterisation of M1/M2 activation was largely based on *in vitro* experiments, where following stimulation M1 and M2 macrophages respectively adopt a pro- and anti-inflammatory phenotype (15). M1 classically activated macrophages are characterised by the secretion of pro-inflammatory mediators including interleukin 1 beta (IL-1 β) and tumour necrosis factor alpha (TNF- α) cytokines, typically stimulated by lipopolysaccharide (LPS) and interferon gamma (IFN- γ) (16). M1 macrophages initiate immune responses, are highly phagocytic, and have cytotoxic and anti-tumour properties (17, 18). In comparison, M2 alternatively activated macrophages mediate wound-healing, tissue repair and suppress or resolve inflammation (19). Though the M1 phenotype also has reparative functions and can

stimulate angiogenesis through secretion of mediators like vascular endothelial growth factor (VEGF) (20). M1 macrophages are activated by cytokines IL-4, IL-10, or IL-13 to secrete anti-inflammatory mediators including IL-10 and transforming growth factor beta (TGF- β) (21). However, it is now largely understood that this strict bipolar view of macrophage activation is restrictive as *in vivo* macrophages are not terminally differentiated and instead respond to highly complex networks of spatially and temporally fluctuating signals which result in what would be more accurately described as a continuum of macrophage phenotypes *in vivo* (2, 22-24).

1.1.2 Origins and Development

Once widely believed to be established from circulating monocytes derived from hematopoietic stem cells in the bone marrow (25), a new understanding of tissue-resident macrophage origins in mice suggests that most are derived from monocytes produced early in embryogenesis (26). Murine studies indicate that there are three main waves of haematopoiesis which lead to the establishment of all adult monocyte and macrophage populations (Figure 1): 1) primitive haematopoiesis, 2) erythro-myeloid progenitor (EMP), and 3) definitive HSC mediated haematopoiesis (27).

The first wave occurs around embryonic day (E)7.25, where primitive hematopoietic cells are derived from blood islands in the extra embryonic yolk sac and give rise to macrophages in the absence of a monocyte intermediate (28, 29). These macrophages establish microglial populations in the brain, which are the only tissue-resident macrophage population derived from this first wave of haematopoiesis retained into adulthood (30). The second wave occurs around E8.25 where EMPs are derived from the hemogenic endothelium in the yolk sac (31, 32). These short-term progenitors migrate to the foetal liver and give rise to monocyte intermediates which establish all adult tissue resident macrophage populations excluding microglia cells in the brain (3, 30, 33). The third wave of haematopoiesis occurs around E9.5 where HSCs, also derived from the hemogenic endothelium, migrate to the foetal liver where they mature and expand rapidly through self-renewal before once more migrating to populate the foetal bone marrow and establish the adult HSC bone marrow compartment and all subsequent blood cell lineages including circulating monocytes, or peripheral blood mononuclear cells (PBMC) (33, 34).

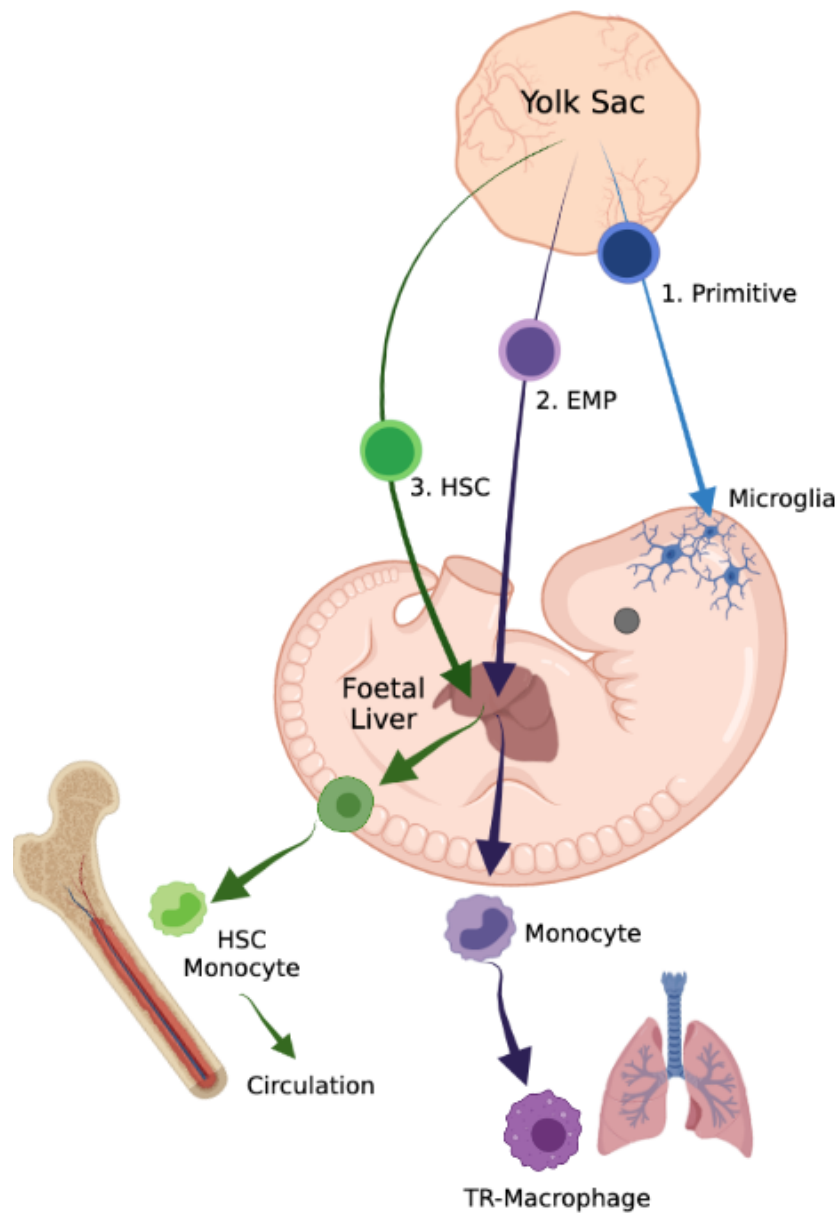


Figure 1 – Foetal haematopoiesis. Three waves of murine haematopoiesis during foetal development: primitive, erythro-myeloid progenitor (EMP), and definitive HSC, give rise to adult macrophage populations. 1) Primitive hematopoietic cells produced in the extra embryonic yolk sac establish microglia in the absence of a monocyte intermediate. 2) EMP cells derived from the yolk sac hemogenic endothelium migrate to the foetal liver where monocyte intermediates then establish all remaining adult tissue resident macrophage populations (TR-Macrophage) including in the lungs. 2) HSCs also derived from the yolk sac migrate to the foetal liver, where they mature to then populate the foetal bone marrow and establish the adult HSC bone marrow compartment and all subsequent blood cell lineages including circulating monocytes. Image created by Nora Clarke in BioRender.

Informed by studies in mice, tissue resident macrophages populations first seeded during the second wave of embryogenesis are no longer believed to be terminally differentiated but instead exhibit marked plasticity and functional heterogeneity in response to their microenvironment (2, 35). As a result, the separate tissue-resident populations are distinct in both function and phenotype and show tissue specific adaptations. This was demonstrated in murine transplantation studies of peritoneal macrophages where following lung delivery these cells lost their tissue specific programming and acquired a new alveolar macrophage (AM) profile (36). Furthermore, studies demonstrated that tissue-specific identity is largely determined by signals from the local microenvironment as following bone marrow transplants in lethally irradiated mice, engrafted donor-derived macrophages acquired tissue specific epigenetic landscapes similar to those of resident macrophages established during embryogenesis (36). Studies show that these macrophage populations established from bone marrow progenitors can not only adapt to the new environment but proliferate locally and self-maintain (37, 38). Under steady state conditions however, EMP derived adult tissue resident macrophage populations are maintained through local self-renewal and limited contributions from circulating monocytes (32, 39, 40). Though studies show that macrophages derived from circulating monocytes of bone marrow HSC origin can coexist with mature resident tissue macrophages (41), and in response to inflammation these cells infiltrate tissues and form polarised macrophage populations that can sustain or resolve inflammation (41). In the heart following myocardial infarct, recruited monocytes can persist for several weeks, and partially renew by local proliferation (42).

1.2 Macrophage Therapy

Primary macrophages can be cultivated *ex vivo* from mature populations or differentiated from HSC derived monocytes in circulation or the bone marrow compartment (43, 44). The *in vitro* differentiation of HSC derived monocytes results in what can be referred to as unpolarised M0 macrophage populations. Their inherent plasticity means that these HSC derived monocyte/macrophages established during the third wave of definitive HSC haematopoiesis can be manipulated *in vitro* and used as models of a diverse range of macrophage phenotypes and functions, though they do not fully recapitulate *in vivo* resident macrophage populations (45-47). These cells are highly positive for both CD11b and F4/80 markers and can be stimulated *in vitro* into M1 (CD11c⁺, CD206⁻) or M2 (CD11c⁻, CD206⁺) activated states (44).

In addition, this has led to a burgeoning field of macrophage-based cell therapeutics contrived on the understanding that they are highly mobile cells and play central roles in the context of inflammation and cancer (48-50). Research first aimed to harness endogenous macrophage behaviours including their ability to home to tumour sites and sites of inflammation, as evidenced by large numbers of monocyte and macrophage tumour infiltrates *in vivo* (51-53). However, the tumour microenvironment can mediate an immunosuppressive phenotype in macrophages through local signalling owing to functional phenotypic plasticity (53-57). Initial macrophage immunotherapies attempting to stimulate a cytotoxic phenotype *ex vivo*, investigated potential anti-cancer/tumour activity following *in vivo* administration and recruitment to tumour sites (51, 58). Clinical trials targeting various different cancers provided proof that administering large numbers of *ex vivo* generated macrophages were safely tolerated in patients who reported only mild side effects (59-62). However, among the various trials performed, patient improvements were modest and transient, and macrophages demonstrated a limited capacity to home to tumour sites when relying on existing recruitment signals (63-65). This was partly due to significant entrapment in the lung and liver, or liver clearance following intravenous administration (61, 64, 66, 67).

1.2.1 Drug Delivery

The field of macrophage cell therapy has now expanded into exciting research utilizing these cells as drug delivery vehicles, where taking advantage of their highly phagocytic capacity, macrophages have been used as alternatives to liposome and polymer drug carriers (66, 68, 69). In the context of cancer, macrophages are loaded with cytotoxic drugs or particles for *in vivo* delivery to tumour sites, acting as so called ‘trojan horses’ (70-72). These therapies in mice rely largely on localized delivery, intrinsic tumour recruitment and homing to sites of inflammation, or alternatively targeting lung and liver diseases/cancers by exploiting entrapment in those organs (73). *In vitro* bone marrow-derived macrophages loaded with a manganese–nitrosyl complex via biodegradable micro-polymers, deeply penetrate spheroid tumour models and release cytotoxic nitric oxide upon stimulation with near infrared light (74, 75). Following *in situ* delivery, these macrophages provided a mechanism for site-specific controlled drug release in a mouse intraperitoneal metastatic model following hypothermic induced release resulting in regulation of tumour development (76). Human peripheral blood mononuclear cells (hPBMC) and human peritoneal macrophages from gastric cancer patients and healthy volunteers, were successfully loaded with anticancer drugs via uptake of oligomannose-coated liposomes and

accumulated in omentum tumour foci following intra-peritoneal injection in mice, and in human surgically resected tumours *in vitro* (77). Macrophages have also proven to be efficient carriers for small molecule drugs with applications beyond anti-cancer therapies, including inflammatory disease, and bacterial or retroviral infections (78-80). The first example of macrophage nanodrug delivery was demonstrated in HIV infected NOD/SCID mice where a single intravenous administration of macrophages loaded with an indinavir nanosuspension resulted in wide-spread distribution, sustained antiviral responses, and partial immune reconstitution (80). In comparison to naked, liposome or polymer delivery, macrophage cargos have reduced immunogenicity, and prolonged half-lives, where passive drug release leads to sustained drug delivery (78). The repertoire of macrophage cell therapies has since been considerably expanded with the application of genetic modifications (81). Macrophages have been engineered to increase targeting of specific disease sites, and in anti-cancer strategies, to reduce local phenotype remodelling by the tumour environment which results in loss of *ex vivo* stimulated anti-tumour activity (82, 83). Excitingly however, macrophages have also been engineered to express therapeutic proteins.

1.2.2 Genetically Modified Macrophages

A growing number of studies have demonstrated that secreted protein therapy with genetically modified macrophages can be efficacious *in vivo*, where gene transfer can mediate long-lasting expression of therapeutic transgenes. Bone marrow derived macrophages (BMDMs) transduced *ex vivo* with a lentivirus to express glial cell-derived neurotrophic factor decreased neuroinflammation and degeneration leading to axon regeneration and improved motor functions in mouse models of Parkinson's disease (79). Significant brain accumulation of transplanted cells was observed in response to inflammation when cells were delivered intravenously, intraperitoneally, and intrathecally, though the latter was the most efficacious. Autologous monocytes expressing GM-CSF by adenoviral transduction, localised to site of injury in a rabbit model of arteriogenesis following ear vein injection (84). The expression of GM-CSF then led to site specific induction of inflammation and monocyte recruitment, aiding in augmentation of collateral vessel growth. BMDMs have also been used as an immunotherapy platform, where through lentiviral gene-modification macrophages expressed transgenic cytokines including IL-12 and full-length checkpoint antibodies in a glioblastoma xenograft mouse model (85). Macrophages were found to traffic and persist at the tumour site where secreted transgenes increased mouse survival and slowed tumour growth. *Ex vivo* gene transfer

to macrophages offers targeted cell transduction and through transcriptional control could also avoid potential silencing of gene expression caused by niche specific remodelling as might occur in the case of tumour immunosuppression (53-55). Harnessing the largely ubiquitous distribution of resident macrophages in most tissues, and their long duration *in vivo* genetically modified macrophages have the potential to provide sustained expression of therapeutic proteins in a range of tissues.

1.2.3 Autologous Sources and Safety Profile

As macrophage populations in the body have distinct ontogenetic sources, the source of cells is a consideration of macrophage-based cell therapy efficacy (3, 40). In patients, monocytes can be derived from both circulating monocytes and those in the bone marrow compartment, and both sources represent large reservoirs that can be tapped for high macrophage yields to enable large dose delivery to patients (86). In mouse studies, murine macrophages are often derived from bone marrow due to the limited availability in the peripheral blood as their approximate circulating blood volume is 1.5-2.5 ml (47, 87). Though murine bone marrow collection is a post-mortem procedure, transplantation within mouse strains is permissible as inbreeding avoids immune rejection. However, readily available sources of monocytes in patients means patients can receive autologous cell therapies, where PBMC can be harvested non-invasively (43).

Indeed, numerous first dose escalation clinical trials have demonstrated the relatively safe efficacy profile of autologous PBMC derived macrophages to patients (61-64, 88-91). No serious adverse effects have been attributed to the experimental delivery of macrophages, though some studies report mild fever and chills. In a recent study phase I safety trial peritoneal infusions of a single high dose (1×10^8 , 1×10^9 , 1×10^{10}) of macrophages was well tolerated in 9 adults who up to a year later showed no evidence of transfusion reactions, or dose limiting toxicity (89). Following the success of phase I trials, a phase II clinical trial was conducted in 43 patients with complete spinal cord injury and though no efficacy was observed, delivery of 4×10^6 autologous macrophages derived from circulating monocytes (in the presence of autologous dermis to stimulate differentiation) was well tolerated (88, 90, 91). Across patient studies, even multiple injections of high doses, either through multiple injections (30-300) of 1×10^6 cells, or weekly doses over 6 weeks of 2×10^8 macrophages for cardiomyopathy (90) and non-muscle invasive cancer (92) also supported the safety and feasibility of autologous macrophage

therapies and repeated administration. Importantly, no evidence of macrophage activation syndrome (MAS) was reported in any of these trials, which is a severe complication of childhood rheumatic diseases whereby toxic uncontrolled activation and proliferation of T-lymphocytes and macrophages can lead to multiple organ failure and death (93).

1.3 Alveolar Macrophages

Macrophages are the most abundant immune cell in the lungs and are key players in regulating lung homeostasis and immunity to support efficient gas exchange. There are two distinct populations of pulmonary macrophages, the first are interstitial macrophages (IM), known thus as they reside in the pulmonary interstitium, which refers the supportive extra-cellular matrix which provides scaffolding for the lung (94, 95). These cells are the lesser studied of the two populations, due largely to their restricted access as they are isolated from digested lung but can be identified by the absence of the lectin Siglec F^(lo) and high expression^(hi) of CD11b, MHC II, CD11c, and CD64 (96, 97). In comparison alveolar macrophages (AMs) are far more common and easily studied as they are isolated in patient bronchoalveolar lavage fluid (BALF) and characteristically express low levels of CD11b and high levels of F4/80, MerTK, CD11c, CD64, and Siglec F, the latter three being used most often in their identification by flow cytometry (98). Baseline AM populations are largely unpolarised (iNOS⁻, CD206⁻), where approximately 31% and 7% display an M1 (proinflammatory; iNOS⁺) and M2 (anti-inflammatory; CD206⁺) phenotype respectively (99). This changes in disease states and in response to environmental exposures like smoking, where unpolarised populations decrease as AMs shift towards either M1 or M2 states (100). AMs are found intimately associated with the luminal surface of conducting airways and terminal alveoli and have a lung specific function and phenotype as a result of environmental signalling (97).

1.3.1 Development and maintenance

AM precursors established during foetal development by monocytes derived from the second EMP wave of haematopoiesis are largely F4/80^{hi}, CD11b^{int}, Ly-6C⁻, and CD64^{hi}. They do not develop into mature Siglec F^{hi}, CD11c^{hi} AMs until shortly after birth (3, 29, 39). This maturation requires signalling of granulocyte-macrophage colony-stimulating factor (GM-CSF) produced non-redundantly by alveolar type II cells (AT2) in the lung epithelium, after which the AM niche is rapidly populated (Figure 2)(101, 102). In the absence of GM-CSF

signalling no AM compartment is established and mature AMs are lost in adult mice (103). Reinforcing the critical role these cells play in maintaining lung function, regulating immune, metabolic and tissue homeostasis, aborted AM maturation through diminished GM-CSF bioavailability leads to the development of an inflammatory lung syndrome known as pulmonary alveolar proteinosis (PAP) (104).

Like most tissue resident cells, they show limited recruitment from circulating monocytes as they are maintained through their marked capacity for self-renewal (3, 29, 39, 40, 105-107). However, in comparison to lung epithelial tissue and other tissue-resident macrophages, like peritoneal macrophages which turnover every 21 days, they are remarkably long-lived as under steady-state conditions AMs have an approximate 40% annual turn-over rate (35, 108). In response to deletion of the AM compartment through ionizing radiation, mature macrophages can be replaced by monocytes recruited from circulation which then adapt to the local niche to resemble tissue resident AMs (32). Circulating HSC-derived monocytes are defined as MHCII⁻, CD64^{low}, CD11b^{high} with subpopulations of ly6C high and low expressing cells and lack the Siglec F expression that is characteristic of AMs (109). Post-injury the AM compartment is also expanded through recruitment of circulating monocytes, demonstrated in studies of acute respiratory distress syndrome (90, 110) and in response to bleomycin induced lung injury (111). Though one study showed that at the resolution of inflammation these recruited cells undergo programmed cell death (35), others have shown that over the ensuing 2 months, a portion are retained and adopt a mature tissue-resident phenotype (Siglec F⁺) (112). This study also showed that in response to influenza infection, recruited monocyte-derived AMs have an initial distinct transcriptional and epigenetic profile to endogenous resident AMs which initially provides antibacterial protection, but which is lost over time as the cells persist and lose this distinctive profile (112). In adults, the AMs niche is therefore composed of a constantly changing heterogeneous population of cells with distinct ontological origins that fluctuate as a result of even mild inflammation as might develop during a common transient respiratory infection (32, 113). This suggests that following lung instillation, macrophage plasticity results in adaptation to the local environment supporting the hypothesis of long-term persistence of transplanted macrophages as the AM compartment supports expansion from external monocyte/macrophage sources.

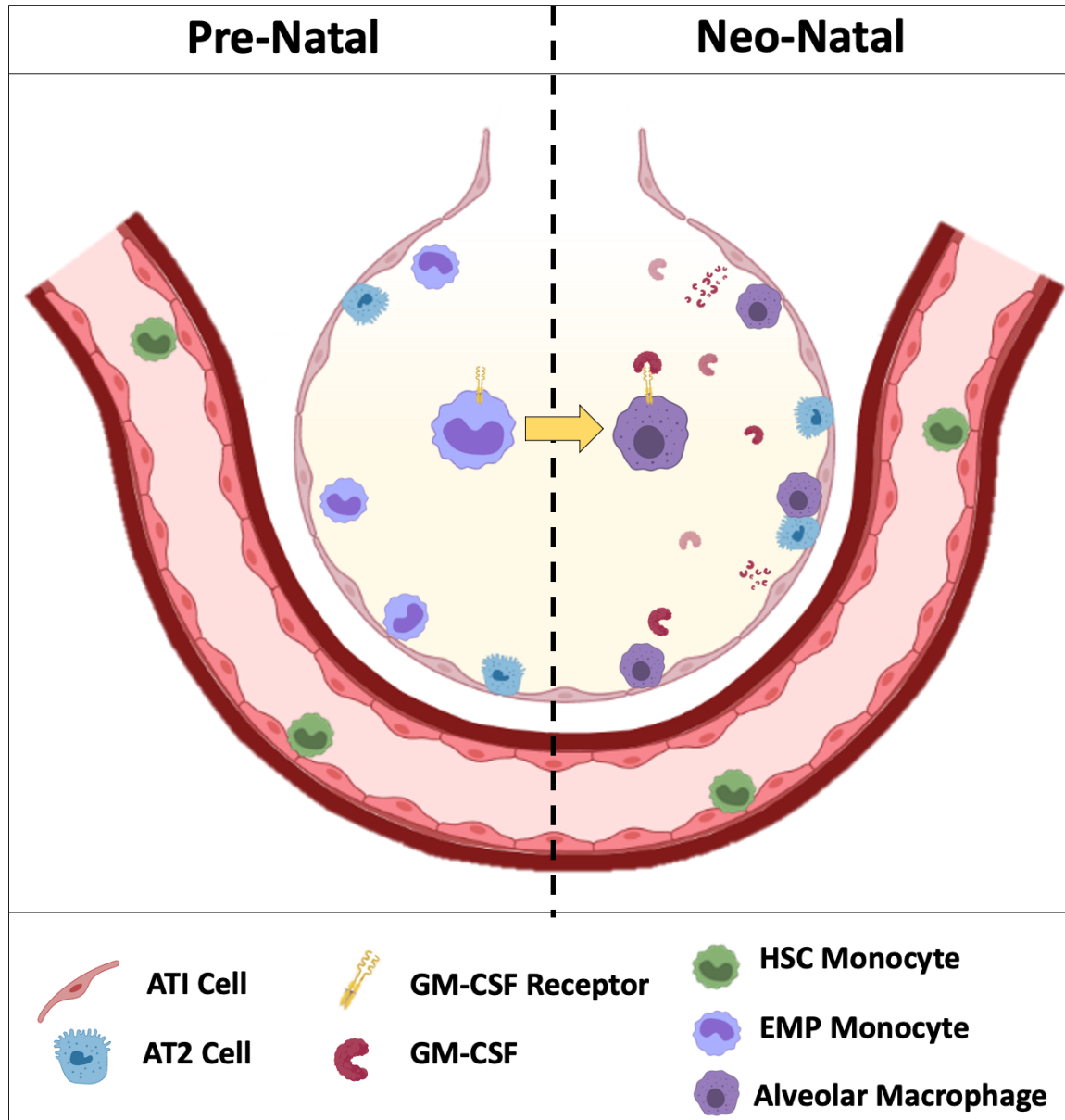


Figure 2 – Post-natal maturation of alveolar macrophages. Erythro-myeloid progenitor (EMP) derived monocytes precursors seed the lung during foetal development. They mature into alveolar macrophages (AM) and populate the AM compartment shortly after birth by granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation. Monocytes in circulation are derived from hematopoietic stem cell (HSC) progenitors in the bone marrow have a separate ontogeny to tissue resident cells. AT2: alveolar type II cells; AT1: alveolar type I cells. Created by Nora Clarke; images sourced from BioRender.

1.3.2 Function of AMs in Lung Homeostasis

Positioned at the airway boundary to the environment, AMs are key players in mediating appropriate responses to innocuous and pathogenic agents. They maintain healthy lung homeostasis through phagocytosis of cellular debris, apoptotic cells, inhaled particles, and pathogens (114). Under steady-state conditions AMs are largely immunosuppressive, overexpressing the CD200 marker associated with an M2 phenotype and downregulation of immune inflammation, regulating innate and immune responses to prevent unwarranted inflammation (115, 116). This is maintained by interactions with the airway endothelium to promote airway tolerance (117). They are poor antigen presenting cells (APCs) and inhibit dendritic cell (DC) migration and antigen presentation (118, 119). Additionally, AMs downregulate T-cell dependent immune reactions by secreting anti-inflammatory mediators that actively suppress their activation, and downregulation of co-stimulatory molecules like CD86 (120). In the absence of this active dampening by AM healthy homeostasis is disrupted, where interruption of AM stimulated FoxP3 induction in T-cells leads to development of asthma (121, 122). However, AMs exist in a naturally hypo-responsive state and are the primary source of pulmonary pro-inflammatory cytokines and chemokines when responding to pathogenic stimuli to initiate innate and adaptive immune responses (114, 123). They can be stimulated through immune recognition of pathogen-associated molecular patterns (i.e., LPS) by pattern recognition receptors (PPRs) including toll-like receptors (TLRs), c-type lectins, and NOD-like receptors (NLRs) (124-128). AMs also directly phagocytose pathogens into cytoplasmic organelles known as phagosomes, which following fusion with lysosomes form phagolysosomes and exposes the engulfed pathogen to a low pH environment and potent anti-microbials effectors including hydrolytic enzymes to mediate its destruction (129-131). Though poor APCs, AMs can transport antigens to lung draining lymph nodes to mediate pulmonary adaptive immunity (132). Additionally, they can release potent anti-microbial inhibitors including reactive oxygen species (ROS) in an oxidative burst following assembly of the NADPH-oxidase complex (133-135). They subsequently also resolve inflammation and in the absence of strict regulation, overproduction of AM pro-inflammatory signals and anti-microbial mediators can lead to development of inflammatory lung diseases like chronic obstructive pulmonary disease (COPD) (136).

AMs also play important roles in metabolic lung homeostasis. AT2 cells and AM both produce pulmonary surfactant, an immunomodulatory monolayer complex of lipids and proteins that

lines the alveolar surface to lower surface tension and prevent collapse during exhalation (137, 138). Surfactant proteins in turn regulate AM phenotype, as immunomodulatory surfactant proteins -A and -D opsonize pathogens and debris for phagocytosis, regulate macrophage activation and inhibit activation of the complement system (139-142). AMs are then also responsible for the catabolism of this monolayer (143). The absence of mature AMs due to decreased GM-CSF bioavailability perturbs proper lung homeostasis leading to alveolar accumulation of surfactant and development of PAP in both patients and mice (104, 144-146).

These homeostatic functions are characteristic and tissue-specific to AM, and we now understand how metabolic signalling can regulate their metabolic profile for lung specific functional phenotypes. Studying the effect local metabolic disturbances have on AMs during chronic lung disease like cystic fibrosis (CF), PAP, idiopathic pulmonary fibrosis (IPF), COPD, and asthma have highlighted how dysregulation of AM functional phenotypes contributes to pathology and suggests their targeting in novel therapeutic approaches (147).

1.3.4 Modulating AMs *In vivo*

Due to their critical and protective role in maintaining lung function, healthy homeostasis, and implication in disease states, AMs are an attractive therapeutic target. Targeting of AMs *in vivo* with aerosolized drugs has been widely investigated to target intracellular survival and persistent bacterial and fungal infections including *Mycobacterium tuberculosis*, *Toxoplasmosis gondii*, *Leishmania* species, *Lysteria monocytogenes* and *Aspergillus* (148-151). These are often delivered in micro- and nano- carriers as passive targeting often fails to result in therapeutic levels of drugs reaching AMs, and carriers can increase drug half-lives and increase efficacy and reduce pleiotropic effects through targeting by modifications of carrier physiochemical properties (i.e. size, charge, surface ligands) (152-159). However, regulation of AM phenotype has shown promise in therapeutic treatment approaches in PAP patients, where inhalation therapy of nebulized GM-CSF increases bioavailability in patients with anti-GM-CSF autoantibodies and rescues AM maturation and surfactant catabolism (160-162).

However, these approaches are limited by their low bioavailability following delivery and off-target effects as non-invasive local delivery to the lungs is not as simple as first believed (163). Though *in situ* pulmonary drug delivery may appear relatively simple owing to a direct interface with the environment, the lung has numerous biological and anatomical/physical barriers to

delivery (164). In the upper respiratory airways ciliated epithelial cells efficiently clear inhaled particles (165). Drug delivery particles and gene-transfer agents can become trapped in the mucous lining the conducting airways through electrostatic forces and subsequently be cleared by muco-ciliary clearance or cough (166). Additionally, nanoparticles can be subject to degradation by proteolytic enzymes in lung epithelial lining fluid (ELF) (167).

In vivo pulmonary gene therapy has also been used to efficiently induce transgene expression, including secreted proteins (168), though studies directly targeting AMs are limited and delivery vectors are still subject to the same anatomical and biological barriers. Gene therapy in the context of hereditary disease can offer solutions that directly target the disease at its genetic origin rather than attempting to regulate symptoms. *In vivo* delivery of a type 2 adeno-associated virus (AAV) vector with a type 8 capsid serotype (AAV2/8) led to transduction of 32% of AMs in BALF (169). However immunofluorescent staining confirmed transduction of multiple endogenous lung epithelial cell types, including club cells, ciliated bronchial epithelial cells, and AT2 cells in addition to AMs. Lentiviral vectors pseudotyped with a vesicular stomatitis virus-G (VSV-G) envelope have a broad vector tropism but targeting expression to the lung epithelium requires first disrupting tight junctions between epithelial cells, to allow the vector access to the basolateral membranes (170-173) (A more in-depth description of lentiviral function and pseudotyping can be found in section 4.1). In the absence of such pre-conditioning studies have shown that, likely due to a combination of transduction and phagocytosis, vectors predominantly transduce AMs (174, 175). Owing to their low turn-over rate under steady-state conditions and vector genome insertion, transgene expression in AMs was long lived (1+ years) and lead to detectable levels of secreted gene expression in BALF. However, gene expression was not strictly limited to AMs, as one study showed 70% of transduced cells were AMs (174). In these studies, AM specific expression was not the experimental aim, as they were designed to secrete therapeutic proteins *in vivo* for expression in the general lung milieu. However, they highlight the issue of off-target transduction with *in vivo* gene delivery to AMs.

1.3.5 Modulating AMs *Ex vivo*

Ex vivo therapy has also been investigated to target AM therapeutically and has the advantage of providing targeted drug delivery *in vitro* (176-181). As previously outlined macrophage therapy has been widely investigated in a general tissue context, where macrophages are either stimulated toward a beneficial phenotype, loaded with drugs and/or nanoparticle carriers, or

genetically modified to deliver a therapeutic cargo or transgene *in vivo*. In the lung, studies directing manipulation of macrophages *ex vivo* could reduce pleiotropic and potentially toxic effects of off-target delivery *in vivo*.

This has proven highly successful in models of disease lacking functional AM compartments, where gene-modified macrophages are transplanted to the lung with long term-survival as they populate the AM niche and adopt tissue specific functional phenotypes. In *Csf2ra*^{-/-} and *Csf2rb*^{-/-} transgenic mice, PAP disease is recapitulated by a lack of functional GM-CSF receptor (*Csf2r*) expression on AMs (176, 182-185). Loss of GM-CSF signalling to prenatally established monocyte populations prevents AM maturation and leads to an empty AM niche. Studies have shown that following *ex vivo* transduction with VSV-G human immunodeficiency type-1 (HIV-1) vectors, bone marrow derived macrophages (BMDMs) can express a functional GM-CSF receptor which following *in situ* lung delivery, allows activation and maturation of transplanted cells by endogenous GM-CSF (176-178). They show that prior to transplant, *in vitro* BMDMs have a CD11b⁺, Siglec F^{low} phenotype which is then converted into an AM-like CD11b^{low}, Siglec F^{hi} phenotype following lung delivery and engraftment (177). These were functional changes to the macrophage phenotype, as transplanted cells rescued PAP phenotypes by establishing a mature and functional AM niche. Over multiple studies this group demonstrated that wildtype (WT) and gene-corrected macrophages are maintained for the duration of a mouse life as a result of rapid proliferation and engraftment (176, 177, 186). A similar study then demonstrated that *ex vivo* macrophage gene therapy can be used to deliver secreted therapeutic proteins to the lung, as a therapeutic strategy for other diseases where pathology is not mediated by AM dysfunction. WT macrophages were transduced to express secreted transgenic alpha-1-antitrypsin (AAT) as proof-of-concept of *in vivo* pulmonary production of secreted transgene which could be therapeutic in patients with alpha-1 antitrypsin deficiency (AATD) (178). However, here macrophages were transplanted into *Csf2rb*^{-/-} and therefore the study does not elucidate the engraftment potential and efficacy of *ex vivo* macrophage approaches to *in vivo* secreted protein delivery in diseases with existing AM compartments, like AATD. This is also the case with studies delivering macrophages transduced to express IFN-γ to the lung of immunosuppressed SCID mice which successfully reversed lung immunodeficiency and resulted in significant levels of IFN-γ in BALF for 14 days (187). A follow-up study demonstrated that in association with increased IFN-γ expression in immunosuppressed SCID mice were partially protected from *Mycobacterium tuberculosis* infection 30 days post transplantation and infection (188). However, the applicability of these findings is

limited as they used J774A.1 immortalised macrophage cell lines and are unlikely to accurately model outcomes of primary macrophages.

As studies transplanting macrophages in the lungs of mice with an established AM niche are limited, the duration of macrophage retention and transgenic protein secretion are unclear. However, their potential for long term delivery of secreted therapeutic proteins *in vivo* is supported by the fact that circulating monocytes of bone marrow origin can coexist with mature resident tissue macrophages (41) and studies that demonstrate the inherent heterogeneity of AM ontogeny (32, 112, 113). One study has shown that transplanting macrophages in the lungs of wildtype (WT) C57BL/6J mice sustained macrophage-driven expression of transgenic alpha-1-antitrypsin protein 1,3 and 7 days post-delivery but did not attempt to detect donor-derived cells directly or investigate further time points (179). Transducing macrophages *ex vivo* and transplanting them to the lung is therefore an exciting potential strategy to for the *in vivo* production of therapeutic proteins but requires further investigation in WT and disease specific models.

1.4 *Ex vivo* Gene Therapy

1.4.1 Lentiviral Biology

Gene therapy has harnessed the ability of viruses including retroviruses, adenovirus (AV) and adeno-associated virus (AAV) to enter cells and deliver genetic material, engineering viral-derived vectors over various generations to make them safer and more efficient (189). Due to their inherent ability to mediate long term gene expression via integration into host genomic DNA, members of the *Retroviridae* family are popular vectors for gene transfer in research and clinical settings (190). There are various retrovirus subfamilies, which can be viewed in three basic groups: oncoretroviruses (for example γ -retroviruses like murine leukaemia virus (MLV)), lentiviruses (including human and simian immunodeficiency viruses (HIV and SIV)) and the spumaviruses (foamy viruses). These viruses are enveloped and have a single-stranded RNA genome that is converted to double-stranded DNA by the viral enzyme reverse transcriptase and then integrated into the host cell genome by viral enzyme integrase (Figure 3) (191). They have similar lifecycles but can also be classed into complex or simple viruses according to their genomic organisation, of which lentiviruses and γ -retroviruses are respective examples. Both are used in the clinic and research development, where one of their key attributes is their large

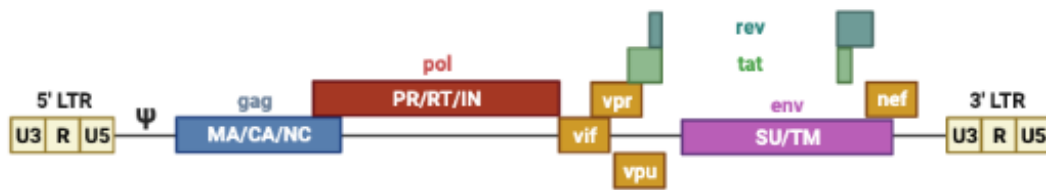
carrying capacity (~9.5kb and ~8 kb respectively) (192). Recently lentiviral vectors have become increasingly popular due to their ability to transduce post-mitotic cells, as gene transfer by other retroviral vectors is restricted to actively dividing cells (193).

The retroviral lifecycle begins with cellular entry of a mature viral particle (as represented in Figure 3). This is mediated by the binding of envelope glycoproteins to cognate cellular receptors either by direct membrane fusion or receptor mediated endocytosis (194). The viral core is then uncoated and released into the cytoplasm, where the viral genome undergoes reverse transcription to form the proviral cDNA (195). This is a multi-priming, multi-step process that results in a dsDNA copy of the viral RNA, flanked by identical long terminal repeats (LTRs) (196). These LTRs are composed of a U3, R and U5 region, that together contain all necessary sequences for transcript initiation and termination, including polyadenylation, enhancer, and promoter sequences (197, 198). This dsDNA is then transported to the nucleus, where unlike other retroviruses, lentiviruses do not rely on the breakdown of the nuclear envelope during cellular division to enter the nucleus. Instead, the proviral dsDNA is actively transported through the nuclear pore complex formed during interphase in a pre-integration complex (PIC) produced from the interaction with viral (including integrase (IN) and capsid (CA)) and host proteins (199). Once inside the nucleus, IN mediates the integration of viral DNA into the host cell genome, from which long-term gene expression is driven. This includes transcription of the full-length viral RNA genome and spliced viral RNA. In the cytoplasm, translation of the latter produces all viral proteins required to package the viral RNA genome for virion assembly and mediate budding of a mature and infectious virion from the cell (200).

The HIV-1 lentivirus particle carries two copies of a single stranded RNA genome tightly complexed with nucleocapsid protein, p7, and enzymes: reverse transcriptase (RT), ribonuclease (RN), protease (PR), and integrase (IN) (Figure 3)(201). The complexed RNA genome is enclosed by a conical capsid (CA) composed of 2000 copies of the viral protein p24, to form the viral core, which is surrounded by an outer layer of matrix proteins that interacts with the cellularly derived lipid membrane envelope (202, 203). During viral production, expression of the *env* gene leads to the expression of viral glycoproteins on the cell's lipid bilayer that are incorporated into a nascent virion (204). These viral glycoproteins determine viral tropism via their interactions with specific cellular receptors to mediate cell entry. They are composed of a surface unit (SU) which binds these cellular receptors, and a transmembrane

unit (TU) that anchors the glycoprotein to the lipid envelope. The HIV-1 genome consists of three major structural genes, *gag*, *pol* and *env*, which encode the structural proteins, replication enzymes (RT, IN, PR, and RN), and envelope glycoproteins respectively (201). Lentiviruses are considered complex retroviruses owing to the expression of additional accessory genes, which in HIV-1 are *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (the latter replaced by *vpx* in HIV-2). These are not expressed by non-lentiviral retroviruses and play roles in the regulation of viral gene expression (including anti-viral responses), assembly and replication (201, 205).

HIV-1 genome



HIV-1 virion

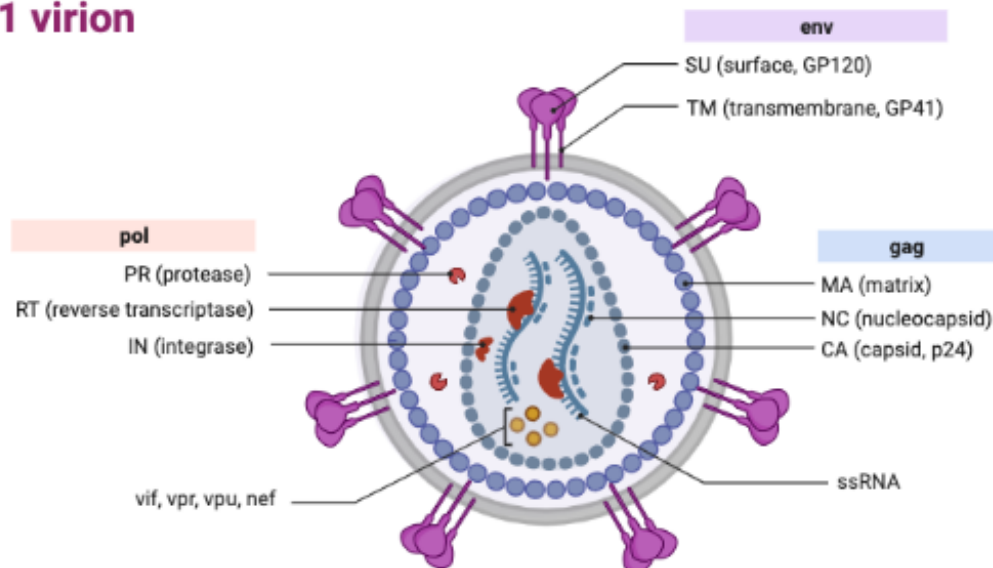


Figure 3 – Schematic of the HIV-1 Genome and Structure of the Viral Particle. The HIV-1 lentivirus particle carries two copies of a single stranded RNA (ssRNA) genome that is converted to double-stranded DNA by the viral enzyme reverse transcriptase and then integrated into the host cell DNA as a provirus. The genome of this provirus is depicted here. The HIV-1 genome is flanked by long terminal repeats (LTRs) that are composed of a U3, R, and U5 region. Three main genes are encoded within the genome, this includes: the structural gene, *gag* which encodes matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, *pol* which encodes replication enzymes protease (PR), reverse transcriptase (RT) and integrase (IN), and *env* which encodes a precursor glycoprotein that is cleaved by PR into surface (SU) protein gp120 and transmembrane (TM) protein gp41. Additional accessory proteins are encoded by genes *tat*, *rev*, *vpr*, *vif*, *vpu* and *nef* which regulate replication and viral pathogenesis. Ψ: Packaging signal. Created with BioRender.

1.4.2 Lentiviruses to Lentiviral Vectors

Various retroviral species have been developed as vectors for gene transfer; designed to deliver an inserted gene of interest (GOI) rather than a functional viral genome, and successive so-called ‘generations’ of these vectors have been engineered to improve the safety and efficiency of gene transfer (Figure 4).

HIV-1 lentiviruses were engineered to create the first lentiviral vectors that delivered a transgene expression cassette to a target cell rather than a viral genome. These vectors are produced through the transient transfection of producer cells with plasmids that encode necessary viral structural proteins and the vector genome. Early iterations of lentiviral vectors used a three-plasmid system for the manufacture of replication incompetent vectors where all structural proteins were provided for in *trans* in a recombinant HIV-1 packaging construct (206). As a result, nascent virions lacked requisite *cis-acting* sequences for producing replication competent virions and reduced the possibility of recombination events resulting in the production of replication competent virus. In addition to the transfer plasmid, these first-generation lentiviral vectors were produced by transfection of envelope and packaging plasmids which encoded the lentiviral genes, *env* and *gag/pol* respectively. The envelope plasmid often encoded the envelope glycoproteins of a different virus to change the vector tropism in a process known as pseudotyping. Pseudotyping of a lentiviral vector was first achieved in 1996 and had previously been successful with MLV-based retroviral vectors (207). In the first instance, the endogenous HIV-1 envelope glycoprotein complex consisting of gp120 and gp41 which mediate cell entry via the CD4 cell surface receptor (208) was replaced with the envelope protein G from the vesicular stomatitis virus (VSV-G) (209). This imparted a broader tropism to the vector as the VSV-G cognate low-density lipoprotein (LDL) receptors are ubiquitously expressed (210)(Further information on VSV-G and pseudotyping can be found in section 5.1). Encoded on the transfer plasmid was the transgene expression cassette (flanked by LTRs) and requisite *cis-acting* elements. These include the RNase H resistant polypurine tract (PPT) that primes plus-strand DNA synthesis during reverse transcription, the rev response element (RRE) that interacts with the accessory protein rev and facilitates posttranscriptional transport of viral mRNA, and a genome packaging signal ψ (192, 201).

Moving towards safer vector design, subsequent generations of lentiviral vectors progressed towards removal of all viral proteins from the viral genome, supplying them in *trans* instead.

Second generation vectors eliminated accessory genes *vif*, *vpr*, *nef* and *vpu* that mediate antiviral responses, therefore ensuring any potential replication competent virus would lack these virulence factors (211). As they are not essential for vector production this was achieved without affecting vector yield or infectivity. To further restrict the odds of recombination resulting in replication competent virus, third generation vectors are produced under a four-plasmid system, where Rev is expressed in *trans* on a separate fourth plasmid (212). These different generations of lentiviral vectors can then have additional deletions in the *tat* gene as well as parts of the 3'LTR U3 region on the transfer plasmid (transferred to the 5' LTR during reverse transcription) that abolish endogenous viral transcriptional activity (213). This includes enhancer and promoter sequences that might otherwise activate neighbouring oncogenes upon proviral integration, including the transactivation-response element (TAR) which acts as a binding site for the viral tat protein required for trans-activation of the viral promoter during replication (192). The resultant lentiviral vectors are known as self-inactivating (SIN) where expression of the vector genome is instead driven by strong internal promoters and have been similarly developed for other retroviral-based vectors (214-216). It has been demonstrated that while not completely abolished, SIN vectors have a reduced transformative capacity (217).

The sustained expression of transgenes during cellular division provided by their stable integration makes retroviruses desirable vectors for *ex vivo* gene therapy as it ensures transgene retention following *in vivo* proliferation. However, as highlighted by a 2000 clinical trial in X-linked severe combined immunodeficiency disease, insertion can be toxic (218). In this study the use of a second generation γ -retrovirus resulted in the activation of the LMO2 proto-oncogene in four patients who developed leukaemia (219, 220). This transformative ability of the retroviral genome was largely driven by the transactivation potential of intact viral LTRs (221). Demonstrating how the strong transcriptional enhancer and promoter sequences in these LTRs can interact with neighbouring cellular gene promoters resulting in cellular transformation and malignancy (222). Subsequent SIN vectors were developed to mitigate these potentially oncogenic interactions (223), however internal promoters used to drive gene expression from within these SIN vectors still have the potential for genotoxic effects and the capacity of an internal promoter to activate neighbouring loci should be considered when designing a vector (224).

Lentiviral vectors are now popular *ex vivo* vectors in the clinic and have been implemented in over 45 clinical trials for monogenetic diseases, including primary immunodeficiencies,

metabolic diseases and haemoglobinopathies (225). Remarkable outcomes have been observed when delivering HSCs genetically modified by lentivirus to children with fatal X-linked adrenoleukodystrophy (ALD), where disease progression has been abrogated for over 10 years with no evidence of insertional mutagenesis or geno-toxicity (226). Evidence of integration-induced genotoxicity by third generation SIN lentiviruses has not been reported in numerous *ex vivo* HSC clinical trials (226-229) and CAR-T cell therapies (230). Analysis of integration site bias in CD4+ T-cells pre-and post-infusion to patients show no bias for integration near proto-oncogenes or tumour suppressor genes (231). Lentiviral vectors are also popular for *ex vivo* macrophage transduction and have been used in all murine pulmonary gene-modified macrophage transplantation studies to date, as they provide long-lasting expression, even in the face of high rates of proliferation following transplantation of gene-modified macrophages into Csf2rb^{-/-} mice with empty AM niches (176). A small number of *in vivo* clinical trials have also implemented intracerebral or subretinal delivery of lentiviral vectors to patients with Parkinson's and macular degeneration respectively (232-234). A phase I trial found subretinal injections of an equine infectious anaemia virus (EIAV)-based vector was well tolerated when used to deliver dual transgenes endostatin and angiostatin to patients with macular degeneration, resulting in stable expression at the last follow-up 4.5 years later (234).

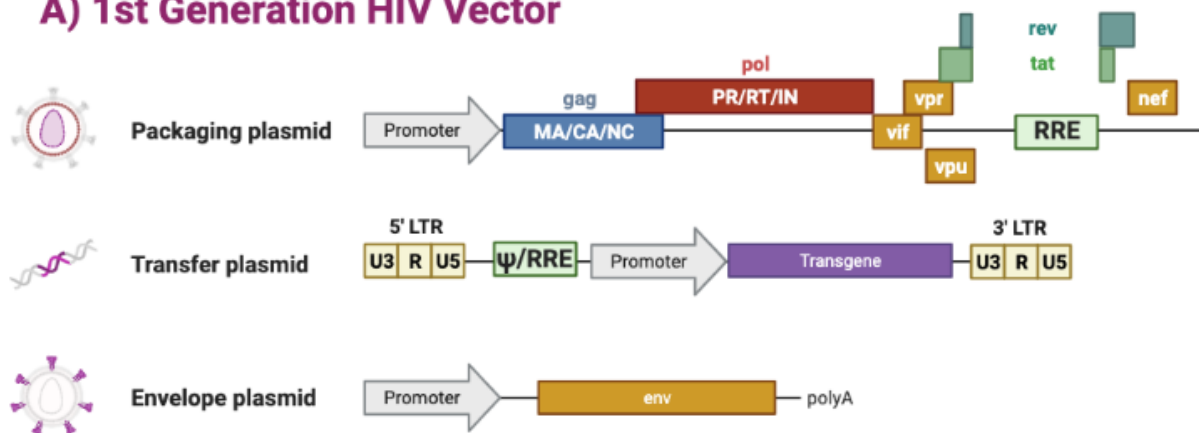
SIN vectors can however still disrupt transcription and induce aberrant splicing (235, 236). In the first β -thalassemia trial (*ex vivo*), clonal dominance was observed in one patient, due to truncated protein expression caused by vector induced premature transcript termination of HMGA2, a gene found to be deregulated in some cancers (227). However, this patient treated in 2010 remains healthy and has not developed malignancies.

These potential genotoxic consequences of proviral integration are dependent on genomic location and different retroviral vectors have different characteristic integration profiles (237). The majority do not integrate randomly, resulting in a higher risk of insertional mutagenesis. Integration of γ -retroviral vectors (best studied in MLV), including SIN vectors, tend to integrate in 5' regulatory regions, preferentially locating within the CpG islands of transcription start sites in actively transcribed areas (222). This potential oncogenic mechanism is untrue of lentiviral vectors (238), though they show the highest preference to integrate within actively transcribed genes (239). Lentiviral vectors may also have lower oncogenic potential as an effect of their ability to target non-dividing cells, as most prefer actively transcribed regions and less transcriptional units involved in cell division will be active in post-mitotic cells (240). Indeed,

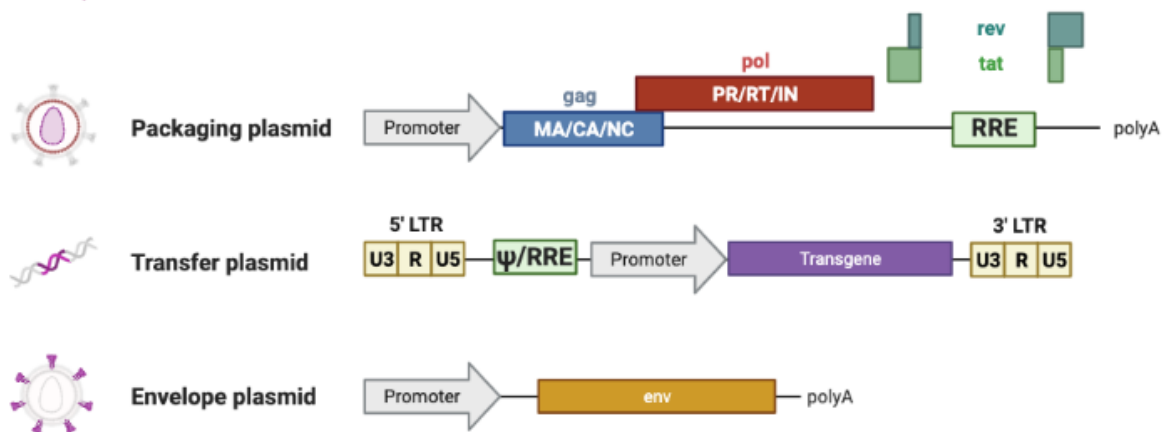
studies of lentiviral vectors in HSC transplants to tumour prone mice have demonstrated their low genotoxicity (241).

Nevertheless, the potential for malignancy remains as SIN vectors, including lentiviral vectors, do not have a random integration pattern (240). This bias is a result of the interaction between the retroviral pre-integration complex (PIC) and host cellular proteins that localize to specific genomic sites (242). Uncovering the key cellular host proteins that tether the PIC to chromatin, via integrase interactions can shed light on genomic distribution biases. For example, integrase-interactor lens epithelium-derived growth factor (LEDGF/p75) is a cellular host protein containing an N-terminal chromatin-reading PWWP domain and a C-terminal Integrase-Binding Domain that binds HIV integrase (243). LEDGF/p75 interactions are specific to lentiviruses but not essential to their integration (244) and when LEDGF/p75 is depleted in host cells lentiviral integration patterns are disrupted demonstrating a determining role for LEDGF/p75 in lentiviral integration (245). One study showed that in cells with depleted LEDGF/P75 expression, fusion proteins where the PWWP N-terminus was deleted and replaced with pan-chromatin binding peptides, a more random pattern of integration was observed (246). The interaction of viral integrase with host cell factors represents a key target towards directed (247) or randomising lentiviral vector integration (248). Alternatively, integrase-deficient lentiviral vectors (IDLVs) have been developed which retain only residual rates of integration (248, 249).

A) 1st Generation HIV Vector



B) 2nd Generation HIV Vector



C) 3rd Generation HIV (SIN) Vector

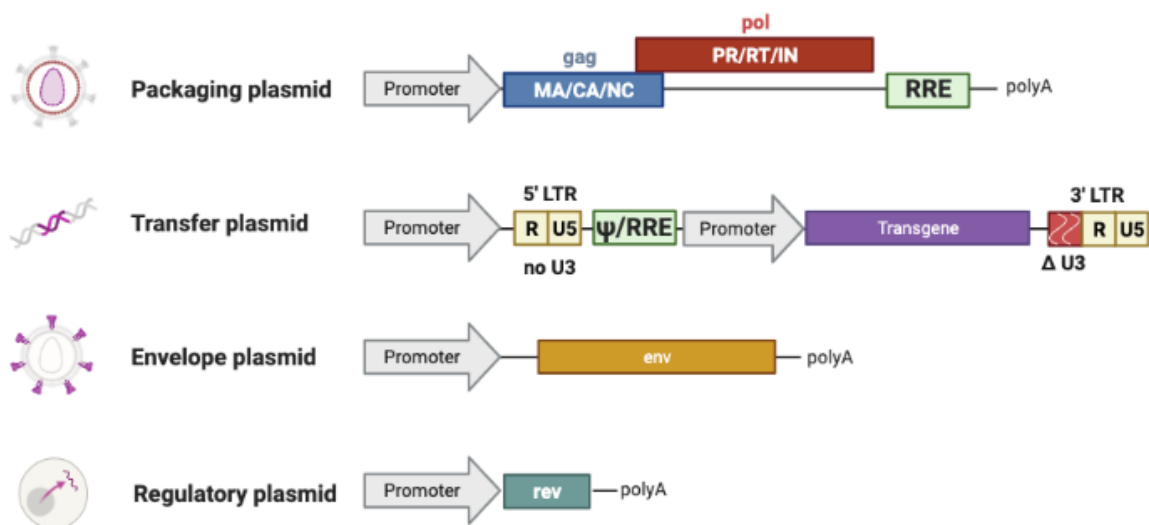


Figure 4 – Generations of Lentiviral Vector Genomes Derived from HIV-1. The HIV-1 genome is flanked by long terminal repeats (LTRs) that are composed of a U3, R, and U5 region. Three main genes are encoded within the genome, this includes: the structural gene, *gag* which encodes matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, *pol* which encodes replication enzymes protease (PR), reverse transcriptase (RT) and integrase (IN), and *env* which encodes the glycoprotein envelope protein expressed on the virion's surface. Additional accessory proteins are encoded by genes *tat*, *rev*, *vpr*, *vif*, *vpu* and *nef* which regulate replication and viral pathogenesis. Ψ: Packaging signal. A) First generation lentiviral vectors used a three-plasmid system for the manufacture of replication incompetent vectors where all structural proteins were provided for in *trans* as the genome is split across a packaging plasmid, envelope plasmid, and transfer plasmid that encodes the transgene. B) Second generation vectors removed accessory genes *vif*, *vpr*, *nef* and *vpu*. E) Third generation self-inactivating (SIN) vectors encoded *rev* on a separate fourth regulatory plasmid and removed the *tat* gene. Deletions to the 3'LTR U3 region on the transfer plasmid transferred to the 5' LTR during reverse transcription, abolished endogenous viral transcriptional activity which is instead driven by an internal promoter. Adapted from Sakuma *et al.* (2012) and created with BioRender.

1.4.3 *Ex vivo* Macrophage Gene Therapy

Transducing macrophages *ex vivo* and transplanting them to the lung is an exciting potential strategy to directly target AM deficiencies or dysfunction, particularly in the context of loss-of-function mutations, and to direct *in vivo* production of therapeutic proteins implicated in a range of pulmonary disorders including autoimmune PAP (aPAP), AATD, COPD, Idiopathic pulmonary fibrosis (IPF) and CF (178, 180, 250, 251). As the use of lentiviral vectors has therefore been successfully established in the context of *ex vivo* gene therapy in the clinic and in the specific context of pulmonary macrophage therapies in mice, they are prime candidates to mediate this *ex vivo* macrophage genetic modification. *In vivo* production of secreted transgenic proteins has also been demonstrated through the *in vivo* pulmonary gene therapy with AAV and lentiviral vectors, where transgenic AAT expression was limited to the airway epithelium in WT mice (168, 169), transgenic AAT was protective in mouse models of emphysema (175), and IL-10 production prevented rejection of intrapulmonary allograft tracheal transplantation (174). However, in addition to the targeted delivery afforded by the nature of *in vitro* transduction, an *ex vivo* approach to secreted protein therapy in the lung has other additional advantages.

Lentiviral vectors are generally less immunogenic than adenoviral (AdV) vectors and have lower rates of pre-existing immunity than AAV (252, 253). However, *ex vivo* transduction avoids direct exposure to patients and can greatly reduce vector specific inflammatory responses which can result in transient expression and severe immune mediated toxicity (254). However, immune responses can still be raised against the transgene and any lentivirus-derived antigens present on transplanted cells as has been observed with HSC transplants in the clinic (255-258). *Ex vivo* delivery of HSCs is often paired with a regimen of transient immunosuppression to allow engraftment and reduce any immune responses (259-262). Though as AM are poor APCs and naturally immunosuppressive, transplanted macrophages with de-novo and viral proteins may be better tolerated in the lung (118-120).

Though it is currently unclear if macrophage transplantation will provide sustained gene expression *in vivo* in the presence of a functional AM niche, the ready availability of autologous patient macrophages makes repeat administration feasible, as clinical trials have demonstrated that repeat administration of high macrophage doses is safely tolerated by patients (90, 92). In comparison, many vectors cannot be repeatedly administered due to the development of neutralising antibodies, and as a result do not provide sustained benefits (263, 264). Several studies have shown that the development of neutralising antibodies after vector delivery and the high incidence of pre-existing neutralising antibodies against AAV (264) and AdV (263) capsids, limit repeat administration and patient pools, and contribute to transient expression as vector specific CD8⁺ T cells recognise viral proteins and eliminate transduced cells (265, 266). Though lentiviral vectors have a lower incidence of pre-existing exposure compared to AAV and AdV vectors, anti-body responses have been observed. In one of the two clinical trials that have tested *in vivo* lentiviral delivery, antibodies against VSV-G were detected 6-months after a single dose administered to the brain (232, 233). In mice and rhesus monkeys, strong antibody responses against a VSV-G HIV and SIV vectors led to vector neutralization of a second intravenous dose (267). However, the effect of neutralizing antibodies is pseudotype specific and lentiviral vectors pseudotyped with fusion (F) and haemagglutinin neuraminidase (HN) from a Sendai virus (268), and in a separate study GP64 from baculovirus (269), have in mice been effective upon re-administration to the lung without loss in transduction efficacy.

It may also be easier to produce the required vector doses for *ex vivo* transduction considering the difficulties associated with up-scaling vector production and processing (270). Due to direct

access to target cells *in vitro*, and a reduction in viral viability during delivery by nebulization or sprayers (271), lower doses may be required *ex vivo* (272, 273). Whether this is the case will depend on the final cell dose delivered to patients and the subsequent vector dose required for efficient macrophage transduction.

1.5 General Aims and Objectives

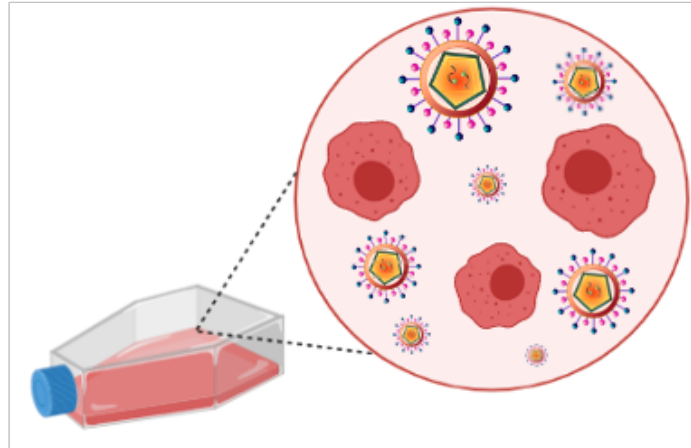
The overarching goal of the studies described in this thesis is to develop a system for *in vivo* production of therapeutic proteins by transplanted *ex vivo* modified macrophages (Figure 5). Here I investigate *ex vivo* macrophage lentiviral gene transfer, and the duration of macrophage retention and gene expression in the lungs of mice, under the hypothesis that macrophage driven gene expression may be therapeutic in disease models requiring either secreted or non-secreted AM protein production.

To achieve this goal, the study aimed to:

- Develop a protocol for successful lentiviral gene transfer in primary murine bone marrow derived macrophages (BMDMs), by specifically:
 - Developing protocols for the isolation and differentiation of BMDMs,
 - Optimising their genetic modification with the GTC's rSIV.F/HN lentiviral vector for the expression of transgenes encoding secreted and non-secreted proteins.
- Deliver genetically modified BMDMs to the mouse lung to determine:
 - The engraftment potential of macrophages and potential for subsequent transgene expression in wildtype mice,
 - Demonstrate proof-of-concept for *in vivo* macrophage secretion of a therapeutic protein in a mouse model of autoimmune PAP, measuring biomarkers of disease to quantify any therapeutic effect.
- Further optimise gene transfer to BMDMs to explore how macrophage expression of transgenes may be increased to make *ex vivo* macrophage gene therapy more efficient by investigating the potential of:
 - Alternative vector pseudotypes and transduction timings to increase transduction
 - Alternative transcriptional regulatory elements to increase gene expression.

- Investigate whether alveolar macrophages in cystic fibrosis (CF) have inherently reduced antimicrobial activity that might be rescued using a genetically modified macrophage transplantation therapy by:
 - Developing assays to measure macrophage antimicrobial activity *in vitro*,
 - Comparing the antimicrobial activity of BMDMs from wildtype and CF mice,
 - Determine that if any inherent defect can be measured, whether it can be corrected by gene therapy.

1. *Ex vivo* transduction of BMDMs



2. Transplantation to the lung



3. *In vivo* production of transgene products

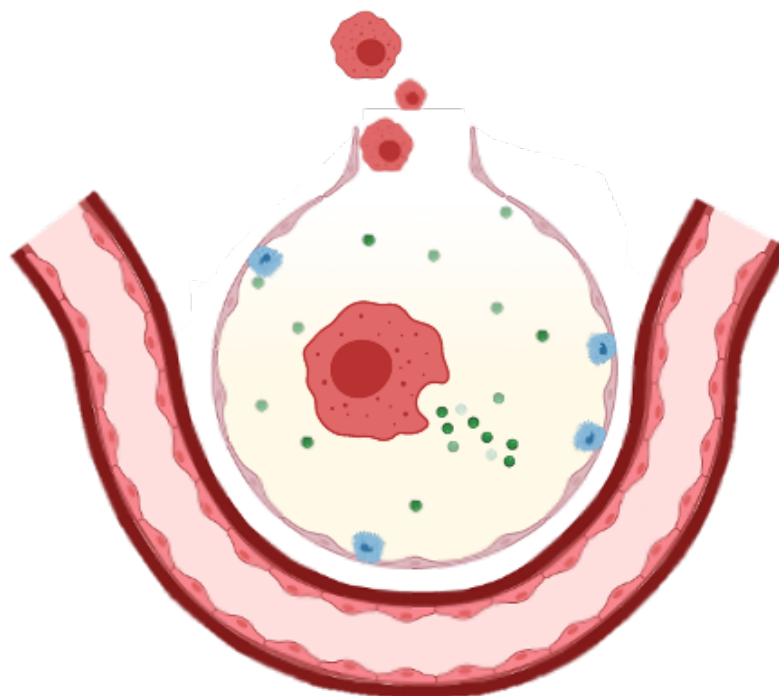


Figure 5 – *Ex vivo* pulmonary macrophage cell-therapy. Bone marrow-derived macrophages (BMDMs) are transduced *ex vivo* with a lentiviral vector and then transplanted to the mouse lung where they can secrete transgenic proteins *in vivo*. Created by Nora Clarke; images sourced from BioRender.

Chapter 2 – General Methods

2.1 Cell Culture

2.1.1 Bone marrow-derived macrophages

Bone marrow (BM) was cultured in complete RPMI (cRPMI) medium: RPMI 1640 Medium (Cat. # 11875093 ThermoFisher Scientific, Loughborough, UK) supplemented with 10% Foetal Calf Serum (FCS) (Cat. #12103C, Sigma-Aldrich, MO, USA) and 1% Penicillin/Streptomycin (Cat. #P4333, Sigma-Aldrich). BM harvesting procedure is described below. Cells were at all times cultured in a 37°C incubator with 5% CO₂. Media was supplemented with 100ng/ml macrophage-colony stimulating factor (M-CSF) (Cat. #300-25, PreproTech, NJ, USA) over 7 days in culture to differentiate BM into BMDMs. Unless otherwise stated, media was replaced on day 4 with fresh cRPMI + M-CSF media. Following 7-day differentiation protocol BMDMs were cultured in cRPMI in the absence of M-CSF. To harvest BMDMs from culture, cells were washed with Dulbecco's Phosphate Buffered Saline solution (dPBS) (Cat. #14190250, ThermoFisher Scientific) and then incubated with warm Versene Solution (Cat. # 15040066, ThermoFisher Scientific) at 37 °C for no more than 30 minutes. The solution was neutralized by dilution in cRPMI and cells were then pelleted by centrifugation at 300 *rcf* for 5 minutes. The efficacy of this differentiation process was then determined by flow cytometry analysis of macrophage phenotype markers as outlined in section 2.5.1.

2.1.2 Adherent immortalized cell lines

Adherent A549 and HEK293T Cells (Cat. #CCL-185 and #CRL-3216 respectively, ATCC, VA, USA) were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) (Cat. # 21969035, ThermoFisher Scientific) supplemented with 10% FCS and 1% Penicillin/Streptomycin and incubated in a 37°C incubator with 5% CO₂. Following thawing cells were cultured for three passages before use and then up to 20 passages before being discarded. Media was changed every two to three days, and cells were passaged every three to four days. Adherent cells were removed from culture by trypsinization. Cells were first incubated for up to five minutes at 37°C with dPBS. Following removal, cells were incubated with 2 mL (75cm² flask – or scaling volume as appropriate) of warm 37°C Trypsin-EDTA

solution (Cat. #T4049, Sigma-Aldrich) and incubated for between five and ten minutes at 37°C to enable cells detachment by brisk tapping of the plate.

2.1.3 Suspension Cells

Suspension HEK 293T/17 SF (ATCC® ACS-4500™) were cultured in animal-free suspension culture in FreeStyle 293 expression media (Cat. #12338018, ThermoFisher Scientific) in 125-ml Erlenmeyer shake flasks (Corning, NY, USA) at 37 °C, 5% CO₂, and 185 rpm. Cells were seeded at 2.5e5 cells per 20ml and to maintain healthy cell culture, densities were kept below 3e6 cells/ml either through media supplementation or passaging. Cells were passaged by pelleting cells at 300 *rcf* for 5 minutes and then resuspending cells in fresh pre-warmed media 37 °C.

2.1.4 Cell Counting

Viable cells were counted manually in a glass haemocytometer or automatically by LUNA-II™ Automated Cell Counter (Logos Biosystems, South Korea) using 10 µL of cell suspension mixed 50:50 with trypan blue viability stain (Cat. #T6146, Sigma-Aldrich).

2.2 Animal Studies

Mice were housed and handled in accordance with the conditions and limitation of the UK Home Office Project and Personal licence regulations under the Animal Scientific Procedure Act (1986). Male and female mice of the following species were used in this thesis:

- Wildtype C57BL/6 (Charles River Laboratories, Margate, UK) mice (6-8 weeks old).
- Mouse model of pulmonary alveolar proteinosis (PAP): GM-CSF knock-out (GM-CSF KO) mice (B6.129S-Csf2tm1Mlg/J, Jackson Laboratories, Bar Harbour, Maine, USA), bred at Imperial College, London (4-7 months old).
- Model of cystic fibrosis (CF): gut corrected FABp-CF-knockout mice (CFTR KO) on a C57BL/6 background were purchased from Drs. M. Drumm and A. van Heeckeren (Case Western Reserve University, Cleveland, OH) and bred at Imperial College, London (2-12 months old) (274).
- Donor CD45.1 mice: C57BL/6-Ly5.1 carry the allele of the SJL mouse in the *Ptprc* gene locus: *Ptprca*/CD45.1/Ly5.1. (2-6 months old).

Daily monitoring was performed by staff of the Central Biomedical Services at South Kensington and Hammersmith facilities of Imperial College London. All mice were euthanized by pentobarbital overdose (Pentobarbitone Sodium 20%, Animalcare, York, UK) administered via intraperitoneal injection. Unless otherwise stated, death was confirmed in the absence of pedal withdrawal reflexes and visible cardiac beating by exsanguination mediated by severing of the femoral artery.

2.3 Bone Marrow Harvesting

Murine bone marrow was collected following previously published procedures (275). Briefly, hind legs were removed at the pelvic joint, taking care not to disrupt the integrity of the bone and expose the bone marrow cavity. Femurs and tibia were then cleaned of soft tissue by mechanical stripping aided by suspension in 70% ethanol. They were then washed in dPBS before careful cutting at either end to expose the bone marrow cavity. Using a 25-gauge needle (BD Microlance, ThermoFisher Scientific) and 15 – 20 mL of cRPMI media, the bone marrow cavity was flushed out into a sterile sterilin tube (Cat. #12693546, ThermoFisher Scientific). Samples were centrifuged at 918 *rcf* for 15 minutes. The cell pellet was then resuspended in 1X Red Blood Cell Lysis Buffer (Cat. #00-4333-57, eBiosciences, ThermoFisher Scientific) for up to 10 minutes which was then neutralized by diluting the buffer in 13 mL of cRPMI. Samples were then filtered through a 70 μ m cell strainer (Cat. #CLS431751, Merck, Feltham, UK) and centrifuged at 918 *rcf* for 15 minutes. Cells were then resuspended in cRPMI for manual cell counting and then appropriately diluted in complete RPMI media supplemented with 100 ng/mL M-CSF and unless otherwise stated, plated in either 6-well or 24-well Nunc™ Non-Treated multidish, or Nunc Non-treated T75 EasyFlask (All from ThermoFisher Scientific) culture flasks at 7.9×10^4 cells/cm² and incubated at 37°C.

2.4 Lentivirus production and nomenclature

rSIV.F/HN lentivirus was produced by the Gene Medicine Research Group at Oxford University at the GTC Core Manufacturing Facility, who selected appropriate plasmids and promoters, and measured viral titres. The different large-scale lentiviruses used in this thesis are described in Table 1 below and where relevant in the thesis. These lentiviruses were produced as previous described (268, 276, 277), through the transient transfection of a HEK293T producer cell line supplied in FreeStyle™ 293 Expression Medium (ThermoFisher

Scientific, USA) with the following plasmids in addition to the relevant genome plasmids listed in Table 1:

- **pGM297** (packaging plasmid): Encodes essential virion components Gag (polyprotein that forms the lentiviral core) and Pol (reverse transcriptase).
- **pGM299** (rev plasmid): Encodes the essential virion component Rev, essential for post-transcriptional transport of viral mRNA that enables synthesis of complementary DNA from viral RNA genome. Note that virus vGM196 was created with a codon optimised version of this plasmid pGM691 (Table 1).
- **pGM301** (envelope plasmid): Encodes the fusion envelope protein (Fct4) that recognises sialic acid residues on target cell receptors and facilitates cell entry via fusion of the viral lipid envelope with the outer cell membrane of the target cell. (F/HN mediated cell entry is discussed in more detail in Chapter 5.1)
- **pGM303** (envelope plasmid): Encodes the virion glycoprotein hemagglutinin neuraminidase (HN) which is also involved in cell attachment. The enzymatic activity of the neuraminidase protein cleaves sialic acid from cell surfaces to promote envelope-membrane fusion.

These plasmids are co-transfected with a fifth viral genome plasmid encoding the gene of interest. Here viruses produced with four different genome plasmids were used:

- **pGM547**: Encodes the murine form of the secreted GM-CSF gene (mGM-CSF or *Csf2*).
- **pGM358**: Encodes the Gaussia luciferase (soGlux) gene, which is a small, secreted protein that catalyses the oxidation of the substrate coelenterazine in a bioluminescent reaction of which the light output is directly proportional to the amount of Glux protein produced (278).
- **pGM357**: Encodes the enhanced green fluorescent protein gene (EGFP) whose excitation peaks at 488 nm and emits light maximally at 507 nm. EGFP is a non-secreted protein that enables identification of EGFP expressing cells at a single cell resolution.
- **pGM020**: Encodes a EGFP and Firefly luciferase (Lux) fusion protein (non-secreted) (279). Luc catalyses the oxidation of firefly luciferin in a bioluminescent reaction of

which the light output is directly proportional to the amount of Lux protein expressed by a cell.

These different genes of interest were all previously codon optimized and cloned into the same lentiviral (SIV) backbone by the GTC Core Facility, a general schematic of which can be seen in Figure 6. Wildtype lentiviral genomes are defined by flanking long terminal repeat (LTR) sequences which contain all necessary sequences for transcript initiation and termination, including a polyadenylation signal, enhancer, and promoter (197, 198). Transcription of the entire lentiviral genome begins in the 5' LTR and polyadenylation of the nascent viral RNA is encoded in the 3' LTR, though both 5' and 3' LTRs have the same sequence arrangement. These LTRs are then processed by the lentiviral integrase to mediate integration into the host genome. To prevent insertional oncogenesis, the lentiviral vectors used here have a deletion of the 3' LTR that abolishes all endogenous transcriptional activity (enhancer and promoter sequences). This deletion is also transferred to the 5' LTR during reverse transcription, and thus transgene expression in these self-inactivating (SIN) vectors is instead driven by internal promoters (223). Here expression is driven by the transcriptional control of the hybrid enhancer/promoter containing the human CMV enhancer (hC) and the human elongation factor 1 alpha (EF) promoter (hCEF). This chimera known as hCEF was previously depleted of CpG islands (280). Vectors were produced in 1L and 5L scale WAVE bioreactor HEK293T suspension cell cultures transiently co-transfected with all five plasmids in a ratio of 20:9:6:6:6 (genome plasmid : pGM279 (gag/pol) : pGM299 (rev) : pGM301 (Fct): pGM303 (HN)) with a total of 0.33 µg/ml of plasmid DNA. Lentiviruses were then purified and concentrated by anion-exchange membrane virus capture and hollow-fibre ultrafiltration/diafiltration (276).

The GTC Core Facility determined the functional titre (TU/ml) of each lentiviral batch following purification and concentration. This was calculated by transducing known numbers of HEK293T cells with serial dilutions of the viral supernatant. Three days later, DNA was extracted from cells and the number of integrated/episomal vector genomes was quantified by TaqMan qPCR using primers binding the WPRE sequence of the proviral DNA. This was normalised to the total amount of DNA in sample to calculate vector titre in transduction units (TU).

Here the GTC lentivirus is referred to generically as rSIV.F/HN when not specifying the transgene and implies the use of a hCEF promoter. Otherwise, lentiviruses are referred to as

rSIV.Pseudotype.Promoter.Transgene, for example vGM107 might be referred to as: rSIV.F/HN.hCEF.EGFP.

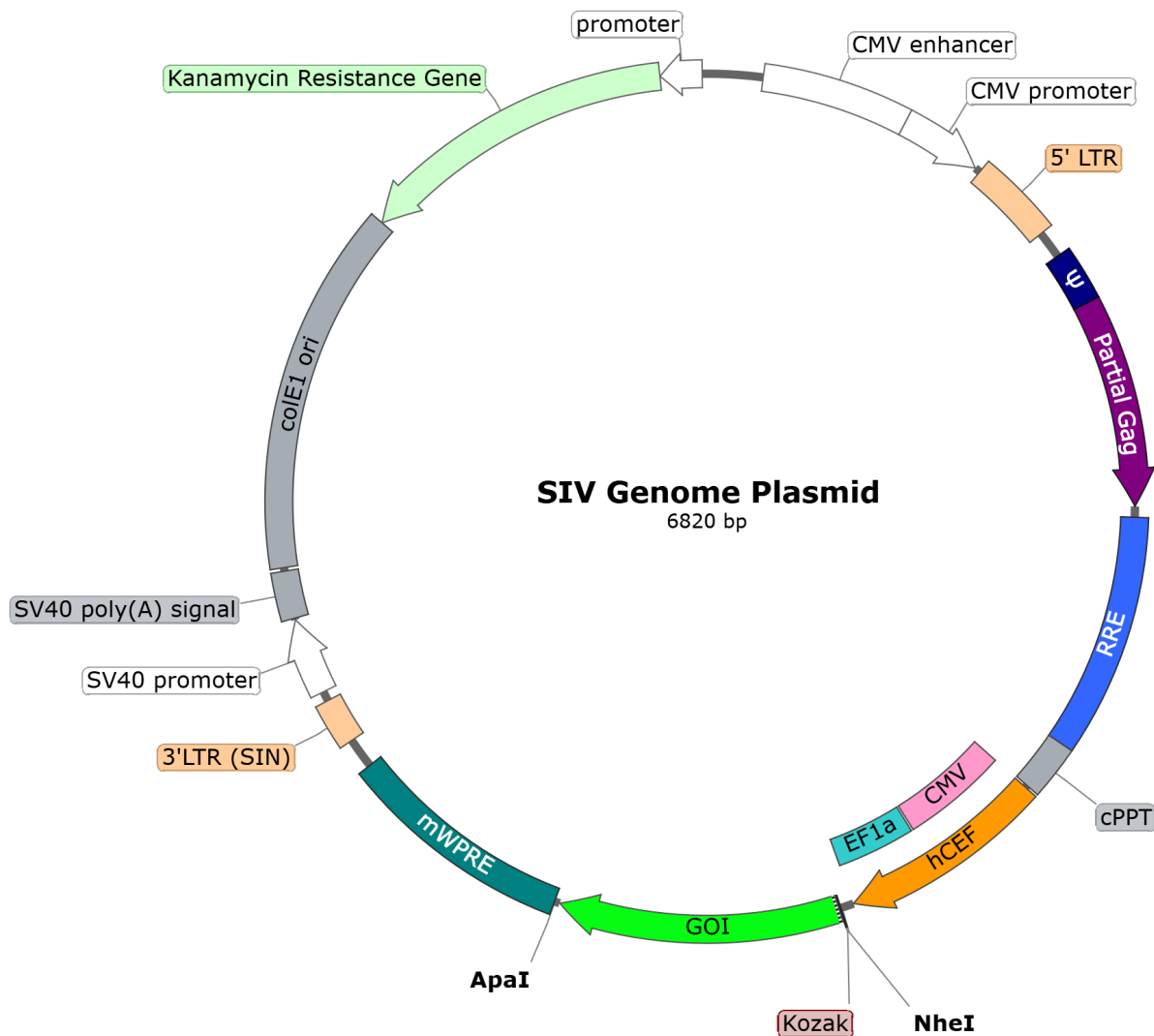


Figure 6 - Schematic of a SIV genome plasmid. The SIV genome plasmid encodes the cDNA of the gene of interest (GOI), which here is either EGFP, mGM-CSF, soGlux, or EGFP/Luc fusion. Transgene expression is under the control of a chimera of the human cytomegalovirus (CMV) enhancer and human elongation factor 1 alpha (EF1a) promoter (hCEF). Self-inactivating long terminal repeats (LTRs) flank the vector genome. Within the expression cassette a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), and a kozak sequence (Kozak) are also encoded. On the backbone but not within the expression cassette are: Gag polypeptide (Gag), a central polypurine tract (cPPT) posttranscriptional regulatory element, a packaging signal (Ψ), and a kanamycin resistance

gene. The image was created using a plasmid encoding EGFP (720 bp) and therefore the size of the empty vector backbone is 6100bp. Image created with SnapGene® (v5.3.2).

Table 1 – List of large-scale purified lentiviruses produced by the GTC Core facility. All Virus types used the SIV backbone. soGLux: Gaussia luciferase; EGFP: enhanced green fluorescent protein; hCEF: promoter/enhancer comprising CpG-depleted human cytomegalovirus enhancer and human Elongation Factor 1a promoter; TU: transducing units.

Name	Batch Name	Promoter	Genome Plasmid	Transgene	Functional Titre (TU/μl)
vGM173	20170607TWP - 134	hCEF	pGM547	mGM-CSF	9.23e9
vGM173	20170816TWP- 138	hCEF	pGM547	mGM-CSF	3.19e9
vGM124	20170222TWP	hCEF	pGM358	soGlux	2.39e9
vGM124	20170802TWP	hCEF	pGM358	soGlux	9.42e9
vGM107	20151201TWP	hCEF	pGM357	EGFP	3.02e9
vGM107	LV2018-20TWP	hCEF	pGM357	EGFP	4.62e9
vGM107	LV2018-33TWP	hCEF	pGM357	EGFP	3.04e9
vGM196	LV2019-36TWP	hCEF	pGM357	EGFP	2.38e9
vGM020	LV2019-54TWP	hCEF	pGM020	GFP/Luc fusion	5.16e9

2.5 Transducing bone marrow-derived macrophages with lentivirus

Unless otherwise stated BMDMs were transduced post differentiation but prior to passaging. For *in vitro* experiments this was performed in 24-well Nunc™ Non-Treated multidishes (ThermoFisher Scientific). When transducing cells for *in vivo* delivery to mice, this was performed in Nunc Non-treated T75 EasyFlask (ThermoFisher Scientific). In each experiment, one representative well or flask was harvested to determine the approximate number of cells per well. In initial *in vitro* transduction experiments however, BMDMs were first harvested from culture on day 7 and plated at 2.5e5 cells in complete media in 6-well Nunc™ Non-Treated multidish (ThermoFisher Scientific), grown overnight and transduced the following day.

Lentivirus was diluted into pre-warmed RPMI media + 10% FCS (37°C) and added to each well at the indicated multiplicity of infection (MOI). Unless otherwise stated, lentivirus was only thawed once directly prior to use. Untransduced controls were exposed to the same treatment, but media contained no virus. Following a 48-hour incubation at 37°C, cells were washed and incubated for 7 days in fresh complete media. Transgenic EGFP expression analysis was confirmed by imaging cells on day 5 using an inverted Widefield Microscope with LED illumination Zeiss Axio Observer inverted microscope and Zen acquisition software at 37°C, and/or quantified by flow cytometry analysis of EGFP expression (Described in more detail where relevant). Secreted transgenes GM-CSF and Gluc expression was quantified in cell culture media removed from cells at defined time intervals. For molecular analysis, BMDMs harvested from culture were resuspended in 300 µl of RNeasy Lysis Solution (Qiagen) and stored for up to two weeks at 4°C or long-term at -80°C.

2.6 Flow Cytometry

During flow cytometry staining and analysis of flow cytometry FACs buffer was used to reduce cell clumping and maintain cellular viability. FACs Buffer was made as follows: 1x dPBS, 1 mM EDTA (Cat. #324504, Merck, Feltham, UK), 25 mM HEPES pH 7.0 (Cat. #15630106, ThermoFisher Scientific), 1% FCS, and stored at 4°C. Throughout flow cytometry procedures, all samples, and reagents were kept at 4°C unless otherwise stated. Data analyses were performed using FlowJo software (v10.7.1, TreeStar, USA). Fluorescence minus one (FMO)

controls were used in every experiment for all fluorophores/dyes/fluorescent proteins and used to establish sequential gating parameters. All samples were first gated by size (FSC-A vs SSC-A), then for single cells (FCS-A vs FCS-H), and viability.

2.6.1 Flow cytometry analysis of macrophage phenotype markers

When analysing BMDMs for macrophage phenotype cell-surface markers CD11b, and F4/80, a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences) was used to perform compensation with Ultra Comp eBeads™ or GFP Bright Comp eBeads™ (Cat. #01-2222-42, #A10514 ThermoFisher Scientific). Where possible between 100,000 – 500,000 cells were stained and analysed in each sample. Cells were first stained according to manufactures protocols with viability dye Zombie NIR™ Fixable Viability Kit (excitation/emission: 633/746) (Cat. #423105, Biolegend) at a 1:250 dilution in dPBS for 10 minutes at room temperature (protected from light). Subsequently BMDMs were stained with fluorophore conjugated antibody mixtures diluted 1:500 in FACs buffer with Fc Receptor Blocking Solution (Cat. #AB_2728082, BD Biosciences, Canada) at a 1:100 dilution for 20 minutes at 4°C (protected from light) (Table 2). Cells were either immediately analysed or fixed for 20 minutes with Fixation Buffer (Cat: 420801, Biolegend) and stored in the dark at 4°C overnight and measured the following day. Lentiviral transduction was quantified by the number of EGFP+ live cells, or EGFP+ macrophages (CD11b⁺, F4/80⁺) (Laser: 488 nm, Bandpass filter: 533/30).

2.6.2 Flow cytometry analysis of EGFP expression

Flow cytometry was used to determine the efficiency of transduction and transfection experiments by analysing cells for EGFP expression using two different protocols. The first was previously described, where macrophages were also analysed for the expression of cell-surface macrophage phenotype markers CD11b and F4/80. When not analysing the phenotype of macrophages experiments analysed unfixed cells using a BD Accuri™ C6 Plus Flow Cytometer and C6 Plus Analysis Software (BD Biosciences, Canada). Cells were analysed live and stained immediately prior to analysis with the TO-PRO™-3 Iodide (1 µM working solution) (excitation/emission: 642/661, detected with a 670 LP filter) (Cat. #T3605, ThermoFisher Scientific) viability dye.

Table 2 - Fluorophore-conjugated antibodies used in flow cytometry analysis. All purchased from Biolegend USA. Antibodies were added to cells in FACs buffer at the indicated dilution and detected by flow cytometry with the indicated laser and bandpass filter configuration.

Antibody	Fluorophore	Laser	Bandpass Filter	Dilution	Catalogue number
F4/80	BV 421	488 nm	450/50	1:500	123131
CD11b	PE	561 nm	582/15	1:100	123109
CD45.1	PE	561 nm	582/15	1:100	110707

2.5.2.1 Quantification of EGFP expression

To determine the efficiency of transduction or transfection, the percentage of EGFP positive cells was analysed by flow cytometry (Laser: 488 nm, Bandpass filter: 533/30), and analysed using FlowJo software (TreeStar, USA). This was either calculated as the percentage of live cells or CD11b⁺, F4/80⁺ Macrophages. Non transduced/transfected controls were always used to gate for positive EGFP expression. The mean fluorescent intensity of the EGFP signal, was quantified for cells positively gated for EGFP expression in the FITC channel.

2.7 Quantification of secreted transgene expression

The production of secreted transgenes was measured *in vitro* in cell culture media, and *in vivo* in lung homogenate, serum, or BALF samples (sample collection described in Chapter 4.2).

2.7.1 Quantifying Glux expression

The amount of Glux protein in undiluted cell culture media and mouse samples was quantified according to manufacturer's instructions by Pierce™ Gaussia Luciferase Flash Assay Kit (Cat. #16158, ThermoFisher Scientific). Briefly 10 µl of undiluted samples were added to black opaque Greiner Bio-One 96-Well Flat Bottom Chimney Style Non-binding Microplates (Cat. #655900, ThermoFisher Scientific). The 100X Coelenterazine enzyme was diluted to a 1X working concentration in Gaussia Flash Assay Buffer. 50 µl of working solution was added by

automatic injection to each well using a FLUOstar® Omega microplate reader (BMG Labtech, Aylesbury, UK) which measured sample luminescence (filter 475nm $\pm$ 30) after a 2 second 250 rpm shaking integration time (Gain: 3600, 95% thresholding).

2.7.2 Quantifying mGM-CSF expression

The concentration of mGM-CSF in 50 μ l of undiluted cell culture media or mouse samples was quantified according to manufacturer's instructions by a solid-phase Quantikine ELISA Mouse GM-CSF kit (Cat. #MGM00, R&D Systems, Abingdon, UK). Briefly, a standard curve from 500 – 7.8 pg/ml was produced by serially diluting a standard 2-fold in calibration diluent. 50 μ l of samples were incubated for 2 hours at room temperature with 50 μ l of assay diluent in a standard 96 well ELISA plate format with gentle shaking. Wells were then washed 5 times with wash buffer and 100 μ l of conjugate solution was added for a second 2-hour incubation at room temperature with gentle shaking, after which wells were once more washed 5 times. 100 μ l of substrate solution made of a mixture of reagent A and B was added to each well for a 30-minute incubation (protected from light) during which time a colourimetric reaction occurs that is directly proportional to the amount of GM-CSF protein in the original sample. To stop the reaction 100 μ l of stop solution was added and the change in colour quantified by reading samples at 450 nm absorbance with a wavelength correction at 570 nm on a FLUOstar® Omega microplate reader. The concentration of GM-CSF in lung homogenate samples and cell culture media was normalised to the total protein concentration in the lung sample and cell lysates respectively.

2.7.3 Protein Assay

To normalise the expression of secreted transgenes by cell number, cells were first lysed according to manufacturer's instructions with Pierce™ IP Lysis Buffer (ThermoFisher Scientific). Briefly, following removal of media, cells were washed once with ice-cold dPBS and then incubated on ice for 5 minutes with 200 μ l of ice-cold lysis buffer and periodic mixing. The lysate was then transferred to a microcentrifuge tube (Cat. #11926955, ThermoFisher Scientific) and centrifuged at 13,000 *rcf* for 10 minutes at 4°C. The total protein concentration of samples was then quantified as a read-out of total cell numbers in culture. If not performed immediately, samples were stored briefly at -20°C.

The total protein concentration of samples including lung homogenate and cell lysate was quantified according to manufacturer's instructions with the Bio-Rad RC DCTTM Protein Assay Reagents Package (Cat. #5000121, Bio-Rad, Hertfordshire, UK). Cell lysate samples were analysed neat, while lung homogenate samples were first diluted 1:40 in dPBS. A standard curve with a 4–0.063 mg/ml range was produced by 2-fold serial dilutions of bovine serum albumin protein (BSA) (Cat. #A9419, Sigma-Aldrich). Samples and standards were measured in duplicate, plating 5µl of onto a 96-well plate. 25µl of buffer A' was added to each well, where the buffer was made immediately prior to adding by mixing buffer S (20 µl) with buffer A (1 ml). Samples were then incubated at room temperature for 10 minutes before adding 200 µl of buffer B and incubated for another 30min (protected from light). Protein concentration is directly related to the colourimetric change that occurs and can therefore be read at 750 nm absorbance on a FLUOstar® Omega microplate reader.

2.8 Digital droplet polymerase chain reaction (ddPCR)

Digital droplet PCR (ddPCR) was performed using the BioRad QX200TM ddPCR system (BioRad, CA, USA) and processing was performed in a Microflow Peroxide Class II ABS Cabinet (Bioquell, Andover, UK) inside a PCR clean room. PCR reactions were emulsified in a droplet generator cartridge using the QX200 droplet generator (Cat. #1864002, Biorad) with 70 µL of droplet generation oil for probes (Cat. #1863005, Biorad) per sample. A C1000 Touch Thermal Cycler (Cat. #1851197, Biorad) was used to perform thermocycling and a QX200 Droplet Reader (Cat. #1864003) was used to PCR positive and PCR negative droplets. Analysis was performed using QuantasoftTM Software (v1.7.4, BioRad). Only samples with droplet numbers above 10000 were included in the analysis. Thresholding of positive droplets was performed automatically by the software and in rare cases, manual thresholding was used if sufficient separation of positive and negative droplets could be confirmed visually.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8) and the statistical test used in each experiment is described in the relevant results section and/or the figure legends where they are reported. Normality of data distribution was assessed using the D'Agostino-Pearson omnibus normality test. In the majority of experiments n numbers were too small for this analysis, and a non-parametric test was used in the subsequent analysis. As a result, data is generally reported as median (range). In instances where normal data distribution was

confirmed data is reported as mean $\pm$ standard error of the mean (SEM). The descriptive value is always defined when reporting data.

When comparing two groups, a student's t-test or Mann-Whitney t-test were performed for parametric and non-parametric data respectively. Multi-group analysis was performed using a Kruskal-Wallis test for non-parametric data with Dunn's post-hoc multiple comparisons test and no multi-group analyses of parametric data was performed in this thesis. Multigroup analysis of two categorical variables were performed using a two-way ANOVA test with Sidak's post-hoc multiple comparisons.

The null hypothesis was rejected at $p < 0.05$.

Chapter 3 – Isolation, differentiation and genetic modification of bone marrow-derived macrophages

3.1 Introduction

The overall aim of this thesis is to develop a platform technology based on the transplantation of gene-modified macrophages for *in vivo* pulmonary production of therapeutic proteins.

Macrophage cell types are considered relatively refractory to gene transfer as they have potent immune functions that can restrict introduction of foreign genetic material (281, 282). They play a key role in anti-viral defence, where in vertebrates this occurs largely through the discrimination of non-self-nucleic acids (283). Macrophages can recognise foreign nucleic acids by their availability, localisation, and structure (including sequence motifs, conformation and chemical modifications (284). They then not only initiate appropriate immune responses but have intrinsic anti-viral activity including abundant intracellular expression of DNases, which in the context of gene therapy can limit the effective delivery of nucleic acids (283, 285, 286). In addition, as primary macrophages are largely non-proliferative in culture, it limits the ability of exogenous nucleic acids to reach the nucleus during mitotic breakdown of the nuclear lamina (287).

In general viruses are considered more effective than chemical or physical transformation/transfection methods, and this is also true when genetically modifying macrophages (189, 288-299). Lentiviral vectors have become increasingly popular as key aspects of their biology make them particularly suited to overcome barriers imposed by macrophages. As previously discussed in Chapter 1, they are popular vectors for *ex vivo* gene therapy due to their integrative ability providing long-lasting gene expression in dividing cells. Though following lung delivery primary macrophages are known to proliferate, *in vitro* cultures are largely non-dividing (176, 287, 290). Lentiviral vectors are unique in that they can actively transduce a wide range of both dividing and non-dividing mammalian cell types by passing through the nuclear pore complex formed during interphase to traffic molecules between the nucleus and cytoplasm (189, 292, 293). Furthermore, simian immune-deficiency viruses (SIV) and human immune-deficiency viruses (HIV) exhibit natural macrophage tropism and use macrophages as viral reservoirs to form latent infections making them excellent candidates by

which to develop vectors for macrophage gene transfer (297). Though studies have noted that *in vitro*-derived macrophage populations can still be refractory to lentiviral transduction and infection (295), macrophages have been successfully transduced by lentiviruses in the literature (180, 298-301).

The UK Respiratory Gene Therapy Consortium developed a propriety lentivirus for *in vivo* lung administration (Figure 7) (277). The vector is a simian immunodeficiency virus (SIV) pseudotyped with fusion (F) and haemagglutinin neuraminidase (HN) from a Sendai virus (268). Transgene transcription is driven by a patent-protected CpG-free, constitutively active hCEF promoter/enhancer chimera composed of the cytomegalovirus (CMV) immediate-early enhancer and promoter and eukaryotic translation elongation factor 1 alpha (EF1 α) (280). *In vivo* this vector has shown efficient transduction of the lung epithelium for cellular and secreted transgene expression, and *in vitro* has successfully transduced numerous immortalized cell lines (CHO, A549, HEK293T, BHK), air-liquid interface cultures, human nasal brushings, human and sheep lung slices, and human intestinal organoids (168, 268, 276, 302). However, it has not yet been evaluated for *ex vivo* transduction of primary macrophages or macrophage cell lines which in the literature have been successfully transduced by other lentiviral vectors, most commonly VSV-G pseudotyped HIV-1 based vectors (298, 300, 301, 303, 304).

In this chapter I aim to characterise a protocol for the isolation, differentiation and genetic modification of murine bone marrow-derived macrophages (BMDMs). I hypothesize that the rSIV.F/HN lentivirus will be able to transduce these cells *in vitro* to express secreted and non-secreted proteins, aiming to establish a protocol as part of a platform technology using gene modified macrophage transplantation to drive pulmonary expression of therapeutic proteins. Here I demonstrate that BMDMs can be cultured and transduced by rSIV.F/HN in a dose dependent manner resulting in significant *in vitro* production of reporter and therapeutic proteins.

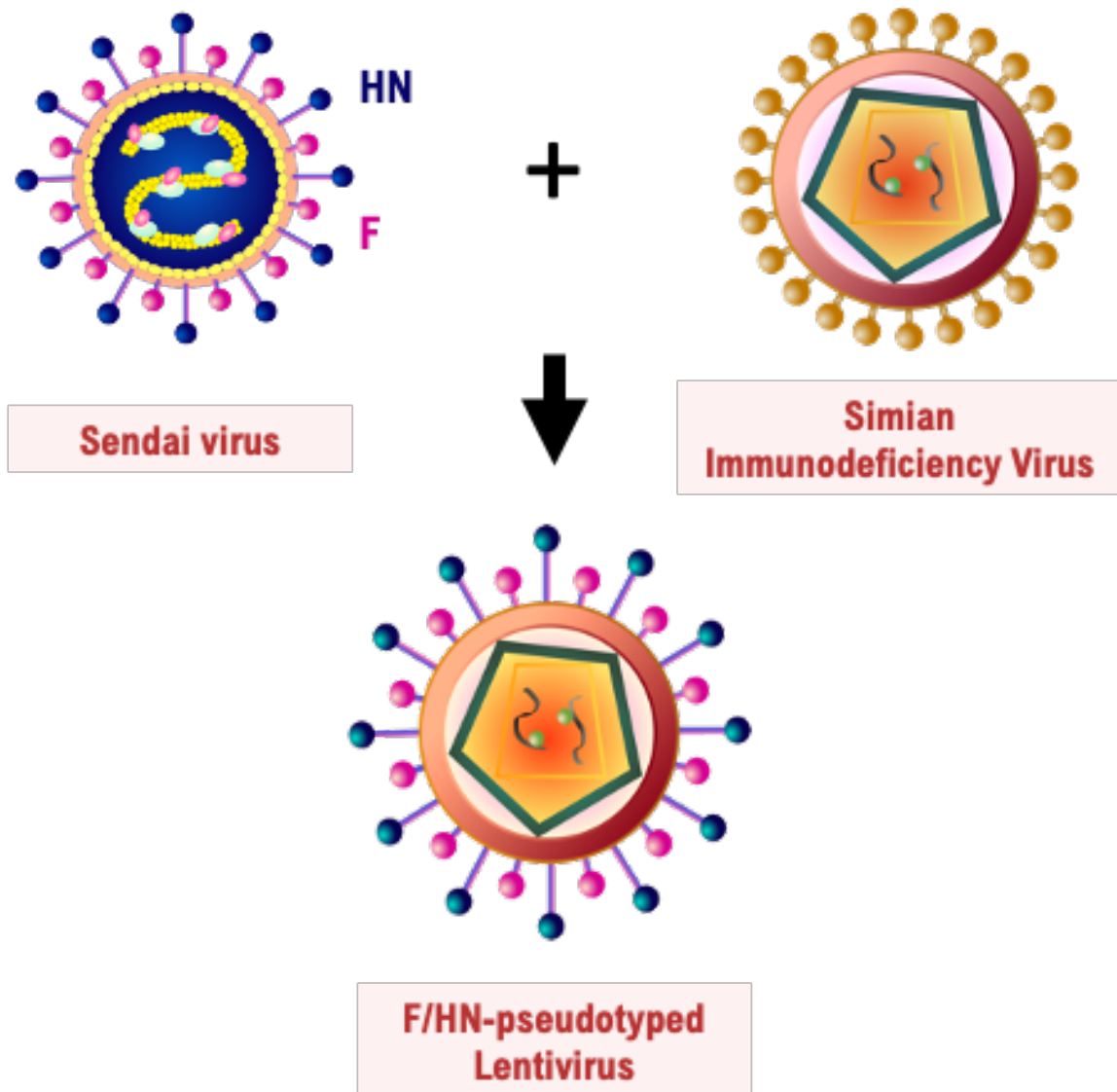


Figure 7 – Proprietary lentiviral vector developed by the UK Respiratory GTC. The rSIV.F/HN vector is a simian immune deficiency virus pseudotyped with the fusion (F) and haemagglutinin (HN) proteins from the Sendai virus with increased tropism for the lung epithelium. Produced by the UK Respiratory Gene Therapy Consortium and adapted with permission.

3.2 Methods

3.2.1 Live cell imaging of EGFP expression

Expression of EGFP in BMDMs was visualised 5 days post-transduction by fluorescent widefield imaging of EGFP expression in live cells using an inverted Widefield Microscope with LED illumination Zeiss Axio Observer inverted microscope and Zen acquisition software. Images were captured with an EC Plan-Neofluar 40x/0.75 Ph2 objective and a Zeiss Colibri 7 light source by a Zeiss Filter Set 10 (FITC), with 525/50 emission filter and phase contrast. During imaging cells remained in culture and were incubated at 37°C. Macrophages were carefully transported in polystyrene boxes to and from the microscopy facility, limiting time outside of incubation as much as possible. Following imaging cells were once more cultured in a 37°C incubator with 5% CO₂. Analysis and image formatting was then performed in FIJI-ImageJ².

3.2.2 Immunocytochemistry labelling of BMDMs

BMDMs were harvested from cell culture and fixed in suspension in 2% paraformaldehyde (Cat. #P6148, Sigma Aldrich, Dorset, UK) for 10 minutes at room temperature and then washed 2x with ice-cold dPBS. Cells were resuspended in ice-cold dPBS at approximately 2e5 cells/ml, and 200 µl were then placed into an EpreDia™ TPX Single Sample Chamber, Caps and Filter Cards set up (Cat. # 11922355), where a reusable cytofunnel and a disposable filter card are assembled and held together by an EpreDia™ Cytoclip™ Stainless-Steel Slide Clip (Cat. #59910052). Cells were transferred onto EpreDia™ Shandon™ Single Cytoslides™ (Cat. #5991056) by centrifuging the assembled cytofunnels at 400 rpm in a Shandon cytofuge (Cat. # 74010121). (All reagents and components produced by ThermoFisher Scientific). Slides were then airdried for 15 minutes and then stained for subsequent fluorescent imaging.

BMDMs were then fluorescently labelled with an anti-F4/80 antibody. All the buffers used in this procedure can be found in Table 3. Sides were successively submerged in 0.1M PBS and 0.1M PBST for 5 minutes each. To minimize unspecific binding, slides were incubated with blocking buffer for 20 minutes. To fluorescently label F4/80+ macrophages, cells were incubated for one hour with 75 µl of an anti-mouse F4/80 rat monoclonal antibody [CI:A3-1] (Cat. #b6640, Abcam, Cambridge, UK) diluted 1:100 in incubation buffer. Cells were

subsequently washed three times for 5 minutes in 0.1M PBST and then incubated with 50 μ l of an Alexa flour-594 conjugated goat anti-rat antibody (Cat. #ab150160, Abcam) (diluted 1:500 in incubation buffer) for 1 hour. Following three washes in 0.1M PBST for 10 minutes, slides were washed in 0.01M PB (10 minutes) and then mounted using 1 to 2 drops of HardsetTM Antifade Mounting Medium with DAPI (Cat #H-1500, Vectorlabs). All antibody incubation steps were performed in a humidity chamber at room temperature.

Fluorescent imaging of F4/80 expression was captured with a Zeiss Telaval 31 Inverted microscope (Carl Zeiss, Cambridge, UK) using Plan-Neofluar 40x/0.75 Ph2 objective and a Micropix Elite 5 Microscope Camera on Cytocam MicroPix software (v1.6.0.0, Microscope Systems Ltd, Glasgow, UK). Images captured in the blue channel (358 nm excitation, 420-490nm emission) for DAPI staining, and in the red channel for F4/80 staining (594 nm excitation, 604-644 nm emission).

Table 3 – Buffers used when fluorescently staining BMDMs. Each buffer was made by diluting components in the indicated base solution/liquid. Final concentration of each component given in molar (M) or as a percentage of the final volume. All reagents were purchased from Sigma-Aldrich.

Buffer	Base	Components	Concentration
PB (0.1M)	ddH ₂ O	Na ₂ HPO ₄	0.08 M
		KH ₂ PO ₄	0.02 M
PBS (0.1 M)	ddH ₂ O	Na ₂ HPO ₄	0.08 M
		KH ₂ PO ₄	0.02 M
		NaCl	8 %
		KCl	0.2 %
PBST	PBS	Triton-100X	0.05 %
Blocking Buffer	PBST	BSA	0.03 %
		Normal Goat Serum	10 %
Incubation Buffer	PBST	BSA	0.03 %
		Normal Goat Serum	5 %

3.3 Results

3.3.1 *In vitro* differentiation of bone marrow derived-macrophages induced by M-CSF produces highly homogenous CD11b⁺, F4/80⁺ cell populations

Murine bone marrow-derived macrophages (BMDM) were used in the development of the *ex vivo* macrophage gene therapy platform in mice. Murine BMDMs (CD11b^{high}, Siglec F^{low}) are commonly used *in vitro* as models of unpolarized macrophages (305) and in the context of transplantation to the lung have shown proof-of-concept in the literature where cells acquire a mature AM phenotype (CD11b^{low}, Siglec F^{high}) (44, 306). Murine BMDMs were utilized here in developing an *ex vivo* cell therapy approach to lung gene therapy owing to the quantity, ease and purity of ensuing macrophage cultures (286).

A BMDM differentiation protocol was first optimised, where resulting cells were characterised by flow cytometry and immuno-histochemistry analysis. This protocol was based on common practices found in the literature where bone marrow collected by flushing the fibula and tibia of mice was grown in culture with media supplemented with macrophage-colony stimulating factor (M-CSF) for 7 days (44, 306). In these experiments, all BMDMs were harvested from wildtype (WT), C57B/6J mice.

In initial experiments, bone marrow was plated onto tissue-culture treated flasks, and initial attempts to characterize these cells by flow cytometry were unsuccessful (data not shown). The hydrophobic polystyrene surfaces of the flasks have been modified to a more hydrophobic surface to encourage cell attachment (307). Cells were difficult to detach following repeat washing, and extended incubation with versene (EDTA), with insufficient cell numbers for analysis by flow cytometry. This was true even following cell scraping, a practice not recommended for macrophage harvesting due to resulting changes in receptor expression. The protocol was therefore adapted and in keeping with published protocols, the use of non-cell culture-treated flasks alleviated this issue and enabled the harvesting of BMDM for analysis (47, 286, 308).

As macrophages change their functional phenotype in response to their microenvironment, their expression of cell surface receptors is also subject to change and exhibits tissue specificity (2, 21). BMDMs can be identified as following M-CSF induced culture they are highly positive for CD11b and F4/80 which are expressed on cells of a myeloid lineage, and monocytes and tissue resident macrophages respectively (47, 306). Here two common variations of the differentiation protocol were tested and when characterised by flow cytometry both resulted in highly homogenous as CD11b⁺, F4/80⁺ macrophage populations (Figure 8a-b). The protocols varied in the washing steps applied during differentiation, either: (A) media was replaced on day 3-5 of differentiation with fresh, M-CSF-supplemented media (100 µg/ml) (309), or (B) media was left on BMDMs and supplemented with M-CSF (200 µg/ml) supplemented media. The experiment was performed twice, each time on BMDMs from a single C57BL/6J mouse, where cells were cultured in separate tissue culture flasks and analysed independently by flow cytometry (n=4/mouse). Flow cytometry analysis identified CD11b⁺ and F4/80⁺ populations through sequential gating (Figure 8c), and fluorescence minus one (FMO) controls (Figure 8d). Though both protocols reported high median percentages of BMDMs (CD11b⁺, F4/80⁺) as a percentage of live cells, (Protocol A: median 97.4% (range 84.8–99.5%); Protocol B: median 98.5% (range 95.4–99.2%)), the latter (B) showed less inter-experimental variation over two experimental iterations and called for lower quantities of expensive M-CSF. Moving forward, BMDMs were therefore cultured under protocol B. BMDM phenotype under these conditions was further visually confirmed through fluorescent microscopy of BMDM cytopins stained with an F4/80 antibody (Figure 9).

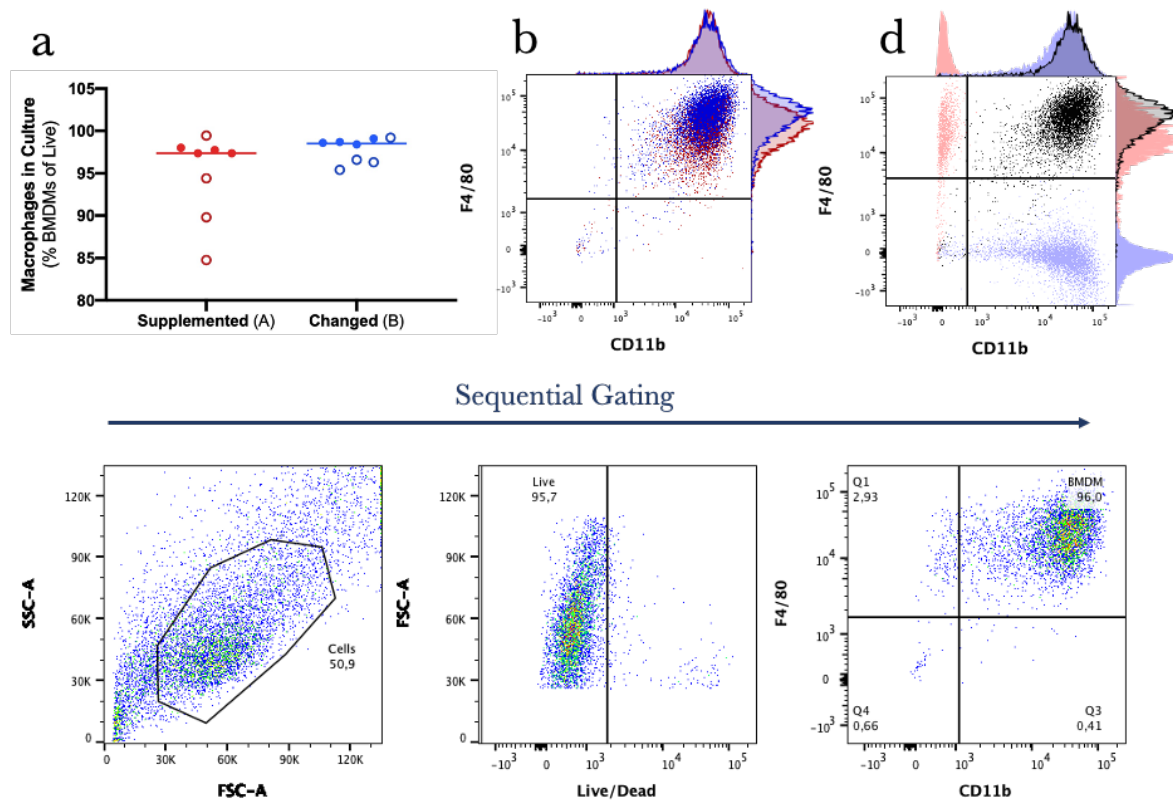


Figure 8 – Flow cytometry analysis of cell-surface phenotypic markers of wildtype bone marrow-derived macrophages (BMDMs). Following M-CSF induced *in vitro* macrophage differentiation of bone marrow cells under two different protocols, where media is either supplemented (A) or changed (B) on day 3. BMDMs were identified through sequential gating analysis (c) as CD11b⁺ and F4/80⁺ cells. (a) The percentage of BMDMs in culture was calculated as the percentage of CD11b⁺, F4/80⁺ live cells in culture. To compare the relative success of each protocol, BMDMs from two wildtype C57BL/6J mice were analysed over two separate experiments. Circles represent BMDM samples differentiated in separate tissue-culture flasks and stained for flow cytometry, where samples from each mouse are either empty or filled circles. Bars represent median value. (b) Expression of CD11b and F4/80 from representative samples for culture condition A (red), and B (blue). (d) FMO controls for each fluorophore were used to accurately gate positive populations, cd11b FMO (light blue), F4/80 FMO (pink), and full stain (black).

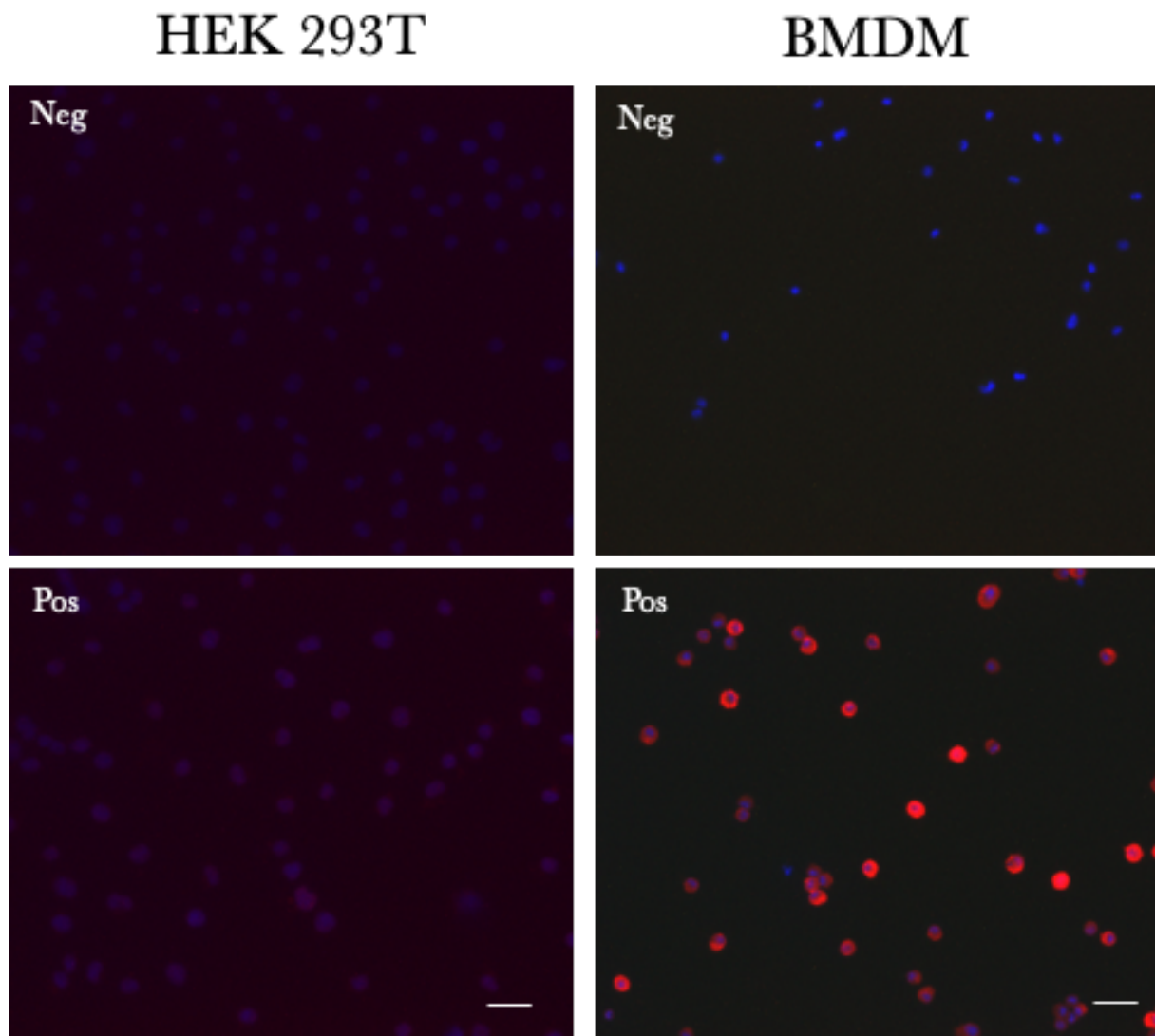


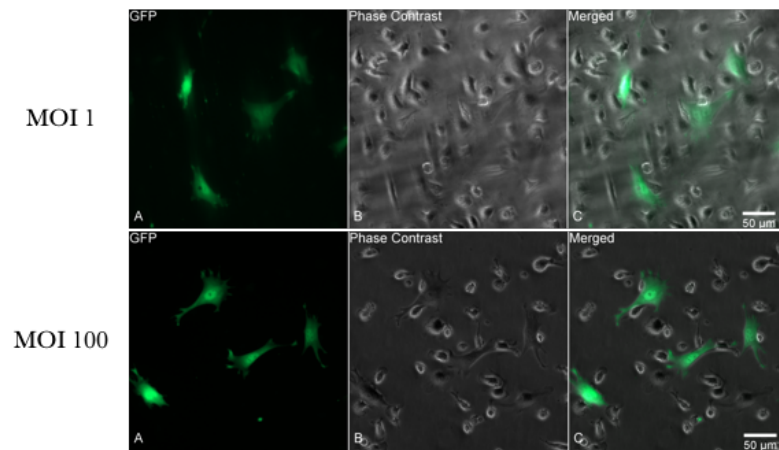
Figure 9 – Specific immunofluorescent staining of bone marrow-derived macrophages (BMDMs) with an F4/80 antibody. Following differentiation, on day 7 BMDMs were fixed for 24 hours in 4% PFA for 10 minutes and cytopun at 40,000 cells/slide. HEK 293T cells were similarly treated and served as a negative control. Incubation with a monoclonal mouse anti-F4/80 antibody ab6640 (abcam) and a secondary goat anti-rat antibody confirmed expression of the F4/80 macrophage/monocyte marker on BMDMs (red channel, 640nm). Composite images of blue channel showing DAPI counterstain (358 excitation, 420-490nm) in stained slides (pos) and negative controls stained incubated with the secondary antibody only (neg). Captured with an inverted light microscope at 40X magnification (Zeiss Telaval 31 Inverted microscope, Carl Zeiss, Cambridge, UK). Scale bar: 50µm.

3.3.2 Passaged BMDMs can be transduced with rSIV.F/HN to express EGFP but cell viability precludes quantitative analysis

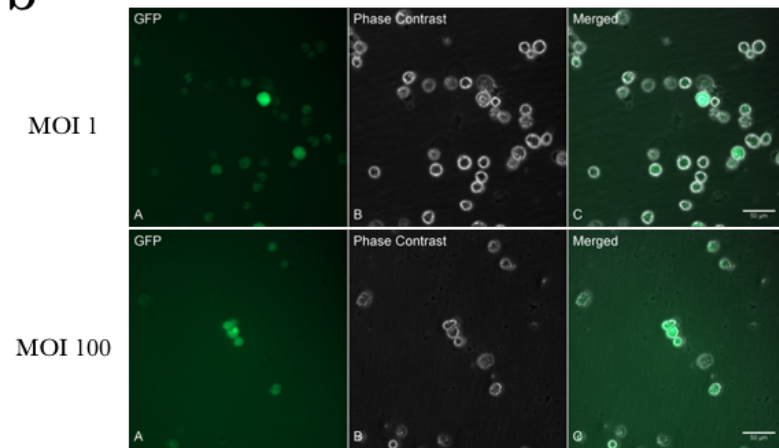
Having established a protocol for the isolation, differentiation and characterisation of BMDMs, I then investigated a protocol for the *in vitro* transduction by lentivirus to produce genetically modified macrophages. In an initial pilot experiment, I sought first proof-of-concept that the rSIV.F/HN.hCEF.EGFP can transduce BMDMs, analysing samples by fluorescent microscopy and flow cytometry ($n=2/\text{MOI}$). Macrophages were differentiated in flasks and then harvested and passaged on day 7 at the conclusion of the M-CSF differentiation protocol. Macrophages were plated into 6-well plates at 2.5×10^5 cells/well and transduced 24 hours later at MOI 1 and 100. Five days later live cell imaging by fluorescent microscopy provided first proof-of-concept that macrophages could be transduced to express transgenic proteins using the Consortium's proprietary lentivirus (Figure 10a). Visually no distinct difference in transduction at MOI 1 and 100 were noted, where in both conditions EGFP positive cells were rare in culture. Quantitative analysis of these images did not prove successful. A lack of fluorescent DNA staining owing to the nature of imaging live cells, phase contrast images were used to visually identify macrophages in culture, and due to the high degree of confluency, macros designed to identify cells and subsequently calculate the percentage EGFP positive cells failed to distinguish individual macrophages.

A large degree of cell detachment and loss was observed over time in both transduced and untransduced control cultures. When harvested for analysis 7 days post-transduction, cell numbers were insufficient for quantitative analysis by flow cytometry as when pooling all transduced wells across conditions a total of 2×10^5 live cells were recovered. Though lacking quantitative data, this experiment provided the first proof that BMDMs are receptive to gene transfer mediated by the rSIV.F/HN lentivirus. The experiment was repeated plating BMDMs at two densities of 2.5×10^5 and 5×10^5 cells/well to observe if this had any effect on cell viability. Unfortunately, there was a large degree of cell detachment across conditions, clearly observable in live cell imaging conditions on day 5 (Figure 10b), flow cytometry analysis on pooled samples demonstrated a lack of viable cells in culture (Figure 10c). This effect was not likely due to the exposure of lentivirus as un-transduced samples demonstrated equally low cell viability. Having previously harvested large quantities of viable BMDMs for flow cytometry on day 7 of the differentiation protocol, I hypothesised that issues with cell viability were owing to a sub-optimal passaging protocol.

a



b



c

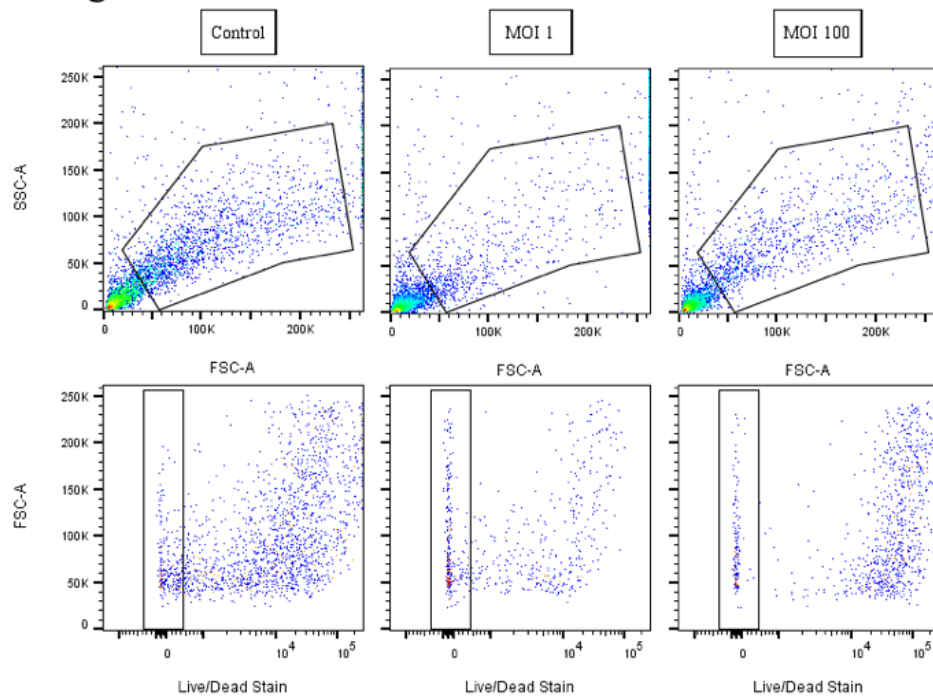


Figure 10 – Bone marrow-derived macrophages (BMDMs) expressing EGFP following lentiviral transduction. Representative images of BMDMs transduced with the rSIV.F/HN.hCEF.EGFP lentiviral vector at an MOI of 1 or 100 and visualized live in culture on day 5 in two separate experiments **(a)** and **(b)**. Captured (A) EGFP expression (green channel: 488 excitation, 510-550nm spectrum), (B) phase contrast by an Inverted Widefield Microscope with LED illumination Zeiss Axio Observer inverted microscope at 40X magnification, (C) composite images of phase contrast and fluorescent images. Merged images created with FIJI-ImageJ2. Scale bar 50µm. **(c)** Flow cytometry analysis of transduced BMDMs visualized in **(b)** when attempting to gate for cells based on size and live/dead staining. BD LSRFortessa Cell Analyser.

3.3.3 Transduction of adherent BMDMs with rSIV/F/HN lentivirus with an EGFP transgene can be quantified by flow cytometry

BMDMs were therefore differentiated and transduced with rSIV.F/HN.hCEF.EGFP at MOI 1 and 20, without an intermediate passage step. Live-cell imaging (Figure 11) and flow cytometry analysis (Figure 12a-b) provided qualitative and quantitative proof of dose-dependent transduction by the F/HN (n=2). Macrophages exhibited good viability and adherence in culture during the 48-hour transduction, imaging on day 5, and harvest for flow cytometry on day 7. This initial test was also used to test the analysis of flow cytometry data to ensure the protocol would enable the determination of transduction efficiency in BMDMs, and any effects on macrophage marker expression. A 32.9% median (range 30.4-33.5%) transduction efficiency at MOI 20 was calculated using FMOs for each fluorophore and sequential gating analysis of EGFP positive BMDMs (CD11b⁺, F4/80⁺, EGFP⁺) (Figure 12c). The percentage of EGFP positive live cells, which includes any live cell regardless of macrophage markers, reported similar results (Figure 12d), which would be expected given that the samples were all indicative of a highly homogenous population of BMDMs (Figure 12e). Importantly transduction did not affect macrophage receptors expression, as the percentage of CD11b⁺ and F4/80⁺ in culture did not change, indicating flow cytometry protocols for the identification of BMDMs is valid in transduced cells (Figure 12e-f). However, this study was just a small initial proof-of-concept trial (n=2) and having established a protocol for flow cytometry analysis of EGFP transduced BMDMs, a larger dose escalation trial was performed.

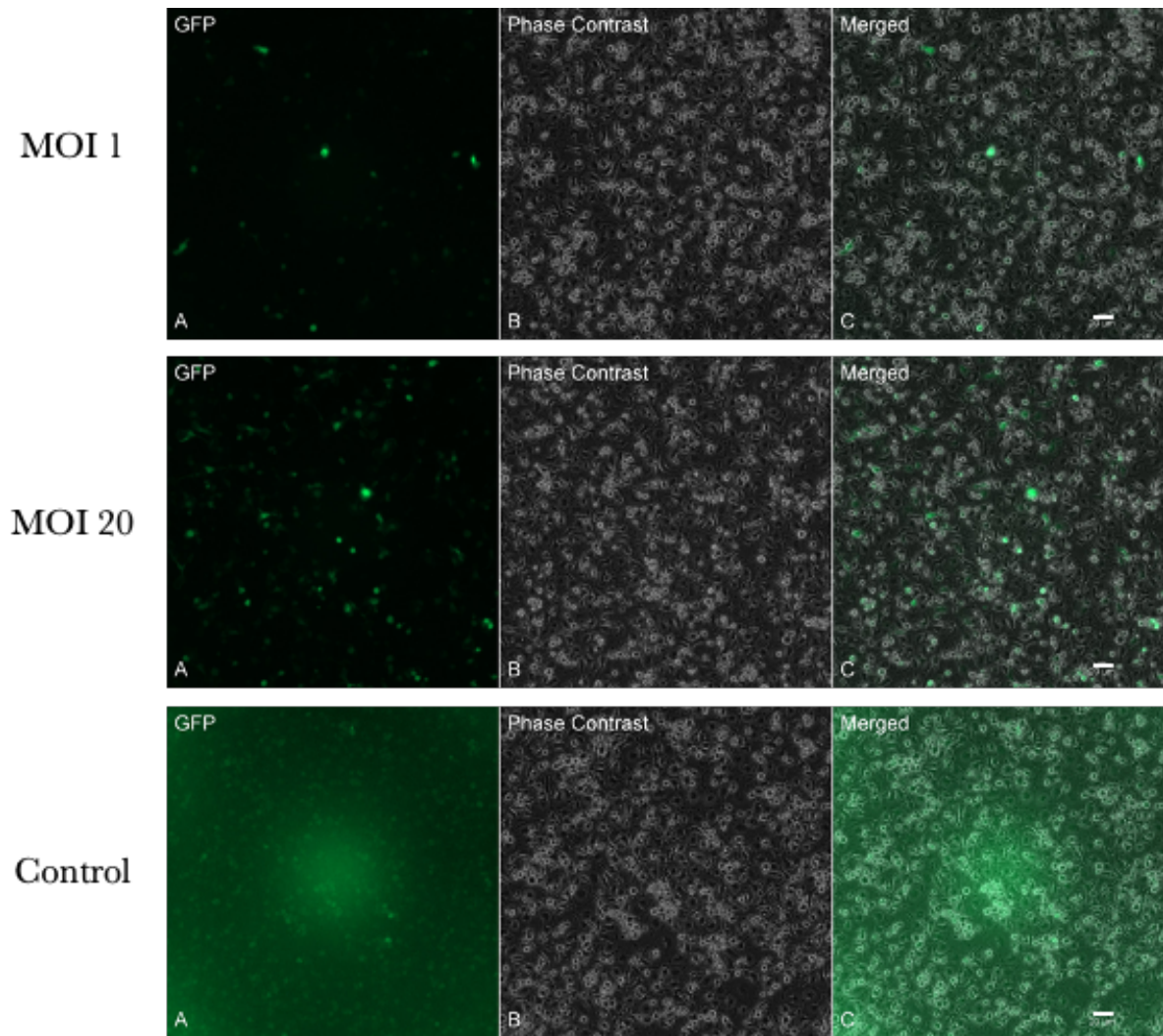


Figure 11 – Bone marrow-derived macrophages (BMDMs) expressing EGFP following lentiviral transduction of adherent cultures. Representative images of BMDMs transduced with the rSIV.F/HN.hCEF.EGFP lentiviral vector at an MOI of 1 or 20, and untransduced controls and visualized live in culture on day 5. Captured (A) EGFP expression (green channel: 488 excitation, 510-550nm spectrum), (B) phase contrast by an Inverted Widefield Microscope with LED illumination Zeiss Axio Observer inverted microscope at 40X magnification, (C) composite images of phase contrast and fluorescent images. Merged images created with FIJI-ImageJ2. Scale bar (white): 50µm.

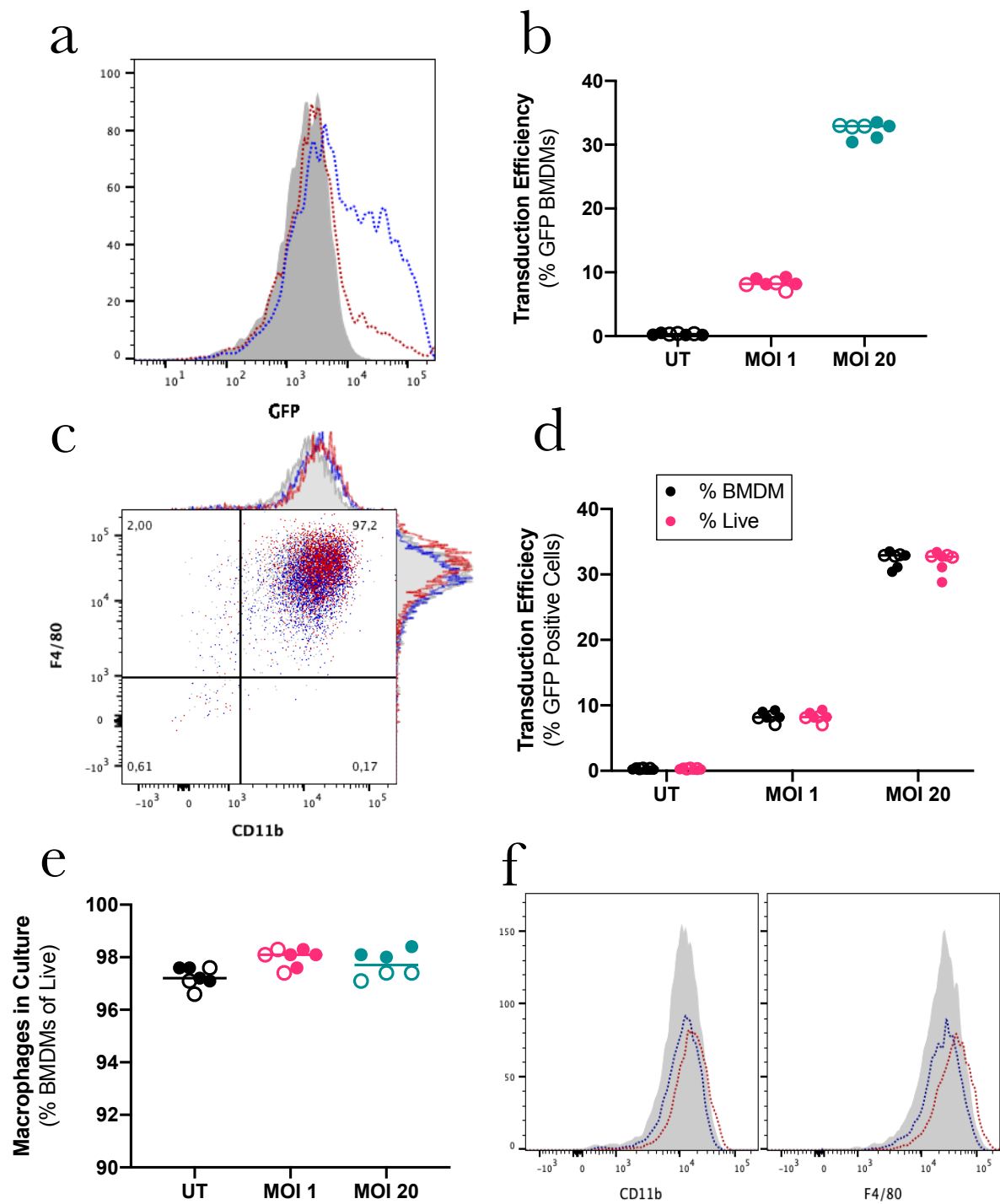


Figure 12 - Bone marrow-derived macrophages (BMDMs) expressing EGFP following lentiviral transduction of adherent cultures. BMDM from C57BL/6 mice were transduced with rSIV.F/HN.hCEF.EGFP at multiplicity of infection of 1 or 20. BMDMs were transduced in duplicate ($n=2$ /MOI, filled/empty circles), measuring four replicates of each sample by flow cytometry. Differentiated macrophages and untransduced (UT) controls were harvested 7 days post viral transduction and analysed by flow cytometry. **(a)** Relative EGFP expression in representative samples

transduced at MOI 1 (red), MOI 20 (blue) in comparison to untransduced controls (grey). **(b)** Transduction efficiency was calculated as the percentage of EGFP positive BMDMs, where macrophages were identified by sequential gating as C11b⁺, F4/80⁺ cells, demonstrated in **(c)** with representative MOI 1 (red), MOI 20 (blue) and to untransduced (grey) samples. **(d)** Comparison between the percentage of total EGFP positive live cells (pink) and EGFP positive BMDMs (black) in culture. **(e)** BMDMs (CD11b⁺, F4/80⁺) as a percentage of live cells in culture following lentiviral transduction, and **(f)** representative images for flow cytometry evaluations of cell-surface macrophage phenotypic markers in MOI 1 (red), MOI 20 (blue) and untransduced (grey) samples. BD LSRFortessa Cell Analyser. Bars represent median value.

3.3.4 Transduction of bone marrow-derived macrophages with rSIV.F/HN lentivirus exhibits dose dependent EGFP expression

To determine the transduction efficiency and maximum feasible dose of the rSIV.F/HN lentivirus in macrophages, BMDMs were transduced at increasing MOIs (1, 5, 20, and 100) with virus expressing EGFP and GM-CSF transgenes under the hCEF regulatory element (n=6). Here, and in all subsequent experiments, BMDMs were differentiated in 24-well tissue-culture plates rather than 6-well plates which reduced the viral cost per well to enable testing at higher n numbers. Transduction efficiency calculations were performed by flow cytometry analysis of EGFP transduced cells and un-transduced controls 5 days after a 48-hour transduction. A clear dose response was observed in the percentage of EGFP positive BMDMs which reached significant levels above that of the un-transduced controls, at MOIs 20 and 100 ($p<0.001$, and $p<0.0001$, respectively, Kruskal-Wallis t-test, Dunn's multiple comparisons) (Figure 13a):

- *Control*: 0.5 (range 0.08-1.1) % transduction,
- *MOI 1*: 4.0 (range 3.4-5.3) % transduction, ($p=0.951$)
- *MOI 5*: 11.0 (range 8.9-16.6) % transduction, ($p=0.0557$)
- *MOI 20*: 28.6 (range 13.1-40.6) % transduction, ($p<0.001$)
- *MOI 100*: 27.7 (range 19.6-51.8) % transduction, ($p<0.0001$, Kruskal-Wallis Dunn's multiple comparisons)

Transduction efficiency increased steadily with increasing MOI, however plateaued between MOI 20 and 100. All samples indicated homogenous CD11b⁺ and F4/80⁺ populations with the exception of MOI 100, where a sharp decrease in the percentage of BMDMs was observed in comparison to controls (median difference: 36.7%, $p<0.05$, Kruskal-Wallis Dunn's multiple comparisons), this correlated with observations of a decrease in cell viability through cell detachment during processing (Figure 13b). This indicates that high lentivirus concentrations may be toxic and result in phenotypic changes in these highly plastic cells. This is recapitulated when comparing the percentage of EGFP positive cells in the total live population to EGFP positive BMDMs (Figure 13c), where only at MOI 100 was there a significant difference between the two values, as BMDM lineage markers were altered (median difference: 7.3%, $p<0.01$, 2wayANOVA, Sidak's multiple comparisons). These results demonstrated gene modification of BMDMs by the rSIV.F/N where maximal efficiency can be reached at MOI 20 without suffering SIV mediated toxicity.

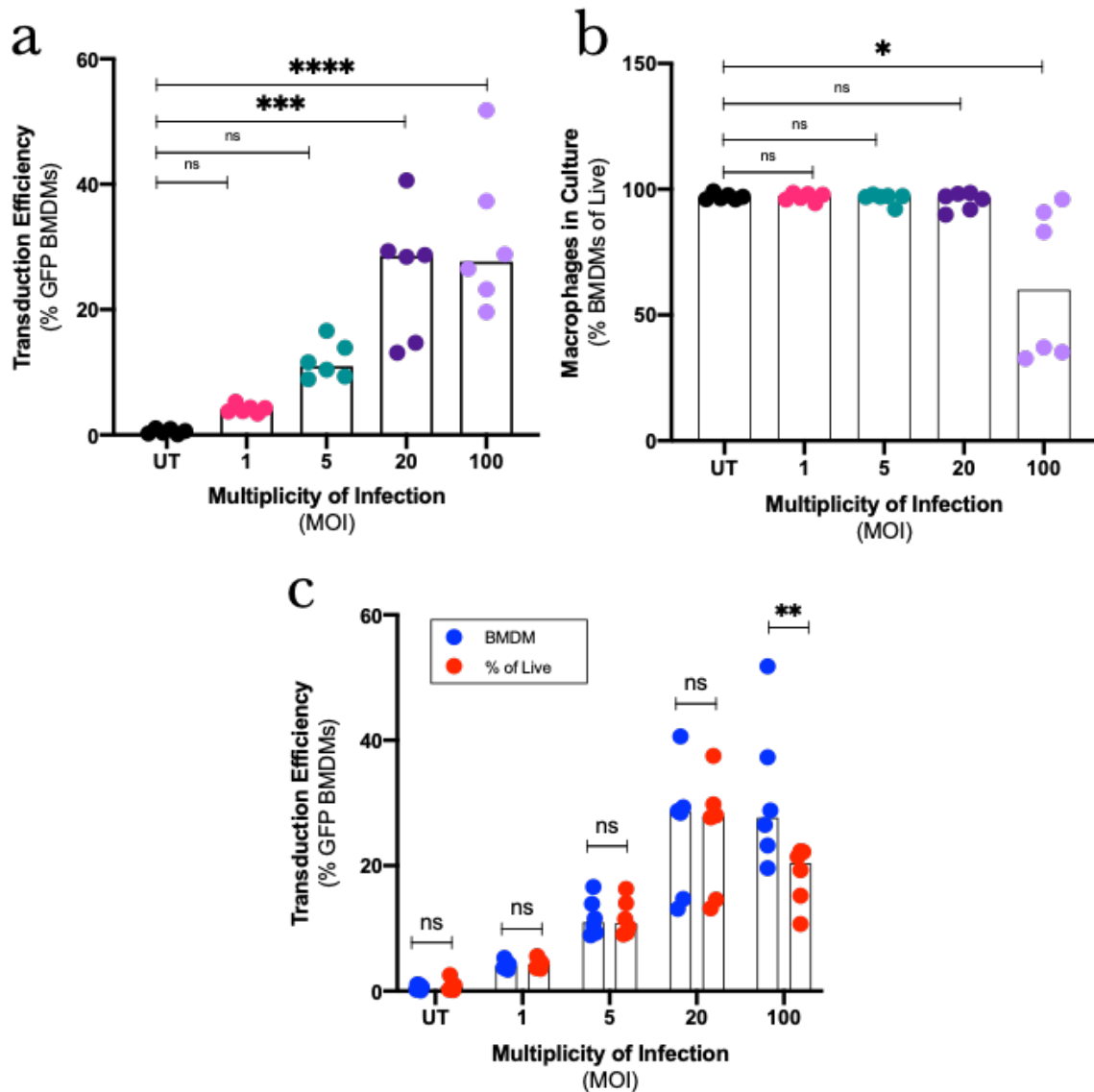


Figure 13 - Dose response in transduction efficiency following rSIV.F/HN lentivirus transduction of bone marrow-derived macrophages (BMDM). (a) The transduction efficiency of BMDMs following transduction with SIV-F/HN expressing an EGFP transgene at increasing multiplicities of infection in comparison to untransduced controls (UT) (n=6/MOI). (b) BMDMs were first gated based on expression of cell-surface phenotypic macrophage markers CD11b⁺, F4/80⁺ to calculate the percentage of macrophages in culture. (*=p<0.05, **=p<0.01, ***=p<0.001, Kruskal Wallis test with Dunn's multiple comparisons.) (c) Comparison between transduction efficiency calculated as either the percentage of EGFP⁺ BMDM (blue), or EGFP⁺ live cells (red) in culture. (**=p<0.01, 2-wayANOVA, Sidak's multiple comparisons). Capture by BD LSRFortessa Cell Analyser. Each data point represents an individually transduced well of BMDMs. Bars represent median.

3.3.5 Bone marrow-derived macrophages transduced with rSIV.F/HN lentivirus express significant levels of a secreted therapeutic protein

Having established that BMDMs permit rSIV.F/HN lentivirus-mediated gene transfer, I then analysed whether following gene transfer macrophages could secrete a transgenic protein. Here a murine GM-CSF transgene was delivered as it is the proposed therapeutic transgene for gene therapy treatments of patients with autoimmune pulmonary alveolar proteinosis (aPAP), which has been proven safe and efficacious with inhalation of exogenous GM-CSF therapy in patients (160, 161, 310, 311). Macrophages were transduced at increasing MOIs for 48 hours with rSIV.F/HN.hCEF.mGM-CSF with un-transduced cells serving as negative controls. Allowing for peak expression, the concentration of mGM-CSF in media was quantified by ELISA in samples collected on day 9 and 11, following a 48-hour incubation. Dose-related mGM-CSF expression was observed at both time points, where expression above the assay's lower limit of detection (LLD) (7.8 pg/ml) was observed above MOI 1 (Figure 14a-b). These findings echoed the dose response previously observed with the EGFP transgene (Figure 13a), as here secreted mGM-CSF peaked at MOI 20 (Day 9: 131.6 (range 30.08-233.7) pg/ml, Day 11: 59.24 (range 22.81-78.47) pg/ml). Attempting to track expression over a longer period of time, cells were kept growing in culture, however a large degree of cell death and detachment occurred before cells could be harvested and used to control for cell number or flow cytometry analysis of BMDM markers. As a result, I could not determine if the drop in expression from MOI 20 and 100 was due to cell toxicity as suggested by the EGFP data or the effect of mGM-CSF expression on macrophage phenotype (median difference day 9: 48.53 pg/ml, day 11: 34.56 pg/ml). Between time points there was a drop in mGM-CSF expression, significant at MOI 20 and 100 (median difference MOI 20: 72.36 pg/ml, MOI 100: 58.39 pg/ml; $p < 0.0001$, and $p < 0.01$, respectively, 2wayANOVA, Sidak's multiple comparisons) (Figure 14c). This may be an effect of cell loss between time points, though no significant difference in cell detachment was noted. Cell viability did decrease noticeably through casual observation over time and therefore analysis of gene expression in future experiments were limited to early time points. These results demonstrate that BMDMs can secrete detectable levels of therapeutic proteins *in vitro* demonstrating they may be suitable cell vehicles for the delivery of secreted pulmonary proteins *in vivo*. They also reinforced previous conclusions that that maximal efficiency of the virus is reached at MOI 20, where at higher MOIs BMDMs suffer from SIV related toxicity.

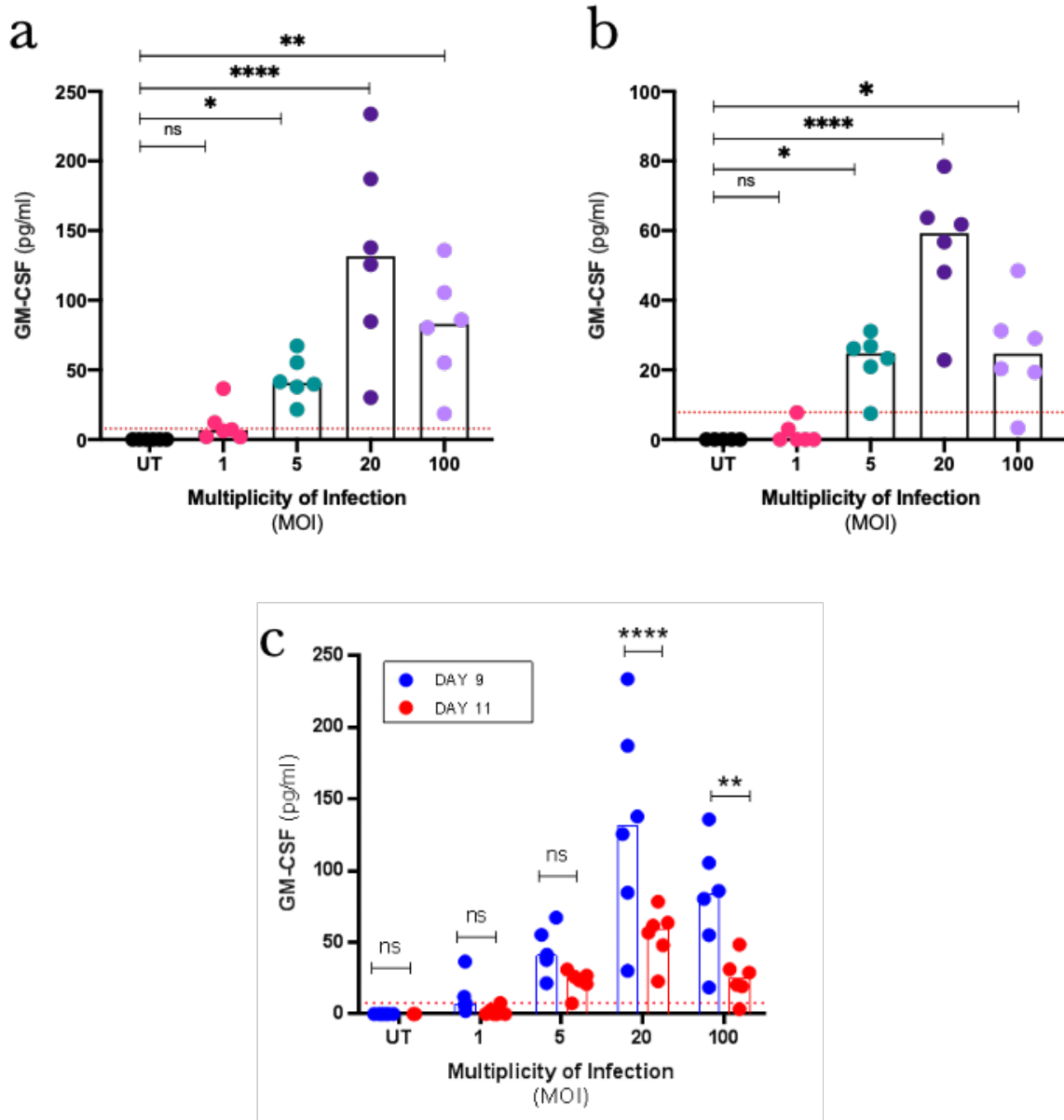


Figure 14 - Bone marrow-derived macrophages (BMDM) secrete transgenic GM-CSF following rSIV.F/HN mediated gene transfer. Expression of transgenic GM-CSF (pg/ml) in cell culture media by BMDMs following transduction with SIV-F/HN at increasing multiplicities of infection (1-100) in comparison to untransduced controls (UT) (n=6/MOI), **(a)** 9, and **(b)** 11 days post transduction. Media was left on cells for 48 hours prior to harvesting for analysis, red line indicates assay's lower limit of detection (7.8 pg/ml) . (*= $p < 0.05$, **= $p < 0.01$, ****= $p < 0.0001$, Kruskal Wallis test with Dunn's multiple comparisons.) **(c)** Comparison of GM-CSF expression on days 9 and 11 (**= $p < 0.01$, ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons). Each data point represents an individually transduced well of BMDMs. Bars represent median.

3.3.6 Establishing rSIV.F/HN maximum feasible dose in bone marrow-derived macrophages

Across EGFP and mGM-CSF transgenes, expression-based analysis indicated that the maximum feasible dose (MFD) falls within the range of MOI 20 and 100. Identifying the MFD of the lentivirus in BMDMs is necessary to implement optimal transduction protocols, where the aim is to transduce as many cells as possible while avoiding toxicity and excessive use of costly virus. BMDMs were therefore transduced at MOI 20 and 50 with rSIV.F/HN virus expressing either EGFP, Gaussia luciferase (Gluc), or mGM-CSF transgenes to determine if peak transduction efficiency and transgene expression could be increased from MOI 20. BMDMs were transduced for 48 hours, and either analysed by flow cytometry on day 7 for EGFP expression and macrophage lineage markers or cultured further to quantify secreted transgenic protein in media.

3.3.6.1 A non-significant increase in transduction and transgene expression is observed between MOI 20 and 50 with rSIV.F/HN in BMDMs

Following rSIV.F/HN.hCEF.EGFP transduction, a significant percentage of EGFP positive cells were detected at both MOI 20 and 50 in comparison to un-transduced controls, (MOI 20: median 2.3% (range 1.3-3.8), MOI 50: median 2.8% (range 1.5-3.5); $p < 0.05$, and $p < 0.01$ respectively, Kruskal-Wallis test, Dunn's multiple comparisons) though there was no significant benefit associated with increased MOI (Figure 15a). In keeping with previous experiments, no difference was observed between the fraction of positively transduced macrophages and transduced live cells (Figure 15b), transduction efficiencies were very low, where at MOI 20, a median transduction efficiency of 2.3 (range 1.3-3.8) %, can be compared to the previous dose escalation trial which reported a mean value of 28.6 (range 13.1-40.6) %. Due to this large discrepancy, in a separate experiment, BMDMs were once more transduced with rSIV.F/HN.hCEF.EGFP at increasing MOIs of 10, 20 and 50, where a mean value of 6.4 (range 3.8-7.2) % was observed at MOI 20 indicating a large degree of inter-experimental variation (Figure 15c). Slight increases in transduction efficiency were observed between MOI from 20 and 50 but these were not significant (median difference: 3.5 % EGFP⁺ BMDMs, $p = \text{ns}$, Kruskal-Wallis test, Dunn's multiple comparisons).

Corroborating these findings, macrophages expressing transgenic mGM-CSF and Glux secreted transgenes demonstrated that increasing MOI from 20 to 50 results in no significant increase in transgene expression (median difference mGM-CSF: 24.8 pg/ml, Glux: 4.87 RLU/mg) (Figure 15d-e). Transgenic protein was quantified in cell media following a 48-hour incubation on day 9 by mGM-CSF ELISA or Pierce™ Gaussia luciferase glow assay kit. BMDMs were harvested on day 9 and either taken for flow cytometry analysis of BMDM markers (mGM-CSF) or lysed to normalize for cell number by total protein content (Glux). Experimental variability was also evident here, as in comparison to previous results lower concentrations of GM-CSF were produced at MOI 20 (median difference: 114.5 pg/ml), though as these samples were not normalised for total protein content, the comparison does not control for differences in cell number.

Taken together, data generated with three different transgenes suggests that regardless of inter-experimental variation, increasing MOI from 20 to 50 can result in slight increases in transduction and transgene expression, but that this difference is not statistically significant.

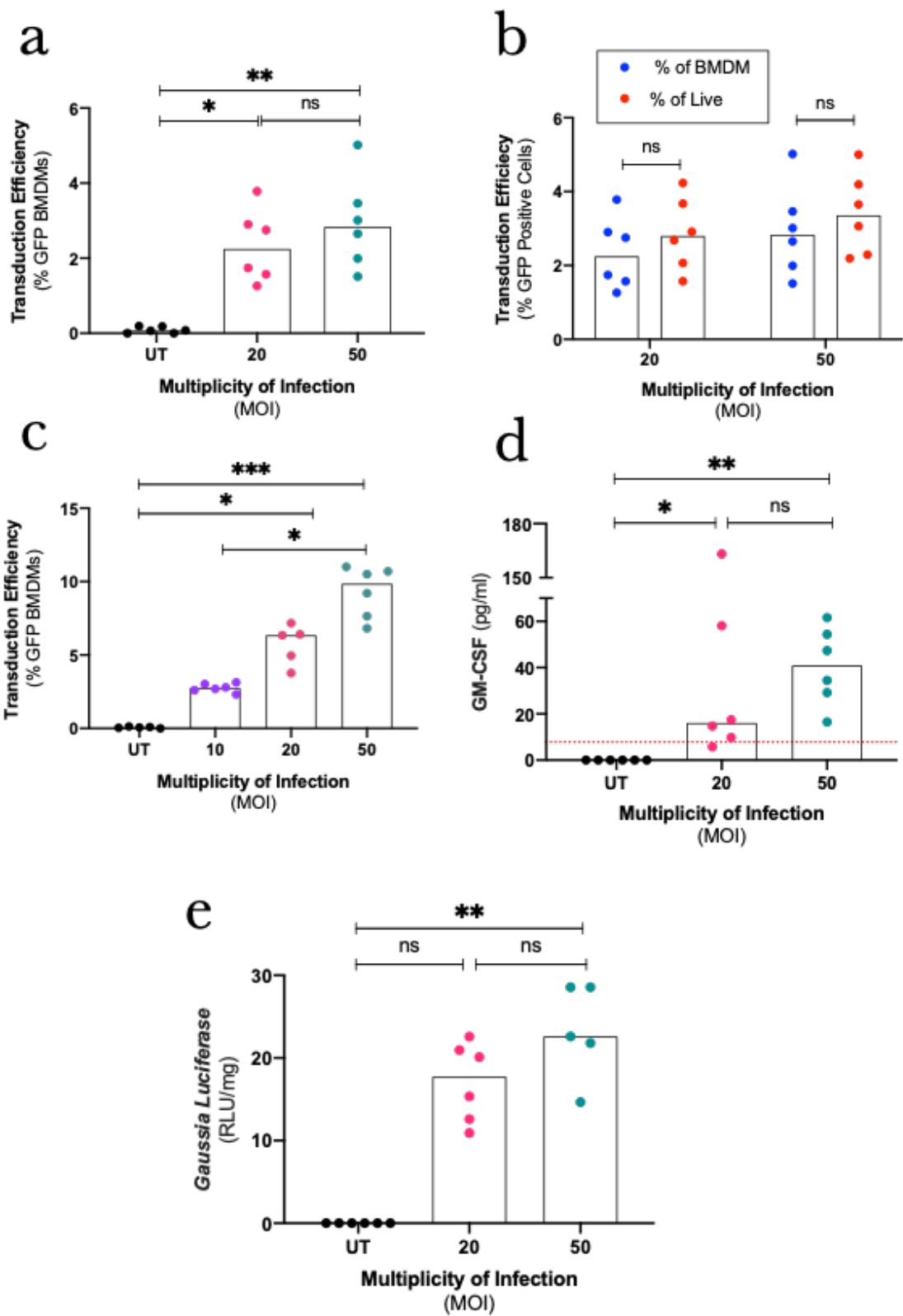


Figure 15 – rSIV/HN multiplicity of infection (MOI) comparisons in bone marrow-derived macrophages (BMDM). Flow cytometry analysis of rSIV.F/H transduction efficiency in BMDMs following transduction with an EGFP transgene at **(a)** MOI 20 and 50, and **(c)** MOI 10, 20 and 50, in comparison to untransduced controls (UT) (n=6/MOI), where BMDMs were first gated based on expression of cell-surface phenotypic macrophage markers CD11b⁺, F4/80⁺ to calculate the percentage of macrophages in culture. (*=p<0.05, **=p<0.01, Kruskal Wallis test with Dunn's multiple comparisons.) **(b)** Comparison between transduction efficiency calculated as either the percentage of EGFP⁺ BMDM (blue), or EGFP⁺ live cells (red) in culture. (2-wayANOVA, Sidak's multiple comparisons). Capture by BD LSRFortessa Cell Analyser. Expression of transgenic **(d)** mGM-CSF (pg/ml) and **(e)** Gaussia luciferase in cell culture media expressed by BMDMs 9 days following transduction with SIV-F/HN at increasing MOI 20 and 50 in comparison to untransduced controls (UT) (n=6/MOI), Media was left on cells for 48 hours prior to harvesting for analysis, red line indicates the GM-CSF ELISA lower limit of detection (7.8 pg/ml) . (*=p<0.05, **=p<0.01, Kruskal Wallis test with Dunn's multiple comparisons.) Each data point represents an individually transduced well of BMDMs. Bars represent median.

3.3.6.2 SIV transduction is largely tolerated by BMDMs with no significant change in phenotype

Following transduction with rSIV.F/HN, EGFP and mGM-CSF transduced cells were analysed for the effect of transduction and transgene expression on macrophage phenotype by flow cytometry. Across conditions, all samples reported highly CD11b⁺ and F4/80⁺ positive populations though in comparison to un-transduced controls, both conditions showed slight decreases in the percentage of BMDMs in culture (Figure 16a-b):

- *EGFP median (range):*
 - *Un-transduced:* 99.8 (99.1-99.9) %
 - *MOI 20:* 99.3 (99.0-99.4) % ($p < 0.05$)
 - *MOI 50:* 99.5 (99.1-99.8) % ($p = \text{ns}$, Kruskal-Wallis test, Dunn's multiple comparisons)
- *mGM-CSF median (range):*
 - *Un-transduced:* 99.8 (99.1-99.9) %
 - *MOI 20:* 99.8 (99.1-99.9) % ($p < 0.05$)
 - *MOI 50:* 99.8 (99.1-99.9) % ($p < 0.001$, Kruskal-Wallis test, Dunn's multiple comparisons)

As this was observed for both vectors, it does not appear to be a transgene specific effect. However, differences were minimal, and all samples consistently reported high values and can likely be attributed to small variations in staining and/or handling, and do not represent a meaningful change. When comparing the percentage of BMDMs identified by flow cytometry across all experimental controls (i.e., un-transduced samples) it suggests that a slight degree of experimental variation is to be expected, where one would expect values above 95% (Figure 16c). I can therefore be assured that the differentiation protocol consistently yields highly homogenous populations of BMDMs which can be accurately detected by flow cytometry, even following lentivirus administration up to an MOI of 50.

These results demonstrate the production of genetically modified, bone marrow-derived macrophages using a rSIV lentivirus pseudotyped with F/HN proteins, where transduction MOIs of 20-50 result in optimal transduction and efficiency gene expression. Across experiments, no effect of SIV or transgene expression was observed on BMDM phenotypes and differentiation protocol consistently produced highly homogenous cultures of CD11b⁺, F4/80⁺ macrophages from murine bone marrow. In subsequent experiments, it is therefore

assumed that under the examined conditions, the majority, if not all cells in culture are BMDMs.

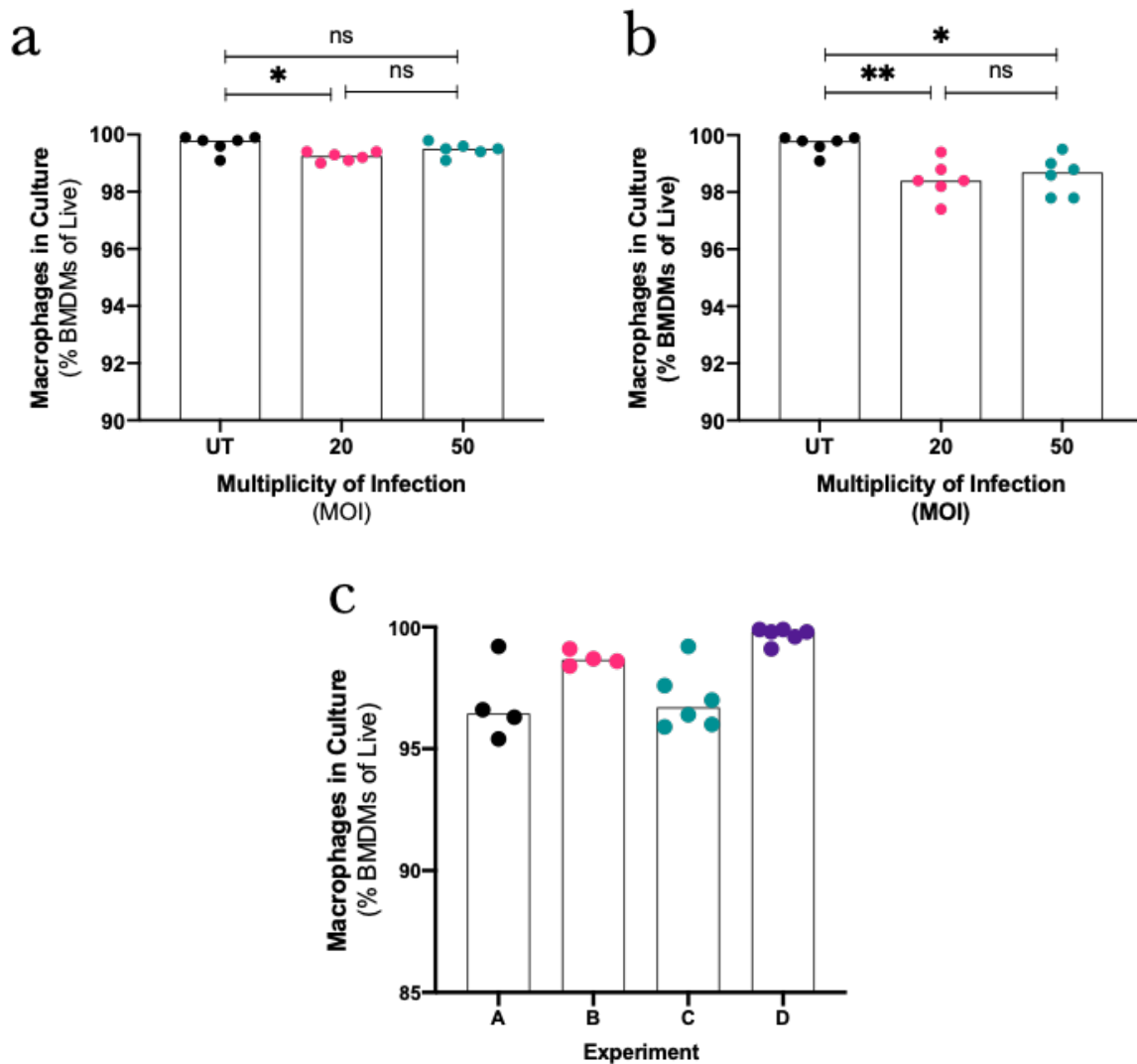


Figure 16 – Effect of rSIV/HN transduction and transgene expression on cell-surface markers of macrophage phenotype. Analysis of the percentage of cells positive for CD11b⁺, F4/80⁺ cell-surface markers of macrophage phenotype (Macrophages in culture) following rSIV.F/H transduction at MOI 20 and 50, when transduced to express (a) EGFP, or (b) mGM-CSF, in comparison to untransduced controls (UT) (n=6/MOI). (*=p<0.05, **=p<0.01, Kruskal Wallis test with Dunn's multiple comparisons.). (c) Comparison of macrophages in culture across untransduced controls in previous experiments (A-D). Capture by BD LSRFortessa Cell Analyser. Each data point represents an individually transduced well of BMDMs. Bars represent median.

3.4 Discussion

In this chapter I optimised a protocol to produce murine bone marrow-derived macrophages (BMDMs) and characterised these cells by flow cytometry. I subsequently demonstrated that the rSIV.F/HN lentivirus produced by the UK Gene Therapy Respiratory Consortium can effectively mediate gene transfer to macrophages *in vitro*. Transduction protocols were first optimised using an EGFP transgene and then *in vitro* proof-of-concept for the secretion of transgenic proteins in the lung was established using the secreted GM-CSF protein and Gaussia luciferase (Gluc) secreted reporter gene.

3.4.1 Producing bone marrow-derived macrophages

As discussed in Chapter 1, there are various autologous sources of monocyte-derived macrophages that in patients can be used to obtain large numbers of macrophages for cell therapy. They are most commonly sourced from peripheral blood mononuclear cells (PBMC) that can be differentiated *in vitro* and have a well characterised safety profile in patients (61-64, 88-92). PBMCs may therefore be the more appropriate source of macrophages to study here. However, as mice have an approximate circulating blood volume is 1.5-2.5 ml this would be highly inefficient in pre-clinical mice studies (47, 286). In contrast, bone marrow monocytes are readily harvested to produce high numbers of BMDMs and as a result are commonly used *in vitro* as models of unpolarized, or M0, macrophages (305). Though circulating and bone marrow monocytes have a distinct ontogeny to tissue resident cells, in the context of transplantation to the lung, studies have demonstrated that BMDMs and induced pluripotent stem cell (iPSC)-derived macrophages can acquire a mature AM phenotype (176, 177, 186). To progress to the clinic this work will require validation in relevant human cell types, but BMDMs are a suitable cell type to study exogenous macrophage transduction and subsequent transplantation to lung in mice.

Initial attempts to transduce macrophages with lentivirus were hampered by low cell viability and adherence following removal from cell culture. As a result, subsequent experiments did not passage BMDMs prior to use. Others have noted that M-CSF derived BMDMs have little to no proliferative abilities following differentiation and similarly concluded that *in vitro* experiments should be performed in the absence of passaging (312). Supporting this hypothesis,

Leyva *et al* adopted a similar non-passaging protocol for lentiviral transduction of BMDMs (299). In contrast others successfully passaged M-CSF induced BMDMs up to four times before cells stopped proliferating (313). Another study demonstrated that BMDMs could be successfully passaged once, but in comparison to the protocol used here where 100,000 cells were plated in a 24 well plate, cells were plated at near confluency (314). A successful, if limited, passaging protocol may therefore be possible, however this was not explored in this thesis and cells were not cultured following removal from cell culture. It is worth noting that initial work described in Chapter 6 was performed in concert with these studies and as a result, initial experiments also attempted to use passaged BMDMs.

3.4.2 rSIV.F/HN Transduction Efficiency in BMDMs

This work tested SIV lentiviral transduction with an F/HN pseudotype in primary macrophages, for the first-time demonstrating proof-of-principle that the Gene Therapy Consortium's (GTC) proprietary vector can be used to produce genetically modified macrophages *in vitro*. Differentiated primary macrophages were optimally transduced by the SIV.F/HN lentivirus at MOI 20, with an approximate 30% efficiency. Subsequent experiments indicated that increasing MOI to 50 resulted in small increases in transduction in comparison to MOI 20, though overall transduction efficiencies were lower overall than in previous experiments as a high degree of inter-experimental variation was observed. Primary macrophages are known to be more resistant to gene transfer than other primary cell types and macrophage cell lines (180). To my knowledge these data represent the first *in vitro* lentiviral transduction of macrophages with an F/HN pseudotype, where the majority of reports transduce macrophages with VSV-G pseudotyped HIV-1 based lentiviral vectors.

HIV-1 based vector transduction efficiencies in the literature show little consistency and there is no consensus on the optimal transduction protocol. For example, the optimal MOI reported varies dramatically, when reporting similar transduction efficiencies as found here, studies used MOIs of 0.65 and reported 40% transduction efficiency (315), while others demonstrated optimal transduction of 30% (316) and 50% (301) at MOI 100. Highly successful transduction of primary macrophages has been reported in the literature, though again they observe little consistency between protocols. Leyva *et al* reported that they could deliver a EGFP transgene to 80-90% of macrophages by transducing human PBMC monocytes early in the differentiation process at MOI 10, and that transduction efficiency decreased as cells become

further differentiated and committed to a macrophage lineage (299). However, they were unable to recapitulate these findings upon repetition with monocytes from a different donor as at MOI 10 transduction fell to 50%. These findings directly contradict studies that demonstrate that freshly isolated monocytes are largely resistant to lentiviral transduction but become increasingly permissive to gene transfer as they are cultured *in vitro* (298, 300, 301, 317). Similar inter-experimental variation was observed with human PBMCs-derived macrophages and murine BMDMs where across experiments transduction efficiencies ranged between 50-80%, however primary cells were transduced as differentiated macrophages on day 6 (304). Successful transduction post-differentiation was also observed in one study on day 9, where transduction was increased from 67.8 to 84.6% by the addition of the cationic polymer polybrene (180). Another study in a similar attempt to increase transduction used polybrene, the addition of the viral accessory protein Vpx and spinfection to achieve 60% transduction of iPSC-derived macrophages (317). However, others have demonstrated polybrene mediated toxicity in macrophages that negate any improvement (299). The transduction efficiency of the SIV.F/HN lentivirus was modest in comparison to some of these other studies, however as culturing conditions, time of transduction, vector, and additional transfection reagents all differ, the mechanism underlying the varied outcomes of lentiviral gene transfer in primary monocytes and macrophages are difficult to pin down. However, they offer many potential avenues by which transduction may be improved, some of which were investigated and discussed further in chapter 5.

Between experiments, the SIV.F/HN lentivirus reported large degrees of variation in transduction efficiency. In early studies, for example where little difference was observed visually between MOIs of 1 and 100 (section 3.3.2) this is likely due to the suboptimal passaging strategy employed prior to transduction and the overall poor condition of the cultured macrophages. The cause of the inter-experimental variation seen in subsequent studies is unclear and no procedural errors were noted, though it is in keeping with other studies in the literature who also reported variation in efficiency between macrophage transduction experiments (299, 304). It is worth noting that one study saw pronounced variation in transduction efficiency when transducing cells derived from different human donors (299). It is possible that donor-differences are at play here as successive transduction experiments were performed using BMDMs harvested from different individual mice. However, as all donor mice were wildtype (C57b6/J inbred strains) it seems unlikely. Other factors like donor age may have had an effect, but again seems unlikely as all mice used were below a maximum of 4 months in

age. Alternatively, the accuracy of the cell counting may have affected MOI calculations to varying degrees across experiments, as when transducing differentiated macrophages in the absence of passaging, cell numbers were estimated from representative wells which were harvested and counted prior to transduction. Furthermore, variation in MOI may have occurred due to the difficulty of accurately pipetting and diluting such highly concentrated vectors ($>1 \times 10^9$ TTU/ml).

As a result of this variation, comparisons between *in vivo* studies delivering macrophages to mice are circumspect as the efficiency of transduction may vary between experiments and highlight the need to characterise samples delivered to mice directly. As macrophages are delivered to mice 24 hours after transduction, assays used to analyse transduction and gene expression on day 7 required validation at early time points. These studies are reported and discussed further in chapter 5.

3.4.3 Secreted transgene expression

Proof-of-concept for *in vivo* expression of secreted proteins was demonstrated with both Glux and mGM-CSF transgenes. In Chapter 4, I investigate the potential of GM-CSF secreted macrophage therapy for autoimmune pulmonary alveolar proteinosis (aPAP) and here I have demonstrated that significant levels of gene expression can be observed *in vitro*. Following gene transfer at MOI 20, 131.6 (median, range 30.08-233.7) pg/ml was secreted into 1 ml of cell media. It is difficult to say whether these levels will be therapeutically relevant in PAP mouse models as *in vivo* concentrations will depend on cell dose and duration of retention in the lungs. Additionally, extrapolating from these *in vitro* studies is made more difficult by the fact that GM-CSF concentration was here not controlled for cell number. To determine whether these levels will be relevant, *in vivo* studies are required. BMDMs were also shown to produce significant levels of Glux, which is a highly sensitive secreted reporter protein (318) which has been used to inform *in vivo* and *in vitro* studies of secreted protein therapy (168, 278, 319). It therefore has the potential to offer a highly sensitive means by which to track gene expression *in vivo* following lung delivery.

3.4.4 Effect of rSIV.F/HN Transduction on BMDM Phenotype

Across investigated MOIs, transduction by the rSIV.F/HN lentivirus only altered the expression of macrophage markers CD11b⁺ or F4/80⁺ at MOI 100, while at lower doses where optimal gene transduction was observed, macrophage markers were not affected. This was then reflected in the fact that the percentage of positively transduced BMDMs (CD11b⁺, F4/80⁺) identified by flow cytometry analysis of transgenic EGFP expression was the same when analysing the percentage of transduced, EGFP positive live cells in culture. As a result, and as all studies indicated the differentiation protocol yields highly homogenous BMDM populations, subsequent transduction efficiency analyses were streamlined and did not analyse macrophage phenotypic markers when measuring transduction efficiencies.

A limitation of this work is that further characterization of the macrophage phenotype and function following SIV gene transfer were not performed. Though macrophage infection by wildtype lentivirus alters gene expression to mediate immune and inflammatory responses (320), studies have shown that following transduction by third generation lentiviral vectors, primary macrophage phenotypes are largely unchanged (176, 178, 321). They retain their phagocytic activity, phenotypic plasticity and can adopt M1 and M2 polarized states in response to stimuli and adopt phenotypes of local tissue resident macrophages following transplantation (177, 180, 321). As the aim of this work is to genetically modify macrophages to act as protein secretion factories rather than exploit their endogenous functions *in vivo*, investigating the functional effects of gene transfer was not considered a priority. Nevertheless, their ability to respond to stimuli that will mediate their retention in the lungs should be addressed in the future. This could include comparisons of phenotype and function between gene modified BMDMs post-transplantation and endogenous alveolar macrophages.

Chapter 4 – Pulmonary delivery of *ex vivo* gene-modified macrophages for *in vivo* protein expression

4.1 Introduction

In Chapter 3 I characterised a protocol for the differentiation of primary murine macrophages and demonstrated that these cells can be transduced by the GTC's proprietary rSIV.F/HN to express secreted and non-secreted transgenic proteins. The aim of this chapter is to establish a cell therapy approach for the *in vivo* pulmonary production of therapeutic proteins based on the transplantation of these gene-modified macrophages. I subsequently aim to demonstrate proof-of-concept that transplanted macrophages can produce therapeutically relevant levels of transgenic protein using a mouse model of autoimmune pulmonary alveolar proteinosis (aPAP) and a GM-CSF transgene. GM-CSF protein therapy has been suggested as a therapeutic approach for autoimmune pulmonary alveolar proteinosis (aPAP) (161, 250, 251) and in the previous chapter I established successful production of transgenic GM-CSF by macrophages following lentiviral gene transfer.

4.1.1 Pulmonary Alveolar Proteinosis

4.1.1.1 Pathophysiology

Pulmonary alveolar proteinosis (PAP) is a syndrome first described by Rosen *et al* in 1958 where patients presented with a characteristic accumulation of Period Acid-Schiff (PAS)-positive proteinaceous material in alveoli histology sections later identified as surfactant (322).

Pulmonary surfactant is a complex of approximately 90% phospholipids (largely phosphatidylcholine) and 10% surfactant proteins (SP-A, SP-B, SP-C, and SP-D) which are produced complexed by type II alveolar (AT2) cells and then secreted into the alveolar spaces (323). The phospholipids, SP-B, and SP-C have hydrophilic regions which reduce surface tension at the air/liquid interface in the lungs (324). This is the main function of pulmonary surfactant which provides alveolar stability and prevents lung collapse during breathing to facilitate gas exchange (323). The hydrophobic SP-A and SP-D afford surfactant its role in host

defence and immune modulation (325). These innate immune proteins can bind to pathogens for opsonization and demonstrate anti -viral -bacterial and -fungal activity (325-327).

In PAP patients, the characteristic accumulation of surfactant in the lungs is caused by disruptions to the granulocyte-macrophage colony-stimulating factor (GM-CSF) signalling pathway (328). The role of GM-CSF signalling in surfactant homeostasis was first realised in transgenic mouse models which recapitulated defining characteristics of then idiopathic PAP. GM-CSF knock out (KO) mice were produced to elucidate the role of GM-CSF in haematopoiesis under steady-state conditions, and researchers instead found that mice demonstrated progressive accumulation of surfactant proteins and lipids (329). These mice developed large interalveolar phagocytic macrophages and presented with subclinical lung infections, local pulmonary inflammation and lobar pneumonia (104). Null mutations in the common beta subunit of GM-CSF receptor in mice (BetaC mice) also resulted in an established PAP disease state, where high-affinity binding of GM-CSF by bone marrow cells was abolished (330, 331). This PAP phenotype could be corrected by rescuing GM-CSF expression in null mice, where daily administration of GM-CSF by aerosol inhalation over a 4/5-week period led to temporary improvements in PAP and clearance of accumulated surfactant (332). Similarly, somatic gene transfer of murine GM-CSF by adenovirus cleared surfactant and enhanced maturation of AMs at 5 weeks (329) and breeding GM-CSF KO mice with transgenic mice where GM-CSF expression is limited to the lung epithelium using SP-C gene promoter regulation also restored surfactant homeostasis (250).

Under steady-state conditions, the rate at which individual components of surfactant are produced by AT2 cells, assembled into complexes in the lamellar bodies and secreted into the alveolar space is balanced by the rate of removal (333). Pulmonary administration of radiolabelled surfactant proteins and phospholipids demonstrated that GM-CSF signalling is necessary for surfactant clearance and catabolism rather than synthesis, as lipids and proteins were rapidly cleared in wildtype but was markedly reduced in GM-CSF KO mice (143, 334). Following direct stimulation by GM-CSF, AT2 cells are responsible for most of surfactant clearance through recycling and lysosomal degradation while AMs contribute to approximately 20% of surfactant clearance through degradation (335-337). Altered AM surfactant catabolism plays a central role in PAP pathophysiology as bone marrow transplants for hematopoietic reconstitution in BetaC mice, reversed PAP disease states and abnormal AM morphology with

donor bone marrow from both WT mice and transgenic mice with mutations rendering them unable to develop lymphocytes (182).

4.1.1.2 Classification

PAP can be classified according to the underlying mechanisms leading to interruption of the GM-CSF signalling pathway. Primary autoimmune PAP (aPAP) accounts for 90% of all cases where autoantibodies biologically inhibit GM-CSF, which can then no longer bind to receptors on AMs or lung monocytes to stimulate maturation their ensuring catabolism of surfactant 2012 (145, 338-340). These autoantibodies are also present in healthy subjects but exceed a critical threshold in PAP (341, 342). This mechanism is supported by studies injecting patient-derived GM-CSF autoantibodies into non-human primates which were able to reproduce PAP disease (343). As a result of inhibited GM-CSF signalling, neutrophil regulation is also impaired, and patients suffer reduced antibacterial activity as a consequence (341, 342).

Hereditary or familial PAP (hPAP) accounts for 5% of cases and is caused by recessive mutations in the cell surface receptors which mediate binding of GM-CSF on myeloid cells (183-185). Homozygous mutations of either the GM-CSF-binding α -chains (CSF2R α) and affinity-enhancing β -chains (CSF2R β) lead to PAP through disrupted GM-CSF signalling, reflecting PAP phenotypes of CSF2R β knock out BetaC mice (182-185). hPAP presents similarly to the autoimmune disease though with earlier onset as it is characterised by insidious progressive dyspnoea in children and failure to thrive (328).

Secondary PAP presents as a consequence of an underlying disease with an ensuing reduction in AM function or number and accounts for 5% of cases. It is most common as an effect of haematological malignancies (340, 344-346), inhalation exposure to occupational dust (347-349), or immunosuppression due to solid organ or allogeneic stem cell transplants (350, 351), or infection (i.e. HIV) (352).

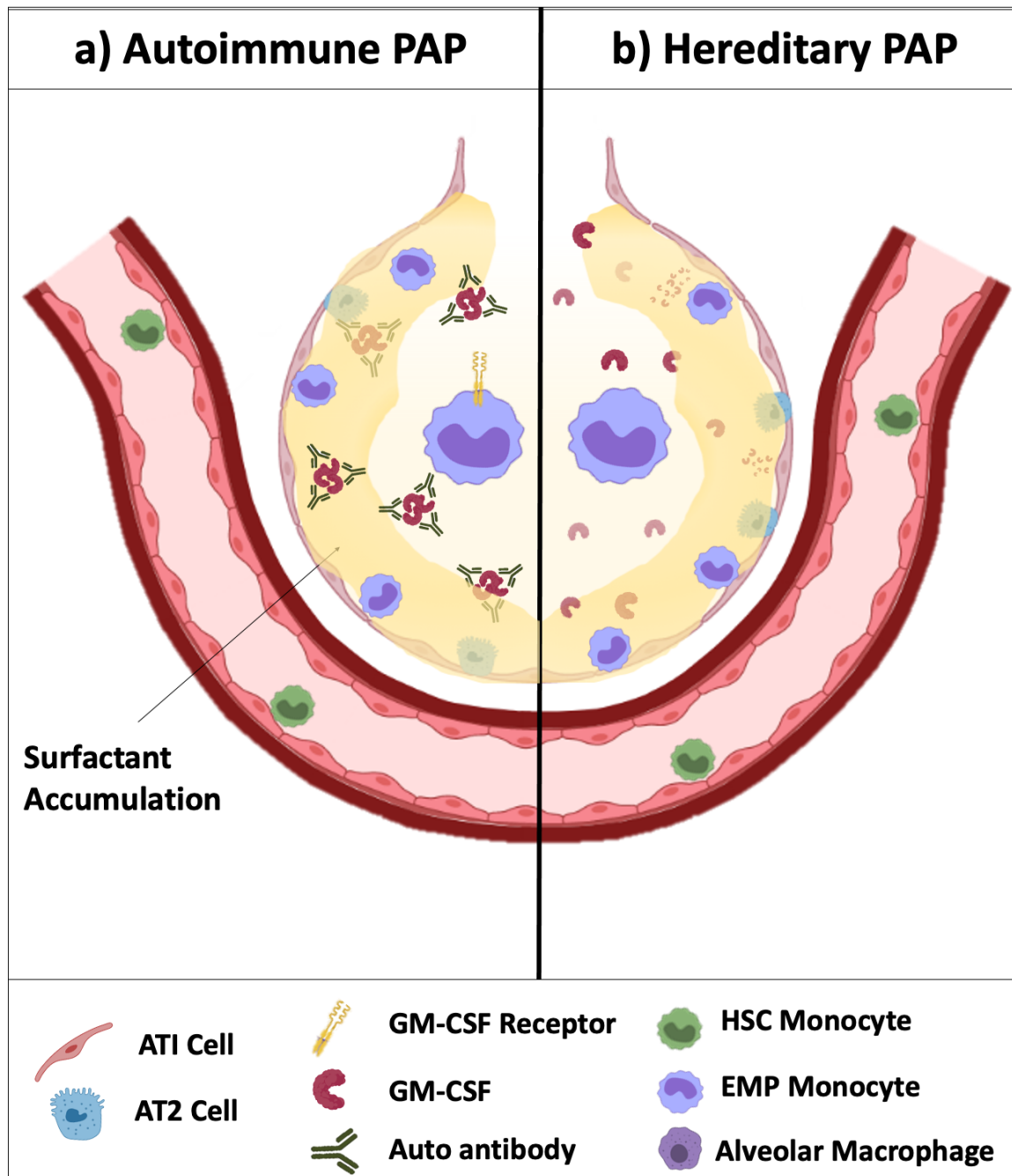


Figure 17 – Hereditary and autoimmune pulmonary alveolar proteinosis (PAP) is occurs in the absence of GM-CSF signalling. Hereditary or familial PAP accounts for is caused by recessive mutations in the cell surface receptors which mediate binding of GM-CSF on myeloid cells. In primary autoimmune PAP autoantibodies biologically inhibit GM-CSF. As GM-CSF signalling is required to initiate erythron-myeloid progenitor (EMP) monocyte maturation, in the PAP lung, monocytes remain immature and do not participate in the catabolism of surfactant which then builds up in the alveolar spaces. AT2: alveolar type II cells; AT1: alveolar

type I cells. HSC monocyte: circulating monocytes of bone marrow origin. Created by Nora Clarke; images sourced from BioRender.

4.1.1.3 Epidemiology and Presentation

PAP is an ultra-rare disease and as a result epidemiological data is sparse, additionally case numbers are likely underestimated due to misdiagnosis as a consequence of disease rarity and non-specific presentation (353, 354). In a large cross-national study in Japan the prevalence amongst the general population was 6.2 cases per million with a 0.49 per million annual incidence rate (339). The median age of patients was 51 and men accounted for 66% of all cases. A comprehensive retrospective study on a large data cohort in the United States of similarly found that the overall prevalence of PAP cases was ~7 patients per million but exhibited no gender bias (183). Incidence peaked between the ages of 45-54 and then later in patients above the age of 75. In both studies, auto-immune PAP represented approximately 90% of all identified cases which was reflected in insights from single centre cohorts in Germany (339, 355). Prevalence increases with age and outside of hereditary PAP is rarely found or diagnosed in children under the age of 10 (339, 356).

Disease progression is highly variable across PAP patients and difficult to diagnose. Over 30% of patients can be asymptomatic at time of presentation (identified by health-screening) while others often present with non-specific symptoms (339) and spontaneous remission is possible in up to 10% of cases (355, 357). Retrospective reviews of Mayo Clinic records found that the major clinical features were dyspnoea and cough, with mild dyspnoea the most common symptom, though only 48% of patients reported significant dyspnoea (356). These findings are also echoed by separate studies of PAP in Chinese populations (358) and autoimmune PAP in Korean populations (359). In the Korean study, 86% of patients were symptomatic at presentation. Presentation is similar in hereditary and autoimmune PAP, though in the former is earlier onset as children present with progressive insidious dyspnoea, and failure to thrive (360).

A mild restrictive ventilatory defect is common across patients, with reductions in lung volumes and diffusion capacity (360). As a result of accumulated surfactant in alveoli, gas exchange and oxygen diffusion are impaired resulting in the development of hypoxemic respiratory failure

(322, 328, 361). Additionally, patients have an increased risk of secondary infections and pulmonary fibrosis, as reduced GM-CSF bioavailability leads not only to the dysregulation of AM, but also neutrophils and lymphocytes can lead to opportunistic infections with at times unusual organisms including *Cryptococcus* and *Mycobacterium* species, which in the past contributed to patient mortality (341, 362-366). Patient diagnosis is commonly based on patient history, high-resolution computer tomography (HRCT), autoantibody detection, analysis of bronchoalveolar lavage fluid (BALF) for immature and enlarged macrophages, and PAS staining of lung histology slices (145, 183, 340, 359, 367, 368). The opacity or turbidity of BALF while characteristic of PAP correlates with outcomes of pulmonary function.

4.1.1.4 Treatment

The current standard of care for PAP patients is whole lung lavage (WLL) to physically wash accumulated surfactant from the lungs (369). This is an invasive procedure where under general anaesthesia patients are intubated with a double-lumen-endobronchial tube to isolate the left and right lungs. While one lung is mechanically ventilated, the other is repeatedly filled with warm saline solution and then drained by gravity. Lungs are repeatedly flushed until upon visual inspection BALF is no longer the has its characteristic opaque and milky white appearance. In general lungs are washed in aliquots of 800ml of saline, however the number of washes required varies greatly between patients as a result of intrinsic intra-patient disease presentation, where one study demonstrated that anywhere between 5-40 L and an average of 15.5 L per lung are required to fully wash out surfactant (370). This procedure is only performed in specialised centres and a global survey demonstrated that there is not a great deal of consensus in practices, procedures and patient outcomes (370). However, across centres the main indication for WLL in PAP patients was worsening lung function and/or gas exchange, while the majority also relied on radiographic evidence (79%) and symptomatic worsening (42%). The procedure is generally safe with only a low rate (18%) of procedure related morbidity reported (370). WLL can result in prolonged remission, and improvement in lung function that can be observed immediately following the procedure, including spirometry measures of forced vital capacity (FVC), partial pressure of oxygen (PaO₂), and diffusing capacity of carbon monoxide (DLCO) (371). Additionally, between surveys and retrospective studies there is little consistency in reported outcome, though the overall trend indicates that while WLL can be effective, in many patients this required multiple interventions and the effect is transient over a period of years (369-373). The global survey reported that of 368 treated

patients, the average number of follow up WLL required was 2.5 ± 1.5 within 5 years, where ~67% of patients only required 1 procedure to see the effect sustained during this time period, while 10% needed in excess of 5 (370). One retrospective study found 70% of patients were free from recurrent PAP for 7 years (371), though a more recent study reported that 57% of patients relapsed within a medium period of 19.9 months, where 40% and 9% of patients underwent a second or multiple lavages respectively (369). Though a study of 149 patients demonstrated that with WLL, survival rate at five years improved significantly from 85% in patients without WLL intervention to 94%, 80% of patients followed beyond 3 years had recurrent PAP manifestations (370). Though remission can be achieved in patients using WLL, this technique is highly invasive, varies in efficacy, is limited to specialised centres, and additionally it does not address the causal limited bioavailability of GM-CSF.

In autoimmune PAP recombinant GM-CSF therapy is currently being evaluated as an alternative non-invasive approach to WLL. The aim of recombinant protein therapy is to outcompete GM-CSF antibodies to increase its bioavailability and stimulate AM maturation and rescue their role in lung surfactant homeostasis. This approach was first presented in 1996 where a patient non-responsive to WLL was treated with recombinant GM-CSF therapy and demonstrated that following subcutaneous injection, GM-CSF improved the alveolar-arterial oxygen gradient (AaO₂) and exercise tolerance (374). Early studies then studied subcutaneous delivery of GM-CSF and saw modest but transient improvements in oxygenation (375, 376).

More recently studies have increasingly found that inhalation therapy with nebulised GM-CSF (iGM-CSF) is more efficient and safer than subcutaneous delivery. One meta-analysis reported that iGM-CSF has a response rate of 89%, an improvement over 71% observed with subcutaneous delivery (377), though as with WLL, the benefit of treatment varies between reports, as one study saw only a 49% response rate (378). This trial was conducted in moderately symptomatic patients and highlights how patient variability in PAP presentation and history makes general understanding of treatment efficacy difficult. Studies have shown that patients respond to iGM-CSF therapy, though the effect is variable, largely transient and requires repeat dosing over extended periods of time (379, 380). This was highlighted in a 2014 study where an “as far as it takes” protocol was implemented to treat 6 aPAP patients (162). Patients received a daily dose of 250 µg of GM-CSF given 4 days on and 4 days off for as long as necessary until reaching remission, which took anywhere between 14 and 65 months. During this time doses were de-escalated, and though remission was maintained in 3 patients at their

lowest effective dose, two patients relapsed, and one required the full 250 µg dose to maintain efficacy. In an 11-patient study in 2006, Wylam *et al* demonstrated a 92% response rate in the main efficacy outcome of improves AaO₂ gradients, though 5 patients required additional therapy within 1 year of discontinuing therapy (379). Here 250 µg of iGM-CSF were delivered twice daily on alternate weeks for 24 weeks total. In contrast two recent placebo-controlled phase II trials showed that at the same regime at a lower dose of 125 and 150 µg no clinical benefits were noted (160, 311). They suggest that this may be due to better lung function in these patients at baseline and that iGM-CSF may be more effective in patients with more severe respiratory presentation. A phase 2/3 double-blind, placebo-controlled trial published in 2020 also failed to meet its clinical endpoint as between placebo-controlled groups and patients treated continuously or intermittently with nebulised GM-CSF, no significant improvement was observed in AaO₂ gradients after 24 weeks (381). As outcomes followed positive trends however, a phase III trial increasing the duration of treatment to 48 weeks is already underway (ClinicalTrials.gov Identifier: NCT04544293) (382).

Some studies have reported toxic effects of GM-CSF therapy due to its pleotropic effects on haematopoietic system (383), where subcutaneous delivery of large doses (10+ µg/kg/daily) resulted in severe side effects including hypoxia, hypertension, and pericarditis (384, 385). A recent clinical trial reported that out of 34 treated patients, 6 suffered adverse events including congestive heart failure, worsening of aPAP, pneumothorax, and influenza type A infection, however 3 untreated patients also reported adverse events and therefore did not differ significantly between groups (380). Many studies however have reported either no, or only mild, side effects suggesting that overall iGM-CSF therapy can be safely tolerated (160-162, 311, 379). Importantly no evidence of increased autoantibodies was observed following treatment.

Thus far the clinical efficacy of iGM-CSF in PAP patients has not been definitively demonstrated and data does not conclusively support its use as a first-line treatment, thus WLL remains the gold-standard of care (377). It is limited by the short half-life of GM-CSF *in vivo* (7.3 hours) (386), the subsequent requirement for repeat administration, relapse following discontinuation of therapy, and high costs as a 6-month regimen costs approximately £30,000 (382).

Overall clinical studies suggest that exogenous sources of GM-CSF can restore AM mediated clearance of surfactant and lead to clinical improvements, but that this requires long term

intervention. Therefore, gene therapy approaches have been suggested as they have the potential to produce a sustained effect through long term expression of a GM-CSF transgene *in vivo* (332, 387). Autoimmune PAP is also therefore an ideal disease model to demonstrate proof-of-principle for an *ex vivo* gene modified macrophage therapy secreting therapeutic proteins *in vivo* as a mouse model is available (GM-CSF KO), and the effect of recombinant protein therapy supports secreted protein therapy. Furthermore, decreased GM-CSF bioavailability in the lung may lead to high levels of donor-macrophage engraftment following transgenic GM-CSF expression and may therefore recapitulate the success of macrophage transplantation observed in mouse models of hereditary PAP (*Csf2ra*^{-/-}, *Csf2rb*^{-/-}) (177, 178, 186, 321).

4.1.2 Hypothesis and Aims

In Chapter 3 I showed proof-of-concept that the rSIV.F/HN lentivirus can transduce BMDMs which subsequently secrete transgenic proteins including murine GM-CSF *in vitro*. As monocytes and macrophages from bone marrow origins have proven able to persist long-term in the lung following *in situ* delivery (177, 178, 321), I hypothesize that genetically modified BMDMs can secrete transgenes *in vivo* following transplantation to the lung. As the degree of macrophage engraftment will be dependent on the state of existing tissue resident macrophage populations (176, 178), it is likely that transplanted cell outcomes will vary between wildtype mice and mouse models of disease. The aim of this work is to establish a platform technology for *in vivo* pulmonary production of therapeutic proteins based on the lung delivery of gene-modified macrophages that could be applied to diseases with established or empty AM niches. This chapter will therefore characterise the engraftment and duration of macrophages and their transgene expression following transplantation in wildtype mice. To provide proof-of-concept that transplanted macrophages can produce therapeutically relevant levels of transgenic protein *in vivo* this system will be tested in a mouse model of aPAP with a GM-CSF transgene (Figure 18).

In this chapter, proof-of-principle experiments in PAP mice are largely informed by an *in vivo* approach to aPAP gene therapy currently under investigation by Helena Lund-Palau and Claudia Juarez-Molina at the UK Respiratory Gene Therapy Consortium (GTC). Their work in establishing and characterising the GM-CSF KO mouse model and outcomes following *in*

in vivo lentiviral administration has been highly valuable, however the work is currently awaiting publication.

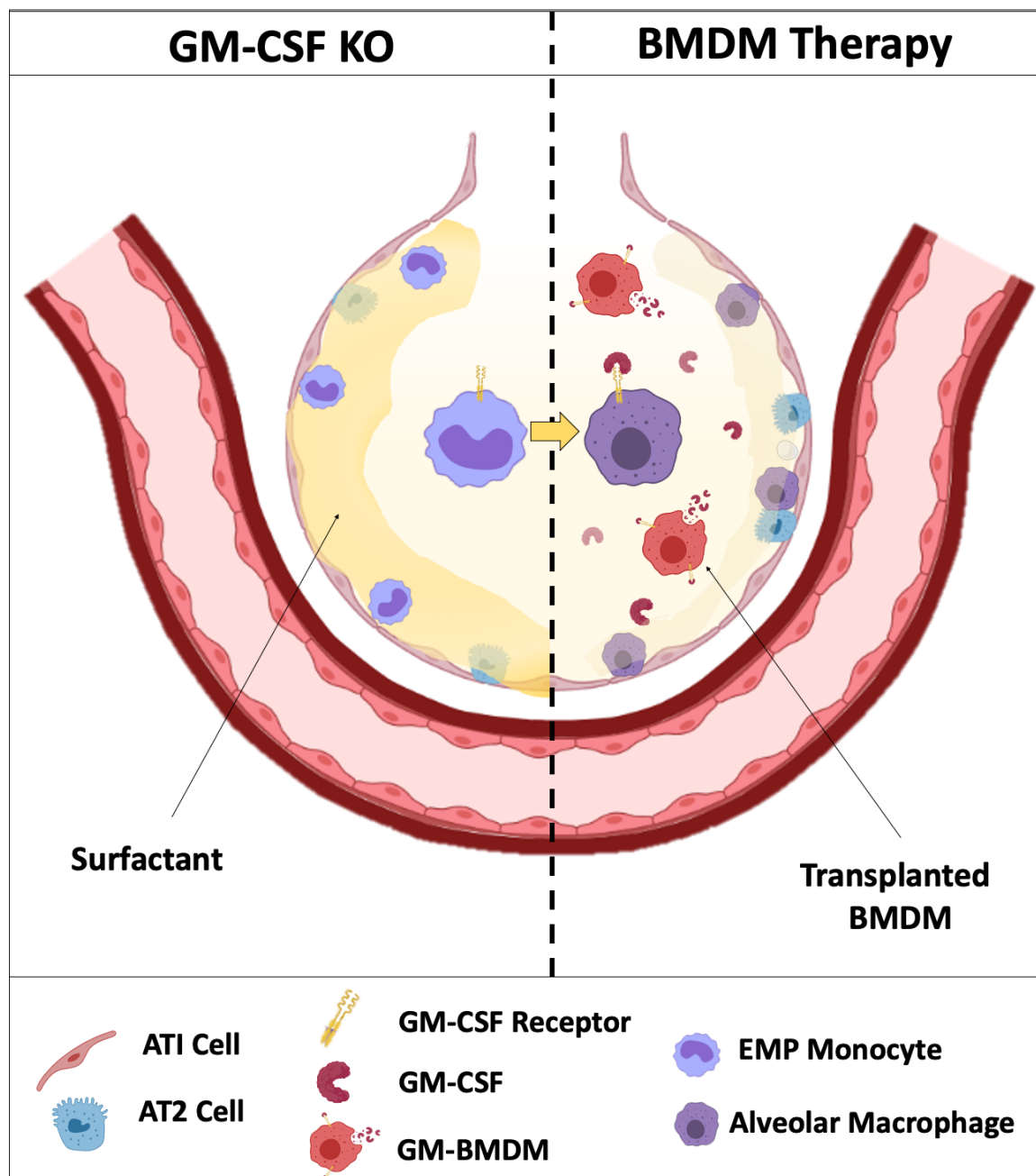


Figure 18 – *Ex vivo* macrophage cell therapy in a mouse model autoimmune pulmonary alveolar proteinosis (PAP). Granulocyte colony-stimulating factor (GM-CSF) knockout (KO) mice recapitulate the PAP phenotype as GM-CSF signalling is required to initiate erythron-myeloid progenitor (EMP) monocyte maturation. As a result, monocytes remain immature and do not participate in the catabolism of surfactant which then builds up in the alveolar spaces. The proposed cell therapy approach would transplant *ex vivo* gene modified macrophages (GM-BMDM) to the lung to

secrete transgenic GM-CSF to rescue macrophage maturation and clearance of surfactant. AT2: alveolar type II cells; AT1: alveolar type I cells. BMDM: bone marrow-derived macrophage. Created by Nora Clarke; images sourced from BioRender.

4.1.3 Declaration

The oropharyngeal delivery protocol was performed with the assistance of Anthony Sinadinos who developed the procedure. He designed the digital proximity ligation assay and assisted in performing the experiment and analysis.

I received assistance in the data collection of the long-term duration study (Chapter 4.3.6) by my colleagues: Cuixang Meng, who harvested mice and performed downstream sample processing, and Ana Sergijenko and Kyriel Pineault who processed and performed flow cytometry on BALF samples at weeks 4, 8 and 16.

4.2 Methods

4.2.1 Dosing mice by oropharyngeal delivery

BMDMs differentiated in 75 cm² tissue culture flasks were transduced for 24 hours with lentivirus at the indicated MOI and then harvested from culture as previously described (Chapter 2.4). This involved two initial wash steps as first adherent cells were washed with 5 ml of dPBS, and a second wash as cells were pelleted out of 13 ml of versene + neutralising media. Cells were then pooled across flasks and resuspended in 5 ml of dPBS. The total number of viable cells in suspension was determined using a LUNA-II™ Automated Cell Counter (Chapter 2.1.4). Cells were then pelleted at 400 *rcf* for 5 minutes and resuspended in the appropriate volume of dPBS to achieve the correct concentration of cells to deliver either 2e6, 3e6, or 6.32e6 cells in a 50 µl dose. Aliquots of each wash supernatant were stored at -80°C for future analysis of transgene expression (Glux or GM-CSF). The concentration of the cell suspension was then confirmed and if within a 10% error margin cell counts were incorrect, volume was adjusted accordingly. Samples were kept on ice until 10 minutes prior to administration to allow temperature to equilibrate.

Mice were anaesthetised via intraperitoneal administration of ketamine (76mg/kg) (Narketan® Ventoquinol, Towcester, UK) and Medetomidine Hydrochloride (1mg/kg) (Dormitor®, OrionPharma, Reading, UK) and kept in a pre-warmed recovery chamber until consciousness was lost. Anaesthetised mice were suspended upright by their upper incisors from taught dental floss strung up on a clamp & stand assembly, ensuring that their feet lightly rested on the workbench surface. The tongue was pulled out with forceps to prevent swallowing. While holding the tongue in this position, 50 µl of cellular suspension (2e6, 3e6, or 6.32e6 cells in dPBS) or dPBS (untreated controls) was administered into the distal part of the oral cavity. A tissue wettened slightly with dH₂O was used to firmly pinch the nose closed to facilitate the inhalation of the cellular suspension. Mice were then recovered from anaesthesia by subcutaneous injection of atipamezole hydrochloride with Anitsedan® (OrionPharma, Reading, UK) (1mg/kg). The animals were carefully monitored during recovery until full consciousness was regained and kept in a heated chamber or wrapped in insulating material to maintain body heat. Unless otherwise stated, mice were provided with wet food for the following 72 hours and closely monitored for the first week after treatment.

4.2.2 Tissue harvesting

Mice were humanely euthanised by pentobarbital overdose (Pentobarbitone Sodium 20%, Animalcare, York, UK) administered via intraperitoneal injection. Following confirmation of the absence of the pedal reflex, death was confirmed by exsanguination via cardiac puncture. Mice were then placed on their backs and taped into place on a surgical pale or sterile field for sample harvesting.

4.2.2.1 Serum

Blood was collected via cardiac puncture by first opening the thorax to exposure the heart and lung. A disposable insulin syringe (Cat. #7050603, Micro-Fine 1ml U-100 Insulin 29G, BD, Berkshire, UK) was used to puncture the heart along the frontal plane and collected blood was transferred into a 1.5ml microcentrifuge tube (Cat. #311926955, ThermoFisher Scientific). Samples were centrifuged at 13,000 *rcf* for 30 minutes in a Fresco™ 17 Microcentrifuge (ThermoFisher Scientific) to separate serum. Sample supernatant, or serum, was collected and stored -80°C pending further analysis of GM-CSF or Glux transgene quantification.

4.2.2.2 Bronchoalveolar lavage fluid (BALF)

To collect BALF, lungs were washed with dPBS as previously described (388). Briefly, the thoracic cavity was opened to expose the trachea by completing dissection of the neck and abdomen. A catheter (Abbocath-T 22G x 25mm, BD, Berkshire, UK) was inserted into the upper section of trachea and stabilised by tying a silk suture thread around both the trachea and catheter. A syringe filled with dPBS was then connected to the catheter and the solution was gently injected into the lungs, and then aspirated, injecting and aspirating the lungs three times in total. The recovered lavage fluid (BALF) was then collected in a 1.5ml microcentrifuge tube and kept on ice for downstream processing. When performing whole lung lavage, a 500 µl volume of dPBS was used to flush the lungs. Where indicated some experiments only lavaged the right lung with 250 µl of dPBS by first firmly tying silk suture thread around the left primary bronchi.

4.2.2.3 Lung tissue for homogenate

Following BALF collection, the chest cavity was further dissected to remove lung tissue. Lungs were cut into separate pieces and transferred into an RNase/DNase free Lysing Matrix D 2ml tube (Cat. #116913100, ThermoFisher Scientific) and immediately placed on dry ice to ‘snap

freeze' samples. Samples were stored at -80°C until further homogenized. Homogenisation was performed keeping samples on ice where possible. For each lung in sample, 180 µl of Cell lysis buffer 2 (Cat. #895347, R&D Systems, Abingdon, UK) was added and tubes were agitated for 30 seconds at 5 m/s using a MP Biomedicals™ FastPrep machine (ThermoFisher Scientific). Following manufacturer's instructions, a Qiagen QIAshredder kit (Cat. #79654, Qiagen, Manchester, UK) was used to isolate tissue lysate (referred to as lung homogenate). Samples were transferred into a QIAshredder disposable cell-lysate homogenizer and centrifuged at 10,000 *rcf* for 10 minutes. The collection column was then removed, and the lysates were centrifuged for an additional 5 minutes at 10,000 *rcf*. Sample supernatant containing the lung homogenate was then collected and stored at -80°C. Glux and GM-CSF transgene expression was quantified in these samples as previously described, and values were normalised by the total protein concentration of samples. This was quantified in lung homogenate samples diluted 1:40 in dPBS using the Bio-Rad DC Protein Assay Reagents Package (Bio-Rad, Hertfordshire, UK) as previously described.

4.2.2.4 Lung tissue for molecular analysis

Though molecular analysis of lung tissue has at this point in time not been performed, where indicated the left non-lavaged lung was collected for future analysis. These lungs were dissected at the bronchiole, just above the silk suture thread used to tie off the lung during the lavage procedure, and transferred into microcentrifuge tubes containing 750 µl of RNeasy Lysis Solution (Qiagen, Crawley, UK) and stored at -80°C.

4.2.2.5 Lung tissue for histology

Though histological analysis of lung tissue has at this point in time not been performed, in GM-CSF KO mice, the left lung was not lavaged and was instead inflated with 4% paraformaldehyde solution (Cat. #P6148, Sigma Aldrich, Dorset, UK). The lung was then dissected at the bronchiole, just above the silk suture thread used to tie off the lung during the lavage procedure. Lungs were submerged in 4% PFA solution for 24 hours at 4°C and then transferred to 70% ethanol and stored at 4°C for up to a month until processing for histological analysis.

Tissues were then processed by Lorraine Lawrence, at the histology Core Facility at Imperial College London. Briefly, lung tissue was dehydrated using the Shandon Hypercentre XP tissue processor (GMI, London, UK) and then embedded in paraffin by the TES Valida Modular

Paraffin Embedding System (Mediate, Dartford, UK). These samples were cut longitudinally at a thickness of 5 µm with a Leica RM2125 microtome (Leica Biosystems, Wetzlar, Germany), each sample was cut at three levels separated by 100 microns and then mounted onto glass slides. These sections were then dewaxed in Aqua-Clear (Electron Microscopy Sciences, Wisbech, UK) and ethanol absolute (VWR, Leicestershire, UK) and are currently awaiting staining by periodic acid-Schiff reagent (PAS).

4.2.3 Downstream processing of BALF

4.2.3.1 *BALF Turbidity*

Where possible, downstream processing of BALF was performed while keeping samples on ice or at 4°C. The turbidity of BALF samples was measured as a biomarker of PAP disease phenotype in GM-CSF KO and WT controls. Samples were pipetted up and down to resuspend any debris and 100 µl were transferred to a clear 96-well plate. Turbidity was measured by optical density measurements at 600 nm wavelength by a FLUOstar® Omega microplate reader. These 100 µl aliquots of each sample were then added back into the remaining sample.

4.2.3.2 *Transgene expression analysis*

BALF samples were then centrifuged at 400 *rcf* for 5 minutes to pellet BALF cells. Supernatant was collected and stored at -80°C until further analysis for transgene expression (Glux or GM-CSF) or surfactant protein-D quantification as a biomarker of PAP disease.

BALF cells were resuspended in 70 µl of cold 1X Red Blood Cell Lysis Buffer and incubated for 5 minutes before neutralisation with 230 µl of ice-cold FACs buffer (recipe as previously described). Cells were once more pelleted at 400 *rcf* for 5 minutes and resuspended in 300 µl of ice-cold FACs buffer. One third of this suspension was frozen at -80°C and used to measure the amount of Glux transgene expression in BALF cells as previously described. The remaining cells (2/3 of sample) were then analysed by flow cytometry to identify donor derived CD45.1⁺ cells.

4.2.3.3 *Flow Cytometry analysis of donor-derived cells in BALF*

Where indicated, BALF samples were cleaned by percoll® density centrifugation prior to flow cytometry analysis. In a Falcon™ 15 mL Conical Centrifuge Tube (ThermoFisher Scientific),

200 µl of BALF cells suspended in dPBS were gently pipetted onto 100 µl of percoll® (Sigma-Aldrich) and centrifuged at 894.6 *rcf* for 30 minutes. The white cell layer was then removed with Fisherbrand™ Gel-Loading Tips (ThermoFisher Scientific), washed with 1 ml of dPBS and resuspended in 200 µl of FACs buffer for subsequent staining for flow cytometry.

Half of the remaining cell suspension was stained with a CD45.1 antibody mixture (Table 2.1) in FACs buffer with Fc Receptor Blocking Solution (Biolegend) at a 1:100 dilution for 20 minutes at 4°C (protected from light). The remaining sample served as an unstained negative control and was used to identify CD45.1 positive populations in sequential gating analysis by FlowJo software. Cells were stained with TO-PRO™-3 Iodide and immediately analysed using a BD Accuri™ C6 Plus Flow Cytometer and C6 Plus Analysis Software. Donor-derived cells were calculated as a percentage of the total number of live cells in each sample. Where indicated, experiments also included 5 µl of CountBright™ absolute counting beads (ThermoFisher Scientific), added just prior to analysis to facilitate quantification of the number of donor-derived cells in each sample. Counting beads were used to calculate the concentration of donor-derived cells in each sample with was used to extrapolate the total number of donor-derived cells in the sample analysed by flow cytometry. As one third of all BALF cells were analysed in each sample, this figure was then multiplied by 3 to determine the total number of donor-derived cells retrieved in BALF.

4.2.4. Quantification of surfactant protein-D in BALF

Surfactant protein-D (SP-D) is a biomarker of PAP disease and was quantified in BALF supernatant using the Quantikine ELISA Mouse SP-D (MSFPD0, R&D Systems, Abingdon, UK) according to manufacturer's instructions. Briefly a standard curve from 4,000–31.25 pg/ml was produced by serially diluting a standard 2-fold in calibration diluent. 50 µl of samples were incubated for 2 hours at room temperature with 50 µl of assay diluent in a standard 96 well ELISA plate format with gentle shaking. Wells were then washed 5 times with wash buffer and 100 µl of mouse SP-D conjugate was added for a second 2-hour incubation at room temperature with gentle shaking, after which wells were once more washed 5 times. 100 µl of substrate solution made of a mixture of reagent A and B was added to each well for a 30-minute incubation (protected from light) during which time a colourimetric reaction occurs that is directly proportional to the amount of SP-D in the sample. To stop the reaction 100 µl of stop solution was added and the change in colour quantified by reading samples at 450 nm absorbance with a wavelength correction at 570 nm on a FLUOstar® Omega microplate reader.

4.2.4 Labelling cells with CFSE dye

BMDMs grown in a Nunc™ MicroWell™ 96-Well Microplates (Cat. #167008, ThermoFisher Scientific) plate were labelled *in vitro* with a CellTrace™ CFSE Cell Proliferation Kit (Cat. #C34554, ThermoFisher Scientific) according to manufacturer's instructions. Briefly the lyophilised CFSE reagent dye was reconstituted in DMSO and then diluted in dPBS 1:1000 to a 5 µM working solution. Cell culture media was removed from BMDMs, and cells were washed once with dPBS, and then incubated with 100 µl of working solution for 20 minutes at 37°C (protected from light). The solution was then removed, and cells were washed twice with dPBS and then cultured in complete media for 7 days before analysis by flow cytometry to determine the percentage of positively stained cells. CFSE dye has a 492/517nm excitation/emission wavelength that can be detected by flow cytometry with a 488 nm blue laser and 533/30 bandpass filter. Therefore, the same protocol as previously described to detect the percentage of EGFP positive cells using an Accuri™ C6 Plus Flow Cytometer and C6 Plus Analysis Software was used.

4.2.5 Live cell imaging of Firefly luciferase expression

Expression of the Firefly luciferase (Lux) transgene was analysed by bioluminescent imaging of live cells 7 days after transduction using an IVIS Lumina XR III (PerkinElmer, MA, USA). BMDMs were differentiated and transduced in a 24-well Nunc™ Non-Treated multidish. To quantify bioluminescence (photons/s/cm²/sr), 100µl of Luciferase assay reagent from the Pierce™ Firefly Luciferase Glow Assay Kit (Cat. #16176, ThermoFisher Scientific) was added each well and measured at a binning of 8 for 60 seconds using Living Imaging Software (IVIS imaging Systems, PerkinElmer, Ma, USA). A pseudocolour image representing light intensity (from least intense (blue) to most intense (red)) was generated and superimposed over a greyscale reference image. To quantify bioluminescence on a per well basis, photon emission was quantified by defining the area of treated and untreated wells. The defined area (circular) was consistent across samples which were marked on the greyscale reference image to avoid bias.

4.2.6 GeneSwitch™ regulated expression system

To establish proof-of-concept of regulated lentivirus-mediated gene expression in macrophages, BMDMs were transduced with lentiviral vectors encoding reporter protein Glux under transcriptional control of the commercial GeneSwitch™ system (Cat. #K106001, GeneSwitch™ Mammalian Expression Kit). (This system is explained in more detail in Chapter 4.3.7). Two different vector systems were designed by Robyn Bell (unpublished) and cloning and subsequent generation of VSV-G vectors was performed by VectorBuilder (Illinois, USA):

- A two-vector system (2V) where the commercial expression cassette was kept in the original configuration, where the pGene and pSwitch plasmids were each cloned into separate lentiviral backbones termed GEV and pSV respectively (Figure 19a-b). In these vectors the polyadenylation signal and transcription termination sequences are in the 3' LTR.
- A one vector system (1V) where the transactivator and inducible gene were cloned into a single vector and referred to as an inducible combination virus (ICV). This vector includes two open reading frames (ORFs) encoding a hCEF-transactivator-polyA and the regulated GLux transgene in opposite orientations to ensure full-length viral genomes were packaged into virions without truncation by inclusion of a premature stop codon (Figure 19c). The Glux transgene ORF terminates at the 3' LTR, while transcription of the transactivator is terminated at a polyA sequence encoded in the transactivator ORF in the opposite orientation.

BMDMs were transduced as previously described (Chapter 2.4) for either 24 or 48 hours. The vectors of the 2V system were added in a 1:2 ratio of GEV:pSV. To control for basal levels of expression, when comparing vector systems, the same MOI was used for the vectors encoding the Glux transgene (MOI 10). To induce transgene expression, cells were incubated in cRPMI supplemented with 10 uM mifepristone for 24 hours.

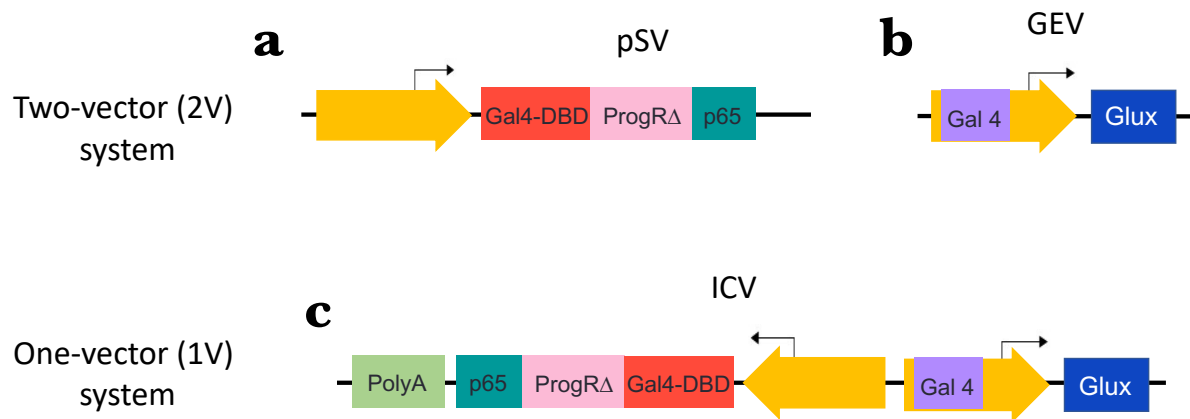


Figure 19 - Schematic of GeneSwitch™ lentiviral genomes. Two lentiviruses make up the 2V system: where (a) pSV encoding the transactivator protein that has three active domains, 1) a Gal4 DNA Binding Domain (Gal4-DBD) that binds the minimal GAL 4 promoter on a separate lentiviral genome, 2) a truncated human Progesterone Receptor Ligand Binding Domain (ProgRΔ) that binds mifepristone, and 3) a NFØB transcription factor p65 (p65) transcription activation domain that activates transcription from the GAL4-E1b promoter. (b) GEV is the second vector of the 2V system and encodes Glux transgene under the transcriptional control of the GAL4-E1b minimal promoter. (c) The ICV vector encodes both components encoded with the GEV and pSV vectors in the same vector backbone in opposite orientations in the 1V system. PolyA: bovine growth hormone poly-adenylation sequence. Images produced by Robyn Bell and adapted here with permission.

4.2.7 dPLA

A digital proximity ligation assay (dPLA) was adapted from published protocols to quantify GM-CSF protein in BALF samples (described in more detail in Chapter 4.3.3.1) (389). Recombinant Mouse GM-CSF protein (taken from the ELISA standard in the Quantikine ELISA Mouse GM-CSF kit from R&D Systems) was serially diluted (1:4) in Lysate Dilution Buffer to make a standard curve ranging from 25 pg/ul to 0.15 fg/µl. Next, 1 µl of each standard concentration was added to 1 µl of negative control BALF samples (untreated wildtype mice).

Mouse-GM-CSF PLA probes were made using the TaqMan™ Protein Assays Open Kit (Cat. #4453745, ThermoFisher scientific) with antibodies Biotin anti-mouse (MP1-22E9, Biolegend, custom biotinylated formulation) and Biotin anti-mouse (MP1-31G6, Biolegend) at 200 nM. To make PLA probes (10nM), 10 µl of each antibody were independently incubated with 10 µl of probes (Cat. #4448549, TaqMan® Protein Assays Oligo Probe Kit) at room temperature for 60 minutes and then mixed with 180 µl of room temperature Assay Probe Storage Buffer (Cat. #4405460 TaqMan® Protein Assays Buffer Kit, ThermoFisher scientific) and incubated at room temperature for 20 minutes. Probes were then kept on ice throughout. Both probes were then added together in Assay Probe Dilution Buffer (TaqMan® Protein Assays Buffer Kit) at a ratio of 190:5:5, (Buffer:Probe-1:Probe-2) to create an assay probe solution. Then 2 µl of BALF samples or recombinant standards were combined with 2 µl of assay probe solution and incubated at 37°C for one hour.

To ligate oligonucleotide probes, 96 µl of ligation solution made of nuclease-free water (Cat. #B1500, NewEngland Biolabs, MA USA), ligase reaction buffer (1X) (TaqMan® Protein Assays Buffer Kit), and DNA ligase (0.001X) (TaqMan® Protein Assays Core Reagents Base Kit, Cat. #4405460, ThermoFisher Scientific) was incubated with each sample at 37°C for ten minutes. To stop the ligation reaction, 2 µl of protease (diluted to 1X in dPBS) (TaqMan® Protein Assays Core Reagents Base Kit) was added to each sample and incubated at 37°C for ten minutes and then 95°C for five minutes.

To quantify the amount of GM-CSF protein, ddPCR was performed with the BioRad QX200™ ddPCR system previously described in Chapter 2.7, to detect the number of ligated oligonucleotides according to manufacturer's protocol. Each 20 µl ddPCR reaction contained 10 µl of the ddPCR™ Supermix for Probes (No dUTP) kit (Bio-Rad, Watford, UK), 9 µl of the protease-treated ligated samples, and 1 µl of the Universal PCR Assay solution (TaqMan™ Protein Assays Core Reagents Base Kit). The thermal cycling conditions used are listed in Table 4.

Table 4 - Thermal cycling conditions used for dPLA digital droplet polymerase chain reaction.

Step	Temperature (°C)	Time	Number of cycles
Enzyme Activation	95	10 minutes	1
Denaturation	94	30 seconds	40
Annealing/Extension	60	1 minutes	
Enzyme deactivation	98	10 minutes	1
Hold	4	Up to 30 mins	1

4.3 Results

4.3.1 Assay development for *ex vivo* cell delivery

To determine how *in vitro* transgene expression by *ex vivo* genetically modified macrophages would translate to *in vivo* production following pulmonary transplant in mice, three assays were assessed for the identification and tracking of cell fate post transplantation to the mouse lung.

4.3.1.1 A CellTrace™ CFSE Cell Proliferation Kit (ThermoFischer Scientific) labelled BMDMs for up to 7 days *in vitro*

The CellTrace™ CFSE dye is used for *in vitro* and *in vivo* labelling of cells to trace generation via dye dilution by flow cytometry with a similar excitation and emission wavelength to EGFP and FITC (492/517nm excitation/emission). The fluorescent dye carboxy-fluorescein succinimidyl ester (CFSE) crosses plasma membranes and covalently binds all free amines on the surface of and inside cells for stable retention (390). BMDMs incubated with the dye *in vitro* and analysed by flow cytometry 7 days later showed a high degree of dye retention, indicating low levels of *in vitro* proliferation post macrophage differentiation (median CFSE⁺ cells) (Figure 20). A degree of variability between samples is likely due to varying degrees of cell loss per well as cells were exposed to multiple washing steps during the protocol, which then may have resulted in inconstant degrees of cell proliferation (391). Only one time point was analysed due to experimental failure at earlier time points, however results indicate that BMDMs can be loaded with the CFSE dye and retained for up to 7 days. Therefore, within this time frame, this dye could be used in BMDMs delivered to the mouse lung to identify donor cells upon recovery in BALF fluid. With further development, it could also be used to measure cell proliferation *in vivo* as the dye is quantifiably diluted over cell divisions.

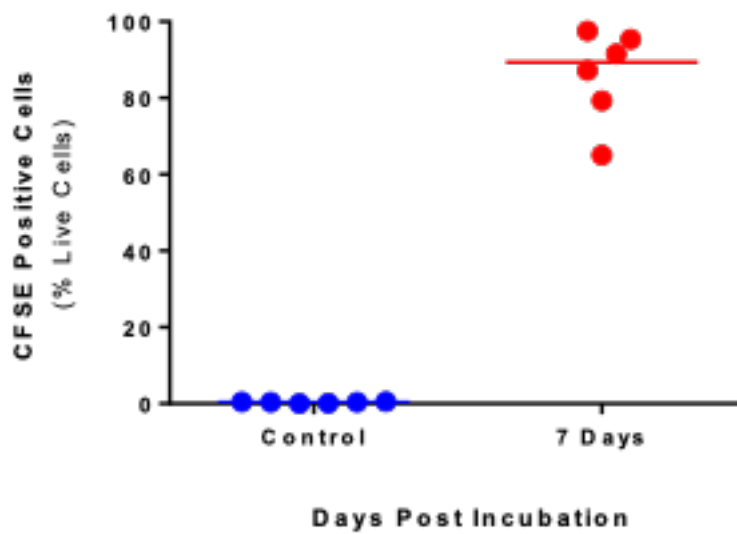


Figure 20 - Fluorescent labelling of BMDM *in vitro* with CellTrace™ CFSE dye. Following a 20-minute incubation with CellTrace™ CFSE dye (Thermofischer Scientific) BMDMs are fluorescently labelled with carboxy-fluorescein succinimidyl ester (CFSE) dye following 7 days in culture. CFSE was detected by flow cytometry (492/517nm excitation/emission) and positive cells were identified by sequential gating on size and live/dead stain with To-Pro-3. Each circle represents BMDM samples independently incubated with CFSE and maintained in culture. Bars represent median.

4.3.1.2 BMDMs express transgenic Firefly luciferase at sub-optimal levels

Identifying transplanted cells through flow cytometry analysis of bronchoalveolar lavage fluid (BALF) is an end-point procedure and thus time course experiments require multiple treated cohorts to track retention and transgene expression over time. Attempting to develop a method for cell fate tracing in live mice, BMDMs were transduced with the rSIV.F/HN.hCEF.GFP/Lux vector (MOI 50), carrying a fusion transgene of EGFP and Firefly luciferase, and compared to untransduced controls (n=6/group). This luciferase is a non-secreted enzyme which can catalyse the oxidation of luciferin resulting in quantifiable light emission which following substrate administration can be used to track transgene expression and localization *in vivo* (302, 392). With sufficient strength of gene expression, I hypothesised that this could be used to track macrophage duration and location in mice following lung administration without requiring animal termination. Gene expression, quantified by bioluminescent imaging of BMDMs in culture, was restricted to rSIV/F/HN.hCEF/Lux transduced cells 7-days post-transduction, however light emissions were low (Figure 21). The sensitivity of this assay is sufficient to detect light emission with viral (302) and non-viral gene therapy (392). However, the degree of light emitted here was such that *in vivo* assessment was thought unlikely to be successful. Though this Firefly luciferase assay has potential to track the duration of transplanted macrophages *in vivo* as it is a non-secreted reporter gene, it would not inform the duration or strength of secreted gene expression.

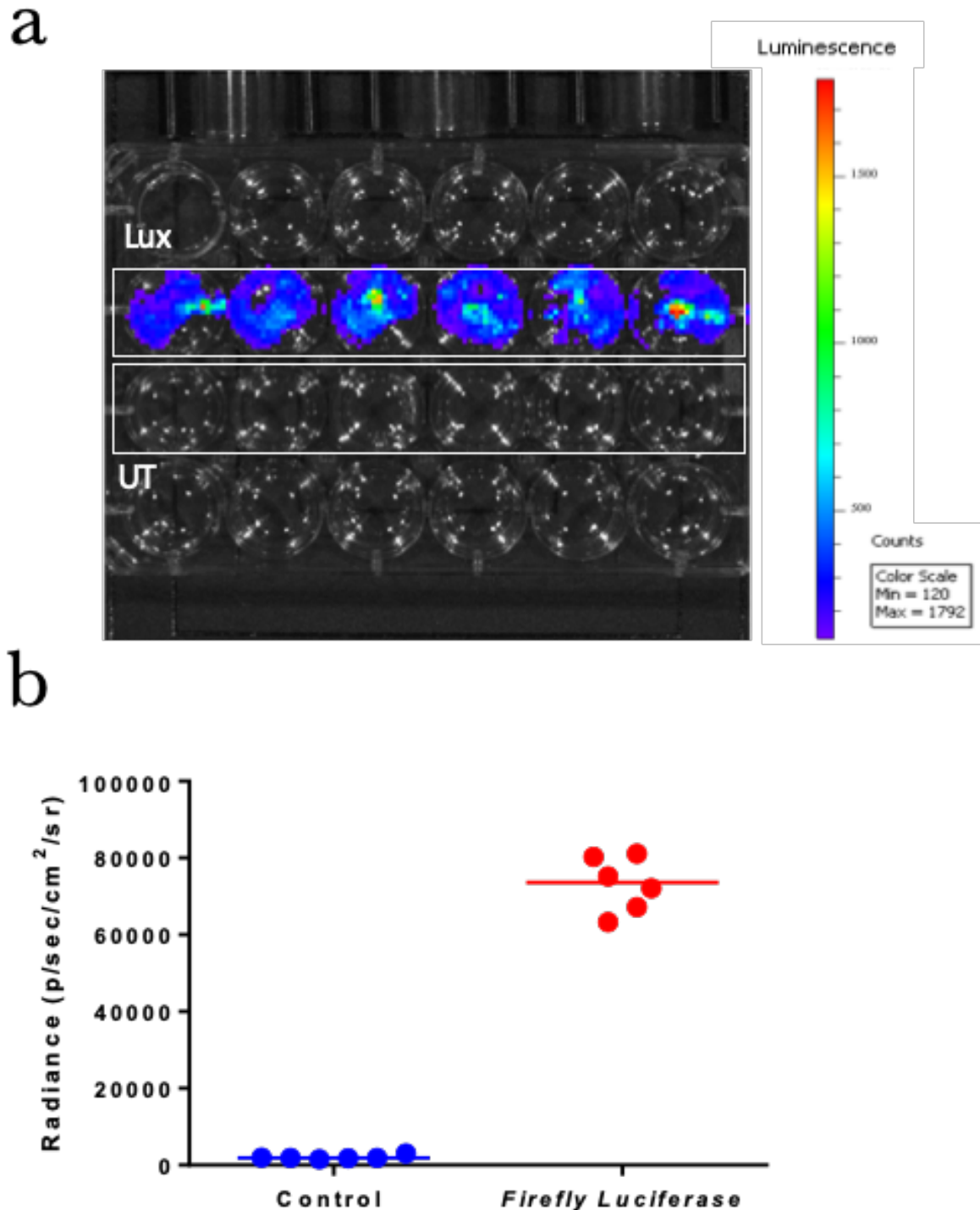


Figure 21 – Bioluminescent labelling of BMDM following *in vitro* transduction with rSIV/F/HN.hCEF.GFP/Lux. Bone marrow transduced macrophages (BMDM) transduced with rSIV/HN lentivirus expressed a Firefly luciferase reporter gene for quantification of gene expression by bioluminescent imaging. **(a)** Live imaging of transduced cells (BMDMs) and untransduced (UT) controls live in adherent culture. **(b)** quantification of photon emission in BMDMs in IVIS software, radiance: (p/sec/cm²/sr). Bars represent median.

4.3.1.3 Macrophages derived from C57BL/6-Ly5.1 donor mice can be specifically detected by flow cytometry in wildtype mouse bronchoalveolar lavage fluid

A common method of identifying transplanted donor cells *in vivo* is based on the identification of the two functionally identical polymorphisms of the transmembrane receptor tyrosine phosphatase protein CD45. CD45 is a leukocyte marker expressed on cells of a hematopoietic lineage including macrophages. Wildtype C57b6/J mice express the CD45.2 allele which differs in its extracellular domains from the CD45.1 allele expressed by C57BL/6-Ly5.1 mice (Charles River) and can be identified by monoclonal antibodies (393). Others have shown that macrophages derived from these mice and transplanted into the lungs can be detected by flow cytometry (176, 177, 186). Staining of BMDMs derived from CD45.1 mice by a CD45.1 specific antibody was first confirmed *in vitro* on day 7 of the differentiation protocol (Figure 22a). To confirm specific staining of donor-derived cells in recipient CD45.2 WT mice, 6.32e6 BMDMs were transplanted to the lungs of a WT mouse through oropharyngeal delivery (n=1). A control mouse received a dose of cell-free PBS (n=1). One week later, BALF was collected, and cells analysed by flow cytometry to confirm specific binding and identification of transplanted cells following lung administration (Figure 22b). This small study provided first proof-of-concept that donor-derived macrophages could be specifically identified by flow cytometry following *in situ* delivery regardless of successful gene transfer.

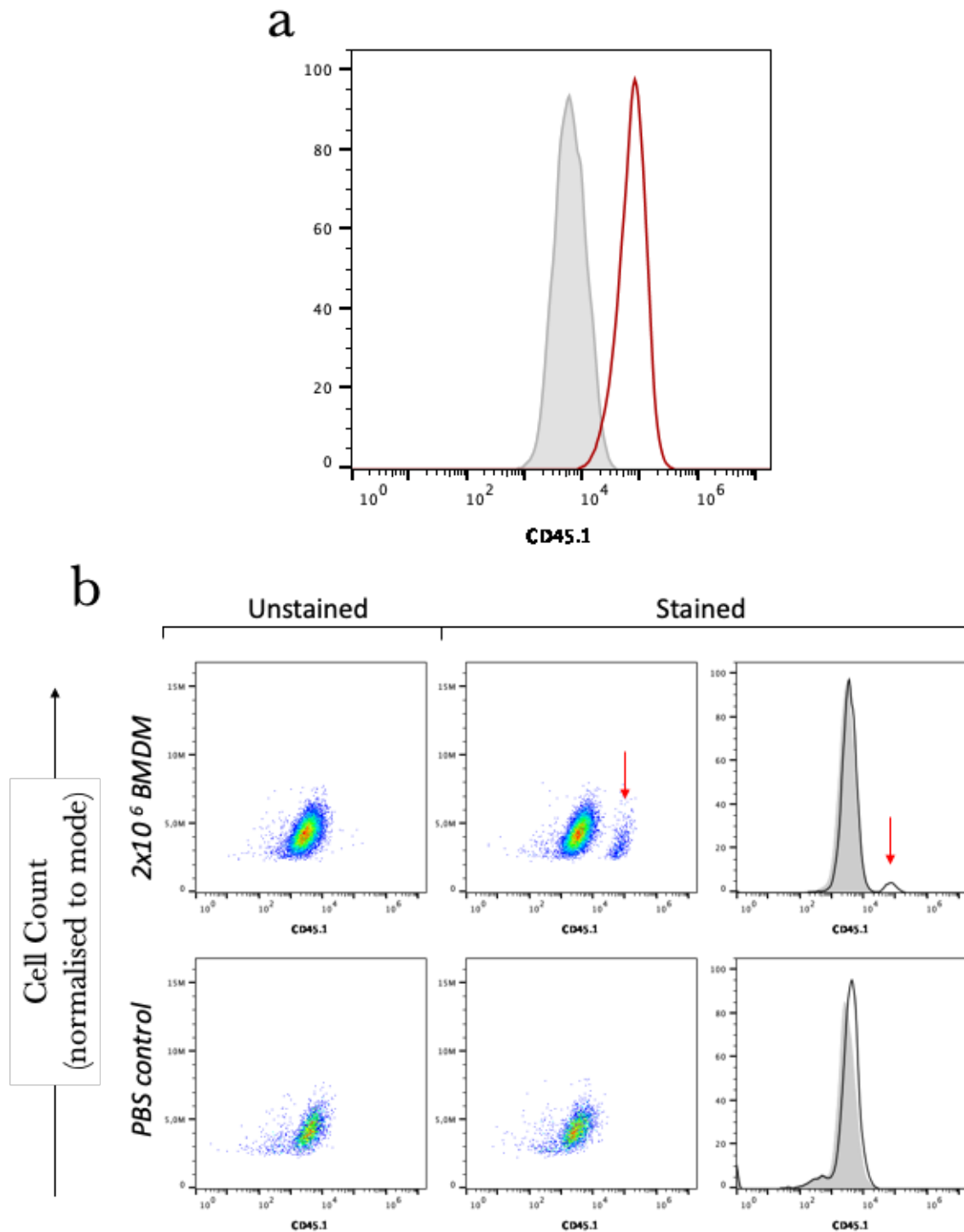


Figure 22 – Specific labelling of donor derived cells from CD45.1 mice. (a) BMDMs derived from CD45.1 (C57BL/6-Ly5.1) mice are detected by flow cytometry with a CD45.1 antibody (red: stained, grey: unstained control). (b) CD45.1 labelling mediates specific labelling of donor derived cells (red arrow) in BALF cell analysis by flow cytometry 7 days after lung delivery to CD45.2 WT mouse. CD45.1 donor-derived cells are absent in PBS controls. Stained: + CD45.1 antibody, Unstained: - CD45.1 antibody.

4.3.2 A single dose of gene-modified BMDMs leads to pulmonary GM-CSF expression in mice

Having established that BMDMs can be transduced to express a secreted therapeutic protein *in vitro*, I performed an initial pilot study to demonstrate proof-of-principle that BMDMs transduced *ex vivo* by rSIV.F/HN can drive pulmonary production of a therapeutic secreted protein *in vivo*. Macrophages were delivered to the lung through an oropharyngeal protocol adapted from similar macrophage transplantation experiments (177), as intratracheal *in situ* administration has proven superior to intravenous (IV) routes in enabling direct delivery of bone marrow-derived epithelial-like cells to the lung (394). Here 50 μ l of cells suspension in dPBS was pipetted into the oral cavity and deposition into the lungs was ensured by extension of the tongue to prevent swallowing and blocking of the nasal airways.

To determine the required number of donor mice per experiment, the average bone marrow yield per mouse was calculated from previous experiments and used to calculate the average number of 75 cm² flasks which can be seeded per mouse (Table 5). In this experiment an approximate yield of 1e6 BMDMs per flask was assumed, while subsequent harvests used averages of previous results.

Table 5 – Bone marrow cells harvested per mouse and the number of 75cm² flasks that can be seeded per mouse. Each row represents an average number of bone marrow cells obtained per mouse on separate occasions. In certain experiments, bone marrow is pooled from multiple mice and then counted, the number below therefore represents yields corrected per mouse.

BM cells / mouse	Flasks / Mouse
5.23e7	9.29
5.8e7	10.31
6.66e7	11.84
5.06e7	9.00
2.83e7	5.03
3.61e7	6.42
3.36e7	5.97
6.3e7	11.20
6.53e7	11.60
4.32e7	7.69
Mean: 4.97e7	Mean: 8.83

Mice were dosed with BMDMs transduced with rSIV.F/HN.hCEF.mGM-CSF or untransduced BMDMs, and BALF was collected 7 days later (n=4/group). Aiming for optimal gene expression, the highest maximum feasible dose tested in *in vitro* studies was used, transducing BMDMs at MOI 50. BMDMs were transduced for 24 hours, washed and then immediately delivered to reduce time in culture. One week was chosen as an initial time point to ensure sufficient time for viral integration and transgene expression (395). BMDMs were delivered in a dose of 2e6 cells per mouse based on a study which found this was the optimal cell dose to deliver for macrophage engraftment following pulmonary macrophage transplant (177).

In treated mice, 11.42 ng/mL (median; range: 5.6-22.19) of mGM-CSF was detected in BALF but was undetectable in control mice (Figure 23). When compared to unpublished data presented at the ASGCT annual meeting by Claudia Juarez-Molina, GM-CSF BALF concentrations were comparable following intra-nasal administration of a cationic

liposome/plasmid complex, GL67a/pIC017, whereby a non-viral expression cassette encoding the same GM-CSF transgene under hCEF transcriptional control was delivered to the murine lungs by non-viral means (387). Importantly we showed that following naïve macrophage administration, there was no detectable GM-CSF in BALF, indicating that endogenous levels in CF mice are below the limit of detection, and that GM-CSF detected in treated mice is transgene specific and not a consequence of macrophage transplantation. Media removed from cells following a 24-hour transduction contained 32.4 pg of GM-CSF (2.7 pg/ml) which at this early time point can likely be attributed to pseudotransduction. Notably no GM-CSF was detected in media from control cells, or in supernatant from subsequent wash steps, indicating GM-CSF present in murine BALF on day 7 is likely the product of transplanted cells secreted *in vivo* during the experimental time frame. This initial study demonstrated that the transduction efficiency of the SIV.F/HN virus in macrophages is sufficient to drive detectable levels of *in vivo* gene expression. Furthermore, expression data suggests that macrophages transplanted into a healthy lung where AMs are present under steady-state conditions can be retained for up to a week in the lungs providing proof-of-concept for macrophage driven therapeutic protein secretion as a platform technology.

As this was an initial proof-of-concept study, excess mice remaining from breeding cystic fibrosis knock out mice were used due to availability, where the donor mouse was a CFTR knock out mouse, three treated mice per group were heterozygotes (CFTR^{-/+}) and one was a CFTR KO. One control mouse (CFTR KO) was found dead three days post-delivery. The lungs from this mouse were collected and fixed in 4% PFA for 24 hours and sent for histopathological analysis by the Imperial College Central Biomedical Services. Following analysis of nine representative sections of the right and left lungs, the pathologist report showed that regardless of marked congestion of the alveolar capillary beds and branches of the pulmonary veins and arteries, “microscopic examination of these lung sections does not reveal histopathological (sic) changes that could explain the sudden death described in the history.” Based off this report and discussion with the named veterinary surgeon, death due to macrophage delivery or subsequent presence in the lungs was deemed unlikely. Furthermore, the named veterinary surgeon in attendance noted no errors or possible contributors to death during oropharyngeal cell delivery. The mouse in question was a CFTR null mouse of +1 year of age, and it was thought that advanced age in combination with contributing stress from the anaesthetic procedure may have had a causative role.

A consideration that has come to light since performing this experiment, is that the use of these GM animals to investigate expression of a therapeutic transgene (GM-CSF) that would not be applicable to their disease is not best ethical practice. Although this study was attempting to reduce the number of mice used experimentally by treating excess mice left over after breeding CF heterozygotes (CFTR^{-/+}) and developing a macrophage cell therapy approach to treatment of CF is also an area of interest in this thesis, GM-CSF is an unlikely therapeutic transgene. In all subsequent experiments and moving forward, studies in GM mice will be limited to relevant therapeutic transgenes and appropriate controls. The use of donor macrophages from CFTR KO mice may also have affected the results of cell engraftment and gene expression and is discussed further in section 4.4.4.

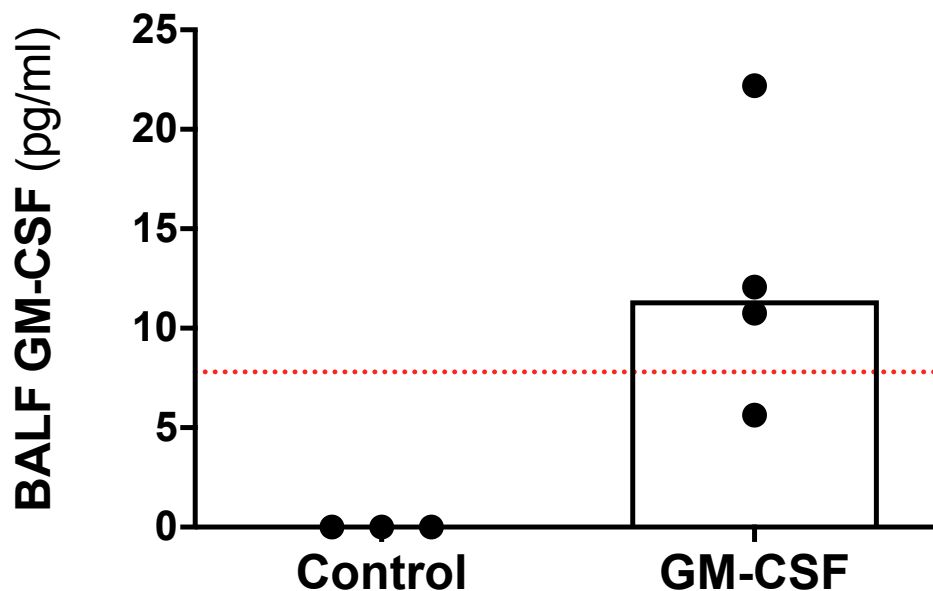


Figure 23 - Following *in situ* delivery, gene-modified bone marrow derived macrophages (BMDM) produce detectable levels of pulmonary GM-CSF *in vivo*. BMDMs were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine GM-CSF under the control of the hCEF promoter at MOI 50. Mice received a single dose of 2e6 gene modified BMDMs, or untransduced BMDMs in control mice, delivered via an oropharyngeal protocol in 50ul of PBS. The concentration of GM-CSF was measured in bronchoalveolar lavage fluid (BALF) collected 7 days later by ELISA. Red line indicates assay's lower limit of detection (7.8 pg/ml). Bars indicate median.

4.3.3 Gene expression and macrophage retention following BMDM delivery in wildtype mice is dose related

4.3.3.1 GM-CSF expression by BMDMs in vivo is dose related and largely below the limit of detection

Having established that gene-modified macrophages produce detectable levels of secreted protein *in vivo*, I investigated whether gene expression could be controlled by varying cell dose. Wildtype C57BL/6J mice were treated with either 2e6 or 6.32e6 genetically modified BMDMs (MOI 50), representing a half log increase in dose, while control mice received 6.32e6 untransduced cells (n=6/group). This high dose was chosen as the maximum feasible dose, since delivering higher cell numbers was found to be economically and logistically unfeasible due to the large number of donor mice and virus required to produce the genetically modified macrophages. BMDMs were once more transduced to express mGM-CSF, and a week after delivery, transgene expression was only detected in lung tissue homogenate samples from mice in the high dose cohort (median: 13.58 pg/ml (range 12.04-19.9) and was not present above the ELISA LLD in BALF regardless of treatment group (Figure 24a-b). mGM-CSF expression in lung homogenate samples from lower dose and control cohorts were similar, where in the majority, samples were faintly positive but not quantifiable as the OD readings on the ELISA were below the assay's LLD. One sample from each cohort was positive for mGM-CSF (4.76 and 6.29 pg/mg respectively), with OD readings just above the LLD. This is not in keeping with historical data as previously Helena Lund-Palau and Claudia Juarez-Molina demonstrated that when characterising the PAP model, no detectable GM-CSF expression was measured in any of the WT controls (387, 396). It might be interesting to re-analyse these samples to confirm these are true positives, however, as the concentration of mGM-CSF in low dose cohorts was below that of the untreated controls (blue line in Figure 24b), I can conclude that no macrophage/treatment specific mGM-CSF was detectable following the low dose delivery of genetically modified BMDMs. Unsurprisingly, GM-CSF was also completely undetectable in the mouse serum (data not shown), though at high levels of expression, transgenic proteins expressed from the lung epithelium following *in vivo* lentiviral administration, can enter circulation (168).

As no GM-CSF was definitively detected in BALF and lung homogenate samples from mice in the low dose cohort above controls, we were unable to reproduce previous findings of positive

GM-CSF signal in BALF at an equivalent dose. This discrepancy and inter-experimental variation were then confirmed when samples from both experiments were once more analysed by GM-CSF ELISA, in addition BALF samples from untreated WT mice were included as an additional negative control. (Figure 24c). It is worth noting in the first experiment only minimal levels of GM-CSF were detected near the ELISA LLD and therefore this difference may not be overly large. Though GM-CSF was below the threshold of detection in BALF following low dose macrophages administration, this may still be sufficient to observe therapeutic outcomes in patients and models of aPAP as wildtype levels of GM-CSF were also below this threshold of detection (387).

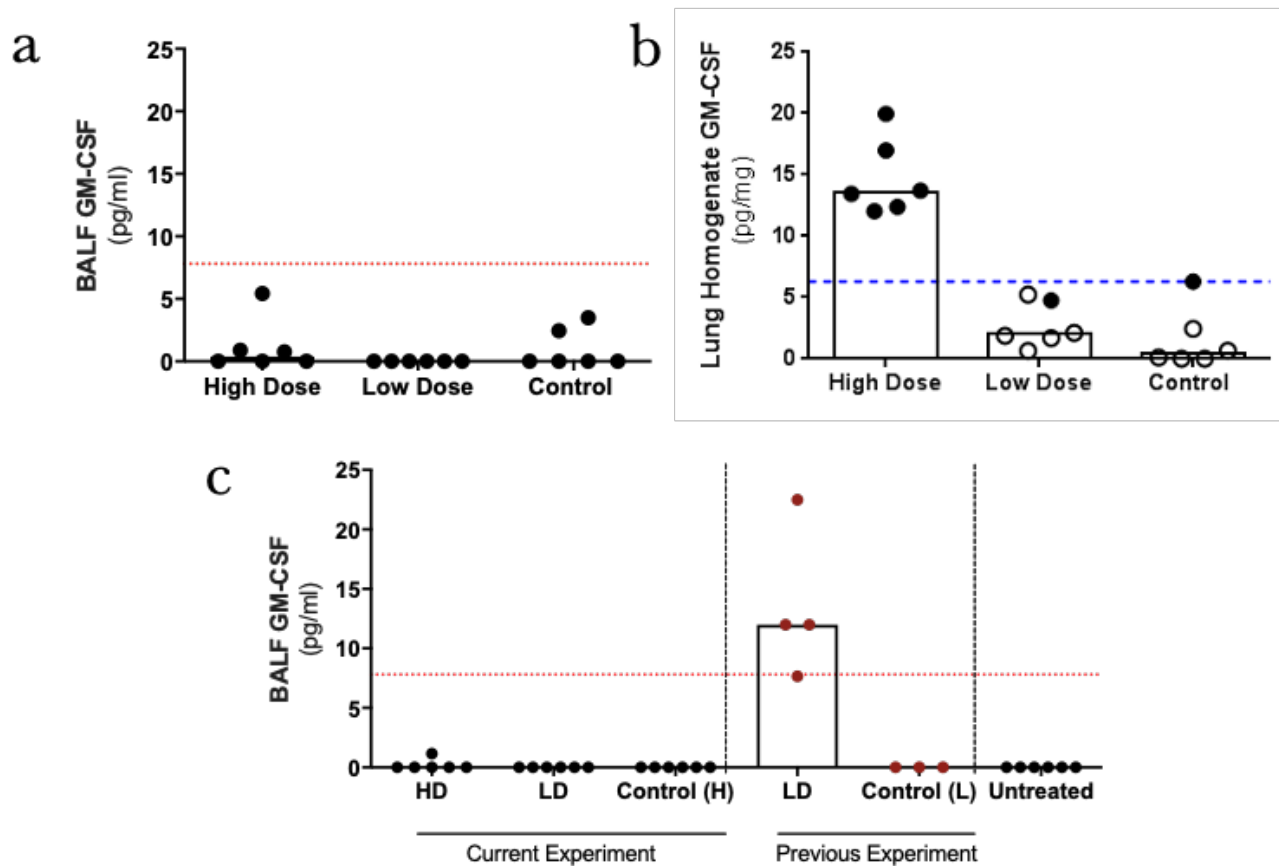


Figure 24 - Dose dependent expression of pulmonary mGM-CSF from gene-modified bone marrow derived macrophages (BMDM) *in vivo*. BMDMs were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine GM-CSF under the control of the hCEF promoter at MOI 50. Mice received a single dose of either 2e6 (low dose/LD) or 6.32e6 (High Dose/HD) gene modified BMDMs, or untransduced BMDMs in control mice, delivered via an oropharyngeal protocol in 50ul of PBS. The concentration of GM-CSF was measured in **(a)** bronchoalveolar lavage fluid (BALF) and **(b)** lung homogenate collected 7 days later using a mouse GM-CSF ELISA. Lung homogenate values were normalised to total protein concentration (mg/mL), samples measuring values below the ELISA lower limit of detection (7.8 pg/ml – red line) before normalisation are indicated by empty circles, where each circle represent a single mouse. Blue line delimits the upper positive value of untreated controls. **(c)** Comparison to previous results, where the GM-CSF detected in BALF was re-analysed in concert with additional samples from untreated WT mice (n=6). Bars indicate median.

An alternative digital proximity ligation assay (dPLA) developed by my colleague Anthony Sinadinos was then characterised to detect GM-CSF at a single molecule resolution (397). The dPLA assay uses the dual action of two oligonucleotide-labelled antibodies that each bind

unique targets on the GM-CSF protein. When the oligonucleotides are in close proximity, a hybridizing connector can bind to both oligonucleotides and enable their ligation. Through sample emulsification, ddPCR can then quantify the number of hybridized oligonucleotides and thereby GM-CSF, at a single molecule resolution. The assay's LLD was determined by standard curve to evaluate its suitability as an alternative, more sensitive assay than ELISA for the detection of GM-CSF in BALF, which claims sensitivity threshold of 3.5 fg/μl. Using the standard curve, the dPLA had a sample limit of detection of 390.6 fg/μl as this was where the assay lost the ability to report changes in standard curve concentration, meaning that even with the correct antibiotic dilution it is less sensitive than the GM-CSF ELISA (Table 6). It is therefore unsurprising that no positive GM-CSF signal was detected across BALF samples from this most recent dose escalation trial (high dose treated and control), the previous initial experiment which were previously positive (low dose treated and control), or untreated WT mice, were all samples reported concentrations below the LLD (Figure 25). In conclusion, the dPLA assay provides no advantage over the ELISA in quantifying secreted mGM-CSF protein in murine samples.

Table 6 – GM-CSF standard curve for dPLA. Concentration recombinant GM-CSF in spiked BALF samples from WT mice and the corresponding copies/μl ddPCR read out, where the lower limit of detection is indicated in bold.

GM-CSF Standard (pg/μl)	ddPCR read- out (copies/μl)
25	6600
6.25	1222
1.5625	280
0.3906	90
0.0977	64.5
0.0244	77
0.0061	80.5
0.0015	74

At all doses the therapy was relatively well tolerated, though evidence of a mild suffering phenotype (hunching and listlessness) was observed in approximately 1/3 of mice in the 24

hours following oropharyngeal delivery but the effect was transient. As this was observed across treatment groups including untransduced macrophages it is unlikely a reaction to transgene expression. As in the previous study death was observed in a PBS cell-free control mouse, it suggests that this mild phenotype is due to the anaesthesia or procedure rather than the presence of macrophages in the lung cell.

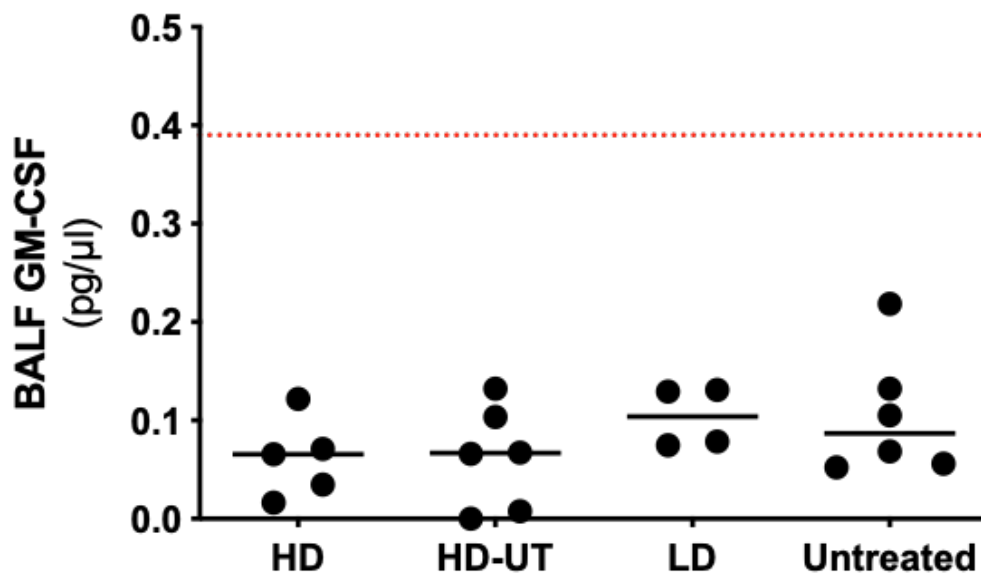


Figure 25 - GM-CSF in BALF is not detectable by a digital proximity ligation assay (dPLA). Here the following samples from previous experiments were analysed: from experiment 4.3.6 where mice receiving 6.32e6 transduced (HD) and untransduced (HD-UT) BMDMs, from experiment 4.3.5 (LD) where mice received 2e6 transduced BMDMs which were previously positive when analysed by ELISA, and untreated WT mice (untreated). Red line indicates the assays lower limit of detection (0.39 pg/μl). Each circle represents one mouse, bars indicate median.

4.3.3.2 Increasing BMDM cell dose leads to a dose related increase of *in vivo* Glux expression

As there was no consensus across experiments, and to see if initial positive findings could be recapitulated, mice were once more dosed with a low (2×10^6) or high (6.32×10^6) dose of BMDMs expressing transgenic GM-CSF ($n=4/\text{group}$). Donor cells were harvested from C57BL/6-Ly5.1 donor mouse to track transplanted cells and determine if any repeated lack of transgene expression is due to a loss of donor macrophages. Additionally, cohorts of mice received either a low or high dose of BMDMs transduced with rSIV.F/HN.hCEF.Glux to express the secreted reporter transgene Glux ($n=4$). Glux is a highly sensitive secreted reporter protein (278) which has been used to inform *in vivo* and *in vitro* studies of secreted protein therapy (168, 319). As production of transgenic GM-CSF may once more fall below levels of detection, to enable characterisation of the effects of increased cell dose, additional cohorts were included treated with BMDMs expressing the sensitive Glux reporter gene. BMDMs were transduced at MOI 20 prior to delivery, while control mice received an equal volume of cell-free PBS ($n=3$). Where previously cells were transduced at MOI 50, here a lower viral load was used due to availability and costs associated with expanded *in vivo* studies, importantly in previous transduction optimization experiments (Chapter 3.3.2) no significant difference was observed in gene expression between BMDMs transduced at MOI 20 and 50.

Again, a mild phenotype was observed in mice within 24 hours of macrophage delivery that was not limited to one treatment or dosage group. This phenotype was reversed in all but one mouse upon the addition of easy-to-reach wet food suggesting that poor recovery is a consequence of dehydration and improper nutrition during recovery from ketamine anaesthesia. One mouse that had received a low dose of Glux BMDMs was terminated as its suffering was observed to have exceeded the severity limit of this procedure.

No evidence of GM-CSF expression was found in serum or BALF samples of any treated or untreated animals, indicating that transgene expression is absent or below the limit of detection (data not shown). In lung homogenate, low levels of GM-CSF were detected in 2 mice from mice treated at a low dose with Gluc expressing BMDMs and 2 mice treated at a high dose with GM-CSF expressing BMDMs (Figure 26a). The positive GM-CSF signal in Gluc treated mice would suggest that transplanted WT macrophages may produce detectable levels of GM-CSF result rather than as a consequence of transgene expression. However, as the cohort treated with a high dose of Glux/BMDMs and control mice from the previous experiment

(high dose un-transduced BMDMs) did not exhibit a similar result it likely that these findings are false positives. As a result, no definitive GM-CSF expression was detected in any sample. When previously dosing mice at this dose, GM-CSF was detected in lung homogenate which suggests that following transduction at MOI 20 there was a decrease in corresponding GM-CSF expression.

In comparison, Glux treatment groups exhibited definitive transgene expression in lung and BALF samples, though again there was no evidence of secretion into the circulatory system (Figure 26b-d). Additionally, a clear dose response in gene expression was seen as a half log increase in dose, equivalent to a 3.16-fold change, resulted in a 2.89- and 1.99-fold change in gene expression in BALF and lung samples respectively:

- *BALF*: median difference 20139 RLU in 10 μ l
- *Lung*: median difference 18737 RLU/mg

Due to low n numbers, statistical analysis was not performed, but trends do indicate that following gene transfer to BMDMs and their subsequent transplantation to the lung, transgenic proteins are secreted into the lung in a dose related manner.

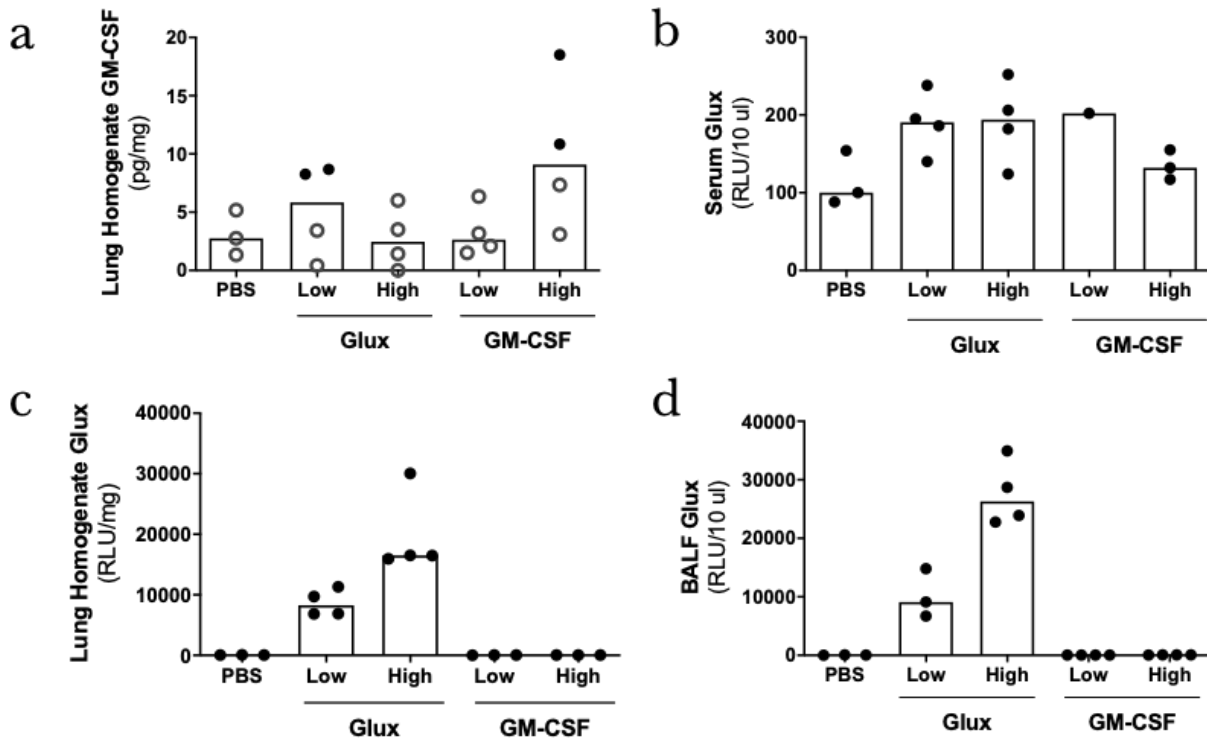


Figure 26 - Dose dependant expression of pulmonary mGM-CSF and Glux from gene-modified bone marrow derived macrophages (BMDM) *in vivo*. BMDMs were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine (a) GM-CSF or (b-d) Glux under the control of the hCEF promoter at MOI 20. Mice received a single dose of either 2e6 (Low) or 6.32e6 (High) gene modified BMDMs delivered via an oropharyngeal protocol in 50ul of PBS. Control mice (PBS) were treated with 50 μ l of cell-free PBS. GM-CSF (pg/ml) and Glux (RLU – relative light units) expression was measured in (a,c) lung homogenate, (b) serum, and (d) bronchoalveolar lavage fluid (BALF) collected 7 days later using a mouse GM-CSF Quantikine ELISA kit or Pierce™ Gaussia Luciferase Glow Assay Kit. Lung homogenate values were normalised to total protein concentration (mg/mL), samples measuring values below the ELISA lower limit of detection (7.8 pg/ml) before normalisation are indicated by empty circles, where each circle represent a single mouse. Bars indicate median.

Transplanted donor macrophages from C57BL/6-Ly5.1 mice were identified in BALF of wildtype mice by flow cytometry. Retention was calculated as the percentage of CD45.1⁺ BALF cells first gated by size and by live/dead stain. Donor-derived cells were present in all treated mice 7-days post-delivery correlating with results of gene expression (Figure 27):

Glux:

- *Low dose:* median 4.44 (range 3.01-9.67) % donor-derived cells in BALF

- *High dose*: median 4.113 (range 3.25-5.69) % donor-derived cells in BALF
GM-CSF:
- *Low dose*: median 4.54 (range 3.52-9.44) % donor-derived cells in BALF
- *High dose*: median 12.86 (range 7.52-17.41) % donor-derived cells in BALF

It is therefore unlikely that the lack of GM-CSF expression is due to a reduced retention of these cells. Indeed, when delivered at a higher dose, cells transduced with a GM-CSF transgene exhibited a higher rate of retention than comparable Glux controls (median difference 8.75 % donor-derived cells in BALF), though due to low n numbers this study is not powered for statistical comparisons. Cell retention was comparable across Glux dosed cohorts, and the low dose GM-CSF cohort. This suggests that GM-CSF is being produced by transplanted cells in a dose related manner, even though it is below the level of quantification, as it suggests an effect of GM-CSF expression only observed at a higher cell dose where here at higher expression levels, GM-CSF stimulates increased engraftment. This is likely a result of proliferation as *in vivo*, GM-CSF regulates AM survival, maturation, and proliferation (39, 103, 398). However, donor retention data does not correlate with the dose response observed in Glux expression (Figure 27b-c). This is likely owing to differences in sensitivity between the two assays, where the flow cytometry detection of CD45.1 cells in BALF is not sensitive enough to detect differences in the number of cells retained in the lungs that has resulted in a dose related increase in Glux expression as detected by the sensitive Glux assay. Having demonstrated that increasing the number of transplanted cells leads to a corresponding increase in gene expression, one might have expected a higher rate of retention. This dose comparison was only performed at one early time point and it may be possible that over time the distinction in gene expression between doses is lost, as the lung only supports retention of a fraction of transplanted cells.

These initial proof-of-concept *in vivo* experiments provided evidence that *ex vivo* delivery of genetically modified BMDMs can result in cellular retention in the murine lungs. Subsequent pulmonary gene expression suggests these cells might then function as protein secretion ‘factories’ of therapeutic and reporter proteins. Though at this point, direct proof of macrophage driven gene expression *in vivo* has not been established and the potential for vector carry-over in the cell suspension has not been eliminated. Having developed a protocol for the modification, delivery, and analysis of this platform technology I then characterised the duration of macrophage retention.

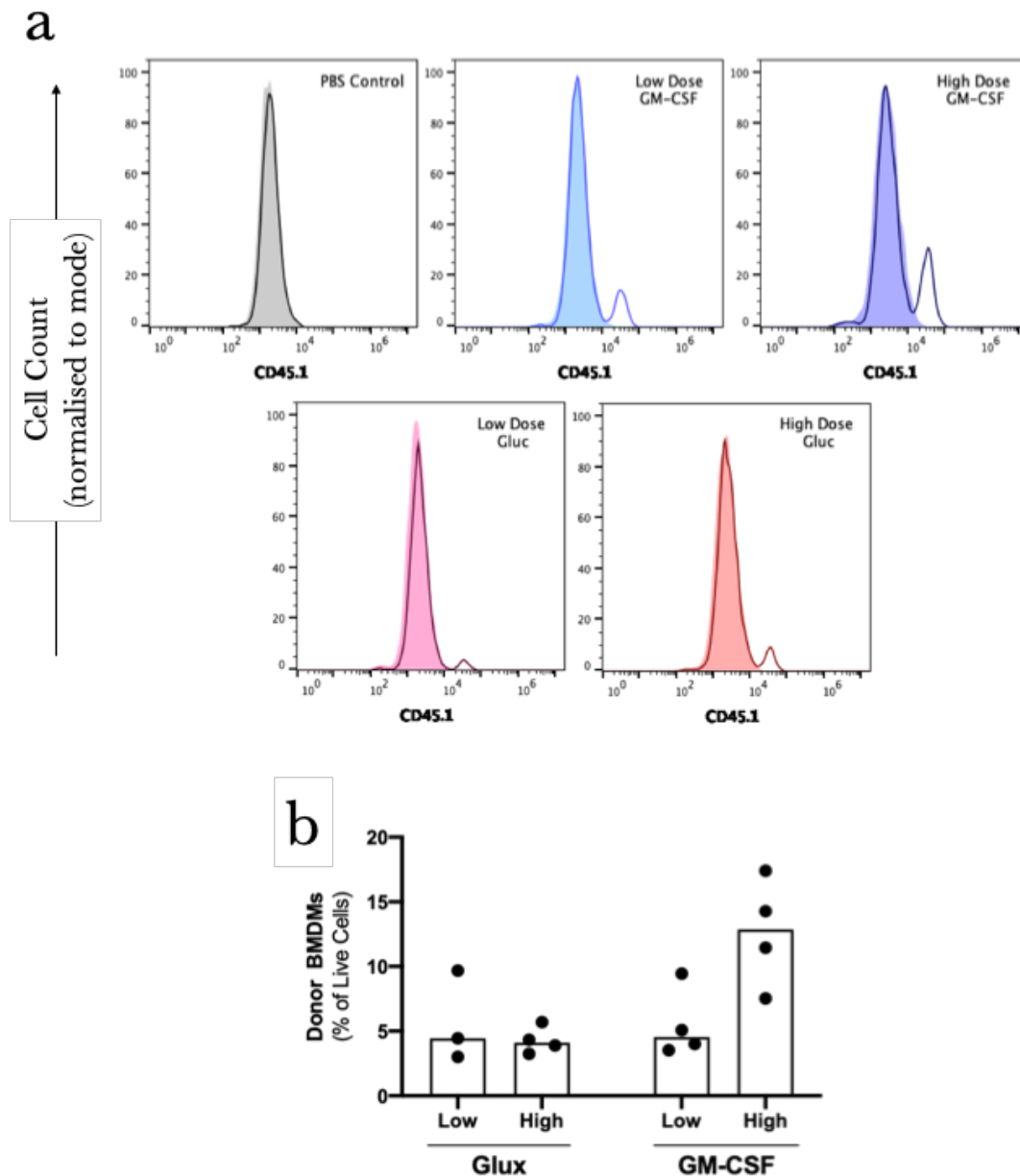


Figure 27 - Pulmonary retention of bone marrow derived macrophages (BMDM) expressing transgenic mGM-CSF and Gluc. (a) Donor-derived macrophages from C57BL/6-Ly5.1 were identified in BALF cells by flow cytometry analysis of CD45.1 expression. Each sample was divided equally and then either stained with a CD45.1 antibody (outlined peak) or used as a gating control and left unstained (filled-in peak). Representative samples for each cohort shown here **(b)** Retention one week after transplantation was quantified as the percentage of CD45.1⁺ cells from a total of all live cells (identified by ToPro-3 staining). Prior to deliver BMDMs were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine) GM-CSF or Gluc under the control of the hCEF promoter at MOI 20. Mice received a single dose of either 2e6 (Low) or 6.32e6 (High) gene modified BMDMs delivered via an oropharyngeal protocol in 50ul of PBS (n=4/cohort). Control mice (PBS Control) were

treated with 50 μ l of cell-free PBS (n=3) and used as negative control when gating for CD45.1⁺ cells. Each circle represents a single mouse, bars indicate median.

4.3.4 Transplanted BMDMs are retained for up to 2 weeks in the lung of wildtype mice

Having established that genetically modified macrophages are retained in the mouse lung one week post-delivery and produce detectable levels of secreted reporter transgenes in the lung, I then investigated the duration of retention and expression in the lung over time in WT mice where studies looking at engraftment and retention of transplanted lung macrophages are limited to short term experiments (176, 178). As a dose effect had been observed with increased gene expression at higher doses, 6.32e6 BMDMs derived from C57BL/6-Ly5.1 donor mice were delivered to wildtype animals. In previous experiments this dose led to detectable levels of the Gluc transgene in BALF and lung homogenate. Though there was no determination of maximal dose, meaningful further increased dosage, while theoretically possible, would require significant increases in donor mice and viral vectors which were not feasible with regard to cost and availability of animals and vectors. Wildtype mice received Gluc/BMDMs transduced at MOI 20 (n=6/time point) or control un-transduced BMDMs (n=2/time point) and were harvested one week or two weeks post-delivery. Due to availability of donor C57BL/6-Ly5.1 mice (exacerbated by the COVID pandemic) mouse numbers in un-transduced BMDM cohorts were low. Therefore, it is not within the purview of this experiment to investigate the effect of transduction on macrophage retention in the lung and instead the experiment focused on comparisons between Gluc treated cohorts, or duration of transduced BMDMs and transgene expression in the lungs.

4.3.4.1 Transgene expression is sustained over 2 weeks post-transplant

Transgene expression was observed in all treated mice and sustained over time in BALF and lung homogenate samples (control mice pooled across time points for Gluc expression analysis) (Figure 28a-b):

Lung homogenate

- *Week 1*: median 10289 (range 6612–19498) RLU/mg
- *Week 2*: median 7613 (range 6481–26230) RLU/mg (p=ns, Kruskal-Wallis, Dunn's multiple comparisons)

BALF

- *Week 1*: median 20802 (range 9970–46881) RLU in 10 μ l

- *Week 2*: median 14231 (range 10113–25830) RLU in 10 μ l (p =ns, Kruskal-Wallis, Dunn's multiple comparisons)

Importantly there was no evidence of Glux expression in mouse serum at either time point indicating that transgene expression was limited to the lung and not entering circulation (Figure 28c). In the first *in vivo* cell therapy experiment, BALF samples were positive for GM-CSF expression, however this finding was not repeated in subsequent experiments, even at higher doses and positive expression was only detected in the lung homogenate. In the first experiment however, BALF was not processed for flow cytometry and therefore cells were not pelleted out prior to expression analysis. I therefore hypothesized that the discrepancy in GM-CSF expression levels between experiments was owing to GM-CSF retention in the pelleted cells that were not removed prior to analysis in the first experiment. Here, when pelleting BALF cells for flow cytometry analysis, a third of the cell suspension was analysed for Glux expression which reported only minimal levels of expression (Figure 28d). This demonstrates that retention of Glux in the cellular fraction of BALF is limited and unlikely to influence expression data in the liquid fraction in the absence of pelleting. Extrapolating these results to a GM-CSF transgene, suggests that GM-CSF retention in transplanted cells was not the cause of discrepancy in gene expression between initial GM-CSF experiments.

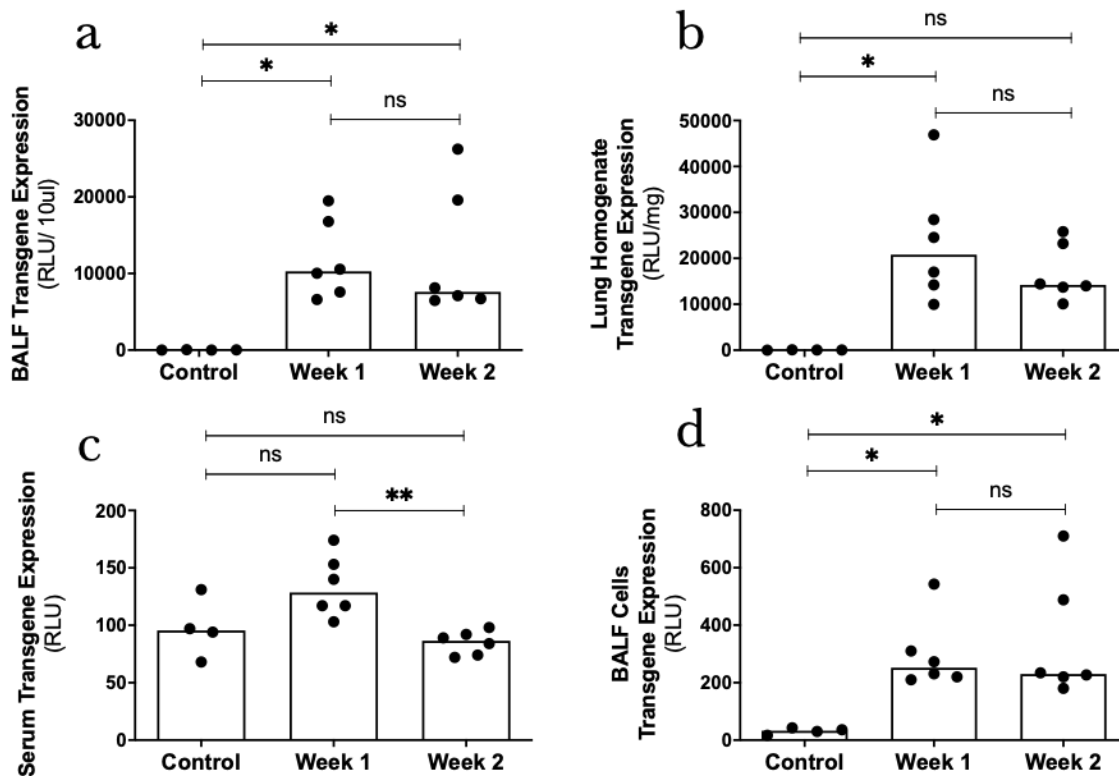


Figure 28 - Sustained expression of pulmonary Glux from gene-modified bone marrow derived macrophages (BMDM) *in vivo*. Glux expression in (a) BALF, (b) lung homogenate, (c) serum, and (d) BALF cells, one and two weeks post-delivery of 6.32e6 BMDMs per mouse. Transplanted cells were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine Glux at MOI 20 and then delivered via an oropharyngeal protocol in 50ul of PBS. Control mice were treated with an equivalent dose of un-transduced macrophages (n=2/time point) and pooled across time points for expression analysis by Pierce™ Gaussia Luciferase Glow Assay Kit. Glux expression quantified as relative light units (RLU), in 10 µl of sample. Lung homogenate values were then normalised to total protein concentration (mg/mL). Each circle represents a single mouse. Bars indicate median.

4.3.4.2 *The number of BMDMs retained by the lung decreases over 2-weeks post-transplant*

Absolute numbers of transplanted donor macrophages in BALF were identified by CD45.1 expression by flow cytometry. The absolute number of cells present in the total BALF sample were then calculated using CountBright™ Absolute Counting Beads and showed a significant decrease in donor cell retention between time points (median difference: 1176 cells retained, $p < 0.01$, Mann-Whitney t-test), though cells were still present after 2 weeks (median: 540 cells retained (range 344–1541) (Figure 29a). This was also true when comparing what percentage of live BALF cells are donor derived (Figure 29b). Control cells demonstrated a higher rate of retention (due to low n numbers statistical comparisons were not performed) though viability of BMDMs across treatment groups were all over 95% upon delivery suggesting SIV transduction or transgene expression may reduce cell engraftment or long-term viability. 1 week post-delivery, the rate of cell retention was on similar to previous results, where Glux/BMDMs were harvested at the same time point at low and high doses (Figure 29c). These results suggest that the lung has an upper limit to the number of donor cells it will support and demonstrate that a single dose of genetically modified bone marrow derived macrophages can sustained *in vivo* transgene expression for up to two weeks post-delivery.

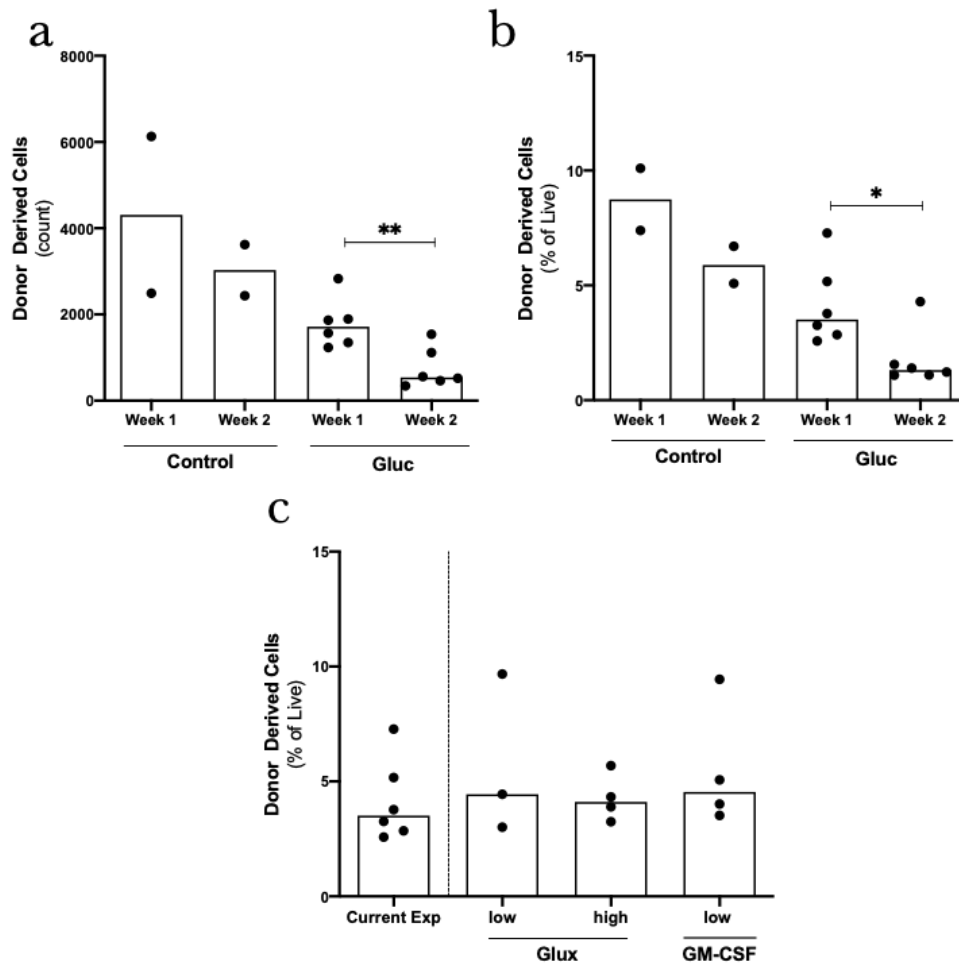


Figure 29 - Pulmonary retention of bone marrow derived macrophages (BMDM) expressing transgenic Glux over time. (a) Donor-derived macrophages from C57BL/6-Ly5.1 transduced to express Glux (rSIV.F/HN.hCEF.Glux, MOI 20) were identified in BALF cells by flow cytometry expression of CD45.1⁺ one week and two weeks following pulmonary delivery of 6.32e6 BMDMs (n=6/group). Control mice were treated with an equivalent dose of un-transduced macrophages (n=2/time point). Absolute counts of donor cells in BALF were extrapolated using counting beads. (b) Retention one week and two weeks after transplantation was also quantified as the percentage of CD45.1⁺ cells from a total of all live cells (identified by ToPro-3 staining). *=p<0.05, **=p<0.01, Mann-Whitney t-test. (c) Comparison of percentage of CD45.1 cells in BALF identified here one week post deliver, to those found at one week in the previous experiment (4.3.4.2) where cells were dosed with either 2e6 (Low) or 6.32e6 (High) gene-modified BMDMs delivered expressing transgenic Glux or GM-CSF. Each circle represents a single mouse, bars indicate median.

4.3.5 GM-CSF secreted by BMDMs ameliorates biomarkers of PAP disease

Having established that macrophages are retained in the lungs for up to two weeks where they mediate sustained transgene expression in the lung, I then investigated whether macrophages could secrete transgenes at therapeutically relevant levels. GM-CSF knock-out (KO) mice recapitulate the phenotype of acquired pulmonary alveolar proteinosis, where a lack of GM-CSF signalling leads to defective surfactant clearance and deposition in the alveoli. Unpublished work by Dr Helena-Lund Palau has characterised this mouse model and demonstrated that expression of transgenic GM-CSF following *in vivo* transduction with the rSIV.F/HN.hCEF.GM-CSF vector leads to a reduction in PAP biomarkers, including BALF turbidity, and the burden of surfactant protein-D (SP-D) in BALF and lung homogenates as detected by SP-D ELISA and the accumulation of surfactant extracellular proteinaceous periodic acid-Schiff (PAS)-positive material in histology lung slices. These mice were therefore used as a disease model for proof-of-concept that *ex vivo* transplantation of genetically modified macrophages can produce therapeutically relevant levels of secreted proteins.

GM-CSF KO (PAP) mice were dosed with 6.32e6 GM-CSF/BMDMs (n=6/time point) or Gluc/BMDMs controls (n=5/time point) transduced at MOI 20 and harvested for biomarker analysis 2 and 4 weeks later. Having confirmed macrophage retention and transgene expression at two weeks, I wanted to test to a further time point. 2- and 4-week time points were chosen for initial proof-of-concept studies based on the window of GM-CSF expression required to observe phenotype amelioration in unpublished *in vivo* viral delivery studies by Helena Lund-Palau and Claudia Juarez-Molina. The PAP phenotype is age related in GM-CSF KO mice and increased surfactant accumulation is established by 2 months and steadily increases as mice age (Helena Lund-Palau, unpublished). Here mice were between 4.47–6.7 months of age which was as close to age matched as possible with the available mice. Untreated WT (n=6) and untreated PAP (n=3) mice were also included for comparison. These mice did not receive any intervention or placebo treatment before harvesting. Due to low availability of PAP mice following COVID pandemic related reduction of breeding stocks, only three untreated PAP mice were available. This initial study is therefore not designed to evaluate the effect of macrophage therapy (regardless of transgene) on natural disease progression. Gluc.BMDM treated mice therefore served as the negative control for GM-CSF.BMDM mice.

In this study only the right lung was selectively lavaged to collect BALF, and the left lung was instead fixed and processed for histological assessment of PAS staining. Due to an unforeseen complication, PAS analysis was delayed and is therefore not included here.

4.3.5.1 In vivo transgene expression is sustained over 4 weeks

BALF and lung homogenate of PAP mice reported sustained Glux expression with no significant decrease over time. Though expression did trend downwards this suggests that following transplantation, macrophages are retained in the lung at a steady state for up to 1 month (Figure 30):

Lung homogenate

- *Week 2*: median 9818 (range 4391–57661) RLU/mg
- *Week 4*: median 5851 (range 1853–8050) RLU/mg ($p=0.4206$ (ns), Mann-Whitney t-test)

BALF

- *Week 2*: median 18050 (range 8665–21963) RLU in 10 μ l
- *Week 4*: median 13864 (range 11104–15775) RLU in 10 μ l ($p= 0.3095$ (ns), Mann-Whitney t-test)

GM-CSF expression similarly exhibited detectable levels in lung homogenate at week 2 (median 23.80 (12.34–53.76) pg/ml), though expression was not uniformly positive across all mice, and was absent in all but one mouse at week 4 (23.63 pg/ml). No conclusive GM-CSF expression was detected in BALF as levels fell largely below the linear range of the standard curve.

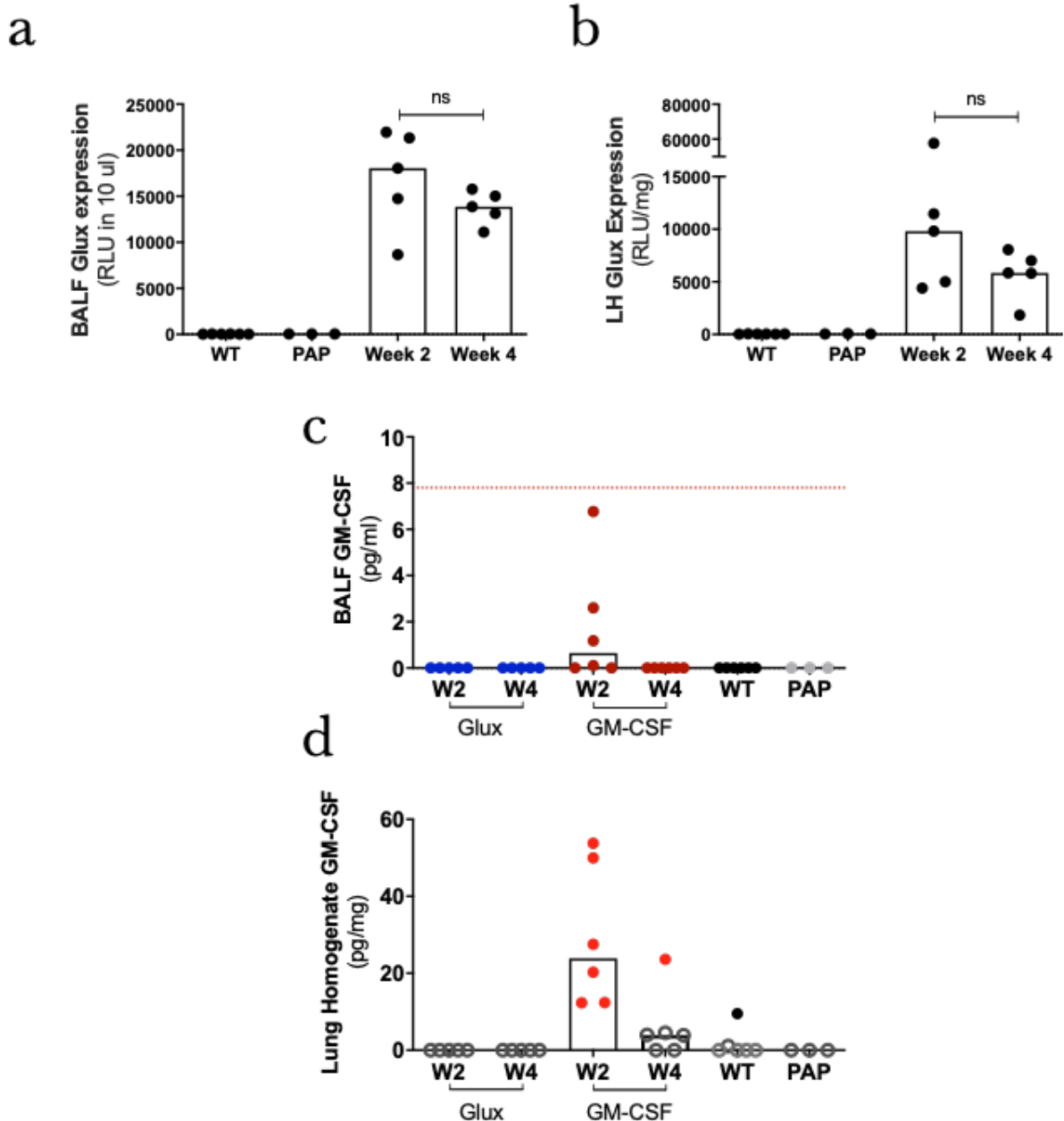


Figure 30 - Transgene expression of pulmonary Glux from gene-modified bone marrow derived macrophages (BMDM) *in vivo*. GM-CSF KO (PAP) mice received a single dose of 6.32×10^6 BMDMs transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine GM-CSF ($n=6$ /time point) or Glux ($n=5$ /time point) transgenes (MOI 20). Untreated C57B/6J (WT) and PAP mice were included as controls but not used in statistical comparisons. Glux (RLU – relative light units) expression was quantified in 10 μ l of **(a)** bronchoalveolar lavage fluid (BALF), and **(b)** lung homogenate by a Pierce™ Gaussia Luciferase Glow Assay Kit. ns: non-significant, Mann-Whitney t-test. GM-CSF (pg/ml) expression was quantified in **(c)** bronchoalveolar lavage fluid (BALF), and **(d)** lung homogenate by a mouse GM-CSF Quantikine ELISA kit. Lung homogenate values were normalised to total protein concentration (mg/mL), samples measuring values below the ELISA lower limit of detection (7.8 pg/ml)

– red dotted line) before normalisation are indicated by empty circles, where each circle represent a single mouse. Bars indicate median.

4.3.5.2 BMDM transplantation therapy ameliorates biomarkers of PAP disease 4-weeks after treatment

Due to surfactant build up, PAP BALF is characteristically milky in appearance and highly turbid which can be quantified by measuring absorbance at 600nm (361). Here I showed that in comparison to Gluc/BMDM treatment controls, at week 4 there was a significant reduction in turbidity indicating a clearance of surfactant (Figure 31a):

- *Gluc*: median 4.38 (range 2.99–5.12) OD 600nm,
- *GM-CSF*: median 2.03 (range 1.39–3.19) OD 600nm, ($p < 0.05$, 2wayANOVA Sidak's multiple comparisons)

In comparison to WT untreated controls (median 0.43 (range 0.37–0.46) OD) BALF turbidity indicates that the surfactant burden has not been completely resolved. PAP untreated controls did report high a BALF turbidity, however n numbers were low ($n=3$, individual turbidity's: 2.06, 3.36, and 4.46). Furthermore, the large spread of data, indicates that larger comparisons between treated and untreated mice are required to evaluate the effect of macrophage delivery on BALF turbidity.

Direct quantification of the surfactant burden in the lungs through SP-D ELISA of BALF samples also demonstrated a trend in reduction of surfactant protein-D over time (Figure 31b):

Week 2:

- *Gluc*: median 6201 (range 2287–7973) ng/ml
- *GM-CSF*: median 2573 (range 1630–6216) ng/ml

Week 4 :

- *Gluc*: median 4987 (range 1516–6416) ng/ml
- *GM-CSF*: median 1435 (range 708–8680 ng/ml)

This reinforced conclusions that the reduced BALF turbidity seen in treated mice at week 4 is as a result of transgenic GM-CSF expression rescuing surfactant homeostasis. Differences between treatment groups were not significant and there was a high degree of variation within cohorts. However, these data support a causative role for functional GM-CSF expressed by BMDMs, as at both time points the SP-D concentration trended downwards in GM-CSF treated mice when compared to Gluc controls. This supports the hypothesis that undetectable levels of BMDM driven GM-CSF expression can still be above the clinical threshold in this model of aPAP. These large data spreads may be due to inconsistent delivery, though there was no evident failure noted during delivery. In the past, unsuccessful lung deposition was observed

when post-delivery, cell suspension exited the oral cavity of anaesthetised mice upon being placed in a prone position on tissue and was not observed here. Lung homogenate samples were also collected, however, due to error during processing SP-D quantification could not be performed.

Though all mice were as close in age, retrospectively it was realised that due to an oversight during dosing, GM-CSF mice were all older than Gluc treated mice when dosed:

Week 2:

- *Gluc*: median age 5 (range 4.7–5) months
- *GM-CSF*: all 6.7 months of age

Week 4:

- *Gluc*: all 4.47 months of age
- *GM-CSF*: all 6.7 months of age

As such the experiment was not optimally controlled for age and therefore disease progression. It is unclear how much of an effect this might have had on disease biomarker analysis. However, as GM-CSF mice are all older, it suggests that if there is an effect, it would be to underestimate the effect of therapy in comparison to Gluc controls.

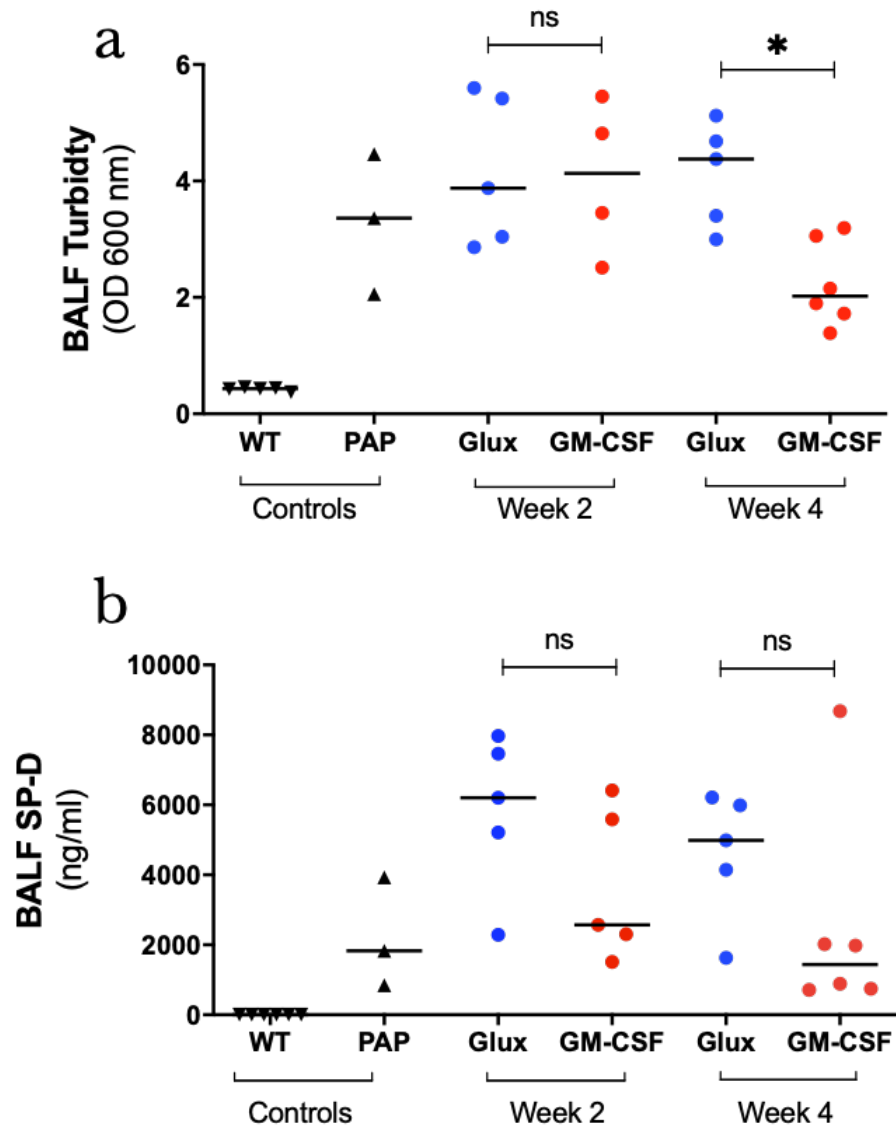


Figure 31 – Biomarkers of PAP disease are reduced following pulmonary delivery of genetically modified bone marrow derived macrophages (BMDMs). GM-CSF KO (PAP) mice were dosed with 3×10^6 BMDMs transduced with an rSIV.F/HN lentivirus encoding either a GM-CSF ($n=6$ /time point) or Glux ($n=5$ /time point) transgene and harvested for analysis of PAP disease biomarkers (**a**) BALF turbidity and (**b**) concentration of surfactant protein-D (SP-D), two and four weeks later. Untreated WT ($n=6$) and PAP mice ($n=3$) were included as controls. The turbidity of BALF fluid was measured as the optical density at 600 nm. Two BALF readings of GM-CSF treated mice at week were not included due to improper processing. $*=p<0.05$, 2wayANOVA Sidak's multiple comparisons. Each circle represents a reading from a single mouse. Bars indicate median.

4.3.5.3 The retention of transplanted cells in the PAP mouse lung could not be quantified by flow cytometry

To determine the retention and degree of macrophage engraftment, BALF cells were stained with a CD45.1 antibody and analysed by flow cytometry with CountBright™ Absolute Counting Beads. In this study only the right lung was selectively lavaged to collect BALF, as a result the estimations of donor derived cells in BALF are not comparable to previous experiments. However, due to accumulation of surfactant and extracellular material build up in the PAP BALF, flow cytometry identification of donor-derived cells was not possible and could therefore not be correlated to gene expression or surfactant clearance. This is evident in SSC vs FSC plot where cells are usually identified by size, as in untreated PAP controls the characteristic lung debris obfuscated identification of cell populations, and using gates drawn using WT controls could not successfully analyse live cell populations (Figure 32). It is also possible however, that due to surfactant build up, removal of macrophages from the lungs was impeded and that therefore the cells were not present in the BALF to begin with.

Similar studies following macrophage transplantation to the lung of mice models of hereditary PAP reported no similar issues with flow cytometry protocols but mentioned the use of percoll gradients to clear BALF samples (177). However, no further details were provided regarding the protocol in the paper referenced (32). I therefore developed a generic percoll gradient protocol using density centrifugation of samples, and though some debris was removed, identification of cell populations including donor-derived cells, was still not possible at 2- and 4- week time points with any sample even using gates drawn in WT mice samples (Figure 32). Thus, in this experiment direct measures of donor-cell retention in the PAP lung were not possible and rely instead on transgene expression, phenotype amelioration and data from WT controls. Before advancing with future PAP studies, alternative methods of donor-derived macrophage detection or PAP BALF analysis need to be developed.

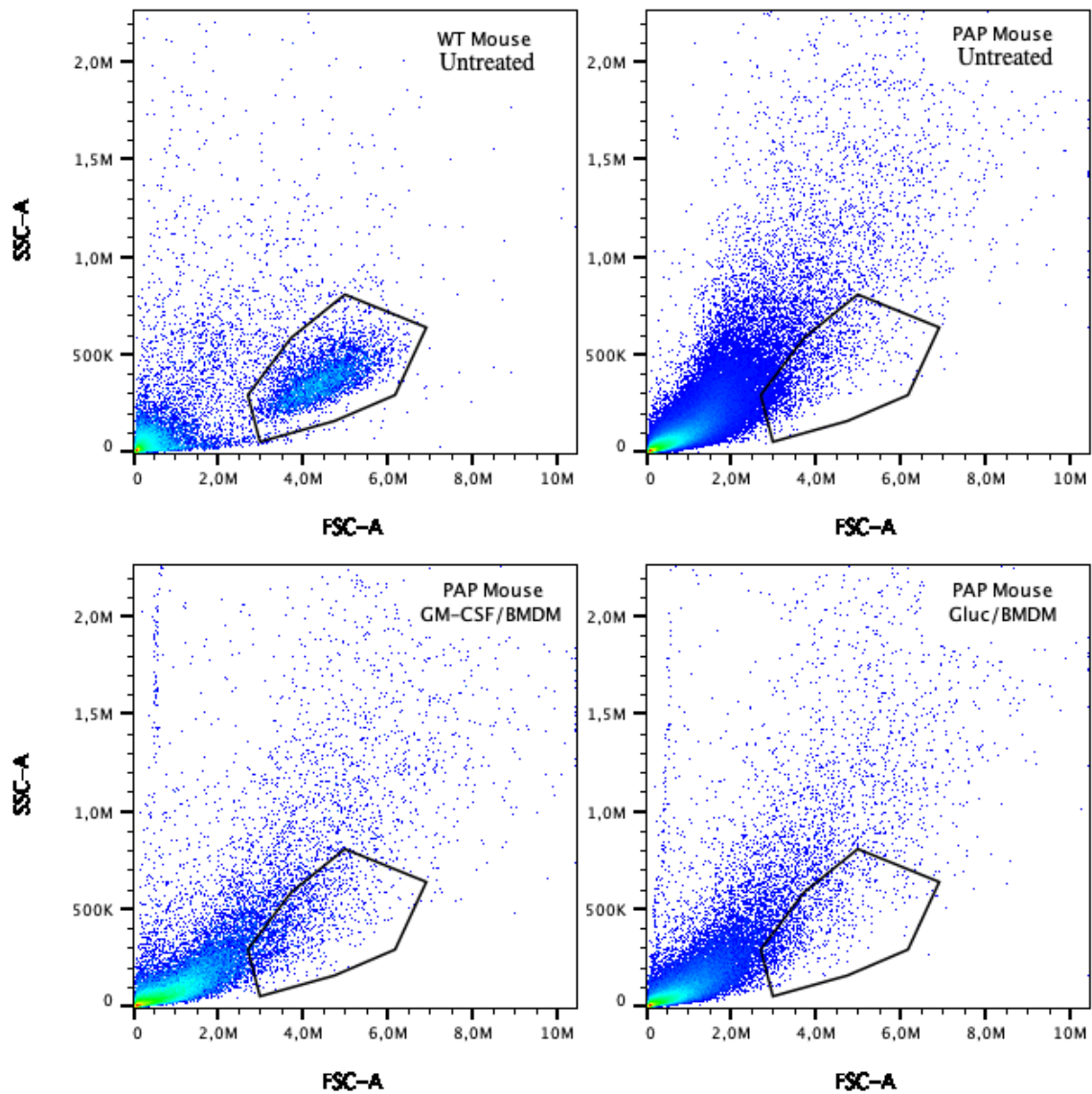


Figure 32 – Flow Cytometry Analysis of BALF cells from GM-CSF KO (PAP) mice. Cell populations in pelleted BALF samples from PAP mice were not present or detectable by flow cytometry. Here representative flow cytometry plots of WT and PAP untreated mice, and PAP mice treated with Gluc or GM-CSF BMDMs at two weeks are shown. Gating based off size identified cell populations in WT mice BALF, but the same gate was unable to isolate corresponding cells in PAP mice which showed a large amount of debris present. BALF cells from PAP mice treated with BMDMs were first subjected to a percoll density gradient which did not enable successful cell analysis. FCS-A: Forward scatter – Area, SSC-A: Side Scatter – Area.

In summary, I have demonstrated that following pulmonary transplantation of BMDMs transduced with lentivirus, therapeutically relevant levels of recombinant proteins are produced *in vivo*. In a mouse model of aPAP, the subsequent expression of GM-CSF showed evidence of decreasing the surfactant burden in the lungs after 4 weeks of treatment. Whether these trends will continue over time and with increasing significance, or whether this effect is short lived will require longer term investigation. However, direct proof of macrophage driven gene expression *in vivo* has not yet been established and the potential for vector carry-over in the cell suspension has not been eliminated. Here this was further obfuscated by a failure of the flow cytometry protocol to identify donor cells in the BALF of PAP mice.

4.3.6 A single dose of BMDMs leads to sustained transgene expression for up to 16 weeks in wildtype mice

Having established that in WT mice, transplanted cells are retained for up to two weeks post-delivery and having observed sustained transgene expression (Glux) for up to 4 weeks in PAP mice, I carried out a longer duration experiment. Glux/BMDMs transduced at MOI 20 were delivered to mice and harvested at 2, 4, 8 and 16 weeks to investigate the duration of cell retention and transgene expression (n=6/group). Overall, the procedure was well tolerated, with no signs of suffering observed following recovery from anaesthesia, or failure to thrive in the following weeks after treatment. Due to availability of donor cells, mice received a dose of 3e6 cells, which is different to previous experiments and unfortunately precludes cross-experimental comparisons. However, the cells dose was here consistent across all time points and controls enabling characterisation of cell retention and transgene expression over time. One cohort of control mice receiving un-transduced BMDMs were harvested at week 2 (n=6). As controls were only included at a single time point, these mice served as a negative control for gene expression at all other time points. These mice were harvested at week 2, to correspond with maximal levels of Glux expression in treated mice. As in the previous study, here only the right lung was selectively lavaged to collect BALF. The left lung was instead stored in RNAlater for molecular analysis of VCN and transgene expression, however this work is still ongoing and therefore not included here.

4.3.6.1 *Glux expression is sustained for up to 16 weeks in bronchoalveolar lavage fluid and lung homogenate*

In treated mice, Glux expression was present between 2 and 16 weeks post-delivery suggesting that *ex vivo* transduced macrophage therapy can provide long-term delivery of secreted proteins *in vivo* (Figure 33a-b). In lung homogenate at week 16 there was a significant drop in Glux expression, as in comparison to untreated controls, expression levels were no longer significant (Control: median 46.06 (32.52–67.08) RLU/mg; Week 16: median 2933 (2602–4247) RLU/mg, p=0.95 (ns), Kruskal-Wallis test, Dunn's multiple comparisons). However, Glux levels in BALF samples were highest at week 16 than at any other time point (median 206981 (182401–26874) RLU in 10 µl). This suggests that when looking at the overall levels of Glux expression in the lung as a whole, across BALF and lung homogenate samples, Glux expression is sustained over a 16-week time-point. It may be that the reduced Glux detection in lung

homogenate is due to differences in BALF sampling technique or success, though across time points the same procedure was performed by the same collaborator who collected samples from all mice with the exception of half the mice harvested at week 2. As flow analysis of CD45.1 was unreliable, further information regarding macrophage location (as might be obtained from histological analysis) was not collected here, and as samples were collected by a colleague when I myself was not present, it is difficult to determine what might be the likely cause of this discrepancy. It is therefore currently unclear what may have caused this differential detection of Glux in the lung homogenate and BALF, and as this was the first pilot long-duration experiment it should be repeated to confirm any conclusions. However, overall, the data indicates transgene expression is maintained for up to 16 weeks post macrophage delivery.

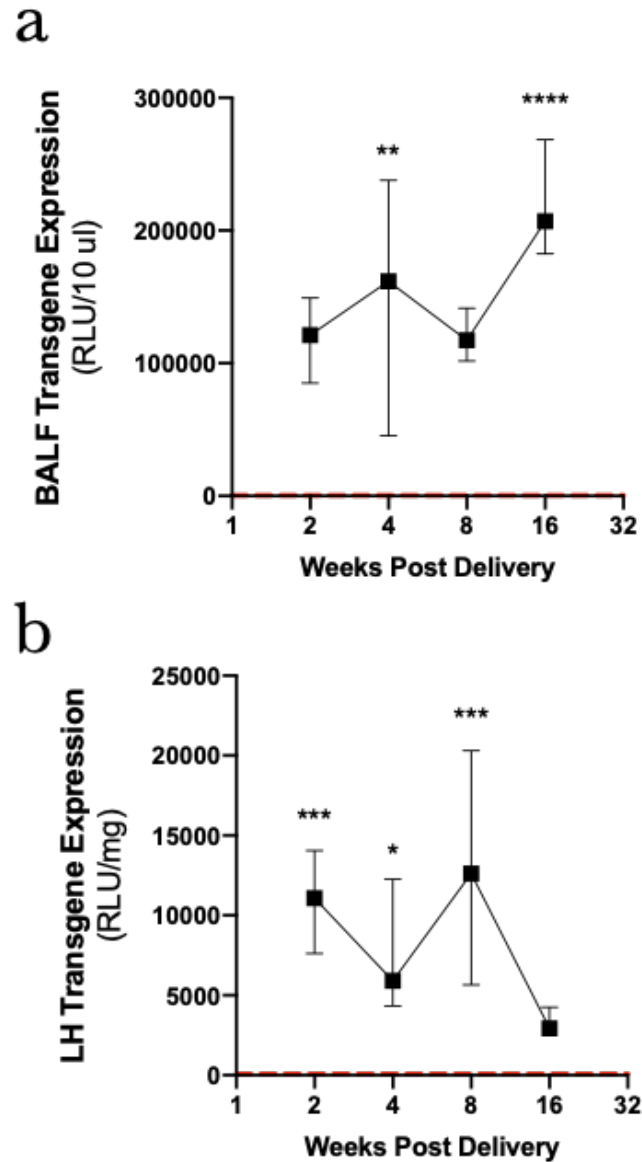


Figure 33 – Sustained Glux expression *in vivo* following bone marrow derived macrophages (BMDMs) transplantation. BMDMs were transduced *ex vivo* with rSIV.F/HN lentivirus encoding murine Glux under the control of the hCEF promoter at MOI 20. Following a single dose of 3×10^6 cells delivered via oropharyngeal delivery Glux expression was detected in **(a)** bronchoalveolar lavage fluid (BALF) and **(b)** lung homogenate (LH) 2, 4, 8, and 16 weeks later (n=6/group). Glux expression was compared to samples from control mice which received a corresponding dose of un-transduced macrophages and were harvested at week 2 (n=6), maximum reported Glux expression in these mice is indicated by red dotted line. Glux was quantified in 10 μ l of BALF and lung homogenate, the latter were then normalised to total protein concentration (mg/mL). Median values plotted; range indicated by error bars. (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$, Kruskal-Wallis, Dunn's multiple comparisons).

4.3.6.2 Quantification of transplanted cells retention in wildtype mice could not be reliably performed at later time points

Though the previous study suggested that un-transduced macrophages were retained better than transduced macrophages, this was not observed here with higher n numbers. There was no significant difference in the absolute number of donor cells (CD45.1) calculated with counting beads or the percentage of CD45⁺ BALF cells harvested at week 2 between control and treated mice (Figure 34a-b):

- *Cell Count*: median difference: 20.96 donor-derived cells (p=ns, Kruskal-Wallis, Dunn's multiple comparisons)
- *% Of BALF*: median difference: 0.67 % donor-derived cells (p=ns, Kruskal-Wallis, Dunn's multiple comparisons)

This suggests that modification by SIV.F/HN and transgene expression is not affecting cell viability or engraftment *in vivo*.

Over time there was a significant reduction in donor-derived cell counts (p<0.05 main effect, 2wayANOVA) where at week 16 donor-derived cells were rare (median 29.36 cells in BALF (range 19.56–39.91). However, at week 16, and to a lesser extent week 8, the total number of cells in BALF (identified by size gating) were extremely low calling the validity of the analysis into question, as at these low numbers of events, calculating donor-derived cells is unlikely to be accurate (Figure 34c-e):

- *Week 8*: median 434.5 (308–719) total cells in BALF
- *Week 16*: median 284.5 (90–420) total cells in BALF

It is worth noting that though the same person performed all the lavages, the downstream processing was not, where week 8 and week 16 were performed by a colleague and therefore differences in processing may have resulted in cell loss during preparation for flow cytometry. As a result, robust conclusions cannot be drawn from these data and new protocols to detect donor derived cells directly are warranted. However, at weeks 2 and 4 when sufficient BALF cells were present in flow cytometry samples, the number of retained macrophages appears consistent:

- *Week 2*: median 1408 (915–3060) donor-derived cells in BALF
- *Week 4*: median 1081 (684–2936) donor-derived cells in BALF

As expression was still sustained up to a 16-week time point, it would suggest by proxy that donor-derived macrophages are still present. However, once more, a limitation of these data is that I have not confirmed that macrophages are driving the observed transgene expression *in vivo*.

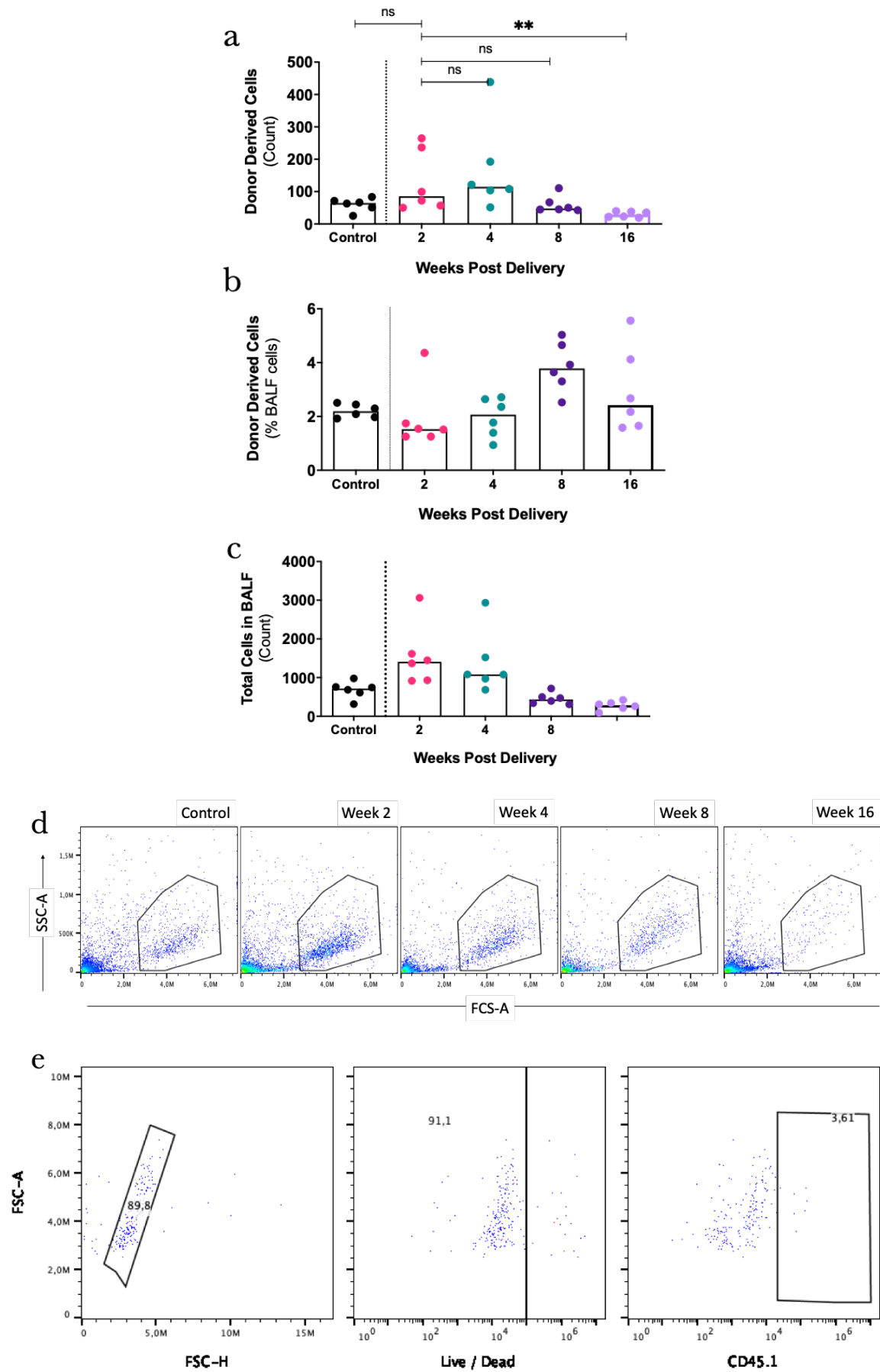


Figure 34 – Retention of bone marrow derived macrophages (BMDMs) in the lung following transplantation. A single dose of 3×10^6 BMDMs transduced *ex vivo* with rSIV.F/HN.hCEF.Glux were delivered via oropharyngeal delivery to WT mice. **(a)** The number of donor-derived cells (CD45.1⁺) in BALF samples were identified by flow cytometry in the BALF of mice harvested 2, 4, 8, and 16 weeks post-delivery (n=6/group). Absolute counts of donor-derived cells in BALF were extrapolated using counting beads. **=p<0.01, Kruskal-Wallis, Dunn's multiple comparisons. **(b)** Retention over time was also quantified as the percentage of CD45.1⁺ cells from a total of all live cells (identified by ToPro-3 staining). **(c)** The total number of cells in BALF identified by gating for size in flow cytometry (FCS-A, SSC-A), and **(d)** representative plots for each treatment and control group demonstrating reduced cell numbers at week 16, and **(e)** a representative analysis of the subsequent gating strategy attempted with low cell numbers in BALF harvested from a mouse at week 16. Sequential gating strategy: size, single cells, live cells, CD45.1⁺. Each circle represents a single mouse, bars indicate median.

4.3.7 Calculating the recovery rate of donor-derived cells following pulmonary transplantation

To determine the success of bronchoalveolar lavage at retrieving transplanted cells, WT mice were dosed with un-transduced BMDM and immediately culled for lavage collection and subsequent quantification of donor-derived cells. Wildtype mice were dosed with 6.32e6 BMDMs from CD45.1 donor mice. They were then harvested 20 minutes later, and lavaged to collect BALF cells for flow cytometry analysis. Here only the right lung was lavaged to control for PAP experiment (though this analysis later failed) and the long-term duration study. If 20 minutes post-delivery, all transplanted cells are still present in the lungs, the 1/2 lung lavage flow cytometry protocol was found to have a recovery rate of less than $0.048 \pm 0.013\%$ (Table 7)

Table 7 – Calculating the recovery rate of donor-derived cells in BALF by flow cytometry.

Flow cytometry analysis of CD45.1⁺ cells was used to calculate the percentage of donor derived cells in BALF, and with counting beads estimate the total number of retired donor-derived cells in BALF (count). The recovery rate of 6.32e6 delivered cells was then calculated for each mouse (row). Last row: mean $\pm$ SEM.

Donor-Derived Cells (% of BALF cells)	Donor-Derived Cells (Count)	Recovery Rate of Delivered Cells (%)
24.9	2300	0.036
51.4	1880	0.030
47.5	6350	0.101
16.0	1590	0.025
19.8	1570	0.025
42.1	4590	0.073
Mean: 33.6 $\pm$ 6.2	3047 $\pm$ 806	0.048 $\pm$ 0.013

4.3.8 A conditional promoter system leads to inducible transgene expression in bone marrow derived macrophages

Following *in vivo* transgene expression driven by a constitutive promoter/enhancers in WT mice, transplanted macrophages sustained expression of a reporter Gaussia luciferase for up to 16 weeks. In PAP mice reporter gene expression was detectable for up to 4 weeks and though GM-CSF fell below levels of detection, it had a measurable impact on the PAP phenotype. Here, constitutively active hCEF drove continuous transgene expression in the lung. However, haematological toxicity is a potential side effect of off-target GM-CSF activity and is a noted concern in recombinant GM-CSF therapy for PAP (384, 385) and has been observed due to the sustained GM-CSF expression achieved following *in vivo* administration of rSIV lentivirus (Helena Lund Palau - unpublished manuscript). PAP gene therapy may therefore benefit from regulated protein expression, where pulmonary GM-CSF secretion *in vivo* can be controlled and turned 'off' or 'on'. This may also enable mimicking of inhalation therapy on/off dosing regimens, where periods of daily GM-CSF treatment delivered at regular intervals can lead to temporary alleviation of PAP symptoms in patients (161, 162, 311, 381, 399). Using macrophages with regulated gene expression to deliver therapeutic transgenes *in vivo* could also expand the therapeutic repertoire of the *ex vivo* macrophage therapy platform to other diseases with narrow toxicity-efficacy windows and make the therapy safer and more attractive to regulators.

To develop a more regulated and fine-tuned expression cassette, an alternative system of transcriptional control in lentivirus was adapted from the GeneSwitch™ system by my colleague Robyn Bell as part of her PhD (unpublished). GeneSwitch™ is a two-vector system composed of a transgene and transactivator gene both under the control of minimal promoters and is summarized in Figure 35. The transgene is under transcriptional control of a GAL4-E1b minimal promoter which according to manufacturers should be transcriptionally silent without additional factors. The GAL4-E1b promoter is activated by GeneSwitch™ regulatory protein (transactivator), encoded in a separate expression cassette and regulated by a minimal HSV TK promoter. The GeneSwitch™ regulatory protein is minimally expressed in the absence of the progesterone antagonist, mifepristone and has three functional domains:

1. A Gal4 DNA Binding Domain (Gal4-DBD) enables binding to the GAL4-E1b promoter,
2. A truncated human Progesterone Receptor Ligand Binding Domain (hPR-LBD),

3. And an NF κ B transcription factor p65 (p65AD) transcription activation domain that activates transcription from the GAL4-E1b promoter.

In the presence of mifepristone, the hPR-LBD domain on the GeneSwitch™ regulatory protein undergoes a conformational change, enabling activation of the GAL4-E1b promoter, resulting in transgene expression. It further upregulates its own expression by binding to a Gal4 DNA Binding Domain upstream of the HSV TK promoter, therefore amplifying the induction of expression of the gene of interest.

The GeneSwitch™ transactivator and inducible GAL4-E1b expression cassette were cloned into a lentiviral backbone to drive expression of reporter gene Glux. Two alternative vector systems were produced by Vector builder, where both elements are encoded separately by two vectors (2V), or where both are cloned into a single vector (1V)(Figure 19):

- 2V: pSwitch™ vector (pSV) encoding the transactivator + GAL4-E1b expression vector (GEV) encoding inducible Glux
- 1V: inducible combination virus (ICV) encoding both the transactivator (pSwitch™) and inducible Glux gene.

As the lentiviral vectors encoding this inducible system were produced by VectorBuilder who do not produce vectors with a SIV backbone or F/HN pseudotype, they have a HIV-1 lentiviral backbone and are pseudotyped with the vesicular stomatitis virus-G (VSV-G) envelope protein.

In collaboration with Robyn Bell, both vector systems were used to transduce BMDMs in pilot studies aiming to produce genetically modified macrophages with an inducible transgene expression system. BMDMs were also transduced with rSIV.F/HN.hCEF.Glux as a positive control of gene expression with a constitutive promoter, however as described in the following section, the experiment was not designed to directly compare the efficiency of the inducible system to hCEF constitutive expression. To control for basal levels of expression, MOI was controlled for vectors encoding the Glux transgene between treatment groups (MOI 10). Following an initial 24-hour transduction, daily media changes were performed over 5 days to measure Glux levels on a daily basis rather than the accumulative effect.

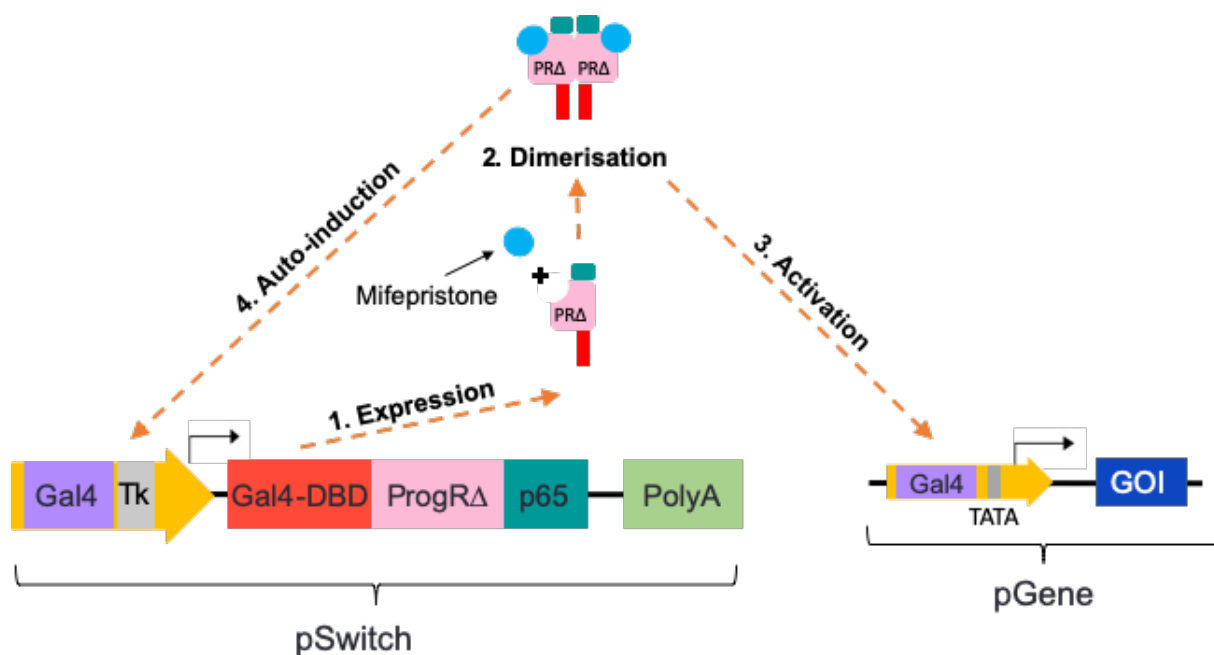


Figure 35 - Schematic representation of the commercial GeneSwitch™ system of regulated gene expression. The system is encoded in two separate expression cassettes on separate plasmids pSwitch and pGene. The former encodes a transactivator protein that has three active domains, 1) a Gal4 DNA Binding Domain (Gal4-DBD) that binds to Gal4 binding sites, 2) a truncated human Progesterone Receptor Ligand Binding Domain (ProgRΔ) that binds mifepristone, and 3) a NFØB transcription factor p65 (p65) transcription activation domain that activates transcription from the GAL4-E1b promoter. The pGene plasmid encodes a gene of interest (GOI) under the transcriptional control of the GAL4-E1b minimal promoter. In the presence of mifepristone, the transactivator protein dimerizes, undergoing a conformational change that permits binding of the GAL4-E1b promoter to stimulate transgene expression. It can also bind four Gal4 binding sites upstream of the minimal HSV TK promoter on pSwitch to auto-induce its own expression. Adapted from Robyn Bell at the Gene Therapy group at Imperial College London with permission.

4.3.8.1 Significant induction of Glux expression following 24-hour stimulation with mifepristone with a single and dual vector system

Glux expression in positive controls (rSIV.F/HN.hCEF.Glux) increased over time showing no evidence of having reached peak expression within the experimental window (Figure 36a). It is likely that vectors in the 1V and 2V system also did not reach peak expression and that subsequently these results may underestimate the degree of leaky and induced expression. Though higher levels of gene expression were observed in the positive controls, this experiment was not designed to allow robust comparisons between inducible and constitutive expression systems. In these initial experiments, the rSIV.F/HN.hCEF.Glux cohort was included only as a positive control of Glux expression and to determine the kinetics of vector entry, integration and expression in macrophages. This is in part because the inducible vectors were produced commercially by the company VectorBuilder who only offer limited options in the way of vector design, meaning vectors were produced with a HIV backbone (vs SIV) and VSV-G envelope (vs F/HN). In addition to having different lentiviral backbones and envelopes, dose was not strictly controlled between systems as the rSIV.F/HN.hCEF.Glux virus had previously been freeze-thawed which reduce viral viability (400). This is less than ideal, however as no direct comparisons were planned between the inducible and constitutive vectors owing to the differences in design, freeze-thawed virus was used to limit viral wastage. Viral titre was estimated by allowing for a ~10% decrease in viability with each freeze-thaw cycle, but as a result MOI was not strictly matched to inducible vectors.

A 24-hour incubation with mifepristone resulted in significant induction of Glux expression in both vector systems when comparing induced samples versus non-induced controls (Figure 36b-c). Gene expression was significantly induced in the 24 hours after mifepristone treatment (1V: $p < 0.001$, 2V: $p < 0.0001$, mixed-effects analysis, Sidak's multiple comparisons) and sustained for at least 48 hours after which expression dropped once more. As daily media changes were performed, this drop in expression was expected and indicated the reversible nature of mifepristone-induced gene expression following removal of the inducer from culture. Both systems therefore produced so-called 'inducible macrophages', which could be useful in providing regulated pulmonary transgene expression *in vivo*.

Though one would assume that dual transduction of two lentiviral vectors would be less efficient than transduction of a single vector, the 2V system resulted in significantly higher Glux expression than the 1V at each time point (Figure 36a), where the mean difference was:

- *Day 1*: 7.4e4 RLU/mg (P<0.001)
- *Day 2*: 1.2e5 RLU/mg (P<0.001)
- *Day 3*: 4.8e5 RLU/mg (P<0.0001)
- *Day 4*: 5.2e5 RLU/mg (P<0.0001)
- *Day 5*: 7.4e4 RLU/mg (P<0.001, Mixed-effects analysis, Sidak's multiple comparisons)

Though the dual vector system had significantly higher rates of 'leaky' Glux expression, (p<0.0001, simple linear regression, elevation comparison) expression was low and over time increased at an equal rate in both systems, (p=0.316, simple linear regression, comparison of slopes) (Figure 36e). As a result, the 2V system had a higher induction ratio (IR) at each time point which peaked at day 3 in both systems (Figure 36f-d):

- *2V*: 64 IR on day 3,
- *1V*: 7.5 IR on day 3

These data suggest that while both the 1V and 2V systems transduced BMDMs *in vitro* to produce inducible macrophages, macrophages transduced with the latter would lead to higher expression levels *in vivo*, while demonstrating comparable levels of leaky gene expression while in the 'off' state. An ideal inducible system exhibits precise regulation of expression, and therefore requires low background activity in the absence of the inducer (here mifepristone) and maximal expression in its presence. Of the two inducible systems tested, the two-vector system showed a lesser degree of deviation from the ideal.

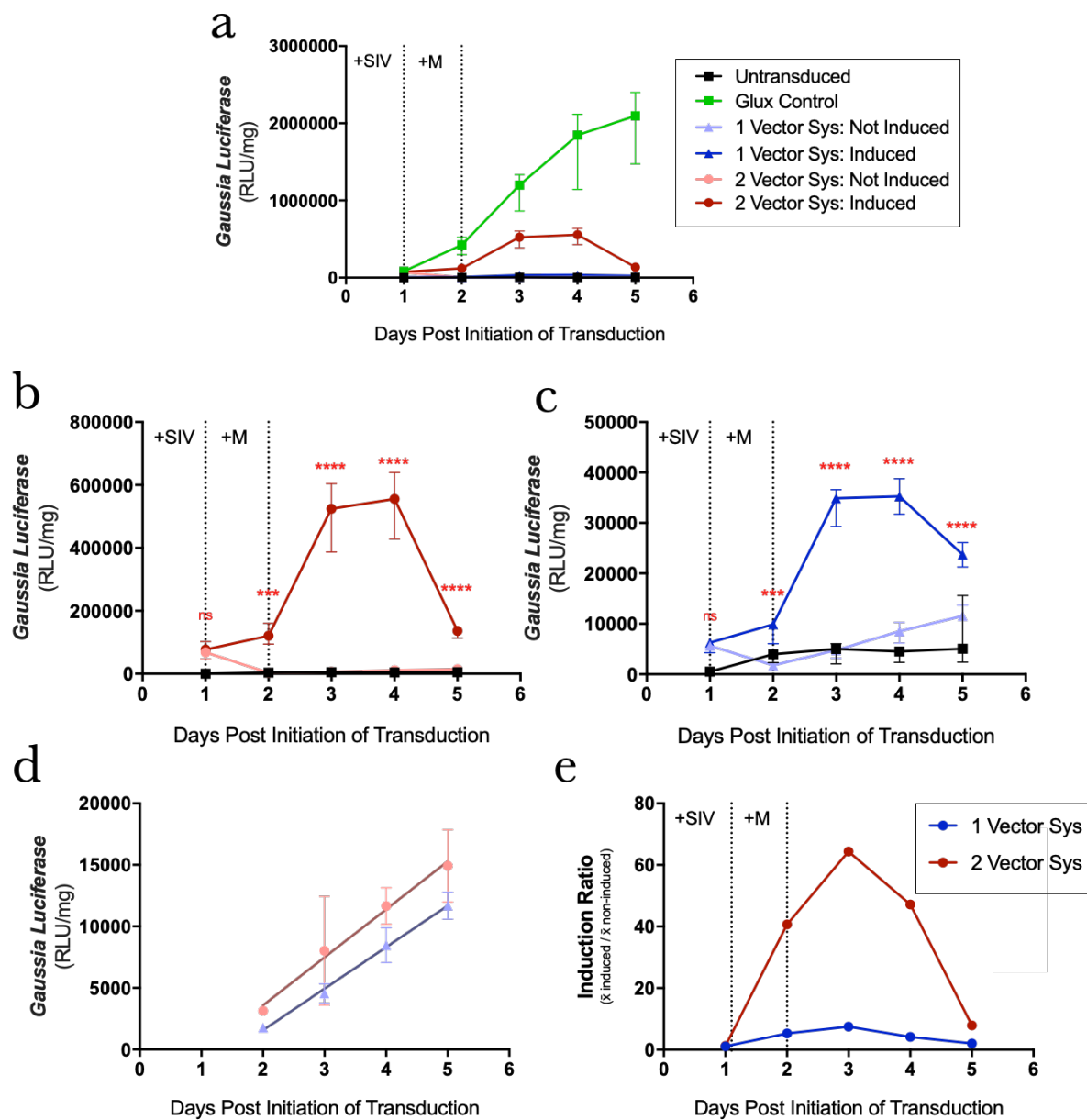


Figure 36 – Induction of Gaussia luciferase (Glux) transgene expression in bone marrow derived macrophages (BMDMs). BMDMs were transduced with one of two regulated expression lentiviral systems: **(b)** a dual (2) vector system or **(c)** a single vector system for 24 hours (+SIV) (MOI 10 – controlled for Glux encoding vector). Gene expression was induced by a 24-hour incubation with 10 μ M of mifepristone (+M), and Glux was quantified in cell culture media every 24 hours for 5 days, where at each time point cells were incubated with fresh media (1 ml). Expression in induced samples was compared to non-induced controls at each time point to determine the degree of induction for each vector system, and significant differences between these conditions is indicated at each time point in **(b)** and **(c)**. ns = $p > 0.05$, ***= $p < 0.001$, ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons). Positive controls were transduced with rSIV.F/HN.Glux whose expression steadily increased over time

and untransduced cells served as negative controls, and **(a)** the results of all test conditions and controls were plotted on one graph (n=6/group). **(d)** Untransduced controls exhibited increasing levels of leaky expression over time, each data set was fit to a model of simple linear regression. **(e)** The induction ratio of each vector system was calculated as the median difference in gene expression between induced and non-induced controls. Glux was quantified as relative light units (RLU) in 10 μ l of media by Pierce™ Gaussia Luciferase Glow Assay Kit and normalised by cell number at the end of the experiment by quantification of total protein (mg/ml). Data plotted as median $\pm$ range.

4.3.8.2 Gene expression can be induced multiple times by mifepristone with a single and dual vector system

To determine if gene expression could be repeatedly ‘turned on’, in a subsequent experiment, macrophages received a second dose of mifepristone once expression had fallen after an initial induction. As positive controls in the first experiment indicated that gene expression is unlikely to be at its peak 24 hours after transduction, cells were instead induced after a 48-hour transduction. Later time points were not possible as under the developed culturing protocol primary macrophages cannot be cultured indefinitely and so have a narrow window with which they can be manipulated *in vitro*.

In both vector systems, gene expression was induced on both day 2 and day 6 evidencing that transgene expression can be repeatedly switched on and that induction lasts for approximately 4 days (Figure 37a-b). In the dual vector system expression peaked following the first induction in media, collected on day 3 (median 5.2×10^5 , range 3.7×10^5 – 6.4×10^5), which correlated with a peak IR of 119 when compared to non-induced samples (Figure 37d). The second induction resulted in higher levels of gene expression, almost tripling levels seen on day 3 (median 1.1×10^6 , range 7.4×10^5 – 1.5×10^6). However, as non-induced samples also showed steady increases in Glux expression (Day 3: median 4.2×10^3 , range 3.0×10^3 – 6.2×10^3 ; Day 7: median 9.7×10^3 , range 7.6×10^3 – 1.3×10^4) the IR was comparable at 113. Additionally, in comparison to the previous test which reported a ratio of 64, both induction events in this second experiment led to higher rates of induction, indicating a longer transduction window or delayed onset of induction is conducive to higher gene expression.

The single vector system (1V) contrastingly exhibited peak gene expression on day 4 following the first induction (median 3.1×10^4 , range 1.5×10^4 – 7.4×10^4) (Figure 37c), with a ratio of 11.5 (Figure 37e). In comparison to the dual vector system, expression was lower and took longer to peak, but still occurred within a 4-day window. Following the second induction on day 6, Glux levels were over 4 times higher than the first induction, however again the IR was comparable at 10.8 (median 1.4×10^5 , range 5.7×10^4 – 2.0×10^5). In comparison to the previous test which reported an IR of 7.5, both induction events led to moderate increases in fold induction. Comparisons between experiments showed that a longer transduction window or delayed onset of induction is conducive to higher gene expression as larger induction ratios were seen in the second iteration.

In the previous experiment, non-induced macrophages demonstrated linear growth in Gluc expression over time, here leaky expression of the 1V system appeared to be increasing exponentially while the 2V system showed a slower rate of growth (Figure 37d). In addition to achieving higher levels of gene expression and induction, if current trends were to continue, un-induced gene expression in the 2V system may be lower than in the 1V.

As evidenced by the positive control, it is unlikely that within the experimental time-frame peak transgene expression was achieved, as following viral incubation, time is required for the vector lifecycle to be completed (395, 401)(Figure 37a). This explains the higher level of expression in induced samples on day 7 and the increase in un-induced samples which we hypothesise would eventually reach a plateau. However, it is possible that the system will continue to become inherently more unstable, or 'leaky', over time and *in vitro* tests in immortalised cell lines or *in vivo* will need to track expression over longer periods of time to confirm.

These initial experiments provided *in vitro* proof that following lentiviral gene-transfer of the Gene Switch™ expression cassettes, macrophages can be strictly regulated to produce a secreted protein.

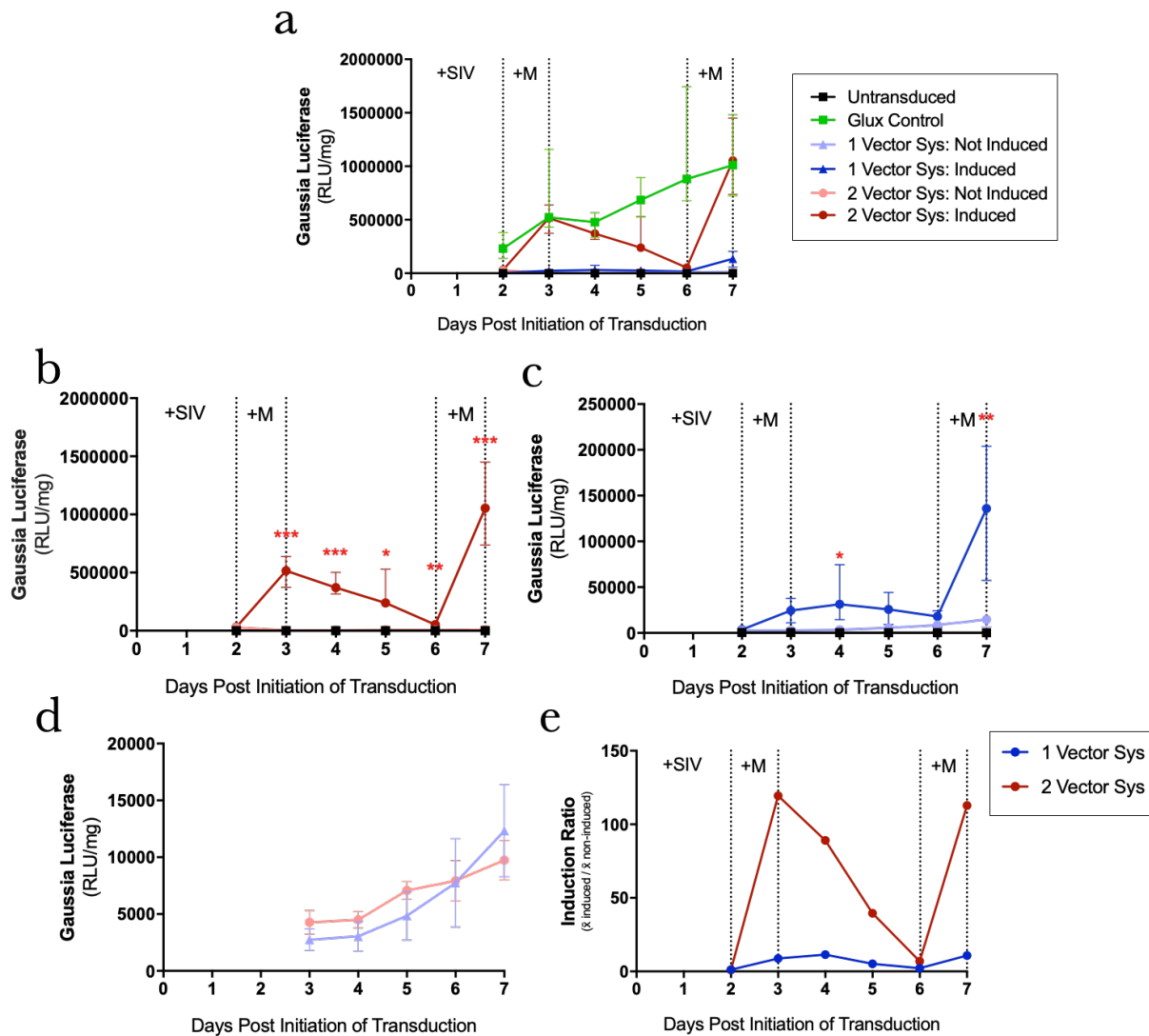


Figure 37 – Induction of Gaussia luciferase (Glux) transgene expression in bone marrow derived macrophages (BMDMs). BMDMs were transduced with one of two regulated expression lentiviral systems: **(b)** a dual (2) vector system or **(c)** a single vector system for 48 hours (+SIV) (MOI 10 – controlled for Glux encoding vector). Gene expression was induced by a 24-hour incubation with 10 μ M of mifepristone (+M) on day 2 and day 6. Glux was quantified in cell culture media every 24 hours for up to 7 days post transduction, where at each time point cells were incubated with fresh media (1 ml). Expression in induced samples was compared to non-induced controls at each time point to determine the degree of induction for each vector system, and significant differences between these conditions are indicated at each time point in **(b)** and **(c)**. *= p <0.05, **= p <0.01, ***= p <0.001, 2-wayANOVA, Sidak's multiple comparisons). Positive controls were transduced with rSIV.F/HN.Glux whose expression steadily increased over time and untransduced cells served as negative controls, and **(a)** the results of all test conditions and controls were plotted on one graph (n =6/group). **(d)** Untransduced controls exhibited increasing levels of leaky expression over time. **(e)** The induction ratio

of each vector system was calculated as the median difference in gene expression between induced and non-induced controls. Glux was quantified as relative light units (RLU) in 10 µl of media by Pierce™ Gaussia Luciferase Glow Assay Kit and normalised by cell number at the end of the experiment by quantification of total protein (mg/ml). Data plotted as median $\pm$ range.

4.4 Discussion

Here the rSIV.F/HN lentivirus produced by the UK Gene Therapy Cystic Fibrosis Consortium has been adapted from its initial *in vivo* specifications for an *ex vivo* approach to respiratory gene therapy. Proof-of-concept for the delivery of bone marrow derived macrophages (BMDMs) and the subsequent secretion of transgenic proteins in the lung was established using the secreted GM-CSF protein and Gaussia luciferase secreted reporter gene. Various protocols were developed to enable the characterization of this technology including the use of BMDMs derived from CD45.1 donor mice which then demonstrated that gene expression can be sustained with for up to 16 weeks in wildtype (WT) mice. A mouse model of adapted pulmonary alveolar proteinosis (aPAP) was also employed to demonstrate that clinically relevant levels of transgene can be expressed in the lung. Lastly an adapted commercial system for regulated gene expression was able to produce so called ‘inducible macrophages’ by providing strict in BMDMs.

4.4.1 Delivering genetically modified bone marrow derived macrophages to the lung

To secrete therapeutic transgenes *in vivo*, macrophages modified by SIV.F/HN were transplanted via oropharyngeal delivery to the mouse lung. The technique, performed under anaesthetic, is minimally invasive in mice as a cell suspension is delivered by pipetting into the oral cavity and requires only brief extension of the tongue to prevent swallowing and covering of the nose to encourage mouth breathing. When considering translation to the clinic, a relatively similar method of cellular deposition to the lung has been employed in a clinical trial whereby autologous adipose derived stromal cells were delivered directly to patient lung through endobronchial infusions using a bronchoscopic channel (402). Alternatively spraying technologies have been developed for cell delivery to the lung, optimized for uniform distribution and high cell viability (403). In the context of PAP, pulmonary cell delivery might therefore be more invasive than inhaled delivery of nebulized recombinant GM-CSF protein or virus nebulization for *in vivo* gene therapy, but would still be an improvement over WLL, the standard treatment of care for PAP.

This lung-targeted approach avoids introduction of transplanted cells into systemic circulation which could lead to off-target delivery. Following IV delivery, mesenchymal stem cells (MSCs) were widely distributed in the kidneys, lung, liver, thymus, and skin (404), and IV administered macrophages were able to cross the blood-brain-barrier (79). Alternatively, *in vivo* administration of vectors targeting the lung epithelium, has successfully employed nasal sniffing, where rSIV.F/HN shows high transduction of both the nose and lung epithelium (302). However, a nasal sniffing route raises concerns over entrapment in nasal passages as the nasal cavity functions to filter large particle deposition in the lungs (405), and off-target delivery as following administration to the nasal cavity, MSCs and neuronal stem cells have been shown to cross the olfactory epithelium and deliver cells to the central nervous system (CNS) (406-409).

4.4.2 Tracking Delivered Cells

Three assays were evaluated *in vitro* as potential assays for detecting transplanted cells *in vivo* in order to track the duration of macrophage pulmonary retention. Initial characterisations of an assay that would use Firefly luciferase expression to track macrophages *in vivo* has not yet been implemented. This was partially due to the belief that the levels of expression observed *in vitro* would not be high enough to translate to *in vivo* imaging. The advantage of such a system, however, is that it can provide *in vivo* tracking over time as luciferase expression can be detected in live animals (410). However, as only transduced cells will express the luciferase transgene, this method would not provide a real rate of engraftment as not all donor-derived cells are positively transduced. It could in the future be used in combination with another technique, such as CD45.1 expression, however in these initial proof-of-concept experiments for *in vivo* transgenic protein production, I chose to use secreted reporter gene instead.

A fluorescent dye-based system using the CellTrace™ CFSE dye was also briefly investigated and has not yet been implemented. This system does not require lentiviral transduction and therefore could be used in conjunction with a secreted transgene. Its use *in vivo* has not been tested; but it has the potential to provide information regarding *in vivo* proliferation of transplanted macrophages as with each cellular division the dye is diluted (411, 412). Other studies have used Ki-67 protein as a marker of AM proliferation as expression strictly correlates with proliferation and is only present during all active phases of the cell cycle (413). Following pulmonary transplantation into *Csf2rb*^{-/-} mice, Arumugam *et al* used Ki67 expression to

calculate AM proliferation, however this measured overall levels and did not specifically measure proliferation of donor-derived macrophages (176). The CFSE dye might therefore offer specific understanding of transplanted macrophage dynamics *in vivo*. Manufacturers claim a minimum of 6 generations can be visualised with this kit, and its use *in vivo* will require optimisation to determine if CFSE can be detected in macrophages and for how long following transplantation.

The third assay is commonly used in literature for the labelling of transplanted cells as cells derived from C57BL/6-Ly5.1 animals with a CD45.1 allele can be specifically detected by flow cytometry monoclonal antibody staining and differentiated from cells of congenic C57B/6J mice expressing the CD45.2 allele (393). Here it was able to detect donor derived cell populations following *in vivo* delivery of BMDMs. One of the limitations of these mice, however, is that recent research implies that in bone marrow transplantation studies CD45.1 cells have a slight competitive disadvantage to CD45.2 cells and may therefore show reduced engraftment in the presence of functional AMs (414, 415). These mice are however still widely used today, and it was determined to be the best method available for tracking donor-derived cells. Here counting beads were used to determine the absolute number of donor-derived macrophages in BALF however, a limitation is that it does not therefore control for the success of the lavage procedure which may vary, as highlighted by the variation in BALF volume that can occur between samples (168, 416). This issue was highlighted in the duration experiment where at weeks 8 and 16, the total number of cells (identified by size gating) in BALF was very low. At these time points, the downstream processing of the BALF for flow cytometry analysis was carried out by a collaborator who had not previously performed or observed the technique and therefore the low numbers may be as a result of inter-researcher variation. As a result, the accuracy of the analysis was poor. Alternatively, the donor-derived cell retention was expressed as a percentage of all live cells in BALF, however this similarly faced issues of accuracy at low total BALF numbers. Other papers have instead first gated BALF cells for F4/80 monocyte/macrophage markers and then expressed the percentage of donor derived macrophages (CD45.2) as a percentage of endogenous AMs (CD45.1) (176, 177). Future experiments should adopt this protocol as it will better control for the success of lavage between mice, and in retrospect should have been used here. As a result, CD45.1 analysis in this work is limited in its ability to inform the relative engraftment of transplanted cells in the AM compartment. However, the low number of BALF cells obtained in samples at week 8 and 16 would still be problematic and suggests the development of an additional non flow cytometry-

based analysis method, such as histological staining of non lavaged lungs for donor-derived macrophages. Furthermore, the retrieval rate of the lavage - flow cytometry procedure was only 0.048% and suggests that a large majority of engrafted cells may not be removed during BAL and that here engraftment may therefore be underestimated. Increased washing of the lungs to further remove cells could potentially increase the retrieval rate of the technique, as here per half lung only 250 µl of PBS were used to flush the lungs 3 times. This protocol was initially developed for the analysis of secreted gene expression in BALF rather than the collection of lung macrophages and therefore increased washes with higher volumes may increase the success of transplanted cell retrieval. For example, one study aiming to isolate AMs for adoptive transfer studies, lavaged airways six times with 0.5 ml of PBS (417). It is also possible however that the low retrieval rate is owing to the success of the delivery technique and future experiments should address the possibility of off-target delivery, such cellular deposition in the stomach of mice when using oropharyngeal delivery.

4.4.3 Lung engraftment following pulmonary macrophage transplantation

4.4.3.1 Duration

Following a single administration of gene-modified macrophages to the lung, sustained levels of secreted transgenic proteins were produced *in vivo*. The duration and retention of donor-derived macrophages and gene expression was only investigated up to 16 weeks post-delivery. Even though analysis of exact retention was not robust, results indicate that donor-derived cells were still present in BALF at this time point, and Gaussia luciferase expression was sustained over time. Assuming gene expression is driven by genetically modified macrophages, as the recovery rate of these cells under optimal conditions was low (<0.05%) these results suggest that though macrophages are lost from the lungs over time, they are still present in low numbers at week 16.

Tissue resident AM populations are established during embryogenesis and show significant tissue specific specialisation in both phenotype and function (39, 40). Under steady state conditions they show minimal contributions from circulating monocytes as they self-renew and proliferate *in situ* (3, 39, 105-107, 418). However, several studies have demonstrated that monocytes and macrophages from bone marrow origins can replace depleted mature tissue-

resident macrophage compartments and adopt tissue specific phenotypes (176, 178). This includes the lung, where owing to their highly plastic nature, exogenous or recruited cells acquire a mature AM phenotype (CD11c^{hi}, Siglec F^{hi}) in response to local environmental cues, which crucially includes GM-CSF signalling (39, 177). Here I demonstrate that exogenous BMDMs are maintained for up to 16 weeks in the lungs of WT mice following *in situ* delivery. This recapitulates studies demonstrating that under steady-state conditions, circulating monocytes originating from HSC precursors in bone marrow can coexist with mature resident tissue macrophages (32, 112, 113). A recent study has demonstrated that the fate and long-term engraftment of monocyte-derived macrophages following inflammation-driven recruitment, is regulated by the local environment (419). They showed that where severe inflammation results in the ablation of resident peritoneal macrophage populations, eventually they are replaced by recruited macrophages as they adopt a mature resident phenotype (CD11c^{hi}, Siglec F^{hi}). However, in the case of mild inflammation where enduring resident macrophages are still present, recruited cells still persist long term in tissue, but in an immature intermediary state of differentiation (419). This suggests that here, BMDMs transplanted to WT mice are being similarly maintained in the presence of an existing populated AM niche, where this transitional state accommodates *in vivo* transgene expression and local delivery of secreted proteins. Here macrophage phenotypes have not been assessed following lung delivery, and assessment of macrophage markers pre- and post-delivery are necessary to confirm this hypothesis and determine whether donor CD11b⁺, Siglec F^{low} BMDMs convert to a mature CD11b^{low}, Siglec F^{high} AM phenotype.

Previous pulmonary macrophage transplantation therapies for hereditary PAP and AATD demonstrated sustained long-term engraftment of WT or gene corrected (*Csf2rb* or *Csfra*) macrophages in the AM compartment as cells adapted to the lung microenvironment and underwent phenotypic changes resembling mature AMs (176, 178, 420). For example, one study using BMDMs demonstrated that prior to transplant, *in vitro* BMDMs have a CD11b^{hi}, Siglec F^{low} phenotype which is then adapted to an AM-like CD11b^{low}, Siglec F^{hi} phenotype following lung delivery and engraftment (176). This was mediated by the fact that *Csf2rb*^{-/-} and *Csf2ra*^{-/-} deficient mice have an empty AM niche as *Csf2r* receptor mediated GM-CSF signalling is required for AM development and maturation (40, 185). One study demonstrated that high levels of engraftment by transplanted macrophages expressing *Csf2ra*, in *Csf2ra*^{-/-} mice (31% ± 3.7 and 40.1% ± 7.1%, at 2 and 12 months post-delivery) is mediated by proliferation of transplanted macrophages in response to high levels of pulmonary GM-CSF (176). In

accordance with findings here, where 4 weeks post-delivery of Glux/BMDMs, 2.1% (median) of BALF cells were donor-derived, this study reported that 1 month post-delivery the percentage of donor-derived macrophages recovered from the BALF cells of WT mice was $4.4\% \pm 0.97\%$ (mean $\pm$ SEM), which was significantly lower than in *Csf2ra*^{-/-} recipients ($46.4\% \pm 5.1\%$) (expressed as a percentage of endogenous AM macrophages) (176). The study also showed that macrophage proliferation was consistent across treated and untreated WT mice demonstrating that there is no proliferative advantage of delivered cells in the presence of an endogenous AM population (176). Proliferation was not directly measured here but could be admitted into future study design to determine the degree to which transplanted cells proliferate in WT mice and how this contributes to their retention over time. Outside of these studies in *Csf2ra*^{-/-} and *Csf2rb*^{-/-} mice, where engraftment following transplantation in WT controls was only reported at a single 1-month time point (176), there is a lack of research characterising the fate of transplanted macrophages in the lung. Evidence of one other study transplanting macrophages in the lungs of wildtype C57BL/6J mice did not directly quantify transplanted cells, and only demonstrated sustained expression of transgenic alpha-1-antitrypsin protein 1,3 and 7 days post-delivery (179).

Other studies have investigated macrophage transplantation to the lung in SCID mice and models of idiopathic pulmonary fibrosis (IPF) and though the retention of transplanted cells was only minimally investigated, they observed only transient retention. Studies transplanting macrophages to the lung of immunocompromised SCID mice to examine the role of AM in lung immunity delivered J774A.1 immortalised macrophage cell lines transduced to express IFN- γ (421). Lung immunodeficiency was successfully reversed, and significant levels of IFN- γ were present in BALF for up to 14 weeks but tapered towards baseline after 1 month (187). Another study transplanted lentiviral transduced IC-21 immortalised macrophages expressing keratinocyte growth factor (KGF) and found that in WT mice cells were lost after 24 hours and were only retained in IPF models with bleomycin induced injury for up to 7 days (180, 422). Overall, no detectable KGF was produced, and expression had no discernible effect on the IPF model. Though these findings contradict my results, these studies used immortalised macrophage cell lines rather than primary cells. They may have chosen these cells as they show increased transduction efficiencies with lentiviral vectors, however it suggests that macrophage cell lines do not accurately model primary macrophage cell fates and that as a consequence of manipulation for *in vitro* culture, these cells lose the ability to engraft and respond to the lung environment *in vivo*. Indeed, studies have documented differences between cell lines and

primary macrophage cytokine production and expression of cell surface proteins required for cell adhesion and activation (423), and responses to bacterial pathogens (424).

4.4.3.2 Mouse Model Comparisons

Here a WT mouse model was used to study long term engraftment of BMDMs and transgene expression in the lungs. However, these results may underestimate the engraftment and therefore duration and transgene expression in GM-CSF KO (PAP) mice, as in the absence of GM-CSF signalling and therefore maturation of foetal monocyte populations, PAP mice have an empty AM niche (39, 102, 425). I would therefore expect that following macrophage driven expression of transgenic GM-CSF, endogenous monocytes and transplanted macrophages would mature and proliferate to populate the AM niche (176). Consequently, PAP mice may exhibit a higher degree of engraftment than WT mice. However, this will not occur when transplanting control Gaussia luciferase (Gluc) macrophages to the PAP lung, as there is no endogenous or transgenic GM-CSF in the lung. Gluc control BMDMs delivered to PAP mice may therefore have a lower degree of engraftment and therefore relative transgene expression in comparison to BMDMs secreting GM-CSF. This is supported by my finding that at high doses, transgenic GM-CSF expression can drive higher rates of macrophage engraftment in WT mice than Gluc controls. Unfortunately, due to the degree of debris and surfactant present in the PAP mouse BALF, I was unable to quantify and compare the retention of transplanted cells by flow cytometry. This highlights the requirement for an adapted flow cytometry protocol that permits analysis of BALF cells from PAP mice, or the development of alternative assays, such as transgene mRNA expression, in future comparative study of *ex vivo* macrophage GM-CSF therapy in PAP mice with WT and Gluc controls. Though we may see higher levels of engraftment in treated PAP mice than WT mice, it is unlikely to recapitulate those seen in *Csf2r^{-/-}* mice, as GM-CSF expressed by transplanted macrophages will also stimulate the maturation of endogenous macrophages which will compete for space in the AM compartment. Additionally, this highlights how future experiments should investigate proliferation and phenotypic changes of transplanted macrophages to understand how these cells engraft to the AM compartment in different mouse models and the effect of transgene expression.

4.4.3.2 Effect of Cell Dose

In an initial dose escalation trial Gluc controls exhibited no difference in macrophage retention between doses which suggests that the mouse lung can only support a limited number of exogenous macrophages when donor mice have an existing mature AM compartment. The

effect of dose was not extensively investigated here, however findings are corroborated by studies showing that repeat dosing and dose escalation has no effect on macrophage engraftment and transplantation efficacy in *Csf2rb*^{-/-} mice for hPAP (177). However, dose-related responses were observed in gene expression. It is unclear why higher levels of gene expression would be observed if the number of remaining donor-derived cells is consistent. Longer duration studies at multiple doses will be required to determine if the increased expression seen at higher doses is a temporary distinction, that as macrophages are lost in the lung, is abrogated. It is also possible that the protocol for the recovery of donor-derived cells by lavage and subsequent identification by flow cytometry is not sufficiently sensitive to detect differences in cell number, but that resultant changes in gene expression can be detected by the sensitive Glux assay. A limitation of these results remains the lack of having established a minimal effective dose which could make this treatment more cost effective.

In comparison to Glux controls and lower dose cohorts, macrophage retention at one week was greater following delivery of a high dose of macrophages expressing mGM-CSF. This experiment was performed at low n numbers, but a clear trend was still observable. This may be driven by GM-CSF induced proliferation or the maturation of transplanted cells as GM-CSF regulates AM survival, maturation and proliferation (39, 398). This suggests that when macrophages are transplanted into mice with established AM compartments, GM-CSF signalling can rescue the transitional monocyte phenotype of transplanted cells leading to maturation and adaptation to an AM phenotype. This is supported by studies of transgenic mice where overexpressing GM-CSF in the airway epithelium leads to *in vivo* AM proliferation and subsequent activation (329, 426). However, as it was not within the purview of this work to investigate macrophage phenotype prior to- and post-transplantation this is currently conjecture and will require confirmation in future experiments. Inhaled GM-CSF therapy in concert with pulmonary macrophage transplantations for hPAP has been suggested to promote survival of transplanted macrophages (177). This theory is supported by these initial findings here and suggest GM-CSF may be useful as an adjuvant therapy if expanding the *ex vivo* platform repertoire to other lung diseases such as AATD, where GM-CSF is not the therapeutic protein. Alternatively, resident AMs could first be depleted to increase the engraftment of transplanted cells which could then repopulate the AM niche as has been proposed using clodronate liposomes to replace inherently dysfunctional AMs in immunodeficient mice (178, 427). This would likely result in increased, long-term engraftment like that observed in *Csf2rb*^{-/-} mice.

4.4.4 Transgene expression in the lung following pulmonary macrophage transplantation

Proof-of-concept for *ex vivo* macrophage therapy was first established by transplanting BMDMs expressing transgenic GM-CSF in the lung. Though transgene expression was detected in BALF above the lower limit of detection in this initial study, subsequent experiments failed to achieve similar results, where expression usually fell below or around the lower limit of detection by the GM-CSF ELISA (7.8 pg/ml). However, at equivalent doses, all mice treated with Glux/BMDMs showed detectable levels of expression in the lung.

It is possible that detectable levels of GM-CSF were only measured in mouse BALF in the first experiment owing to the use of donor macrophages from CFTR KO mice. Dosed mice were either heterozygotes (CFTR^{-/+}) or knockouts (CFTR^{-/-}) and therefore donor macrophages lacking CFTR expression were chosen to avoid potential recognition of CFTR as a novel antigen following transplant to CFTR^{-/-} mice. The absence of CFTR expression on these cells may have altered the regulation of macrophage engraftment and fate post-transplant and resulted in higher levels of engraftment and/or proliferation and therefore increased transgenic GM-CSF levels in BALF. As is extensively discussed in chapter 6, growing evidence demonstrated that AMs in CF have altered inflammatory and antibacterial responses owing to either an inherent lack of CFTR expression and/or conditioning by the CF lung environment (428-432). The CF lung is characterised by increased AM infiltration, an inability to resolve inflammation and an overall pro-inflammatory environment (433). In children (median age 3.1 years) the number of AMs in BALF was increased in comparison to non-CF children even in the absence of infection (434). Furthermore, studies transplanting macrophages from CFTR KO mice into the lungs of WT mice were able to partially recapitulate a CF exaggerated immune response to *Pseudomonas aeruginosa* (435). This suggests that the absence of CFTR expression on the transplanted cells may have led to increased donor engraftment and transgenic GM-CSF expression, perhaps as a result of a proinflammatory phenotype caused by dysregulated immune signalling.

Direct comparisons with the success of pulmonary transgene expression following *in vivo* SIV.F/HN delivery were not performed here. However, in comparison to previous work by Helena Lund-Palau and Claudia Juarez-Molina where the SIV.F/HN.hCEF.GM-CF virus

was delivered *in vivo* to directly target the lung epithelium, lower levels of transgene expression were produced here following macrophage transplantation. The lowest tested dose of SIV.F/HN.hCEF.GM-CSF delivered directly to the lung in this previous work was 1×10^7 TU/mouse which led to sustained expression of 3.33 ng/mL (median) of GM-CSF, and thus far above the GM-CSF ELISA lower limit of detection (7.8 pg/ml) (387, 396).

In vivo delivery of the SIV.F/HN virus has also led to stable gene expression that lasted for up to 15 months in mice (268, 277, 302), but as an equivalent duration study was outside the scope of this work it is unclear if macrophages will achieve similarly long-lasting expression. Non-viral *in vivo* gene therapy also offers an alternative intervention strategy as secreted protein expression of Gaussia luciferase and hAAT was also seen in the lung following delivery of non-viral cationic lipid formulation GL67A (168, 319). However, in comparison to macrophage therapy, non-viral delivery results in transient expression of the transgene, where repeat administration of GL67A was required to sustain expression of a human CFTR transgene for at least 56 days in mice (280).

The discrepancy in magnitude of transgene expression between GM-CSF and Glux is not likely to be due to a higher relative retention and accumulation of Glux in the lungs, as both GM-CSF and Glux have short *in vivo* half-lives of approximately 7.3 hours (436) and 20 minutes (278) respectively. Instead, it is likely a result of either reduced expression of the transgene, or the relative insensitivity of GM-CSF detection. Though the former was not measured here, following *in vivo* administration of the rSIV.F/HN lentivirus a similar discrepancy was observed in the detection of secreted alpha-1-antitrypsin (AAT) and Glux however where there was no significant difference in transgene mRNA levels (168). This study concluded that the Glux is likely just more sensitive than protein detection by ELISA, due to the large degree of signal amplification which occurs in the luminescent reaction used to measure Glux. This theory is supported here, as though only minimal levels of GM-CSF were detected across experiments in PAP and WT mice, GM-CSF expression successfully resulted in the improvement of the PAP phenotype in GM-CSF knock out mice, in keeping with undetectable wildtype levels of GM-CSF in WT mice. That the effect is due to consistent secretion of GM-CSF by BMDMs over this 4-week period, is supported by the short half-life of GM-CSF. If we extrapolate from Glux studies, where the protein has an even shorter half-life, we can assume GM-CSF expression will be similarly sustained over time. The validity of this comparison is reinforced by clinical trials delivering recombinant GM-CSF protein to PAP patients which suggest that

the phenotypic improvements seen here in PAP mice would require continuous transgene expression (161, 381). Future comparisons could attempt to measure transgene mRNA levels in lung homogenate, which if detectable could determine if there are transgene specific differences in gene expression.

4.4.5 Proof-of-Concept in a PAP Mouse Model

4.4.5.1 PAP Mouse Model and Outcome Measures

GM-CSF knockout mice recapitulate PAP-like lung disease with manifest accumulation of surfactant in alveolar spaces. They exhibit a mild phenotype and develop lung abnormalities in the 3rd week, or second month of life (104, 437). These mice provided initial insights into the mechanistic role of GM-CSF signalling in PAP and have since been used as mouse models of the disease when investigating therapeutic interventions. One of the defining characteristics of PAP in patients is the milky appearance and increased opacity, or turbidity, of BALF as a result of surfactant build up which is recapitulated in PAP mice and can be used to establish a PAP diagnosis in approximately 75% of clinically suspected cases (361). Water soluble SP-D and SP-A are components of the surfactant complex and whose prominent increase in sputum and BALF has been used to diagnose PAP in patients (438, 439). Correspondingly SP-D is markedly increased in BALF and PAP mice which recapitulate the PAP phenotype (334, 437, 440). Previous work by Helena Lund-Palau as part of her PhD extensively characterised the phenotype of this mouse model and developed assays to study markers of disease in response to treatment (387, 396). In agreement with other studies, she demonstrated that BALF turbidity, and SP-D accumulation in BALF and lung homogenate samples are robust markers of disease. At the lowest viral dose tested of 1×10^7 TU/mouse, one week was sufficient to observe significant normalisation of BALF turbidity in PAP mice treated with rSIV.F/HN-Csf2 (median 1.675) in comparison to rSIV.F/HN.Glux controls (median 4.742). This was shown to corroborate with decreased surfactant levels as SP-D measured in the BALF and lung also demonstrated prompt and significant decreases following viral administration (median decrease 2.33 ng/ml and 68.15 ng/ml respectively).

I therefore employed these biomarkers of disease to determine the therapeutic effect of gene-modified macrophage transplantation in PAP mice, though due to a processing error, SP-D in lung homogenate samples were not available. Helena Lund-Palau had tested additional biomarkers and found that in comparisons with WT mice, phosphatidylcholine (PC) levels in

BALF and quantification of period-acid-schiff (PAS) staining material in lung histology samples were robust markers of PAP disease and supported positive outcomes following gene therapy interventions (396). In this study the left lung of each treated mouse was not lavaged and instead fixed and processed for histological assessment of PAS staining. However due to an unforeseen complication, this analysis was delayed and is therefore not included here. As a result, in this initial PAP study only two disease markers were used to characterise the PAP phenotype, turbidity and SP-D burden in BALF, and through they support GM-CSF mediated clearance of the lung, the study is limited by the number of biomarkers used in the analysis. The additional markers characterised by Helena Lund-Palau will be included in future macrophage experiments, though it is important to note that these untested markers showed good consistency with SP-D and BALF turbidity outcomes. In the clinic however, spirometry function tests and radiographic imaging are used to inform disease progression and efficacy outcomes of treatment and one study argued that quantification of surfactant components may not accurately represent the level of respiratory impairment (340, 370, 399). Though Helena Lund-Palau characterised these tests in both GM-CSF KO and WT mice they could not be powered to measure effect of treatment (unpublished PhD thesis). Therefore, though macrophage GM-CSF therapy equated to positive outcomes in BALF turbidity and SP-D burden, they do not provide a complete picture of how lung function and patient outcomes may be affected.

Furthermore, though the mouse model used here has been employed extensively in the literature to research PAP treatment and pathophysiology, it does not fully mimic autoimmune PAP where decreased GM-CSF bioavailability is due to anti-GM-CSF antibodies. An alternative mouse model of autoimmune PAP has been described and could in future be used to fully elucidate the potential therapeutic effect of the GM-CSF levels achieved *in vivo* by macrophage therapy. However, testing these mice was not within the scope of this project, due to lack of availability and as only older mice above 1 year in age develop a disease phenotype .

4.4.5.2 Efficacy of Gene-Modified Macrophage Therapy

In this chapter, results indicate that delivery of genetically modified macrophages secreting GM-CSF protein to the lung can reduce disease biomarkers 1 month after transplantation as they trend towards baseline. Whole lung lavage (WLL) is the gold standard of care for PAP patients and though between studies and patients the success rate and rate of relapse following WLL varies greatly, it effectively reduces the concentration of SP-A in BALF (441) and leads to

improvements in lung function and survival (371). However, WLL interventions do not address the causal lack of GM-CSF bioavailability in the way GM-CSF inhalation or gene therapy might.

Inhalation of recombinant GM-CSF protein has successfully corrected PAP phenotypes in PAP mice, where daily administration of GM-CSF by aerosol inhalation over a 4–5-week period led to temporary improvements in PAP and clearance of accumulated surfactant (250, 251, 329). Patient trials administration of GM-CSF recombinant protein demonstrate that in the presence of autoantibodies, GM-CSF therapy increases bioavailability to a therapeutic effect, even in patients previously unresponsive to WLL (374) and importantly demonstrate that serum anti-GM-CSF antibody levels remain unchanged (161). However, mouse and patient studies both indicate that sustained repeat dosing is required to observe therapeutic effects which are temporary following discontinuation of therapy. In PAP mice, daily but not weekly administration of recombinant protein over 4-5 weeks led to therapeutic benefits which relapsed 5 weeks after treatment was discontinued (250, 251). In patients, improved efficacy has been observed by increasing duration of GM-CSF treatment. Relapse following withdrawal of treatment can be rescued in patients with reoccurring conditions using a 6-month maintenance regimen delivering a lower dose of GM-CSF than in initial treatments, indicating that sustained GM-CSF expression is required to sustain therapeutic outcomes (160, 161). In corroborating unpublished results by Helena Lund-Palau, treating PAP mice with SIV.F/HN.Csf2 led to sustained high levels of GM-CSF expression and rescue of PAP manifestations within a week of treatment that were sustained for up to 9 months (unpublished). In comparison, macrophage GM-CSF expression only diminished PAP manifestations in PAP mice after 1 month of treatment, where BALF SP-D and turbidity were not yet normalised. This is due to lower levels of GM-CSF secretion, as here GM-CSF was largely undetectable in BALF and lung homogenate samples (lower limit of detection 7.8 pg/mL), while the lowest viral dose tested (1×10^7 TU/mouse) produced sustained expression of 2.782 ng/mL (median) of GM-CSF. As these results are similar to inhalation studies in mice, where delivering 2ng of nebulised GM-CSF effectively reduced PAP phenotypes after 4 weeks of daily administration, it suggests that macrophage therapy is achieving a comparable level of *in vivo* expression (251). However, this study did not report on SP-D or BALF turbidity outcomes and direct comparisons are not possible. These results suggest that the *ex vivo* macrophage platform developed here has the potential to recapitulate the dosing regimen of GM-CSF inhalation therapy and its therapeutic benefits with a single macrophage dose.

This initial study in PAP mice was not powered to sufficiently address the effect of macrophages on phenotype in the absence of GM-CSF signalling, as the number of untreated PAP controls was low. However, while it is possible that transplanted macrophages may affect the surfactant burden, in the absence of GM-CSF signalling *in vivo*, and stimulation *ex vivo*, it is unlikely that macrophages not expressing transgenic GM-CSF (i.e., Glux controls) will be able to adopt a mature AM functional phenotype to mediate disease presentation.

4.4.5.3 GM-CSF Toxicity

Though lentiviral studies showed faster onset of GM-CSF driven therapeutic benefits, they also demonstrated evidence of GM-CSF induced toxicity, where at high doses mice presented with unexpected death 2- and 4- weeks post-treatment (Helena-Lund Palau, unpublished results). Similar results in transgenic mouse model expressing elevated levels of GM-CSF from a retroviral promoter, suggest that there is a threshold above which GM-CSF expression is detrimental due to its pleiotropic hematopoietic activity, as between the ages of 2-4 months these mice exhibit muscle wasting and subsequent death (442). Additionally, transgenic mice using the SP-C promoter to drive overexpression of GM-CSF (SPC-GM-CSF^{+/+}) specifically in the lung epithelium spontaneously developed emphysema and secondary polycythaemia, and in comparison, to WT mice exhibited increased mortality (426). Transplantation studies of bone marrow overexpressing GM-CSF from a retroviral promoter led to high levels of circulating GM-CSF and a fatal myeloproliferative syndrome (443). In patients, daily doses of 10-30 µg/kg were toxic in patients with aPAP as patients presented with fever, elevation of liver enzymes, and pericarditis (384), while a similar study demonstrated severe adverse effects above 10 µg/kg daily doses of GM-CSF including hypoxia and hypertension (385).

These studies caution delivery of high doses of recombinant proteins or sustained overexpression of GM-CSF *in vivo*. Though toxicity was not directly measured, no evidence of toxicity or GM-CSF related mortality was observed in following GM-CSF.BMDM delivery to the lungs. Whether the relatively low levels of GM-CSF expression achieved here will induce toxicity needs to be investigated over longer duration studies but is unlikely to recapitulate the toxic concentrations produced *in vivo* following lentiviral delivery or following exogenous delivery in patients (384, 385). Should GM-CSF mediated toxicity arise however, as the transgenic producer cells are not part of the lung epithelium, research could investigate the

inclusion of a suicide gene into the lentiviral expression cassette to allow selective ablation of transgenic donor macrophages without major side effects to lung function (444).

4.4.6 Macrophage Specific Expression

One of the major limitations of this work that became apparent when analysing the final long-term duration study is the lack of direct evidence supporting that expression of secreted transgenes *in vivo* is directly produced by transplanted macrophages. Though cells were washed 4 times prior to delivery, this was an overlooked consideration, and I did not study the degree of viral carry over when dosing macrophages with the cellular product. This could explain why transgene expression was sustained when macrophage cell engraftment decreased in time course studies. However, as secreted transgenes were used, no evidence of direct transduction was performed. The likelihood of significant carry-over may be low however, as one considers the number of washes employed, the effect of versene exposure during detachment, and the stability of the vector particles in cell culture media at 37 °C for 24 hours prior to delivery. Though the half-life of the F/HN.SIV vector under has not been studied these conditions and stability is temperature sensitive in a pseudotype-specific manner (445), two separate studies of VSV-G indicate a half-life of approximately 35 hours at 37 °C , while another reported it to be 10.4 ± 1.4 hours (446). This indicates that prior to washing, the amount of transduction-capable vector in the cell media is likely already reduced. Regardless, future work should address this issue by testing the amount of transduction capable vector in cell supernatant upon delivery and the effect of washing to strengthen conclusions of macrophage-driven expression *in vivo*. Secondly alternative methods to detect transplanted macrophages in the lungs and demonstrate macrophage specific expression should be developed to allow more robust conclusions as to the fate of transplanted cells and any transgene expression. This could be achieved by transduction with the EGFP transgene used in initial transduction efficacy experiments to show EGFP expression in BALF cells following delivery or in histological analysis. Molecular analysis of vector copy numbers in BALF cells harvested during the long duration experiment is already underway but not included here.

4.4.7 Regulated Expression in Gene-Modified Macrophages

4.4.7.1 GeneSwitch™ Regulatory System

An alternative approach to reduce GM-CSF mediated toxicity, has been to develop lentiviruses with regulated gene expression. For initial proof-of-concept studies, this was achieved by adapting the GeneSwitch™ expression system to the SIV lentiviral backbone by Robyn Bell as part of her PhD (unpublished). While this system is in parallel being tested for *in vivo* delivery to the lung epithelium, I showed here that it can successfully produce so called ‘inducible macrophages’ which expressed the secreted reporter transgene Glux only when prompted. This provided proof-of-concept for regulated expression of a secreted protein *in vivo* following macrophage transplantation and could be used to control GM-CSF production to avoid exceeding the toxicity threshold in PAP. Similar approaches have been successful where tetracycline dependent expression of a Glux reporter in hemopoietic cells *in vivo* was achieved following transplantation of cord blood CD34⁺ cells transduced with two lentiviral vector system into NOD/SCID mice (447).

Here the commercial GeneSwitch™ system was employed to provide proof-of-concept for regulated gene expression in macrophages following lentiviral gene transfer and future steps will be to test induction *in vivo* and adapt the system for human use (448). In this system, expression is induced by the synthetic mifepristone, which is a competitive glucocorticoid and progesterone receptor agonist, proven safe in clinical use for treatment of high blood sugar in adults with Cushing’s, as an emergency contraceptive or early-stage medical abortion drug (449, 450). Administration of mifepristone to induce gene expression *in vivo* may therefore exert pleiotropic effects against these endogenous receptors, however rat studies suggest the concentrations required to induce gene expression is significantly lower than the dose required to antagonize progesterone receptors (451). A further limitation of this system is the reliance on the yeast GAL4 DNA binding domain to drive gene expression, therefore, to avoid enhanced immunogenicity in patients, work is being undertaken by Robyn Bell to exchange the functional binding domains for ‘humanised’ or synthetic equivalents.

4.4.7.2 Dual vs Single Vector System

The commercial GeneSwitch™ system was adapted for SIV lentiviral packaging, in one of two articulations, a two-vector (2v) and single vector (1V) system. Both transduced macrophages to express a reporter gene under tight transcriptional regulation, though the 2v system was more effective. Initial tests by Robyn Bell reflected similar findings in immortalised cell lines and air-liquid interface cultures (unpublished). Though direct comparisons with *in vivo* administration

have not been performed, this suggests that this system of regulated transgene expression may be more efficient in an *ex vivo* system. The success of the 2V system in macrophages may be surprising as it requires co-transduction of two vectors, and experiments with VSV-G pseudotyped lentivirus in Chapter 5, indicate that at an MOI of 10, the percentage of transduced cells in culture is unlikely to rise above 10%. However, the vectors are third generation HIV-1 vectors with VSV-G pseudotypes and therefore cannot expect similar outcomes to those observed with the rSIV.F/HN vector. As transduction efficiencies were not analysed, it is possible that transduction exceeded efficiencies seen with the GTC's vector, or alternatively that expression is driven by a select few positively transduced cells. Alternatively, we have not ruled out the unlikely possibility that the transactivator, pSwitch™ regulatory protein is secreted and therefore capable of driving transgene expression from the GAL4-E1b promoter in a separate cell only transduced with a single vector.

Co-transduction of viral vectors *in vitro* has been used as a strategy to overcome packaging constraints that limit the number and size of inserts that may be packaged into a single vector, or as in this case where a regulated gene and a controlling gene need to be co-expressed in a single cell (452-454). Insert size did not preclude packaging both elements of the GeneSwitch™ system in *cis*, but when compared to the original two-vector conformation the single vector led to reduced induction of transduction in both macrophages and HEK293T cells. Successful expression of multiple transgenes from a single vector can be achieved through the use of, multiple promoters (455), bicistronic transgenes with internal ribosome entry site (IRES) elements (456, 457), self-processing polyproteins (458, 459), alternative splicing (460, 461), or fusion genes (462). Regulated expression systems are constricted to the use of different promoters as each transgene requires separate transcriptional control. Though the use of multiple promoters in gamma retroviruses and lentiviruses has historical success, promoter or transcriptional interference, suppression and rearrangements are not uncommon roadblocks to efficient transgene expression and may explain the reduced efficiency of the single vector system (463-468). Interference between internal heterologous promoters has been well documented and can be bidirectional as dual promoters simultaneously influence expression of adjacent transgenes (467). Transcriptional interference may occur due to read-through of transcription from an upstream gene into the regulatory region of another (469), however in attempts to negate this issue, here the two open reading frames were cloned in opposite orientations (470). Alternative mechanisms of promoter interference may still explain the reduced activity of the single vector system, such as competition for transcription factors or enhancers, and their

binding proteins (467, 471), or epigenetic silencing as upstream or downstream transgenes are transcriptionally inhibited by the chromatin remodelling of another promoter (470). Post-transcriptional competition or interference is a less likely cause of the single vector's low transgene expression as this would likely also affect the two-vector system. Future optimisation of the system, especially if considering *in vivo* administration, could however measure the relative transcript levels of each transgene to investigate potential promoter interference and determine how efficient expression of the 1V system may be rescued. However regardless of the reduced expression from the 1V system and the underlying mechanism, strict and efficient gene expression was seen in 'inducible macrophages' with the 2V system.

Moving forward from these initial proof-of-concept studies in macrophages, direct comparisons can be made with other constitutive candidate promoters, hCEF, EF1 α , CMV, in *in vivo* and *in vitro* models of macrophage cell therapy, as well as *in vivo* delivery of the 1V and 2V systems directly to the mouse lung. Having here established that macrophages can be induced and that transplanted gene-modified macrophages secrete sustained levels of transgenes in the lung, work to characterise the delivery of these cells and provide proof-of-principle for *in vivo* macrophage induction are already underway. These findings represent exciting initial work that could make macrophage pulmonary therapy safer by limiting potential toxic effects of transgene expression.

4.4.8 Safety of pulmonary transplantation of genetically modified macrophages

Across *in vivo* macrophage delivery experiments I found no evidence of transgene or macrophage mediated toxicity, though this was not directly tested for. Though one case of unexpected mortality occurred, it was seen in an untreated PBS control. Furthermore, suffering phenotypes observed in the 24 hours following the delivery procedure were abrogated when supportive wet food was provided indicating they were caused by poor recovery from anaesthesia. As such, macrophage delivery and transgene expression of Gluc and mGM-CSF was well tolerated, and mice followed over 16 weeks demonstrated no outward ill effects of treatment. Similarly, long term studies of Gluc expression in mice showed no ill effects of expression, and in the literature, murine studies transplanting gene-modified macrophages to the lung reported no adverse effects. Clinical trials delivering macrophages to the intraperitoneally, intravenously, and intrathecally also only reported mild side effects of

treatment even following high dose, and repeated administration (63, 64, 88-91). These findings therefore support the theory that autologous macrophage cell therapy has a favourable safety profile. However as discussed previously, longer term studies specifically measuring different toxicity outcomes are required to determine if macrophage expression can produce toxic levels of GM-CSF previously reported with subcutaneous delivery of large doses as here mice were only followed up to a 1-month time-point.

4.4.9 Conclusion

In this chapter I have demonstrated proof-of-concept of an *ex vivo* macrophage platform technology for *in vivo* pulmonary transgene expression. Following pulmonary transplantation of genetically modified BMDMs in wildtype mice, sustained levels of reporter gene expression were measured in the lungs for up to 16 weeks. Gene expression data suggests transplanted cells are retained throughout this time period, though cell recovery and analysis by flow cytometry at later time-points was unreliable. The therapeutic potential of this system was demonstrated in a mouse model of aPAP disease, where one month after lung delivery of a single dose of BMDMs genetically modified to express transgenic GM-CSF protein, disease biomarkers were ameliorated. Though direct evidence of macrophage driven expression *in vivo* is yet to be seen and the detection of transplanted cells over time was unreliable when analysing BALF cells by flow cytometry, these data provide support for a macrophage-based system aiming to secrete therapeutic transgenic proteins in the lungs. Though no evidence of toxicity related to macrophage or transgene expression was observed, I have also shown proof-of-concept for regulated gene expression in macrophages *in vitro* that could circumvent any potential transgene related toxicity and provide strict control of *in vivo* protein expression. The degree of macrophage retention was increased by GM-CSF expression and is dependent on the mouse model and the state of the AM niche, but duration experiments suggest that in wildtype mice only a fraction of transplanted cells are retained in the lungs. Therefore, the best strategy to increase *in vivo* transgene production will likely be to increase the efficiency of gene transfer and strength of transgene expression in BMDMs to increase the effective cell dose.

Chapter 5 – Optimising transgene expression in macrophages following gene transfer

5.1 Introduction

As previously discussed in Chapter 3.1, lentiviral vectors are popular gene transfer vectors in *ex vivo* clinical trials and pre-clinical macrophage cell therapies (88, 89, 91, 174, 177, 180). In this work I have previously demonstrated that the GTC's proprietary lentivirus can transduce bone marrow-derived macrophages (BMDMs) which when transplanted to the lung lead to sustained gene expression *in vivo*, achieving clinically relevant levels in mouse models of aPAP.

5.1.1 Pseudotyping Lentiviral Vectors

Lentiviruses are enveloped, single-stranded RNA viruses that belong to the *Retroviridae* family (471). Host cell entry is mediated by the surface proteins expressed on the envelope lipid bilayer which also largely determines vector tropism (472, 473). This envelope is formed around a viral capsid shell during viral budding and therefore is composed of the cellular surface membrane of its producer cell. Virions encode specific envelope proteins that are expressed on the surface of these cells and therefore incorporated into the virion. These proteins then mediate host cell entry by specific receptor recognition. By a process known as pseudotyping, the envelope proteins expressed on the surface of producer cells and therefore virions can be exchanged with the envelope proteins from other viruses to change vector tropism (473).

For *in vivo* delivery, pseudotyping studies aim to provide target specificity to increase efficient transduction of target cells while limiting off-target transduction. To this end the UK Respiratory Gene Therapy Consortium's proprietary rSIV.F/HN.hCEF lentivirus was developed to target gene transfer to the lung epithelium (268, 277, 473). However, for *ex vivo* applications like the one proposed here, essentially no specific tropism is required as the target macrophages are the only cells present in culture. As a result, it is unsurprising that the majority of *ex vivo* lentiviral strategies, in macrophages or otherwise, use the pan-trophic vesicular stomatitis virus G (VSV-G) protein (176, 180, 298-301, 474). VSV viruses belong to the *Rhabdoviridae* family and cell entry is largely mediated by VSV-G binding to receptors in the

LDL-receptor family (210). These receptors are important in regulating cholesterol homeostasis and are ubiquitously expressed in mammalian cells (210). Recognition by VSV-G mediates cell binding and entry via clathrin-dependent endocytosis (475). VSV-G undergoes a pH dependent conformational change which anchors the virion particle and mediates fusion of the viral and endosomal lipid bilayers by bringing them into close proximity (476). This then mediates release of the capsid enclosed RNA genomes into the cytoplasm.

In comparison the fusion (F) and haemagglutinin (HN) envelope proteins used to pseudotype the Consortium's lentivirus are derived from the murine Sendai virus (SeV) belonging to the family *Paramyxoviridae* and genus *Respirovirus* (268, 277, 473). Cell entry is mediated by an alternative pH-independent mechanism. HN recognises sialic acid residues on surface glycoproteins or ganglioside receptors, preferentially α 2-3-bound N-acetylneuraminic acid, and naturally occurring lectins to attach and bind the virion to a target host cell (477-479). Like VSV-G target receptors, these are also widely expressed on most mammalian cell types, including monocytes (480). The F protein then undergoes a conformational change that anchors the virion and mediates membrane fusion by close proximity (481). In comparison to VSV-G mediated entry, this occurs at the plasma membrane rather than in an endosome. *In vivo* transduction with SeV or F/HN pseudotyped lentivirus leads to the efficient transduction of the lung epithelium (268, 482). This is mediated by abundant expression of α 2-3 sialic acid on alveolar cells and bronchiolar epithelial cells (483). However recombinant SeV can also mediate gene transfer in other cell types and *ex vivo* was shown to mediate highly efficient transduction of human cord-blood-derived HSCs (484). To my knowledge F/HN pseudotyped have not previously been evaluated for *ex vivo* lentiviral gene transfer in macrophages.

5.1.2 Regulation of transgene expression

Including alterations to vector tropism, studies also investigate how to target lentiviral vector expression to specific target cells through transcriptional control. Targeted transgene expression can be mediated by tissue- or cell- specific promoters that limit gene expression in off-target transduced cells (485). However, with *ex vivo* gene transfer this is again less relevant as off-target transduction is largely nullified. Promoters with constitutively strong expression that are active in a broad range of cell types are often popular in *ex vivo* gene therapy as a result (62, 226, 486). These commonly include the cytomegalovirus (CMV) immediate-early enhancer and promoter and eukaryotic translation elongation factor 1 alpha (EF1 α) of which the hCEF

promoter/enhancer used to drive expression in the Consortium's rSIV.F/HN lentivirus is a patent-protected chimera (276, 277, 280, 473, 482). As different promoters have varied levels of activity, show cell-specific patterns or strength of expression, and varied susceptibility to chromosomal positioning effects and epigenetic gene silencing specific optimisation in a relevant cell type or model is required when selecting the most appropriate regulatory element for a specific therapeutic approach (451, 487-489).

5.1.3 Sleeping Beauty Transposon

In comparison to lentivirus, non-viral approaches often exhibit low or non-existent rates of transgene integration (490). Often, they result in a short burst of transgene expression better suited to therapeutic strategies requiring acute expression such as anti-cancer drugs, therapeutic genes with small toxicity-efficacy windows, or vaccine delivery (491-493). However, non-viral approaches are generally more affordable to produce in large scale manufacturing, demonstrate reduced immunogenicity (492). Nevertheless, other common limitations are also low efficiency of gene transfer and an increased propensity of gene silencing due to the formation of DNA concatemers (494, 495). Transposon based gene transfer however offers an alternative to viral vectors which could overcome many of the limitations associated with non-viral therapies, including short duration of expression though transposase mediated integration, while profiting from the many advantages to a non-viral approach.

DNA transposons are discrete genetic elements characterised by their ability to move between segments of DNA via the 'cut-and-paste' activity of a transposase (496). Sleeping beauty (SB) is an example of a DNA transposon and is a non-viral 'vehicle' that moves as DNA from one genomic locus to another without an RNA intermediary (Figure 38) (497). SB is so called as it was resurrected through molecular reconstruction from an inactive transposon fossil found in fish genomes (498). It is composed of two parts, a transposase and a transposon. The transposon is defined as a fragment of DNA flanked by terminal inverted repeats (IRs). These IRs are recognised *in trans* by the transposase polypeptide which catalyses the enzymatic transposition of DNA. A synthetic expression cassette flanked by these IRs can be constructed and co-delivered with the transposase (in DNA/RNA form or as a protein) for the integration of therapeutic genes into host DNA (499). In the SB system transposase binding sites in each IR are required to be in duplicate in a direct orientation (DR) and are therefore referred to as IR/DR. In comparison to lentiviral vectors, its integration is thought to be more random as

integration is targeted to any one of the multitudes of TA sites in mammalian genomes (500). This is a key safety feature as the insertion site preferences of viral vectors can lead to genotoxicity (217, 501, 502). This is emphasized by early gene therapy trials where γ -retrovirus insertional mutagenesis led to the development of acute lymphoblastic leukaemia in patients treated *ex vivo* for X-linked SCID (219).

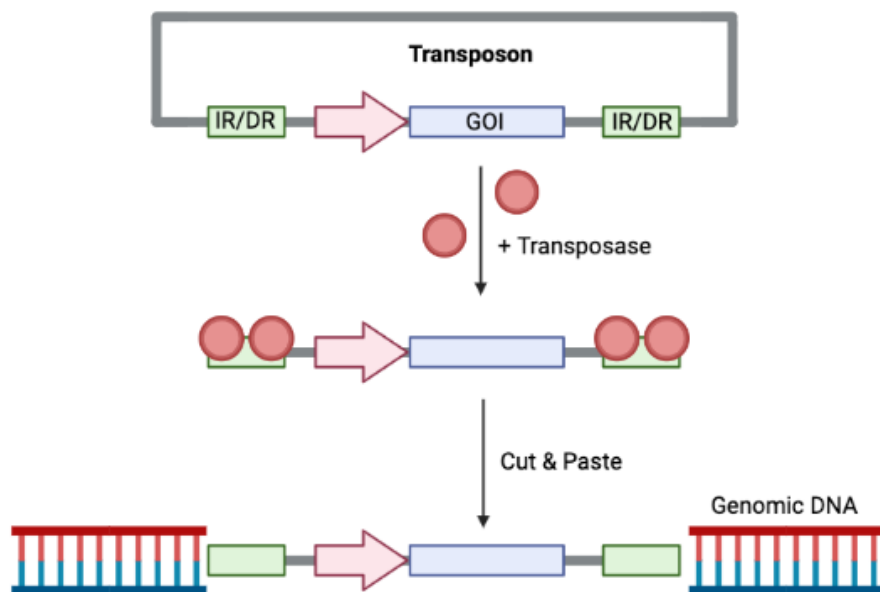


Figure 38 – Sleeping beauty transposon system. The sleeping beauty transposon is defined by flanking sequences of inverted repeats (IR). These IRs contain duplicate repeats in direct orientation (IR/DR) that are bound by the sleeping beauty transposase. Following recognition of these IR/DRs, the transposase excises the transposon and pastes the sequence into genomic DNA. GOI: gene of interest. Image produced by Nora Clarke in BioRender.

The SB system has been used extensively *in vitro* to generate animal models and in insertional mutagenesis screens, however it has also been trialled in the clinic since 2011 (503). There are currently >14 clinical studies in progress largely targeting B cell malignancies (504). In addition to the safety profile of the SB system, one of the major advantages is its reduced cost of manufacturing as current estimates suggest that in comparison to viral vectors the cost per patient may be reduced by as much as 90% (505).

In macrophage electroporation has successfully been used to introduce the SB system into primary cells and immortalized cell lines, though extensive characterization is lacking (506, 507). Indeed, electroporation of the SB system has been gainfully employed across cell types to allow efficient gene transfer and transposition both *ex vivo* and even *in vivo* (508, 509). While many non-viral approaches to macrophage gene therapy are inhibited by macrophage anti-viral responses, electroporation and nucleofection may avoid degradation of foreign nucleic acids by bypassing the phagolysosome system through membrane permeabilization (510). This has been successfully implemented in macrophage cell lines, though use in primary macrophages has been inconsistent. Some groups have shown that in bone marrow-derived macrophages, electroporation efficiency is limited due to excessive apoptosis as the procedure is not well tolerated (286). Others however have demonstrated that nucleofection may be more efficacious, with one group reporting 90% transfection efficiency in primary murine macrophages (511). Nucleofection is a technology based on the principle of electroporation, that pairs unique cell-specific solutions, reagents and pre-programmed electrical parameters to enable efficient gene transfer (512). Though again its success varies in the literature, as one study still observed excessive apoptosis and negligible gene expression in murine macrophage-like J774A.1 cells (513). Though efficient non-viral gene transfer in macrophages still requires optimization, sleeping beauty transposon system offers an exciting safe and cost-effective alternative to lentiviral vectors that unlike other non-viral alternatives, could mediate long lasting gene expression.

5.1.3 Hypothesis and Aims

In chapter 3, I demonstrated for the first time that primary murine macrophages derived from bone marrow can be successfully transduced *in vitro* with the Consortium's proprietary F/HN pseudotyped lentivirus. These macrophages were then able to secrete therapeutic levels of transgenic proteins *in vivo* following transplantation to the mouse lung. However, expression was limited by the vector's transduction efficiency as according to best case estimates from flow cytometry analysis of transgenic EGFP expression, only 30% of delivered cells are transduced. Though definitive dose escalation studies have not been completed, data suggests that only a small portion of delivered macrophages are retained in the WT mouse lung over time.

I hypothesize that increasing the 'effective dose' of transplanted macrophages by optimising transduction and gene expression could lower costs and/or increase the maximum level of

transgene expression *in vivo*. To this end, this chapter aims to compare the relative efficiency of different lentiviral pseudotypes (VSV-G and F/HN) and promoter/enhancers (hCEF, CMV and EF1a), as well as investigate the timing of lentiviral transduction and the use of a potential alternative non-viral vector (SB). Additionally, this chapter further characterises the activity of the SIV lentivirus in macrophages to inform *in vivo* studies and results.

5.1.4 Declaration

The mRNA, VCN and encapsulation assays used to study lentiviral transduction by ddPCR were developed by Ana Sergijenko in the Imperial Gene Therapy Group. Additionally, she performed the encapsulation assay and contributed significantly to the subsequent data analysis.

5.2 Methods

5.2.1 Lentivirus Production

5.2.1.1 Small-scale production of lentivirus

Six different lentiviral vectors were produced in house or by at the GTC Core Facility by Oxford collaborators, a summary of which can be seen in Table 8. These lentiviruses were produced in house in London as previously described (279), through the transient transfection of a HEK293T producer cell line supplied in FreeStyle™ 293 Expression Medium (ThermoFisher Scientific). Plasmids used in the production of F/HN lentivirus were previously described in Chapter 2.3. VSV-G pseudotyped lentiviruses were produced through transient transfection of four plasmids: a genome plasmid, pGM299 (gag/pol), and pGM297 (rev) which have been previously described, and pMD2-G which encodes the vesicular stomatitis virus G (VSV-G) protein that mediates lentiviral cell entry (discussed in detail in Chapter 5.1). In addition to the previously described pGM357 (EGFP transgene under hCEF transcriptional control), two other vector genome plasmids were used:

- **pGM311:** Encodes the enhanced green fluorescent protein gene (EGFP) under transcriptional control of the CMV promoter/enhancer.
- **pGM354:** Encodes the enhanced green fluorescent protein gene (EGFP) under transcriptional control of the EF1a promoter.

All three plasmids encode EGFP on the same SIV lentiviral backbone and expression cassette as previously described in Chapter 2.3 but differ in the promoter used to drive transgene expression. All plasmids used were provided by collaborators at the GTC Core Facility in Oxford.

As previously described (279), a 250 mL Erlenmeyer shake flask (Corning, Flintshire, UK) was seeded with 20 mL of HEK293T cells at 5×10^5 cells/mL. These cells were allowed to double overnight and before being transiently transfected with a “transfection mix” of plasmid DNA and a polyethylenimine (PEI-Pro) transfection reagent (Cat. # 101000017, Polyplus, Reading, UK) for 24 hours at an N:P ratio (ratio of moles of amine groups of cationic polymers to moles of phosphate groups of DNA) of 15:1. F/HN pseudotyped lentivirus was produced through the

transient transfection of 0.33 µg of plasmid DNA per 1e6 cells in culture where the five production plasmids were at a ratio of 20:9:6:6:6, genome plasmid : pGM279 (gag/pol) : pGM299 (rev) : pGM301 (Fct): pGM303 (HN). VSV-G pseudotyped lentivirus conditions varied and are specified when discussed in the results. The following day cells were supplemented with 30 ml of FreeStyle™ 293 Expression Medium (ThermoFisher Scientific) and sodium butyrate (Cat. #193-01522, FUJIFILM Wako Chemicals, Germany) at a final concentration of 5 mM, to enhance transfection efficiency and transgene expression. 48 hours later virus was harvested in the cellular supernatant by pelleting of producer cells. F/HN pseudotyped viruses were then activated by incubating viral supernatant with Benzonase® Nuclease (25 U/ml) (Cat. #E1014, Sigma-Aldrich), 1M of MgCl₂ (Cat. #AM9530G ThermoFisher Scientific), and 10X TrypLE™ Select Enzyme (Stock: 1X) (Cat. #10272625, ThermoFisher Scientific) at 37°C for 1 hour. To inactivate the TrypLE™ Select a Defined Trypsin Inhibitor (Cat. #11588846, Stock: 1X) (ThermoFisher Scientific) was added at a final concentration of 0.1X. VSV-G pseudotyped vectors did not require this activation step. Viral supernatants were then filter sterilized with a 0.45µm filter (Cat. #Z768707, Merck, Feltham, UK) and stored in aliquots at -80°C.

Table 8 – List of small-scale purified lentiviruses produced in house in London and the GTC Core facility. All Virus types used the SIV backbone and encoded an enhanced green fluorescent protein (EGFP); hCEF: promoter/enhancer comprising CpG-depleted human cytomegalovirus enhancer and human Elongation Factor 1a promoter; TU: transducing units.

Name	Envelope	Promoter	Genome Plasmid
vGM216	F/HN	CMV	pGM311
vGM233	VSV-G	CMV	pGM311
vGM196	F/HN	hCEF	pGM357
vGM245	VSV-G	hCEF	pGM357
vGM250	F/HN	EF1a	pGM354
vGM251	VSV-G	EF1a	pGM354

5.2.1.2 Calculating vector titre

The functional titre (TU/ml) of small-scale lentiviral vectors was measured in once thawed virus. Known numbers of suspension HEK293T cells were transduced with serial dilutions of the viral supernatant in triplicate. Three days later, cells were analysed by flow cytometry as described in Chapter 2.5.2. The number of transduction units (TU) in each viral batch was calculated by multiplying the number of cells originally transduced by the percentage of EGFP positive cells and the dilution factor used. This is then divided by volume final volume of HEK293T cells + virus in culture. To avoid counting cells that have multiple integrations only samples where less than 30% of cells were positively transduced to express EGFP were used.

5.2.1.3 Concentrating lentivirus by high-speed centrifugation

Where indicated, lentiviral vectors were then concentrated by high-speed centrifugation. During an initial optimisation experiment this was performed by spinning 30 ml of virus in a 50 ml falcon conical centrifuge tube (Cat. #352070, ThermoFisher Scientific) at maximum of 26,200 *rcf* for 3 hours at 4°C in a Multifuge X3R TX-1000 Cell Culture Centrifuge with a fixed angle rotor (ThermoFisher Scientific). Viral supernatant was decanted by inversion and pipetting, and then resuspended in 250 µl of FreeStyle™ 293 Expression Medium and immediately re-titred. The established protocol concentrated vector by aliquoting virus into 1ml Eppendorf Tubes® (Eppendorf ltd, Germany) prior to centrifugation at 17,000 *rcf* for 3 hours at 4°C in a Fresco™ 17 Microcentrifuge (ThermoFisher Scientific). Viral supernatant was then decanted by inversion only and blotted gently on tissue paper. Viral pellets were resuspended in the remaining volume of supernatant in each Eppendorf tube and pooled across aliquots and stored at -80°C.

5.2.2 Automated RNA and DNA Extractions

RNA and DNA were extracted in bulk from BMDMs stored in RNeasy™ Stabilization Solution (Cat. #AM7020, ThermoFischer Scientific) using Maxwell® RSC Instrument with Maxwell® software (Promega, WI, USA). Cells were first removed from suspension by diluting samples 1:10 in dPBS and centrifuging samples at 400 *rcf* for 5 minutes and then removing supernatant by pipetting.

RNA was then extracted using Maxwell® RSC simplyRNA Cells Kit (Cat. #AS1390, Promega) according to manufacturer's instructions. The invisible cell pellet was resuspended in 200 µl of homogenisation buffer (Cat. #Z305H) and 200 µl of lysis buffer (Cat. #MC501C), and vortexed for 15 seconds. Suspensions were then added to the first well of a Maxwell® RSC Cartridge (RSCD) (Cat. #AS134A), and 5 µl of DNaseI (Cat. #Z358A) were added to the fourth well. An RSC plunger was added to well 8 (Cat. #AS1670). The 'simplyRNA Cells' programme was then selected on the Maxwell® RSC Instrument to initiate automated RNA extraction. RNA was eluted in 50 µl of nuclease-free water.

DNA was extracted using Maxwell® RSC Cell DNA Purification Kit (Cat. #AS1370, Promega) according to manufacturer's instructions. The cell pellet was resuspended in up to 400 µl of dPBS and added to the first well of a Maxwell® RSC Cartridge (RSCD) (Cat.

#AS137A) pipetting up and down to mix samples. An RSC plunger was added to well 8 (Cat. #AS1670). The 'Cell DNA method' programme was then selected on the Maxwell® RSC Instrument to initiate automated DNA extraction. DNA was eluted in 100 µl of elution buffer (Cat. #A828D).

RNA and DNA concentrations were then determined by NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher Scientific) as ng/µl, and samples stored at -80°C

5.2.3 Quantification of viral RNA and transgene mRNA by ddPCR

RNA was converted into cDNA using SuperScript IV VILO kit (ThermoFisher, Waltham, MA, USA), where 16 µl of RNA samples diluted to 50 ng/µl in nuclease-free water (Cat. #B1500, NewEngland Biolabs) were mixed with 4 µl of SuperScript™ VILO™ Master Mix (Cat. #11755050). In thermocycler samples were then incubated for 10 minutes at 25°C, room temperature for 10 minutes, and then 85°C for 5 minutes. Samples were then stored at -80°C.

Target specific RNA was quantified using the One-Step RT-ddPCR Advanced Kit for Probes (Cat. #1864022, BioRad, Watford, UK). Reactions were performed in multiplex using primer/probe qPCR (Integrated DNA Technologies, IA, USA). In each 20 µl reaction, 0.2 ng of cDNA was mixed with 10 µl of supermix, nuclease-free water (Cat. #B1500, NewEngland Biolabs), and 1 µL each primer probe mix (final concentration 250nm) (Table 9). The thermocycling conditions were previously optimised by Ana Sergijenko and can be seen in Table 10.

Virus specific RNA was quantified in an RRE/B2M RNA multiplex as the number of target specific copies of RNA per total ng of RNA in samples. As RRE is encoded in the viral genome but is not transcribed during transgene expression this quantifies vector-specific mRNA (Figure 6). To quantify transgene mRNA a WPRE/B2M RNA multiplex was performed. WPRE is expressed in transgene mRNA but as it is also present in the viral genomic RNA it is not specific to the former (Figure 6). Quantification of housekeeping mRNA was used as a positive control but was not used to normalise concentration of WPRE or RRE.

Table 9 - Primer and probe sequences used for RRE and WPRE RNA multiplex reactions with a B2M housekeeping probe by digital droplet polymerase chain reaction. Dye refers to the 5' fluorophore conjugated to the TaqMan® probe with a 3' TAMARA quencher.

Target	Sequence	Dye
WPRE	Forward primer: TGGCGTGGTGTGCACTGT	HEX
	Reverse primer: CCCGGAAAGGAGCTGACA	
	Probe: TTGCTGACGCAACCCCCACTGG	
RRE	Forward primer: AGTGGCCCTGGACAAATC	HEX
	Reverse primer: CCTCTTGTTCTCTAGCCTTCAC	
	Probe: TGACTTGTTGGAGTGGGAAAGACA	
B2M RNA	Forward primer: GGGTGGAACTGTGTTACGTAG	FAM
	Reverse primer: TGGTCTTTCTGGTGCTTGTC	
	Probe: CCGGAGAATGGGAAGCCGAACATAC	

Table 10 - Thermal cycling conditions used for RNA quantification by digital droplet polymerase chain reaction.

Step	Temperature (°C)	Time	Number of cycles
Enzyme Activation	95	10mins	1
Denaturation	94	30 sec	40
Annealing/Extension	58	1 min	
Enzyme deactivation	98	10 min	1
Hold	4	Up to 30 mins	

5.2.4 Quantification of vector specific DNA

Vector specific DNA was quantified using the One-Step RT-ddPCR Advanced Kit for Probes (BioRad, Watford, UK) in a WPRE/B2M multiplex. Reactions were performed in multiplex

using primer/probe qPCR (Integrated DNA Technologies, IA, USA). In each 20 µl reaction, 20 ng of DNA was mixed with 10 µl of supermix, nuclease-free water (Cat. #B1500, NewEngland Biolabs), 0.5 µl of *HindIII* (Cat. #R3104S, NewEngland Biolabs, MA, USA), and 1 µL each primer probe mix (final concentration 250nm) (Table 11). The thermocycling conditions were previously optimised by Ana Sergijenko and can be seen in Table 12.

To calculate vector copy number, the concentration of WPRE DNA copies was then divided by the number of housekeeping B2M copies. As each cell genome should contain two alleles of the housekeeping gene, the calculation is as follows:

$$\frac{\text{WPRE copies per ng}}{0.5 \times \text{B2M copies per ng}} = \text{VCN}$$

Table 11 - Primer and probe sequences used for detecting vector cDNA in a WPRE/B2M multiplex reaction by digital droplet polymerase chain reaction. Dye refers to the 5' fluorophore conjugated to the TaqMan® probe with a 3' TAMARA quencher. gDNA: genomic DNA. Probes produced by Integrated DNA Technologies, IA, USA.

Target	Sequence	Dye
WPRE	Forward primer: TGGCGTGGTGTGCACTGT	HEX
	Reverse primer: CCCGAAAGGAGCTGACA	
	Probe: TTGCTGACGCAACCCCCACTGG	
B2M gDNA	Forward primer: GTCTCTGGGTATGAGGCTTATTG	FAM
	Reverse primer: CCTCCTCTAGTTCCCTGTCTTA	
	Probe: TGCTGAGGACCTTGTGAGCTTCTT	

Table 12 - Thermal cycling conditions used for vector cDNA quantification by digital droplet polymerase chain reaction.

Step	Temperature (°C)	Time	Number of cycles
Enzyme Activation	95	10 mins	1
Denaturation	94	30 sec	40
Annealing/Extension	58	1 min	
Enzyme deactivation	98	10 min	1
Hold	4	Up to 30 mins	

5.2.5 Calculating transduction efficiency using a ddPCR-based encapsulation assay

The encapsulation assay was developed and performed by Ana Sergijenko based on previously published protocols by BioRad, to quantify the number of cells in a sample that contain a copy of vector cDNA (i.e., post reverse transcription) by cell-based ddPCR (514). Prior to analysis BMDMs were stored in RNeasyTM for up to two weeks at 4°C. Cells were prepared for analysis as follows. Cells were first removed from suspension by diluting samples 1:2 in dPBS + 0.1% BSA (Cat. #A4918, Sigma-Aldrich) and centrifuging samples at 400 *rcf* for 5 minutes at 4°C. Supernatant was then removed by careful pipetting. Samples were then washed in 300 µl of dPBS + 0.1% BSA and pelleted at 400 *rcf* for 5 minutes at 4°C. Pellets were then resuspended in 50 – 100 µl of dPBS + 0.1% BSA (dependent on cell concentration) and counted using automated cell counting (as previously described in Chapter 2.1.4). Cells were then diluted to 1000 cells/µl and stored on ice.

This is a cell-based ddPCR assay that detects viral genomes post-reverse transcription (including integrated provirus and episomal vector cDNA copies) at a single cell resolution by mixing cells directly with PCR reagents for direct encapsulation in droplets generated in the standard ddPCR workflow (Chapter 2.7). Cells are lysed within droplets and PCR amplification enables detection of reverse transcribed genomes and housekeeping genes on a per cell/droplet basis. A schematic representation of this process can be seen in Figure 49. PCR reagents (all purchased from BioRad) are mixed in a 20 µl reaction containing 6.25 µl of ddPCR Multiplex

Supermix (Cat. #12005910), 0.34 µl of DTT (Cat. #12012171), 0.5 µl of ddPCR Enhancer Reagent A (Cat. # 12013425), 8.41 µl of nuclease-free water (Cat. # B1500), 1 µl of each of restriction enzymes *Sma*I and *Bsr*I (Cat. #R0141S and #R0527S, NewEngland Biolabs), and 1.25 µl of each primer probe mix (final concentration 250nm) (Table 12). To this 20 µl reaction, 5 µl of cell suspension (100 cells/µl) is added, where each sample was measured in duplicate reactions. The downstream emulsification and processing were then performed according to the general ddPCR protocol outlined in Chapter 2.7. The thermocycling conditions can be seen in Table 13.

5.2.5.1 Transduction efficiency

The Quantasoft™ Software reports a value of how many droplets were positive for both targets (RRE/WPRE) in copies/µl which can then be expressed as a percentage of all droplets positive for Ppia (copies/µl) to calculate transduction efficiency. This calculation assumes that all droplets containing Ppia encapsulated one intact cell.

5.2.5.2 Linkage calculations

The software also reports the percentage linkage as an indicator of assay accuracy. This value represents the number of droplets that were positive for both targets (RRE/WPRE) in copies/µl, expressed as a percentage of all droplets positive for RRE (copies/µl). A low linkage percentage is an indicator of poor accuracy as viral genomes are present in droplets in the absence of a housekeeping gene.

Table 12 - Primer and probe sequences used in the cell-based ddPCR encapsulation RRE/Ppia multiplex reaction. Dye refers to the 5' fluorophore conjugated to the TaqMan® probe with a 3' TAMARA quencher. gDNA: genomic DNA. Probes produced by Integrated DNA Technologies, IA, USA.

Target	Sequence	Dye
RRE	Forward primer: AGTGGCCCTGGACAAATC	HEX
	Reverse primer: CCTCTTGTTCTCTAGCCTTCAC	
	Probe: TGACTIONGGTTGGAGTGGGAAAGACA	
Ppia gDNA	Forward primer: TACCCATCAAACCATTCTCT	FAM
	Reverse primer: AAGAACTTCAGTGAGAGCAGAG	
	Probe: CCCATCTGCTCGCAATGTCCTGTA	

Table 13 - Thermal cycling conditions used for integrated viral DNA quantification by digital droplet polymerase chain reaction.

Step	Temperature (°C)	Time	Number of cycles
Lysis/Digestion	37	1 hour	1
Enzyme Activation	95	10mins	1
Denaturation	94	30 sec	60
Annealing/Extension	60	2 min	
Enzyme deactivation	98	10 min	1
Hold	4	Up to 30 mins	

5.2.6 Transducing monocytes during macrophage differentiation

To determine the transduction efficiency of rSIV.F/HN lentivirus in monocytes during differentiation into macrophages, freshly harvested bone marrow cells plated in pre-warmed cRPMI + M-CSF (100 ng/μl) were co-incubated with lentivirus for the first 48 hours of the differentiation protocol previously described in Chapter 2.2. To end transduction, media was carefully removed from cells and replaced with 0.5 ml of fresh cRPMI + M-CSF (100 ng/μl). To pellet any cells removed with the viral media, samples were centrifuged at 400 *rcf* for 5 minutes. The viral supernatant was discarded, and cells were resuspended in 0.5 ml of pre-warmed cRPMI + M-CSF (100ng/μl) and gently pipetted back into culture (final volume 1 ml). The differentiation protocol was then followed according to the standard method and cells were harvested on day 7 as BMDMs for flow cytometry analysis of transgene expression and macrophage cell-surface receptors (Chapter 2.5.2).

5.2.7 Cloning Procedures

5.2.7.1 Ligation

Ligation reactions were performed using a T4 DNA Ligase (Cat. #M0202, NewEngland Biolabs) according to manufacturer's instructions. Briefly, in a 20 μl reaction, 2 μl of T4 DNA Ligase buffer, and Vector and Insert DNA at a 2:1 molar ration, using 50ng of vector DNA, nuclease free water, and 1 μl of the T4 DNA ligase. The mixture was then incubated at room temperature for 10 minutes and then heat inactivated at 65 °C for 10 minutes. Ligation mixtures were then chilled on ice until transformation.

5.2.7.2 Bacterial Transformation

Plasmids and ligation mixtures were transformed into NEB® 5-alpha Competent *E. coli* cells (Cat. #C2987H, England Biolabs) by heat shock, according to manufacturer's instructions. 50 μl of competent cells were mixed with 2 μl of ligation reactions or 50 ng of plasmid DNA and incubated on ice for 30 minutes on ice. Bacteria were heat-shocked in a heat-block at 42°C for 30 seconds and then incubated on ice for 5 minutes. 450 μl of pre-warmed SOC medium was added to the mixture and incubated in a shaking incubator at 37°C for 1 hour, 220 rpm. The mixture was then spread on pre-warmed selective LB (Luria Bertani) agar plates and incubated overnight at 37°C.

5.2.7.3 Bacterial Propagation and Plasmid Extraction

Selective LB agar was made by dissolving 35 g LB Agar (Sigma-Aldrich) per 1 L ddH₂O, which was then autoclaved for 15 minutes at 121°C and cooled to approximately 50°C before the addition of the appropriate antibiotic. To make selective LB agar plates, approximately 20 ml of autoclaved warm selective-LB Agar was poured into 90 mm sterile petri dishes (ThermoFisher Scientific) and cooling at 4°C until set. Plates were stored at 4°C.

Single colonies were picked from selective agar plates and propagated in 5 ml LB broth cultures (Sigma-Aldrich) with the appropriate antibiotic and incubated overnight in a shaking incubator at 37°C and 220 rpm. Bacteria were then pelleted by centrifuging of culture broth at 13,000 *rcf* for 5 minutes, and plasmid DNA was extracted from the bacteria using a Monarch® Plasmid Miniprep Kit (Cat. #T010, NewEngland Biolabs) according to manufacturer's instructions. Extracted plasmid DNA was then quantified and checked for purity (A₂₆₀/280 ratio) using a NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher Scientific).

5.2.7.4 Restriction Digests

Restriction digests were used excise fragments of interest and cut open vector backbones. The restriction enzymes used in each reaction are indicated. Digestion reactions were performed in 12.5 µl reactions using 250 ng of plasmid DNA, 0.25 µl of each restriction enzyme, and 1.25 µl of the appropriate CutSmart® Buffer (NewEngland Biolabs) supplied with the enzyme. Samples were then incubated at the temperature and time indicated for each enzyme and 12.5 µl of the digest product was loaded on a 1% agarose gel. These gels were made by dissolving, 1g of agarose (Sigma-Aldrich) in 100 ml of Tris-EDTA buffer solution (Cat. #93283, Sigma-Aldrich) and microwaved for ~2 minutes. 10 µl of SYBR™ Safe DNA Gel StainGelRed™ (Cat. #S33102, ThermoFisher Scientific) nucleic acid gel stain was added, and the gel was poured into gel form to set. Following loading of the restriction digest, and 1 kb or 100 bp DNA ladders (Cat. #B7025, and #B7025 , NewEngland Biolabs), gels were electrophoresed at 80 volts to separate DNA fragments and visualised by blue or UV light. DNA was purified from agarose gels using visualisation by blue light and a Monarch® DNA Gel Extraction Kit (Cat. #1020, NewEngland Biolabs) according to manufacturer's instructions. Extracted DNA was then quantified and checked for purity (A₂₆₀/280 ratio) using a NanoDrop™ 2000/2000c Spectrophotometer.

5.2.8 Sleeping Beauty (SB) transposon system

5.2.8.1 Sleeping Beauty Plasmids

Two plasmids encoding the sleeping beauty transposon system were obtained from Addgene (MA, USA):

- **pCMV(CAT)T7-SB100**: encoding the sleeping beauty transposase enzyme was a gift from Zsuzsanna Izsvak (Addgene plasmid #34879; <http://n2t.net/addgene:34879>; RRID:Addgene_34879) and carries a chloramphenicol resistance gene (Cat. #C0378, Sigma-Aldrich). (Referred to as SB100X)
- **PT2/HB**: encoding the IR/DR sequences that are recognised by the SB transposase and define the transposase genome was a gift from Perry Hackett (Addgene plasmid #26557; <http://n2t.net/addgene:26557>; RRID:Addgene_26557) and carries an ampicillin resistance gene (Cat. #A0166, Sigma-Aldrich).

Sequence confirmation of both plasmids was performed using restriction digests and Sanger sequencing by Genewiz (Leipzig, Germany).

Table 14 – Sanger sequencing primers and restriction enzymes used to confirm plasmid identity. Primer synthesis and Sanger sequencing were performed by Genewiz. All restriction enzymes were purchased for NewEngland Biolabs.

Plasmid	Sequencing Primers	Restriction Enzymes
SB100X	T7 Forward primer: AGTGGCCCTGGACAAATC	<i>Apa</i> I (#R0114), <i>Eco</i> RI (#R0101), <i>Nde</i> I (#R0111)
PT2/HB	T3 Reverse primer: CCTCTTGTTCTCTAGCCTTCAC	<i>Apa</i> I, <i>Eco</i> RI, <i>Bam</i> HI (#R0136)

5.2.8.2 Cloning genes of interest into the *Sleeping Beauty* transposon

Glux, EGFP, and GM-CSF genes of interest were first cloned into non-viral expression cassettes and then cloned into the sleeping beauty (SB) transposon plasmid PT2/HB, to be flanked by the terminal inverted repeats containing duplicate transposase binding sites in direct orientation (IR/DR) that bind the SB system transposase and define the transposon genome.

pGM169 is a non-viral expression plasmid that has previously been delivered to patients with cystic fibrosis in a clinical trial aiming to transfer a wildtype CFTR gene to the lung epithelium (515, 516). CFTR is encoded in a non-viral expression cassette where the CFTR gene of interest is flanked by a hCEF promoter/enhancer and a bovine growth hormone polyadenylation sequence.

CFTR was excised from pGM169 by *NheI* (Cat. #R3131, NewEngland Biolabs) and *ApaI* (Cat. #R0114S, NewEngland Biolabs) restriction digest. Glux, GM-CSF, and EGFP genes of interest were also excised from lentiviral backbone plasmids pGM358, pGM547 and pGM357 respectively using *NheI* and *ApaI* restriction digest. These fragments were then ligated using a T4 DNA Ligase (Cat. #.M0202, NewEngland Biolabs) into the non-viral expression cassette of the empty pGM169 backbone to create three new non-viral plasmids encoding Glux (pGM206), GM-CSF (pIC017), and EGFP (pIC064) (Note: pIC017 had previously been cloned by Robyn Bell). A plasmid map of a general lentiviral backbone plasmid from which the genes of interest were obtained can be seen in Figure 6, and a representative non-viral plasmid encoding a gene of interest can be seen in Figure 39a. Successful cloning was confirmed by diagnostic restriction digest and Sanger Sequencing (Table 15)

The three non-viral expression cassettes encoding Glux (pGM206), mGM-CSF (pIC017), and EGFP (pIC064) were then cloned into the PT2/HB sleeping beauty transposase backbone using NEBuilder® HiFi DNA Assembly (Cat. #E2621, NewEngland Biolabs). Briefly, HiFi primers with large overhangs corresponding to the PT2/HB backbone were synthesized by Integrated DNA Technologies (IA, USA) (Table 16). PCR fragments were created in a 12.5 µl PCR reaction using a Q5® Hot Start High-Fidelity 2X Master Mix (Cat. #M0494, NewEngland Biolabs), 10 µM of each forward and reverse primer, and 0.25 ng of non-viral plasmid DNA (pIC064, pGM206, pIC017). The PT2/HB vector backbone was digested with *HindIII* (Cat. #R3104S, NewEngland Biolabs) and *XbaI* (Cat. #R0154S, NewEngland Biolabs). Fragments were then ligated into the open backbone by NEBuilder® HiFi DNA Assembly

according to manufactures instructions. Briefly, fragments and the digested PT2/HB vector backbone at a 2:1 ratio of insert mass DNA and vector (0.03–0.2 pmols of total fragments) were incubated at 37 °C with 10 µl of NEBuilder HiFi DNA Assembly Master Mix, for 1 hour, followed by a 20-minute incubation at 80 °C. The reaction was then transformed into Dh5a competent cells and plated on ampicillin selection agar overnight to then select colonies. Successful cloning was confirmed by restriction digest with *EcoRI* and *KpnI* (Cat. #R3142, NewEngland Biolabs) and Sanger sequencing (Table 15). These plasmids were then referred to as: PT2/HB-EGFP, PT2/HB-mGM-CSF, and PT2/HB-Glux, and a representative plasmid can be seen in Figure 39b.

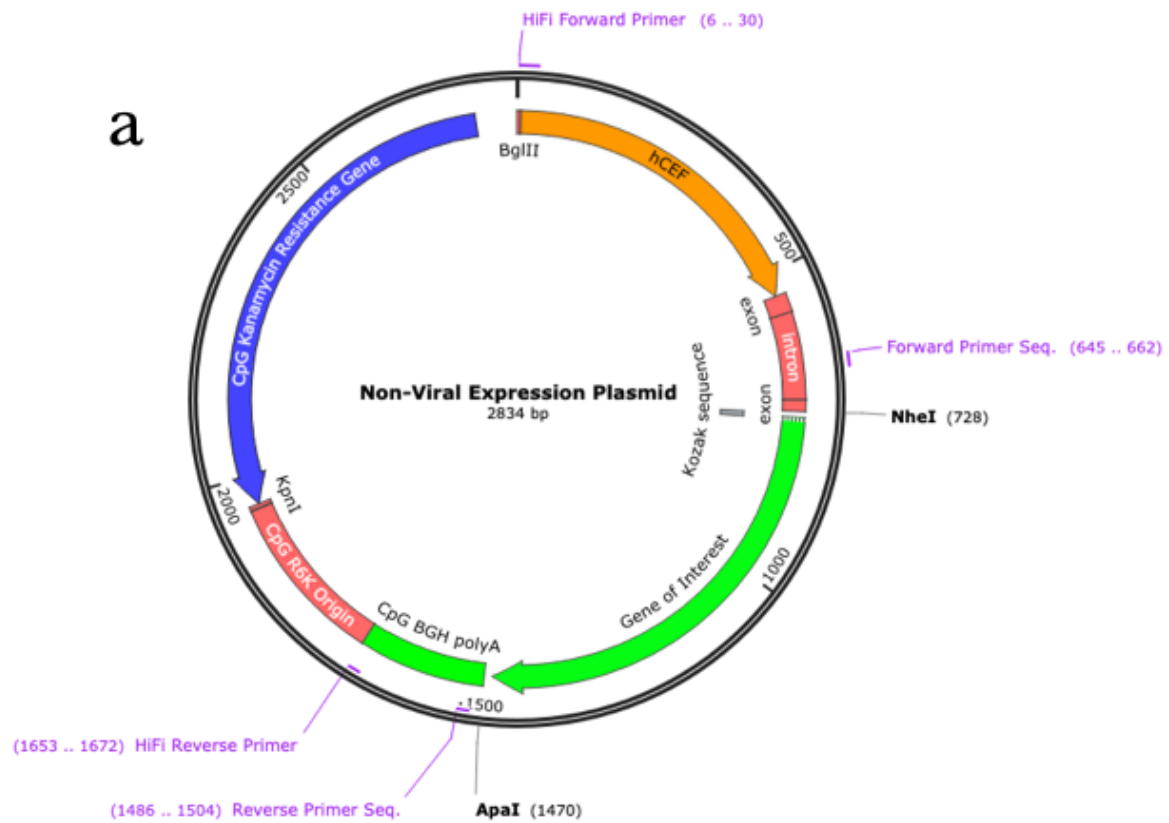
Table 15 – Primers used by Sanger sequencing. Primer synthesis was performed by Integrated DNA Technologies, and Sanger sequencing were performed by Genewiz.

Plasmids	Primers
PT2/HB-EGFP/mGM-CSF/Glux	T7 Forward primer: AGTGGCCCTGGACAAATC T3 Reverse primer: CCTCTTGTTCTCTAGCCTTCAC
pGM206, pIC017, pIC064	Forward primer: CCCTGCAGCCCTAGGAAT Reverse primer: CAAACAACAGATGGCTGGC

Table 16 - Primers used in HiFi Assembly. Underlined sequences are overhands that are complementary to the PT2/HB backbone.

Name	Primers
HiFi Forward Primer	AATGATGTCATGGCTTTAGAAAGCTTTGTTACATAACT TATGGTAAATGGC
HiFi Reverse Primer	ACAAGCTAGATCTGGCCATCTAGAGCATGCCTGC TATTGTCTTC

a



b

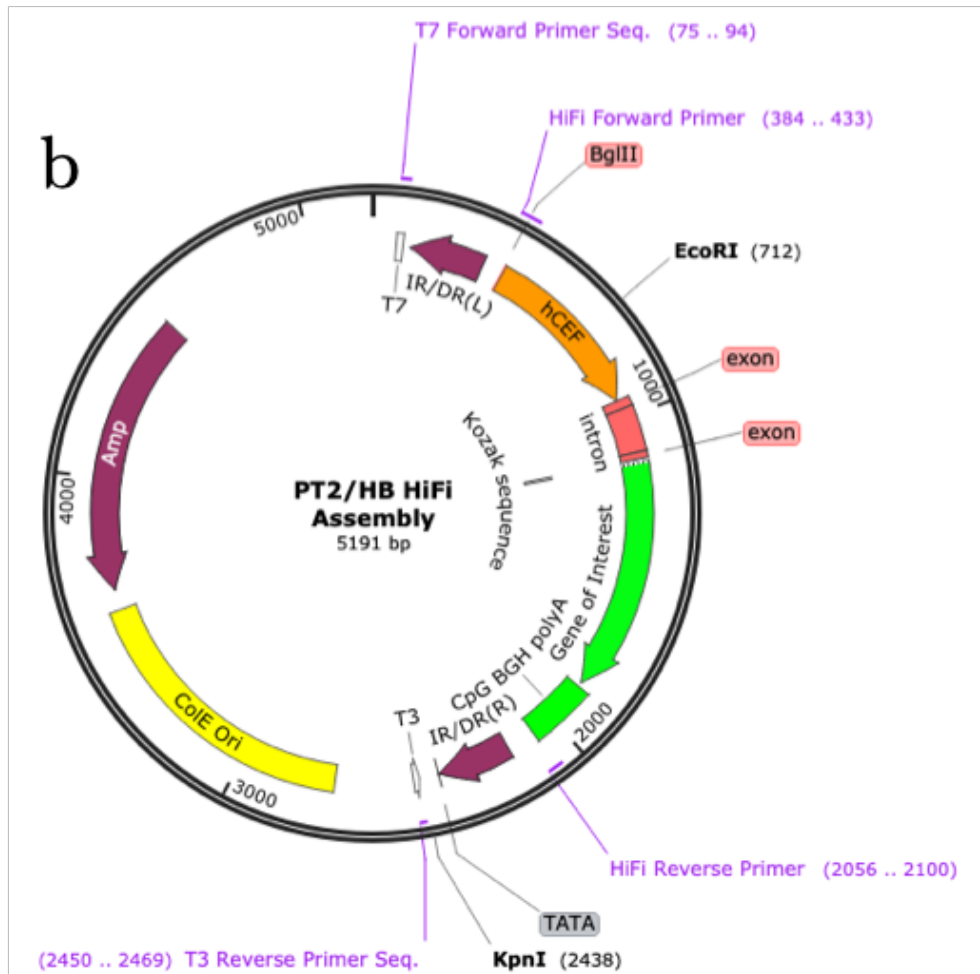


Figure 39 – Representations of a non-viral expression plasmid and a sleeping beauty transposase plasmid with a non-viral expression cassette. (a) Represents plasmids pGM206, pIC017, and pIC064, which encode Glux, mGM-CSF, and EGFP as the gene of interest respectively. (b) Representative plasmid map of PT2/HB- EGFP, PT2/HB-mGM-CSF, and PT2/HB-Glux where non-viral expression cassettes are flanked by terminal inverted repeats containing duplicate transposase binding sites in direct orientation (IR/DR) that bind the SB system transposase. Transgene expression is under the control of a chimera enhancer/promoter hCEF. Encoded within the expression cassette are a bovine growth hormone poly-adenylation sequence (polyA) and a kozak sequence (Kozak). Amp: ampicillin resistance gene, CpG R6K origin and ColE Ori: bacterial origins of replication. Primers used in sequencing are indicated, as well as the binding of primers used in HiFi assembly. Image created with SnapGene® (v5.3.2).

5.2.9 Transfection of the Sleeping Beauty system

The sleeping beauty transposon system was transfected into BMDMs and A549 cells using polyethylenimine (PEI-Pro) transfection reagent (Polyplus, Reading, UK). A549 cells were plated seeded onto a Costar 24- well plate (Sigma Aldrich) at 50,000 cells per well and transfected 24h later. BMDMs were transfected without passaging, following differentiation in a 24-well Nunc™ Non-Treated multidish. Cells were transfected using 2:1 ratio of PEIPro to DNA with either single plasmid transfections, or co-transfections of the SB100X and PT2/HB expression plasmids. Co-transfections were performed at a 2:1 ratio of SB100X to PT2/HB with a total of 0.25 µg/ml. Single transfection controls were transfected at 0.167 µg/ml and were controlled to the amount of PT2/HB transgene expressing plasmid in the co-transfection. Transductions were halted after 24-hours by media change.

5.3 Results

5.3.1 Optimising small scale VSV-G lentivirus production

In order to compare the efficacy of VSV-G and F/HN pseudotypes and the relative strength of hCEF, CMV, and EF1a in BMDMs, I first manufactured the candidate rSIV lentiviral vectors with the following configurations:

- F/HN.hCEF.EGFP
- F/HN.CMV.EGFP
- F/HN.EF1a.EGFP
- VSV-G.hCEF.EGFP
- VSV-G.CMV.EGFP
- VSV-G.EF1a.EGFP

In initial experiments, the six vectors required for the optimisation of transduction and gene expression in macrophages were made in house without further concentration or purification. This avoided the high cost associated with large-scale vector production, purification and concentration. Therefore, these vectors were made in small scale 50 mL productions. The GTC Core Facility in Oxford had provided a protocol for the production of F/HN lentivirus based on the transient transfection of suspension HEK 293T cells, as reported in previous publications (268, 276, 279). However, in the laboratory there was no pre-existing protocol for the production of SIV virus with a VSV-G pseudotype. I therefore first optimised the production of VSV-G pseudotyped SIV, with a protocol based on that of F/HN pseudotyped lentivirus.

5.3.1.1 Optimisation of plasmid concentration increases vector yields

Production was optimised using rSIV.VSV-G virus expressing EGFP under hCEF transcriptional control (genomic plasmid: pGM357 hCEF-EGFP), and successful transduction was determined by flow cytometry analysis of EGFP protein expression. 50ml batches of virus were only produced in duplicate or triplicate (in the first and second experimental runs respectively) due to the substantial associated costs of time and resources. As a result of low n numbers these comparison studies are not powered for statistical analysis.

I first compared the Oxford provided protocol for rSIV.F/HN production (268, 279, 517), which used 0.33 μg of total plasmid DNA per $1\text{e}6$ cells ($\mu\text{g}/1\text{e}6$ cells) in the transient transfection, to rSIV.VSV-G production protocols published by Rebekka Harding-Smith as part of her PhD project with the Oxford arm of the Consortium, where 1.5 μg of total plasmid DNA was used per million cells in culture (517). Increasing the plasmid concentrations from 0.33 $\mu\text{g}/1\text{e}6$ cells (total 6.6 μg) to 1.5 $\mu\text{g}/1\text{e}6$ cells (total 30 μg) lead to an approximately 100-fold increase in vector production (Figure 40), indicating that the concentration of plasmid DNA was a limiting factor in production (Table 17). To determine whether further increases in concentration would further increase production, transfection with 1.5 $\mu\text{g}/1\text{e}6$ cells was compared to 2 $\mu\text{g}/1\text{e}6$ cells which is the upper limit of the PEIpro transfection DNA payload. However, vector yields decreased indicating that past 1.5 $\mu\text{g}/1\text{e}6$ cells the amount of transfected DNA is no longer the limiting factor in vector production or that at higher concentrations there is cytotoxicity associated with the transfection complex (Figure 40, Table 17).

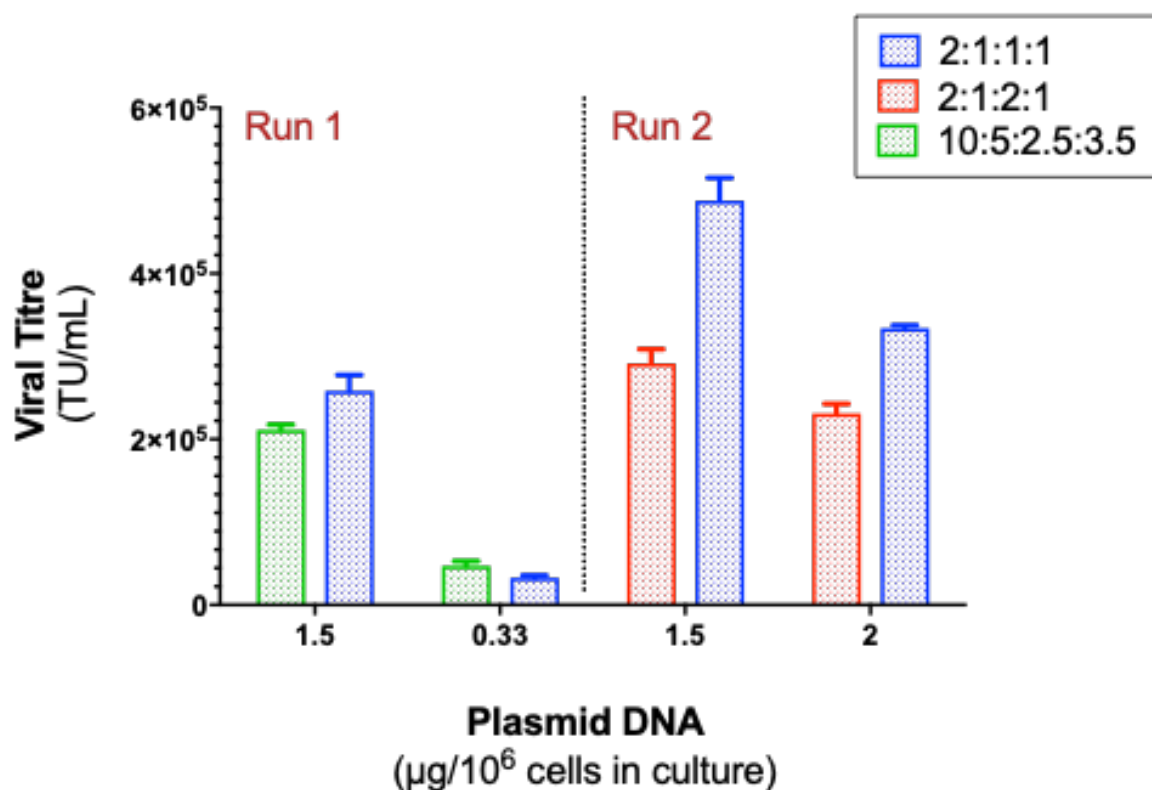


Figure 40 – Titres of rSIV.VSV-G.hCEF.EGFP vector with a VSV-G pseudotype produced under different production protocols. Over two separate small-scale production runs (50 mL) (separated by dotted line) rSIV virus was produced through transient transfection of HEK293T suspension cells with 0.33, 1.5, or 2 µg of plasmid DNA per 1e6 cells in culture. Four producer plasmids encoding the vector genome (pGM357), gag/pol (pGM297), rev response element (pGM299), and the VSV-G envelope (pMD2.G) were used at one of three ratios 2:1:1:1, 2:1:2:1, or 10:5:2.5:3.5 (genome:gag/pol:rev:env). Each virus was produced in duplicate (run 1) or triplicate (run 3) and titred in HEK293T suspension cells by flow cytometry analysis of EGFP signal. Bars represent group medians ± range. TU/mL: Transduction units per mL.

5.3.1.2 *Optimisation of plasmid ratios*

rSIV production with a F/HN pseudotype is based on the transient transfection of 5 producer plasmids as envelope proteins F and HN are expressed on separate plasmids. VSV-G pseudotyped virus is produced by transient transfection with 4 producer plasmids as one plasmid encodes the VSV-G envelope protein. Therefore, the ratio used in F/HN viral production is not applicable and I needed to determine the optimal ratio for VSV-G SIV production. The ratio of producer plasmids used in transfection vary greatly across the literature and using the correct ratio is important for optimal viral yields as it determines the efficiency of virion assembly and genome packaging (518). When describing the relative ratios of each plasmid used in lentiviral production, they are referenced in the following order as the plasmids encoding: the lentiviral genome, gag/pol, the rev response element and envelope proteins. In these optimisation experiments they are pGM537:pGM297:pGM299:pDM2.G (Plasmids described in Chapter 2, section 2.2.1). I first compared the ratio 10:5:3.5:2.5 used by Rebekka Harding-Smith (517), to produce VSV-G SIV to ratios used in standard protocol in the literature (2:1:1:1 and 1:1:2:1) (n=3/group) (519-521).

At high plasmid concentrations a 2:1:1:1 ratio resulted in highest vector yields (Figure 40). At the higher plasmid concentration (1.5 µg/1e6 cells), a ratio of 2:1:1:1, increased titres by 18% in comparison to 10:5:3.5:2.5. In comparison to a 2:1:2:1 ratio, transfection plasmids at a 2:1:1:1 ratio led to a 40% increase when using the optimal 1.5 µg of plasmid DNA per 1e6 cells (Table 17).

Between production runs a degree of inter-experiment variation was observed when comparing vector titres from produced virus under the same protocol (30 µg of plasmid DNA at a 10:5:3.5:2.5 ratio) (Figure 40, Table 17). However, within production runs there was little variation between duplicates or triplicates. This may be due to differences in cell viability which can significantly impact production (276, 279), as between the first two productions, the second reported higher titres and used ‘fresh’ cells which had undergone only 4 passages, in comparison to the >10 in the first (exact number not noted). Direct comparisons of the different protocols were therefore limited to viral batches produced in the same runs and highlighted the importance of using not-overly passaged cells with high viability.

As altering ratios led to small differences in vector titres, and in the interest of time, I decided to move forward with the production of the different experimental vector configurations for

initial testing in macrophages, producing VSV-G vectors through transfection of 30 µg of plasmid DNA at a 2:1:1:1 ratio.

Table 17 - rSIV.VSV-G.hCEF.EGFP titre in 50 mL yields following small batch production optimisation. Production protocol variations for each virus produced over two separate production runs, where viruses were produced in duplicate (run 1) or triplicate (run 2). Ratio of plasmids used in transient transfection given as genome:gag/pol:rev:env.

Production Run	Plasmid DNA Concentration (µg/1e6 cells in culture)	Ratio	Viral Titre Median ± Range (TU/mL)
1	0.33	10:5:3.5:2.5	4.7e4 ± 7.8e3
1	0.33	2:1:1:1	3.3e4 ± 3.8e3
1	1.5	10:5:3.5:2.5	2.1e5 ± 9e3
1	1.5	2:1:1:1	2.6e5 ± 2.7e4
2	1.5	2:1:1:1	5.0e5 ± 5.3e4
2	1.5	1:2:1:2	2.9e5 ± 3.3e4
2	2	2:1:1:1	3.3e5 ± 7e3
2	2	1:2:1:2	2.3e5 ± 2.3e4

5.3.2 Titres of VSV-G and F/HN pseudotyped SIV lentivirus produced in house are too low for macrophage transductions

Three rSIV.VSV-G viruses each encoding a EGFP transgene under transcriptional control of a different regulatory element were then produced under the optimised protocol using 1.5 µg of plasmid DNA per 1e6 cells, in a 2:1:1:1 ratio. Titres were similar to previous production iterations, with vector yields to the order of 1e5 TU/ml. Three rSIV.F/HN viral configurations were also produced using a specific protocol for rSIV.F/HN production provided by the GTC Core Facility in Oxford (268, 276). When comparing titres achieved by F/HN and VSV-G vectors produced with the same expression plasmid (i.e., regulatory element), titres of F/HN vectors were 91%, 69.5%, and 77.5% lower than corresponding VSV-G viruses for hCEF, CMV, EF1α respectively (Table 18). Across all production iterations, vector yields were to the order of 1e4-1e5 U/mL in magnitude, at least one log order lower than the $\geq 5e6$ TU/mL titre reported by Oxford collaborators in small scale (40-400 mL) productions of crude rSIV.F/HN vectors (276).

As described in Chapter 3, the transduction efficiency of SIV.F/HN.hCEF.EGFP (produced and concentrated at Oxford) was optimal at MOIs of 20-50. In optimising the differentiation and transduction of bone marrow-derived macrophages it was found that transduction was most successful in adherent cells that were not passaged following the differentiation protocol. As such transductions were performed without harvesting cells from the wells they were plated in initially as bone marrow. The protocol developed for macrophage differentiation plates bone marrow cells in 24 well plates at a 1.5e5 cells per well which results in 3-4e5 macrophages per well on day 7 of the differentiation protocol. At these cell densities, achieving the MOIs found to be efficient in experiments with F/HN-hCEF virus (MOI 20-50) with low viral titres of even 5e6 TU/mL would be difficult or impossible due to constraints on the maximum volume capacity of the cell culture wells. Therefore, the titres of all vectors produced in house were lower than required for effective comparisons in bone marrow-derived macrophages.

Table 18 - rSIV.VSV-G.hCEF.EGFP titre in 50 mL yields following small batch production optimisation. Production protocol variations for each virus produced over two separate production runs, where viruses were produced in duplicate (run 1) or triplicate (run 2). Ratio of plasmids used in transient transfection given as genome:gag/pol:rev:env.

Virus	Vector Genome Plasmid	Envelope Plasmid(s)	Crude Titre (TU/mL)	Concentrated Titre (TU/mL)	Oxford Titre (TU/mL)
rSIV.F/HN.EFI α	pGM354	F: pGM301, HN: gGM303	2.2e5	1.05e6	1.01e7
rSIV.F/HN.CMV	pGM299	F: pGM301, HN: gGM303	1.15e5	1.71e6	3.57e6
rSIV.F/HN.hCEF	pGM357	F: pGM301, HN: gGM303	2.68e5	6.87e5	2.13e7
rSIV.VSV-G.EFI α	pGM354	VSV-G: pMD2.G	5.29e5	5.10e7	1.43e8
rSIV.VSV-G.CMV	pGM299	VSV-G: pMD2.G	8.39 e5	1.21e8	1.52e8
rSIV.VSV-G.hCEF	pGM357	VSV-G: pMD2.G	4.48e5	1.26e7	5.36e8

5.3.3 Titres of crude vector productions can be concentrated by high-speed centrifugation

As the reported titre achieved in the GTC Core Facility would still be sup-optimal for macrophage transduction, I then attempted to concentrate the virus rather than further attempts at optimising production. By concentrating virus and increasing effective viral titres, sufficient quantities of concentrated virus may be achieved through the pooling of virus from multiple production batches. Oxford collaborators produce highly concentrated rSIV.F/HN virus through the concentration of large 1L and 5L production runs. This purification and concentration protocol is based on anion-exchange and hollow-fibre ultrafiltration/diafiltration size exclusion (268, 276). Without access to the required equipment, I instead relied on high-speed centrifugation.

VSV-G virus produced when optimising production were used to test the efficacy of high-speed centrifugation to concentrate virus. In the first attempt 30 mL aliquots of two different virus preps were pelleted in sterile 50 mL falcon tubes at a maximum of 26,200 *rcf* for 3 hours at 4°C and resuspended in 250 µl of FreeStyle™ 293 Expression Medium. Titres successfully increased from 2.6e5 to 5e6 TU/mL and 2.4e5 to 4.4e6 TU/mL representing an 18- and 19- fold increase in concentration respectively. However, as virus was spun in 30ml aliquots and then resuspended in 250 µl, this represents a large degree of viral loss, where only ~ 15% of viral transduction units were recovered in the concentrated fraction. As the supernatant was discarded, it is unclear if this is due to incomplete pelleting (i.e., due to insufficient *rcf*), or loss of function as a result of centrifugation.

To reduce viral loss, the protocol was adapted by aliquoting virus 24 -aliquots of 1mL prior to high-speed centrifugation, this was performed in a different centrifuge with a maximum speed of 17,000 *rcf*. Viral supernatant was manually decanted by inversion of Eppendorf tubes in one smooth motion rather than through pipetting to prevent viral pellet detachment. As a result, the remaining supernatant in each tube determined the new volume of the concentrated virus which was then pooled across all aliquots. Again, a VSV-G virus produced during the optimisation of VSV-G vector production was used. Following concentration, 24 mL of crude virus was concentrated into a new volume of 820 µl. Here the single virus prep tested was successfully concentrated from an initial titre of 4e5 TU/ml to 1.1e7 TU/ml, representing a 27.7-fold increase in titre, and only a 5.23% loss in total transduction units. Viral particles were

still present in the viral supernatant and could account for the 5.23% loss suggesting that the centrifugation protocol was well tolerated by viral particles. Having established a successful concentration protocol which resulted in low loss of functional virus, the three rSIV.F/HN and rSIV.VSV-G viruses encoding EGFP under transcriptional control of either hCEF, CMV or EF1 α were successfully concentrated 28–144 fold in 1ml aliquots (Table 18, Figure 41).

The process was, however, highly labour intensive, and the overall transduction units required for macrophage transduction would require pooling multiple production batches which would be highly inefficient. The concentration potential was also limited by the volume in which the virus was resuspended in, which is determined by the supernatant remaining after decanting. Furthermore, even following concentration, when testing this virus in macrophages, only rSIV.VSV-G.CMV and rSIV.VSV-G.EF1 α concentrated titres were high enough to transduce macrophages at relevant MOIs.

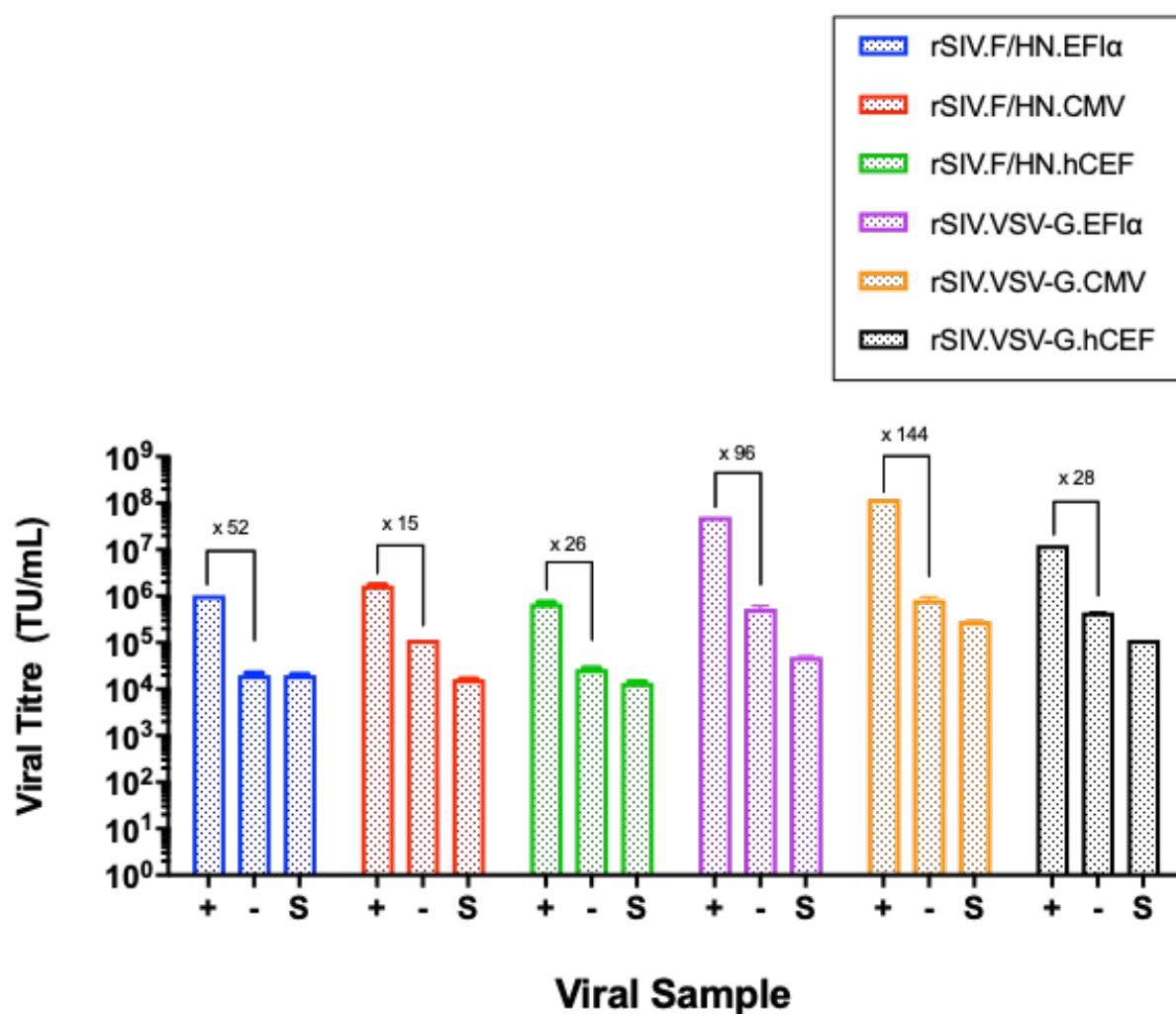


Figure 41 – Titres of six different rSIV lentiviral vectors produced in London in small-scale production runs (50 mL) following concentration by high-speed centrifugation. The fold change in titres between concentrated (+) and crude (-) virus is indicated for each virus configuration, in addition to the titre of discarded supernatant following centrifugation and viral pelleting (S). Each virus was produced as a single batch and titred in duplicate. Bars represent group medians $\pm$ range. TU/mL: Transduction units per mL.

5.3.4 Initial study suggests a VSV-G SIV pseudotype is more efficient in bone marrow-derived macrophages than F/HN

Having produced two VSV-G lentiviral vectors which when concentrated by high-speed centrifugation resulted in sufficiently high vector titres for *in vitro* transduction of BMDMs, I compared VSV-G and F/HN pseudotyped SIV vector efficiency in BMDMs to see if an alternative pseudotype could be used to increase the effective dose of genetically modified macrophages delivered to the lung. To enable this comparison, I used rSIV.F/HN.hCEF.EGFP virus produced and purchased from the GTC Core Facility in Oxford. This was not an optimal comparison as the F/HN lentivirus was produced under a different protocol in a 5L wave-bioreactor and purified via anion-exchange and ultrafiltration/diafiltration size exclusion (473). BMDMs were transduced at MOI 20 and analysed by flow cytometry for EGFP positive cells (n=6 wells/virus). The VSV-G pseudotype effectively doubled the transduction efficiency of the SIV lentivirus with a significant difference in EGFP positive cells between the F/HN.hCEF and VSV-G.CMV vectors ($p < 0.001$, Kruskal-Wallis, Dunn's multiple comparisons) (Figure 42):

- *VSV-G.CMV*: median 40.27 (range 38.92–46.12) % EGFP⁺ cells
- *VSV-G.EF1 α* : median 47.97 (range 42.22–52.02) % EGFP⁺ cells
- *F/HN.hCEF*: median 18.32 (range 15.12–20.82) % EGFP⁺ cells

Though promoters were not consistent between pseudotypes, this pilot study indicated VSV-G may transduce macrophages more efficiently than F/HN.

Having determined that SIV lentivirus may transduce BMDMs more efficiently with a VSV-G envelope, I reached out to collaborators at Oxford who kindly agreed to produce the six vectors at the GTC Core Facility in small-scale 50 mL preps. The vector preparations made at Oxford had higher viral titres when compared to our in-house produced crude and concentrated virus (Table 18 and Figure 43).

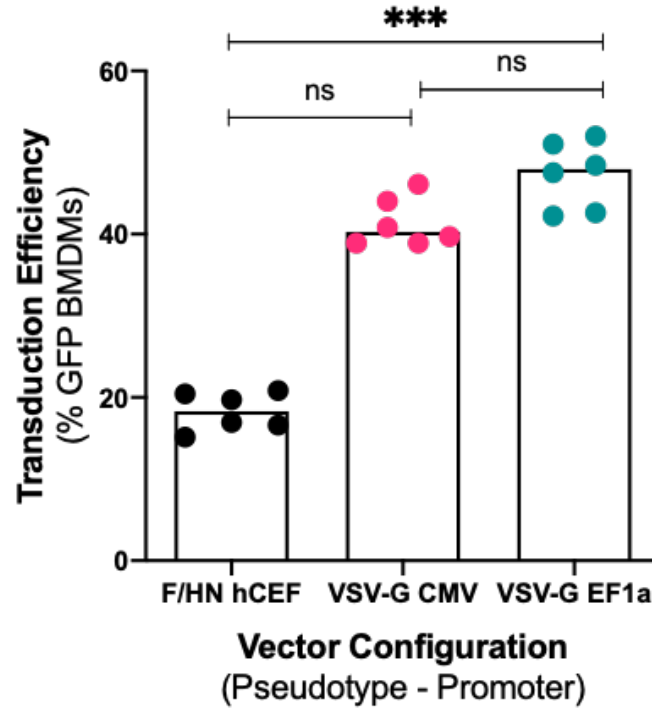


Figure 42 – Relative transduction efficiency of VSV-G and F/HN pseudotyped vectors in bone marrow-derived macrophages (BMDMs). The relative transduction efficiency of BMDMs following transduction with rSIV vectors pseudotyped with F/HN or VSV-G envelopes expressing an EGFP transgene under the control of the hCEF, EF1a or CMV promoter at MOI 20 (n=6/MOI). Transduction efficiency was calculated as the percentage of EGFP positive cells in culture by flow cytometry 7 days post-initiation of transduction (48 hours). Capture by BD Accuri™ C6 Plus Flow Cytometer. Each data point represents an individually transduced well of BMDMs. Bars represent median. ***=p<0.001, Kruskal-Wallis test, Dunn's multiple comparisons.

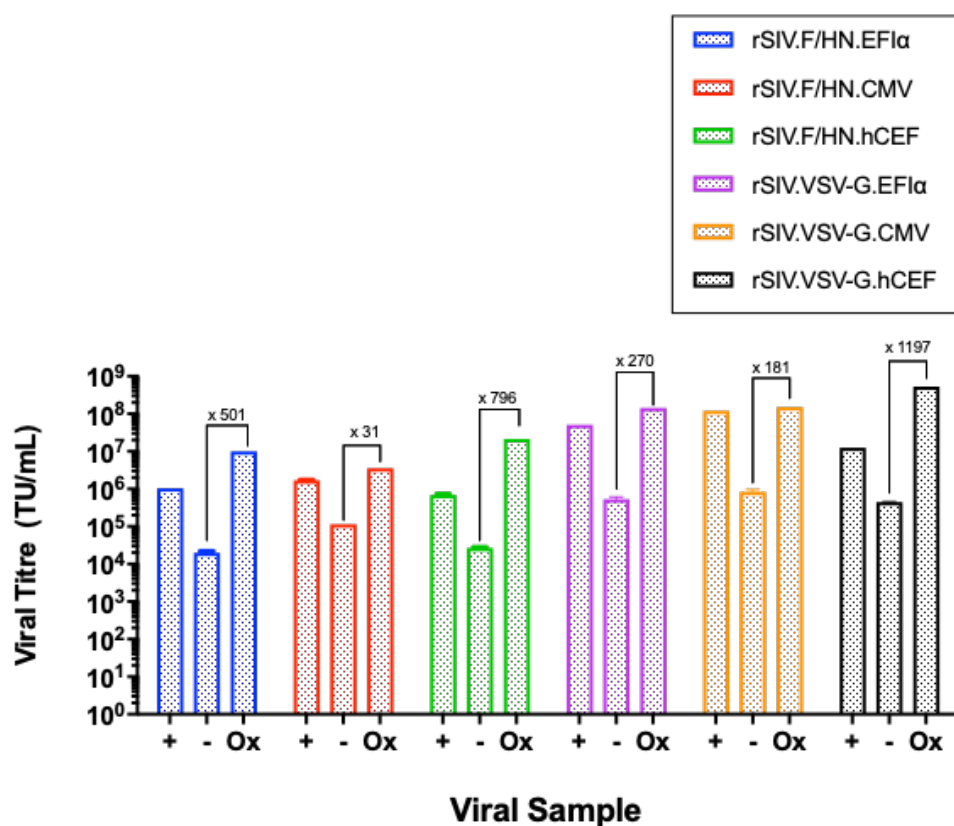


Figure 43 – Comparison vector titres of six different rSIV lentiviral vectors produced in London and Oxford in small-scale production runs (50 mL). London virus was concentrated by high-speed centrifugation, and the concentrated or crude titre (-) and the resultant concentrated titre (+). The fold change in titres between crude London virus (-) and crude Oxford virus (Ox) is indicated for each virus configuration. London virus was produced as a single batch and titred in duplicate. The single value provided for final Oxford titres is reported here. Fold increase in viral titre produced by oxford in comparison to London is indicated for each vector. Bars represent group medians $\pm$ range. TU/mL: Transduction units per mL.

5.3.5 The efficacy of SIV transduction in macrophages is promoter and pseudotype dependent

As small-scale virus produced by Oxford had higher titres, they enabled the direct comparison of VSV-G and F/HN enveloped SIV in macrophages at MOI 10 (n=6/group). This MOI was chosen as it was the closest possible titre to the optimal MOI 20-50 with the available F/HN vectors. However, even at MOI 10, the rSIV.F/HN.hCEF.EGFP titre was insufficient and could therefore was not included in the comparison. In addition, macrophages were also transduced at MOI 50 with the three VSV-G vectors to determine the extent of any response to increasing dose (n=6/group). To determine the effect of envelope on transduction, comparisons were made between viruses with the same regulatory elements.

5.3.5.1 SIV transduction of macrophages is more efficient with a VSV-G pseudotype

In these comparisons the effect of lentiviral envelope was minimal and inconsistent, but statistically significant ($P < 0.01$, 2-wayANOVA, main effect) (Figure 44). VSV-G enveloped virus had a significantly higher rate of transduction than its F/HN counterpart when transgene expression was driven by EF1 α but not CMV:

- *EF1 α* : median difference 4.8 % transduction efficiency ($p < 0.01$)
- *CMV*: median difference 1.7 % transduction efficiency ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

However, the effect was minimal and did not recapitulate the dramatic increases seen previously.

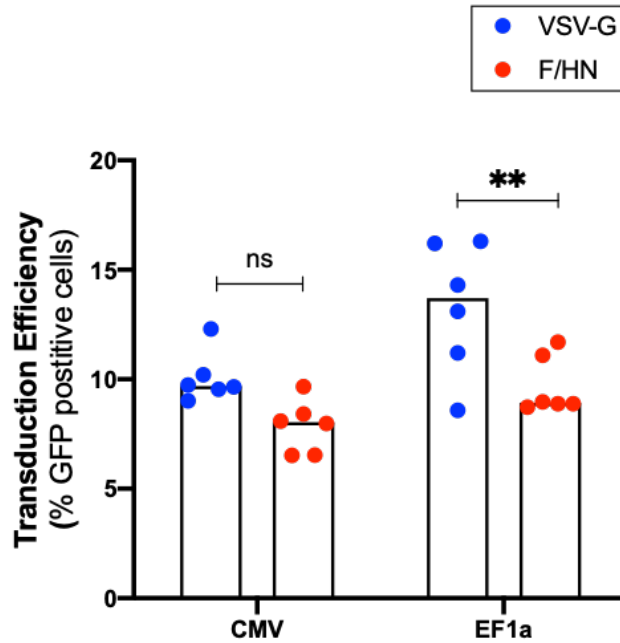


Figure 44 – Relative transduction efficiency of VSV-G and F/HN pseudotyped rSIV vectors in bone marrow-derived macrophages (BMDMs). The relative transduction efficiency of BMDMs following transduction with rSIV vectors pseudotyped with F/HN or VSV-G envelopes expressing an EGFP transgene under the control of the CMV or EF1a promoter at MOI 20 (n=6/MOI). Transduction efficiency was calculated as the percentage of EGFP positive cells in culture by flow cytometry 7 days post-initiation of transduction (48 hours). Capture by BD Accuri™ C6 Plus Flow Cytometer, analysis in FlowJo software. Each data point represents an individually transduced well of BMDMs. Bars represent median. **=p<0.01, 2-wayANOVA, Sidak's multiple comparisons.

5.3.5.2 *Lentiviral constructs encoding an EF1 α promoter report higher transduction efficiencies than hCEF or CMV.*

A distinct dose response was seen between MOI 10 and 50 when transducing macrophages with VSV-G vectors, where both the vectors pseudotype, regulatory element, and their interaction significantly impacted transduction efficiencies ($p < 0.0001$, 2-wayANOVA, main effects) (Figure 45a). Though MOI 50 led to significant increases in transduction across all three regulatory constructs ($p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons), at MOI 50 significantly more EGFP positive cells were detected when expression was driven by EF1 α transcriptional control (Figure 45b):

- *hCEF*: median difference 19.7% transduction efficiency ($p < 0.0001$)
- *CMV*: median difference 20.0% transduction efficiency ($p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

(Note that comparisons within and between MOIs were performed in the same single 2-wayANOVA test with Sidak's multiple comparisons).

Transduction with the VSV-G.EF1 α virus at MOI 50 (median 51.9 (range 46.9–57.1) %) was comparable to the previous initial proof-of-concept experiment with the same vector at MOI 20 (median 47.9 (range 42.2–52) %). Though no direct comparison of F/HN pseudotypes was made at MOI 50 in this experiment, extrapolating from previous data suggest that at higher MOIs VSV-G pseudotyped virus may have a higher transduction efficiency than F/HN, but that the effect may be dependent on the regulatory element driving transgene expression. The regulatory element is unlikely to directly impact transduction but can impact the number of silent integrations as different regulatory elements are prone to varied levels of silencing (522). This suggests that the transduction efficiency of SIV may be higher than determined by flow cytometry expression, but that a number of them are silent. This also suggests that part of the large difference in gene transfer between F/HN and VSV-G pseudotyped virus seen in the first pilot experiment may be due to inconsistent the different regulatory element driving transgene expression.

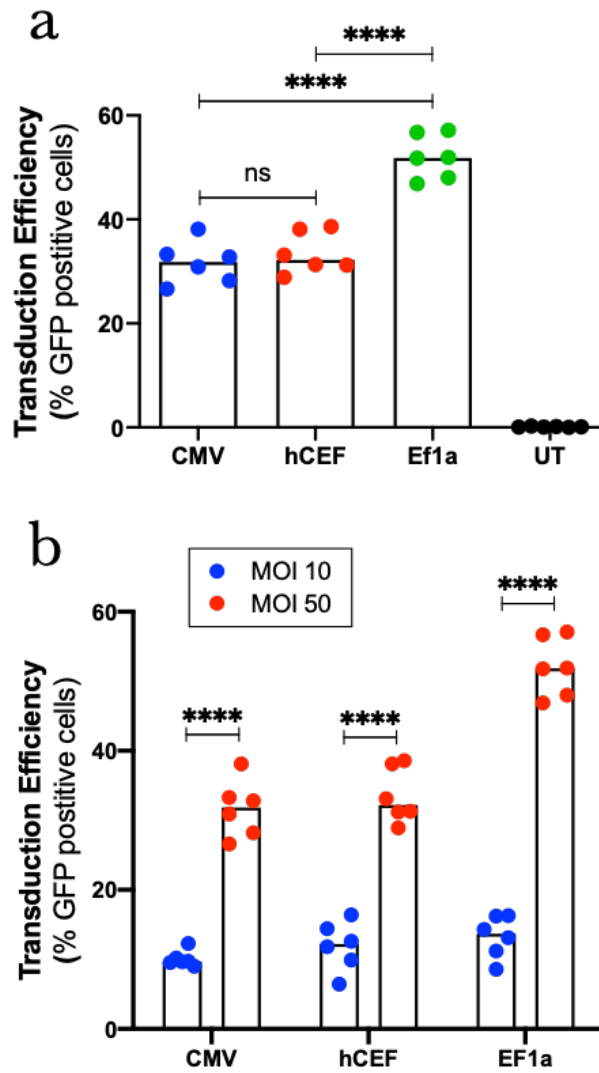


Figure 45 – Relative transduction efficiency of rSIV vectors encoding CMV, hCEF, and EF1a promoters in bone marrow-derived macrophages (BMDMs). (a) The relative transduction efficiency of BMDMs following transduction with rSIV.VSV-G vectors expressing an EGFP transgene under the control of the CMV or EF1a promoter at MOI 10, and (b) dose-related expression seen between MOI 10 and MOI 50 (n=6/MOI). Transduction efficiency was calculated as the percentage of EGFP positive cells in culture by flow cytometry 7 days post-initiation of transduction (48 hours). Capture by BD Accuri™ C6 Plus Flow Cytometer, analysis in FlowJo software. Each data point represents an individually transduced well of BMDMs. Bars represent median. ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons.

5.3.6 Increased SIV transduction efficiency is observed with an EF1a promoter, and in a dose-dependent fashion with a VSV-G pseudotype

Following differentiation of bone marrow, as a result of natural error in the counting and diluting of highly concentrated cell suspensions, a natural degree of variation is present in the number of macrophages found in culture on day 7. As macrophages are not passaged after differentiation and before transduction, the maximal MOI permissible by vector titre and volume capacity can also vary. To enable direct comparisons of F/HN and VSV-G pseudotypes at higher MOIs, the experiment was repeated when the final macrophages density in culture on day 7 was lower than in the previous experiment. Macrophages were transduced at increasing multiplicities of infection (10, 20 & 50) for 7 days and then analysed by flow cytometry. Crude small-scale virus produced by Oxford collaborators were compared in addition to a purified rSIV.F/HN.hCEF.EGFP virus purchased from the Oxford gene therapy group. Due to varying titres, not all viruses were used to transduce cells at all 3 MOIs. The viruses used here and their respective MOIs can be seen in Table 19. Small scale produced F/HN.hCEF vector was not used due to low titres, and while transduction with F/HN.CMV vector at MOI 10 was attempted these results were not used as cells exhibited a large degree of toxicity and cell detachment in culture, likely due to the high volume of viral suspension required.

Table 19 - Configuration of SIV lentiviral vectors used in pseudotype comparison experiments and the various multiplicities of infection (MOI) used. S: small-scale (50 mL) crude virus, L: large-scale, concentrated and purified virus.

No.	Pseudotype	Regulatory Element	Production Scale	MOI
1	VSV-G	hCEF	S	10, 20, 50
2	VSV-G	CMV	S	10, 20, 50
3	VSV-G	EF1α	S	10, 20, 50
4	F/HN	hCEF	L	10, 20, 50
5	F/HN	CMV	S	10
6	F/HN	EF1α	S	10, 20, 50

5.3.6.1 VSV-G pseudotyped lentivirus is more efficient than F/HN at transducing macrophages at high multiplicities of infection

The only comparison possible at all MOIs between pseudotypes where both the regulatory element and production method were consistent was between vectors with the EF1 α promoter (viruses 3 and 6 in Table 19). Transduction efficiencies did not differ significantly at MOI 10 or 20, however VSV-G showed a significant, though slight, increase in transduction efficiency at MOI 50 (Figure 46a):

- *MOI 10*: median difference 0.4 % transduction efficiency (p= ns)
- *MOI 20*: median difference 0.3 % transduction efficiency (p=ns)
- *MOI 50*: median difference 4.2 % transduction efficiency (p<0.0001, 2-wayANOVA, Sidak's multiple comparisons)

When comparing envelopes between viruses encoding hCEF, though produced under different protocols (viruses 1 and 4 in Table 19), a similar trend was observed, with a significant increase at MOI 50 in VSV-G transduction efficiency when compared to F/HN (Figure 46b) suggesting a dose dependant increase in transduction efficiency with the VSV-G pseudotype:

- *MOI 10*: median difference 1.2 % transduction efficiency (p= ns)
- *MOI 20*: median difference 0.4 % transduction efficiency (p=ns)
- *MOI 50*: median difference 4.2 % transduction efficiency (p<0.0001, 2-wayANOVA, Sidak's multiple comparisons)

In comparison to the previous experiment however, here all vectors showed a reduced transduction efficiency, indicating a large degree of interexperimental variation, where here at MOI 50 rSIV.VSV-G.EF1 α .EGFP had a median transduction efficiency of 16.4 (range 13.1–19.4) %, and in the previous experiment under the same conditions the vector had a median transduction efficiency of 51.85 (range 46.9–57.1) %.

Overall, the pseudotype comparisons suggest that VSV-G may have a different maximum feasible dose to F/HN, at which increasing MOI no longer leads to a substantial subsequent increase in transduced cells. Therefore, increasing MOI above 50, though not effective with F/HN may result in higher transduction efficiencies with a VSV-G pseudotype.

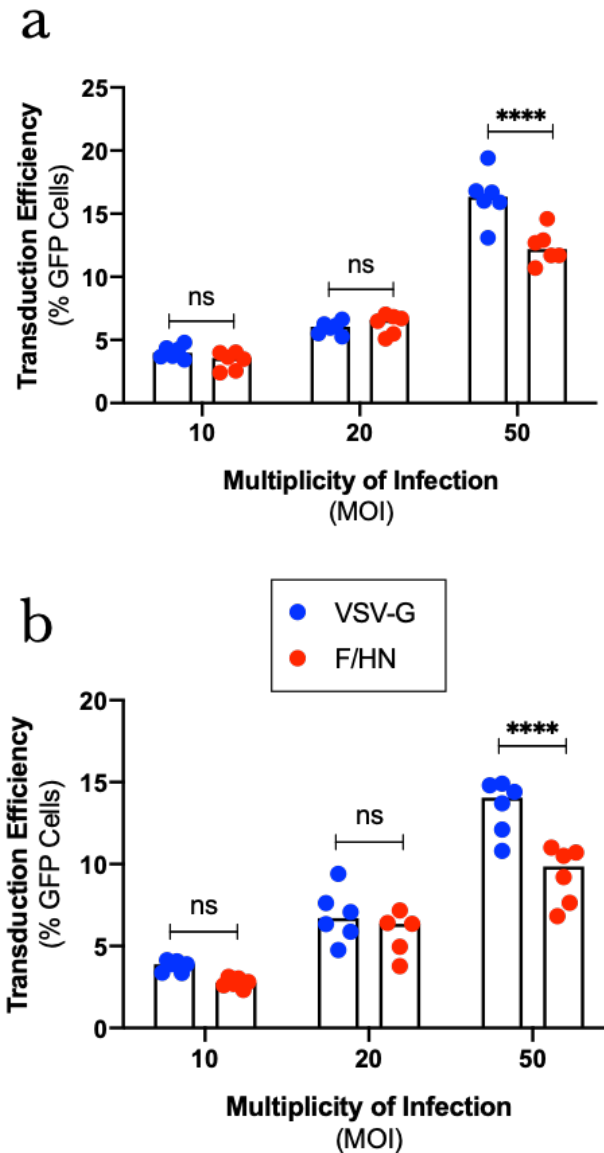


Figure 46 – Dose-related transduction efficiency of VSV-G and F/HN pseudotyped rSIV vector in transducing bone marrow-derived macrophages (BMDMs). The relative transduction efficiency of BMDMs following transduction with rSIV vectors pseudotyped with F/HN or VSV-G envelope proteins expressing an EGFP transgene under the control of the **(a)** EF1a promoter and **(b)** hCEF at increasing multiplicities of infection (MOI) (10, 20, 50) (n=6/MOI). Transduction efficiency was calculated as the percentage of EGFP positive cells in culture by flow cytometry 7 days post-initiation of transduction (48 hours). Capture by BD Accuri™ C6 Plus Flow Cytometer, analysis in FlowJo software. Each data point represents an individually transduced well of BMDMs. Bars represent median. ****=p<0.0001, 2-wayANOVA, Sidak's multiple comparisons.

5.3.6.2 Higher transduction efficiencies observed by lentiviral constructs encoding an EF1 α promoter are not dose-dependent

Across VSV-G pseudotyped vectors transduction efficiency was significantly different between vectors with different regulatory elements, ($p < 0.05$, two-way ANOVA, main effect), where at MOI 50, virus with EF1 α driven expression had significant higher transduction efficiencies to CMV and hCEF virus ($p < 0.05$ and $p < 0.001$ respectively, two-way ANOVA, Sidak's multiple comparisons test) (Figure 47). This suggests that part of the discrepancy between F/HN and VSV-G pseudotyped virus seen in the first pilot experiment may be due to inconsistent use of hCEF, CMV and EF1 α . However, at the same MOI of 20 used in the initial experiment where there was a 126% and 159% increase in mean transduction efficiency between F/HN.hCEF and VSV-G.CMV and VSV-G.EF1 α respectively, here with the corresponding comparisons only a 33% and 4% increase in mean transduction efficiency was observed. Therefore, though transgene regulation can significantly affect transduction efficiencies as reported by flow cytometry it is unlikely to explain the entirety of the variation observed.

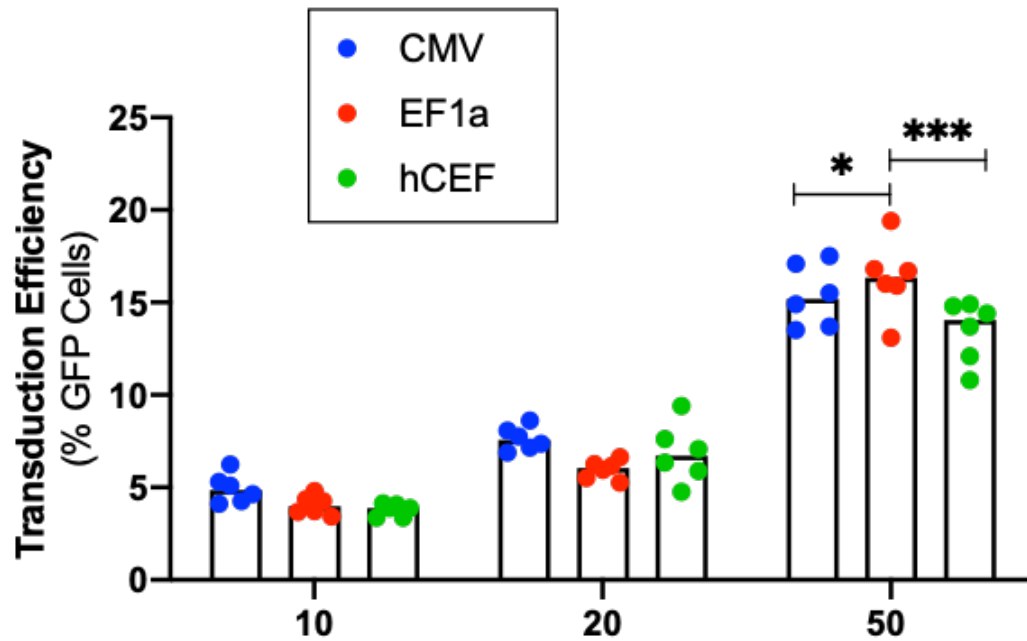


Figure 47 – Relative transduction efficiency of rSIV vectors encoding CMV, hCEF, and EF1a promoters in bone marrow-derived macrophages (BMDMs). The relative transduction efficiency of BMDMs following transduction with rSIV.VSV-G vectors expressing an EGFP transgene under the control of the CMV, EF1a, and hCEF promoters at increasing multiplicities of infection (MOI: 10, 20, 50) (n=6/MOI). Transduction efficiency was calculated as the percentage of EGFP positive cells in culture by flow cytometry 7 days post-initiation of transduction (48 hours). Capture by BD Accuri™ C6 Plus Flow Cytometer. Each data point represents an individually transduced well of BMDMs. Bars represent median. *= $p < 0.05$, ***= $p < 0.001$, 2-wayANOVA, Sidak's multiple comparisons.

5.3.7 CMV promoter/enhancer has a higher activity in bone marrow-derived macrophages than the chimeric regulatory element hCEF or EF1 α promoter

In addition to trying to increase *in vivo* transgene expression following macrophage delivery by increasing transduction efficiency, I also compared the relative level of gene expression driven by three different candidate regulatory elements to see if the average amount of protein expressed by each transduced cell could be increased. The hCEF chimeric regulatory element used in pilot *in vivo* tests of the *ex vivo* lung cell therapy platform (chapter 3) was developed for long term transgene expression in the lung epithelium. As promoter activity is tissue specific, I compared the activity of three candidate regulatory elements, the hCEF chimera, the CMV promoter/enhancer, and the eukaryotic elongation factor 1 α (EF1 α) promoter for their relative strength of gene expression in BMDMs to potentially increase *in vivo* pulmonary production of secreted transgenes. The strength of transgene expression was quantified as the mean fluorescent intensity (MFI) of EGFP positive macrophages following transduction as it correlates with the level of protein expression. This analysis was performed on samples from three experiments analysed in sections [5.3.4](#), [5.3.5](#) and [5.3.6](#) comparing the relative transduction efficiency of VSV-G and F/HN pseudotyped SIV.

In pooled samples from three transduction experiments with VSV-G enveloped lentivirus the relative strength of the CMV promoter/enhancer was significantly higher than hCEF and EF1 α at each MOI ($p > 0.0001$, in all CMV vs EF1 α or hCEF comparisons, 2-wayANOVA, Tukey's multiple comparisons) (Figure 48a). Meanwhile, hCEF and EF1 α exhibited a similar level of activity in BMDMs. MFI was consistent across experiments showing robust reproducibility of the assay. Only at MOI 20 was there a discrepancy within the MFI reported for CMV expression, however within experiments the same trend across promoter/enhancers was seen.

Expression strength analysis of different F/HN pseudotyped lentiviruses recapitulated the same results where CMV drove the highest level of expression (Figure 48b). Due to an incomplete data set, MOI 50 data was excluded for statistic comparisons. In both analyses MOI was a significant factor in determining MFI, where generally higher intensities were seen with increasing viral dose. As this was calculated for cells already gated positively for EGFP expression, this is likely due to cells having more VCNs following transduction with higher

lentiviral doses. Indeed, previous VCN analysis indicated a high propensity for multiple vector insertions in BMDMs. These data suggest that while EF1 α may minimise the effect of neighbouring chromatin, CMV drives higher levels of transgene expression on a per cell basis.

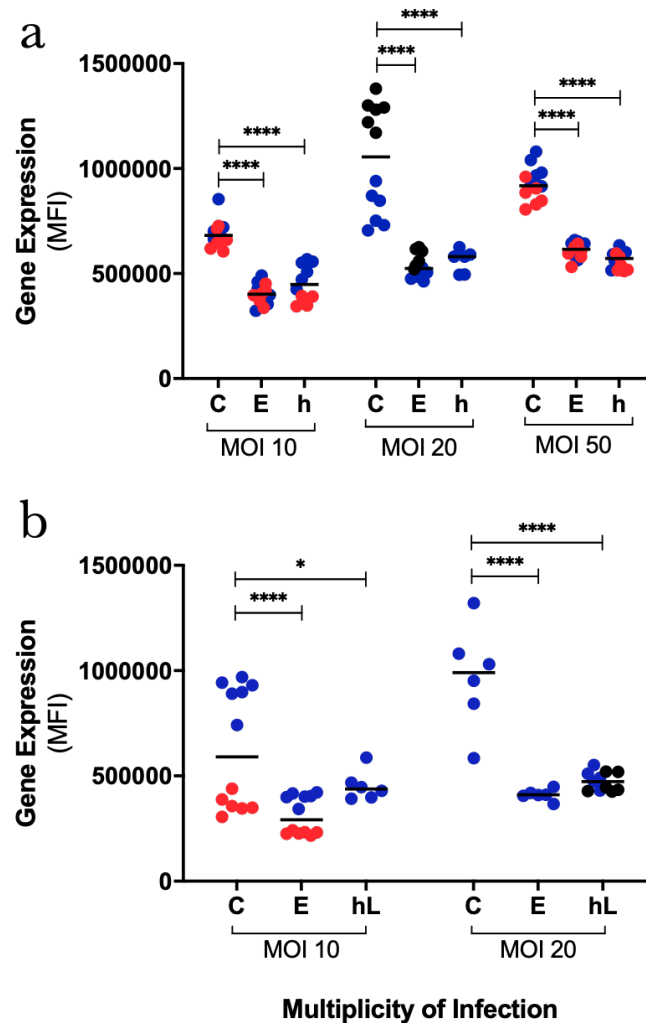


Figure 48 – Relative gene expression of CMV, hCEF, and EF1 α promoters in bone marrow-derived macrophages (BMDMs). BMDMs were transduced with (a) rSIV.VSV-G and (b) rSIV.F/HN vectors expressing an EGFP transgene under the control of the CMV (C), EF1 α (E), and hCEF (h) promoters at increasing multiplicities of infection (MOI: 10, 20, 50) (n=6). Gene expression quantified as mean fluorescent intensity (MFI) of EGFP expression by flow cytometry across previous transduction experiments. Colours relate to different experiments, black: experiment 5.3.4, red: experiment 5.3.5, blue: experiment 5.3.6. Capture by BD Accuri™ C6 Plus Flow Cytometer. Each data point represents an individually transduced well of BMDMs. Bars represent median. All crude vectors produced in small batches, except the large scale purified rSIV.F/HN.hCEF vector in experiment 5.3.4 (hL). *= $p < 0.05$, ****= $p < 0.0001$, 2-way ANOVA, Sidak's multiple comparisons.

5.3.8 Molecular ddPCR analysis reports no difference in VSV-G and F/HN pseudotype transduction efficiencies or subsequent transgene expression

Flow cytometry analysis of EGFP positive cells is commonly used to determine a vectors efficiency of gene transfer and here determined that at high MOIs (≥ 50) VSV-G enveloped lentivirus is more efficient than F/HN in macrophages. I wanted to confirm EGFP based pseudotype comparisons with molecular data on transduction and expression and understand whether EGFP expression is indicative of the real rate of transduction. Furthermore, as transduction efficiency varied between vectors as a result of their different regulatory elements driving gene expression, I wanted to determine the degree to which rSIV integrations in macrophages are silent by comparing flow cytometry and molecular assays.

Macrophages were therefore transduced with a maximum feasible dose of MOI 46 with either rSIV.VSV-G.hCEF.EGFP (small-scale crude prep) or rSIV.F/HN.hCEF.EGFP (large-scale purified prep) for 24 hours and analysed on 7 days post initiation of transduction (n=6/group). Transduced samples were divided and analysed for:

- Transduction efficiency by flow cytometry results of EGFP expression,
- Transduction efficiency by ddPCR encapsulation assay,
- Vector copy number in bulk extracted DNA samples by ddPCR,
- Transgene expression levels by ddPCR of bulk extracted RNA samples.

The encapsulation assay uses ddPCR multiplex detection of vector specific cDNA using a probe that binds the rev response element (RRE), and another that recognises the recognises the peptidylpropyl isomerase A (Ppia) housekeeping gene (Figure 49). As each droplet in the ddPCR emulsion should in theory contain a single cell (or be empty), this assay provides high-throughput quantification of vector transduction efficiency at a single cell resolution (514, 523). Transduction can be determined by calculating the number of droplets positive for both targets and expressing them as a percentage of all droplets positive for only the housekeeping gene. This assay was developed and optimised for single cell analysis of the rSIV vector by my colleague Dr Ana Sergijenko and offers a molecular based alternative to quantification of EGFP expression by flow cytometry which relies on gene expression.

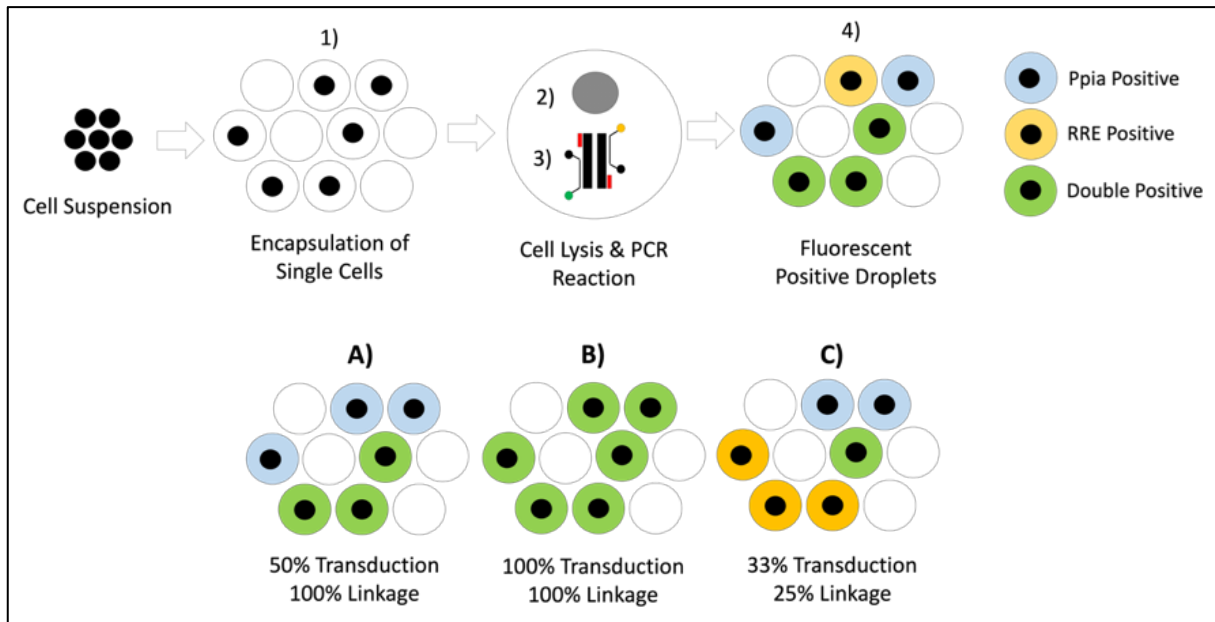


Figure 49 – Single cell digital droplet PCR (ddPCR) encapsulation assay quantification of transduction efficiency is dependent on linkage. Transduced cells in suspension with ddPCR reagents are encapsulated in droplet emulsions. Following cell lysis, independent PCR reactions occur within each droplet, with multiplex detection of vector specific cDNA using a probe that binds the rev response element (RRE), and host cellular DNA with a probe recognising the peptidylpropyl isomerase A (Ppia) housekeeping gene. Following TaqMan hydrolysis each probe fluorescently labels droplets that contained target sequence cDNA. As each droplet in the ddPCR emulsion should either be empty or contain a single cell this assay provides high-throughput quantification of vector transduction efficiency at a single cell resolution. (**a-b**) Transduction is calculated as the percentage of Ppia positive droplets that are also positive for vector cDNA. Low linkage (**c**) occurs when the two probes dissociate, and a significant number of droplets are only positive for vector cDNA (514).

5.3.8.1 Transduction efficiency quantified by flow cytometry and ddPCR reports no pseudotype specific differences, but suggest a high rate of silent integrations

Flow cytometry analysis at day 7 revealed no significant difference in transduction efficiency between VSV-G and F/HN pseudotyped lentivirus, though there was a larger spread in the data set with VSV-G virus ($p=ns$, Mann-Whitney test) ($n=6$) (Figure 50a):

- *VSV-G*: median 13.8 (range 6–19.3) % EGFP positive cells
- *F/HN*: median 10.8 (range 7.8–12.7) % EGFP positive cells

This was also seen in molecular analysis determination of transduction efficiency with the encapsulation ($p>0.999(ns)$, Kruskal-Wallis test, Dunn's multiple comparisons) (Note Kruskal-Wallis analysis performed here is the same test reported in section 5.3.9 where these data are also compared to a third test group) ($n=5$, due to sample loss) (Figure 50b):

- *VSV-G*: median 44 (range 27.8–56.4) % WPRE positive cells
- *F/HN*: median 37.4 (range 27.4–46.2) % WPRE positive cells

VCN analysis of day 7 further indicated no significant difference between the two pseudotyped EGFP viruses (Figure 50c) and indicated the likelihood of multiple insertions per cell ($p>0.999(ns)$, Kruskal-Wallis test, Dunn's multiple comparisons):

- *VSV-G*: median 1.49 (range 0.92–13.16) VCN
- *F/HN*: median 1.37 (range 1.09–1.89) VCN

This was also true when correcting for the percentage of cells positive for vector cDNA as measured by the encapsulation assay and comparing the effective VCN in the transduced cells however, the large spread of data in all groups indicates a high degree of variability. (Figure 50d) ($p>0.746(ns)$, Kruskal-Wallis test, Dunn's multiple comparisons):

- *VSV-G*: median 4.84 (range 4.41–9.11) effective VCN
- *F/HN*: median 3.78 (range 2.42–5.88) effective VCN

This correction leads to high calculated VCNs indicating the likelihood that though not all cells are transduced successfully, those that are, likely carry multiple insertions of the virus. A caveat is that these data were calculated from a bulk population of macrophages rather than single cell analysis, but it is in keeping with literature that suggests lentiviral vectors are often present at high VCN (502, 520).

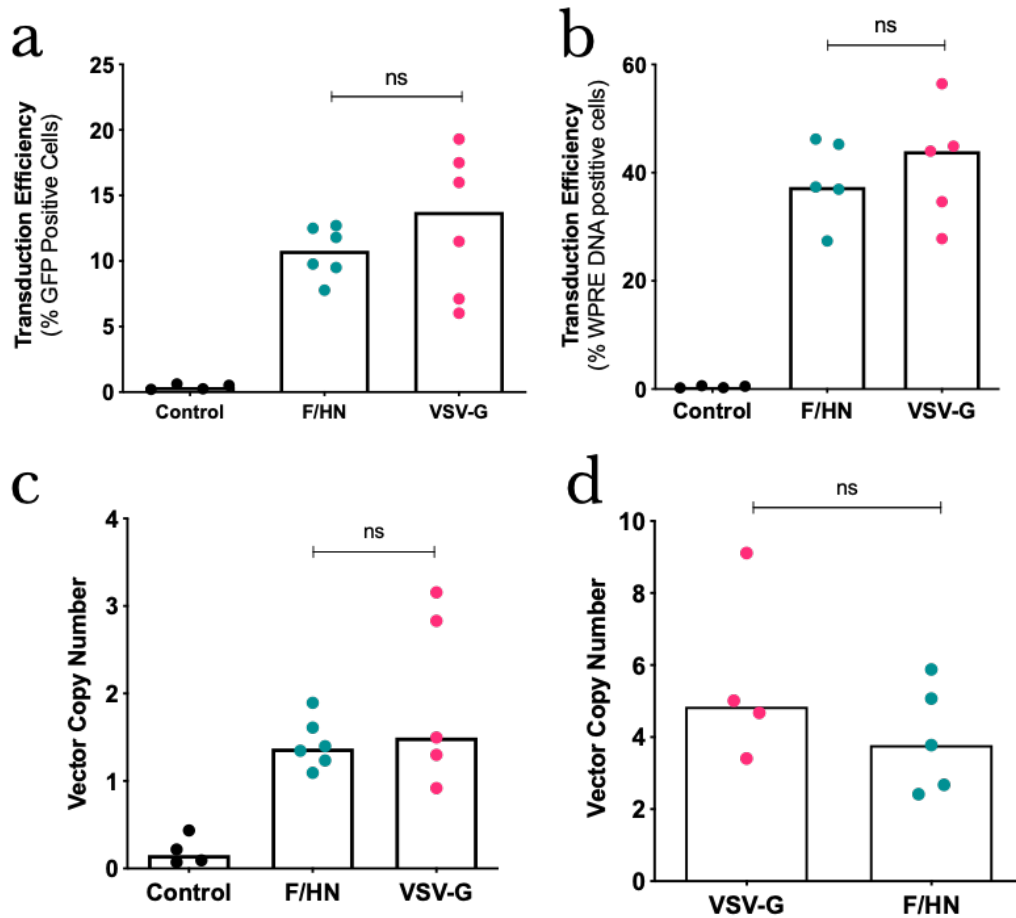


Figure 50 – Relative transduction efficiency and integration of VSV-G and F/HN pseudotyped rSIV lentivirus in bone marrow-derived macrophages (BMDMs).

Comparisons between rSIV vectors with a VSV-G and F/HN pseudotype in BMDMs transduced at MOI 46. Untransduced cells served as control samples. Transduction efficiency was calculated by **(a)** flow cytometry analysis of EGFP transgene expression (n=6), and **(b)** a ddPCR encapsulation assay quantifying cells positive for viral cDNA (n=5). **(c)** Vector copy number (VCN) ddPCR analysis of reverse transcribed lentiviral genomes was performed from bulk extracted DNA samples (n=6), and then **(d)** corrected for transduction efficiency as quantified by the encapsulation assay. BMDMs were transduced for 24 hours and then analysed on day 7. Each sample was split into thirds, 1/3 was then analysed by flow cytometry, another 1/3 by encapsulation. The final 1/3 was divided equally into two and used to procure RNA and DNA extractions. Flow cytometry capture by BD Accuri™ C6 Plus Flow Cytometer, analysis in FlowJo software. Each data point represents an individually transduced well of BMDMs. Bars represent median. **(a)** Mann-Whitney t-test, **(b-d)** Kruskal-Wallis, Dunn's multiple comparisons, ns = not significant.

5.3.8.2 VSV-G and F/HN pseudotyped vectors express similar levels of EGFP transgene expression

Transgene mRNA was detected using a probe for the Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element (WPRE), found on the lentiviral genome downstream of the transgene and functions to enhance gene expression. As a result, it is present on both the viral RNA genome and transgene mRNA. However, vector specific RNA can be detected using a probe for the rev response element (RRE) which is not present in the mRNA. We therefore used both these ddPCR based assays to characterise the levels of transgene expression, the duration of vector genomes post transduction and how they compare between pseudotypes.

Seven days post transduction we would not expect to detect high levels of viral genomes, as all viruses are replication deficient and were removed after a 24-hour incubation period (395, 524). Analysis of RRE copies in bulk extracted RNA indicated significantly lower levels of remaining viral genomes than WPRE copies in RNA (Figure 51), indicating that transgene mRNA is present above the detection threshold dictated by obfuscating RRE/ viral genomes (F/HN: $p < 0.001$. VSV-G: $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons). Importantly RRE RNA copies were comparable, indicating that should any differences be seen at day 7 in WPRE copies, it would not be due to differences in viral genomes ($p > 0.9990$). No difference in WPRE RNA levels was observed, reflecting that there was no difference in transduction efficiency between the two pseudotypes ($p = 0.875$).

In contradiction to previous findings, here extensive molecular and flow cytometry analysis of gene expression and vector integration failed to find any differences between F/HN and VSV-G SIV enveloped viruses, though these studies utilized a lower MOI. Nevertheless, the degree of transduction observed here by flow cytometry was low in comparison to previous experiments. It may be that due to error either in pipetting and diluting of the lentivirus, or counting of macrophage cell numbers in culture, the actual MOI used was lower than MOI 46, or vice versa in the previous experiments. Due to this large interexperimental variation it is difficult to say whether there is a definitive difference in transduction efficiency of SIV owing to pseudotype, and if there is a difference at higher MOIs, what this actual threshold is.

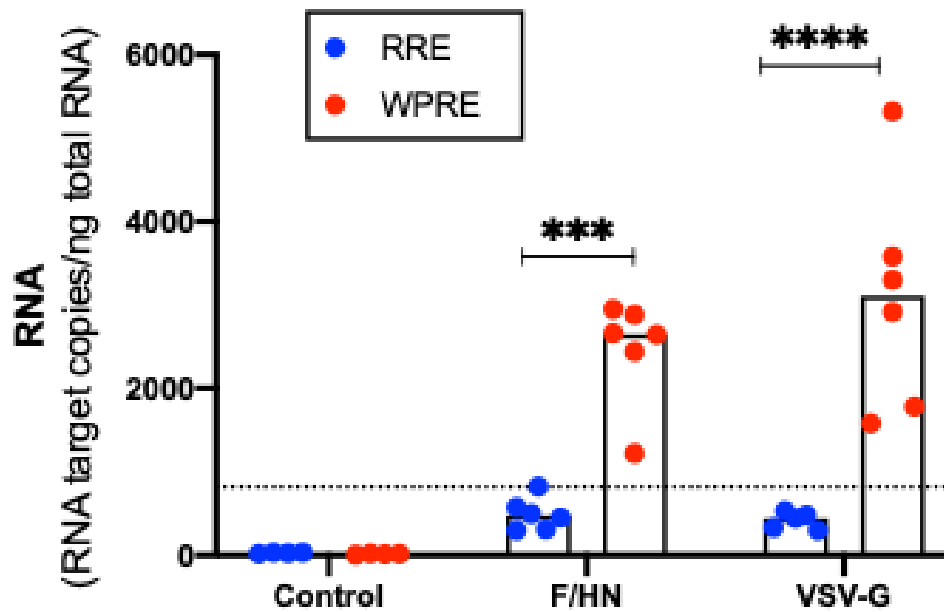


Figure 51 – Transgene mRNA and vector specific RNA comparisons in BMDMs transduced with VSV-G and F/HN pseudotyped rSIV lentivirus. The concentration of RRE and WPRE positive RNA copies were quantified by ddPCR in BMDMs transduced by rSIV vectors with a VSV-G or F/HN pseudotype (MOI 46). Untransduced cells served as control. RNA analysis performed on bulk extracted samples of RNA, where RRE is specific to viral genomes, and WPRE is present on both viral genomes and transgene mRNA. BMDMs were transduced for 24 hours and then analysed on day 7. Each sample was split into thirds, 1/3 was then analysed by flow cytometry, another 1/3 by encapsulation. The final 1/3 was divided equally into two and used to procure RNA and DNA extractions. Bars represent median. ***= $p < 0.001$, ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

5.3.8.3 Higher transduction efficiencies reported by ddPCR over EGFP flow cytometry analysis suggests viral integrations are largely silent

Though both the encapsulation and flow cytometry assays reported similar findings regarding differences between pseudotypes, the former reporting significantly higher transduction efficiencies (VSV-G: 28.64 % , F/HN: 27.96 % mean difference, $p < 0.0001$ respectively, 2wayANOVA, Sidak's multiple comparisons) (Figure 52) (Note that comparisons between pseudotypes and assays were all performed in the same 2-wayANOVA comparison with Sidak's multiple comparisons, and have just been separated graphically for ease of viewing). While molecular analysis identifies all integration events, EGFP analysis only identifies insertions that lead to successful gene expression. Therefore, this suggests that SIV is integrating at a higher efficiency than previous experiments reported, and that the majority of these integrations are silent (VSV-G: 69 %, F/HN: 71.2 %).

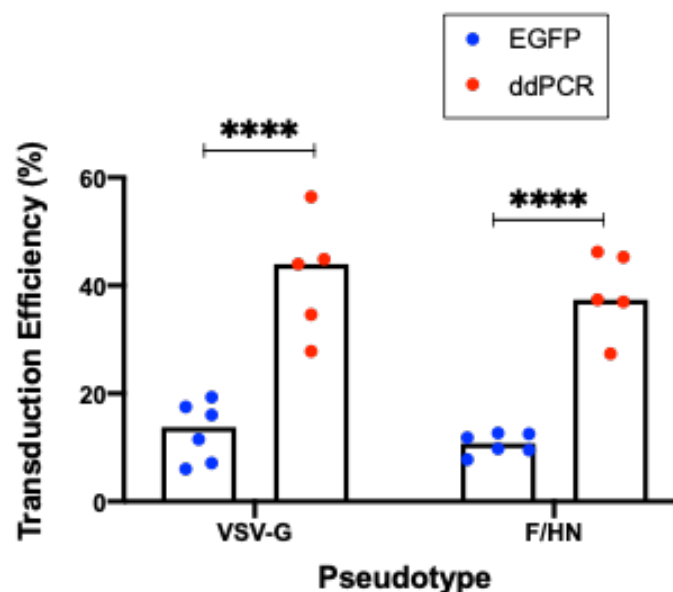


Figure 52 – Evidence of silent rSIV vector integrations in bone marrow-derived macrophages (BMDMs). Comparison of the transduction efficiency of the rSIV vector with a VSV-G or F/HN pseudotype in BMDMs calculated by either the EGFP flow cytometry-based analysis (EGFP) (n=6) or the ddPCR encapsulation assay (ddPCR) (n=5). BMDMs were transduced for 24 hours and then analysed on day 7 (MOI 46). Untransduced cells served as control samples. Bars represent median. ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

5.3.9 Transduction efficiency and transgene expression of the SIV vector is not transgene specific

Having developed assays for the characterisation and optimisation of SIV lentivirus in BMDMs, I then wanted to understand how applicable these findings are when using lentiviruses encoding alternative transgenes to EGFP, specifically mGM-CSF as it is the therapeutic transgene proposed for aPAP therapy. The majority of work characterising SIV lentivirus transduction of macrophages was performed using a EGFP reporter transgene largely due to the ease of assaying transduction efficiency by flow cytometry. I therefore used molecular ddPCR based analysis to compare the relative transduction and expression of rSIV vectors expressing the EGFP reporter and therapeutic mGM-CSF. This experiment was run in concert and under the same protocol as the pseudotype comparisons in section [5.3.3.2](#), however with the caveat that cell transduced with rSIV.F/HN.hCEF.mGM-CSF were not analysed by flow cytometry. The same data gathered using the rSIV.F/HN.hCEF.EGFP vector for the pseudotype comparisons was here compared to data gathered following transduction with rSIV.F/HN.hCEF.mGM-CSF. Note that all comparisons between pseudotypes and transgenes for each variable were all performed in the same statistical test and are just presented separately for ease of viewing and understanding. Kruskal-Wallis tests with Dunn's multiple comparisons were performed with the F/HN EGFP data set as a control.

In comparison to the EGFP vector, the mGM-CSF virus had a reduced transduction efficiency, though the difference was not significant (Figure 53a):

- *EGFP*: median 37.4 (range 27.4–46.2) % transduction
- *mGM-CSF*: median 25.2 (range 12.7–33.1) % transduction, $p < 0.05$, Kruskal-Wallis test, Dunn's multiple comparisons, $n = 5/\text{group}$),

This was reiterated in VCN analysis of bulk data indicating no differences between assayed viruses in the raw data output or the calculated effective VCN (normalized to number of transduced cells) (Figure 53b-c):

VCN

- *EGFP*: median 1.4 (range 1.1–1.9) VCN,
- *mGM-CSF*: median 1.1 (range 0.9–1.1) VCN, $p = 0.6623$, Kruskal-Wallis test, Dunn's multiple comparisons, $n = 6/\text{group}$),

Effective VCN:

- *EGFP*: median 3.8 (range 2.4–5.9) effective VCN,

- *mGM-CSF*: median 4.2 (range 3.4–8.2) effective VCN, $p < 0.811$, Kruskal-Wallis test, Dunn's multiple comparisons, $n = 5/\text{group}$),

However, the copies per ng of WPRE RNA produced in samples transduced to express GM-CSF was significantly lower than in samples expressing EGFP (Figure 53d):

- *EGFP*: median 2650 (range 1218–2950) WPRE copies/ng RNA,
- *mGM-CSF*: median 1101 (range 1024–1620) WPRE copies/ng RNA, $p < 0.05$, Kruskal-Wallis test, Dunn's multiple comparisons, $n = 6/\text{group}$),

These findings indicate a reduction in overall gene expression as RRE copy levels (specific to the viral genome) were minimal and consistent across both EGFP and GM-CSF viruses and were significantly lower than WPRE (detects transgene mRNA and vector genome) indicating that transgene mRNA is present above the detection threshold dictated by obfuscating RRE/viral genomes (Figure 53e):

- *EGFP*: median difference 2175 RNA copies/ng ($p < 0.01$, Mann-Whitney test, $n = 6/\text{group}$),
- *mGM-CSF*: median difference 606 RNA copies/ng ($p < 0.01$, Mann-Whitney test, $n = 6/\text{group}$),

However, this difference was likely an effect of the slight reduction in transduction efficiency. As RNA analysis was performed on a population level in bulk extracted RNA samples, WPRE signal was corrected for the percentage of transduced cells (calculated by the encapsulation assay), and here there was no significant difference in effective WPRE copies ($p = 0.715$, Kruskal-Wallis test) (Figure 53e).

Overall, there were no robust differences in transduction efficiency, integration or gene expression between vectors with different transgenes. Though the transduction efficiency of the GM-CSF vector trended downwards, the difference was not robust and likely within the margins of error that accompanies pipetting highly concentrated viral volumes during transduction (3.19×10^9 TU/mL).

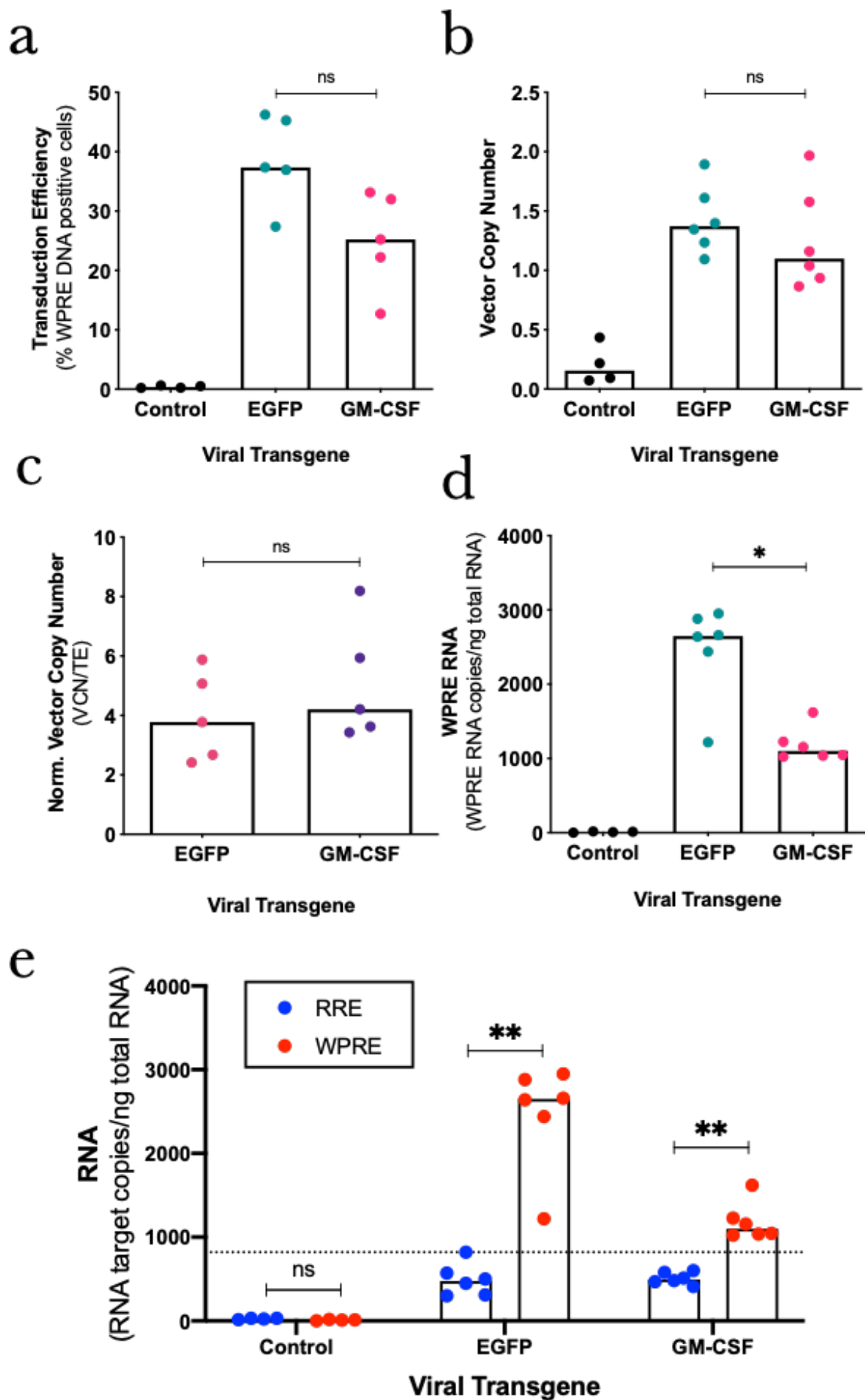


Figure 53 – Comparison between rSIV.F/HN lentiviral vectors with GM-CSF and EGFP transgenes in bone marrow-derived macrophages (BMDMs). Transduction efficiency was calculated by **(a)** a ddPCR encapsulation assay quantifying cells positive for viral cDNA (n=5). **(b)** Vector copy number (VCN) ddPCR analysis of reverse transcribed lentiviral genomes was performed from bulk extracted DNA samples (n=6), and then **(c)** corrected for transduction efficiency as quantified by the encapsulation assay. **(d)** The concentration of WPRE positive RNA copies were quantified by ddPCR analysis performed on bulk extracted samples of RNA, where WPRE detects both viral genomes and transgene mRNA. **(e)** WPRE concentrations were then compared to the concentration of RRE positive RNA which is specific to viral genomes (n=6). BMDMs were transduced for 24 hours at MOI 46 and then analysed on day 7. Untransduced cells served as control samples. ns = not significant, *=p<0.05, **=p<0.01, **(a-d)** Kruskal-Wallis, Dunn's multiple comparisons, **(e)** 2-wayANOVA, Sidak's multiple comparisons. Bars represent median.

5.3.10 Transduction calculations at 24 hours are inconclusive

Having robustly characterised the viral transduction in BMDMs with different pseudotypes and lentiviruses 7 days post-transduction, I wanted to determine if analysis at a 24-hour time point could reproduce findings or give an indication of the success of transduction. The activity of the SIV lentivirus in these cells was previously characterised at a 7-day time point to allow sufficient time for transgene integration and robust expression (395, 401, 525), however when delivering gene-modified macrophages to mice for *in vivo* gene expression, cells are delivered 24 hours post-transduction. As there was a degree of inter-experimental variability observed in the strength of transgene expression in *in vivo* experiments the aim would be to use these protocols for the analysis of delivered cells at a 24-hour time point to conduct cross-experimental comparisons of transduction. I therefore transduced cells for 24 hours with two EGFP viruses with F/HN and VSV-G pseudotypes, as well as an F/HN GM-CSF virus and compared ddPCR molecular and flow cytometry protein-based analysis to data gathered 7-days post transduction. For this experiment cells were transduced in concert with the previous samples analysed at day 7 to allow accurate comparisons.

5.3.10.1 Transduction efficiency cannot be quantified by flow cytometry or ddPCR 24 hours post-transduction

Analysis of transduction efficiency at 24 hours post-transduction was unsuccessful. No positive EGFP signal was detected by flow cytometry in macrophages pseudotyped with either the F/HN or VSV-G EGFP vectors suggesting the onset of transgenic protein expression occurs at a later time point and that therefore expression-based analysis is not appropriate after 24 hours. Unfortunately, transduction efficiencies for the two EGFP viruses and rSIV.VSV-G.hCEF.mGM-CSF could not be determined by the ddPCR-based encapsulation assay as all samples suffered from low linkage making any transduction calculations inaccurate (Figure 54):

- *VSV-G.EGFP*: median 56.2 (range 40.2–72.2) % linkage,
- *F/HN.EGFP*: median 1.4 (range 1.1–1.9) % linkage,
- *F/HN.mGM-CSF*: median 0.0 (range 0.0–3.1) % linkage.

Linkage analysis calculates the percentage of droplets which are positive for vector specific cDNA (which should only be present post reverse transcription, and therefore intracellular), but negative for the housekeeping gene (Figure 49). Low linkage indicates a high degree of dissociation between housekeeping and virion DNA sequences. The assay calculates

transduction by quantifying the number of droplets positive for both targets and expressing them as a percentage of all droplets positive for only the housekeeping gene. It assumes therefore that all housekeeping signal originates from an intact cell and therefore when linkage is low this analysis is highly inaccurate. According to the manufacturer's instructions, linkage should be >90% for accurate results (BioRad). Interestingly, F/HN.mGM-CSF transduced samples had the lowest percentage readout of intact cells, while samples transduced with VSV-G.EGFP had the highest.

One of the possible explanations for low linkage is low cell viability leading to vector cDNA dissociating from housekeeping DNA as a result of cell death. When macrophages from the *in vivo* experiments delivering macrophages to mice, where cells were transduced for 24 hours prior to delivery, the encapsulation assay also failed to quantify the transduction efficiency due to low linkage:

- *F/HN.Glux*: 28.1 % linkage (single sample), delivered to PAP mice (Chapter 4.3.4)
- *F/HN.mGM-CSF*: 32.5 % linkage (single sample), delivered to PAP mice (Chapter 4.3.4)
- *F/HN.Glux*: 15.5 % linkage (single sample), delivered to WT mice (Chapter 4.3.5)

The viability of these cells, as determined by trypan blue staining indicated that these cells had a high cell viability of 95%, 92% and 98% respectively, prior to suspension in RNAlater. This suggest that linkage failure is not due to low cell viability prior to processing.

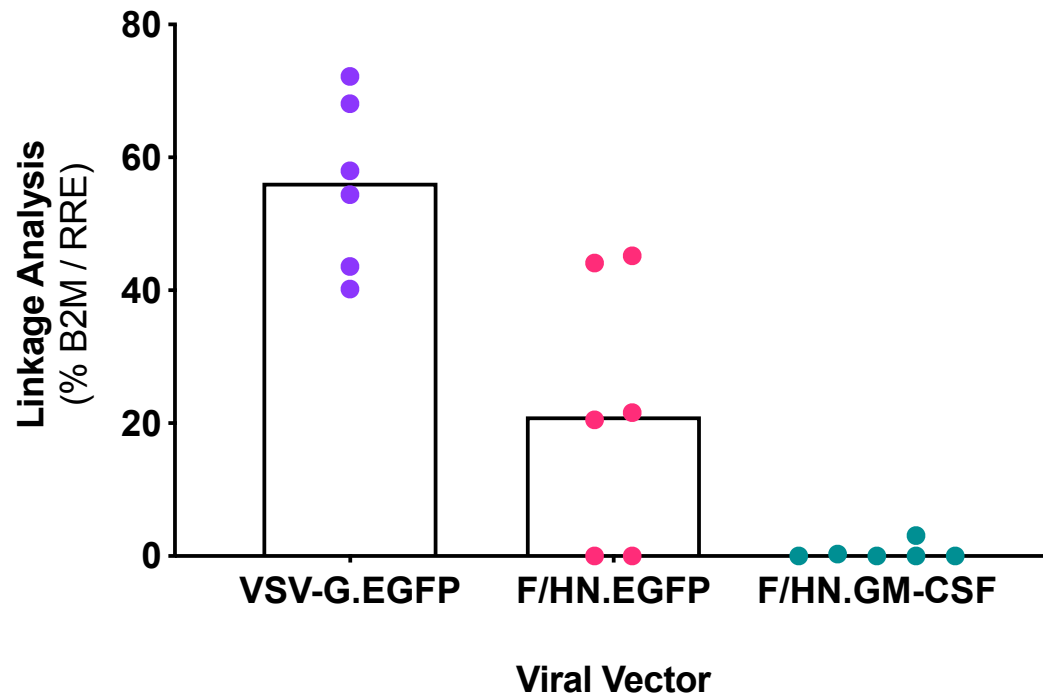


Figure 54 – Linkage analysis of the ddPCR based encapsulation assay in BMDMs transduced by rSIV vectors. Following a 24-hour incubation with rSIV vectors pseudotyped with either F/HN or VSV-G, and encoding either EGFP or GM-CSF transgenes, calculation of transduction efficiency by the ddPCR encapsulation assay failed due to low linkage of the viral RRE cDNA and mammalian housekeeping gene B2M in all samples. Linkage is calculated as the percentage of ddPCR droplets positive for RRE DNA but not B2M, indicating dissociated viral cDNA. Bars represent median.

5.3.10.2 Vector copy number of F/HN pseudotyped vectors is consistent 24 hours and 7 days post-transduction

I was however able to calculate the VCN of each vector at these early time points from bulk DNA extractions indicating that reverse transcription has at least begun 24 hours after initiation of transduction. When comparing the VCN of the three vectors at 24 hours to VCN at 7 days, only the VSV-G pseudotyped virus differed significantly between time points where VCN was higher after 7 days (2-way ANOVA, Sidak's multiple comparisons) (Figure 55):

- *Control*: median difference -0.088 VCN, ($p < 0.999$)
- *VSV-G.EGFP*: median difference 0.53 VCN, ($p < 0.05$)
- *F/HN.EGFP*: median difference -0.81 VCN, ($p = 0.086$)
- *F/HN.mGM-CSF*: median difference -0.56 VCN, ($p = 0.568$),

VSV-G virus showed a significant increase in VCN between the two time points, suggesting early time points may underestimate VCN, though due to low n numbers (24h: n=4/group; 7d: n=6/group) these statistical comparisons are not robust. Samples transduced with either of the F/HN pseudotyped viruses showed an inverse relationship, as samples trended towards a lower VCN on day 7. This pseudotype specific difference is likely a result of different transduction kinetics which have been observed in lentiviruses (401, 526). As VCN was similar across time points for both F/HN pseudotyped vectors, VCN analysis could be used at early time points for inter-experimental comparisons of *in vivo* experiments.

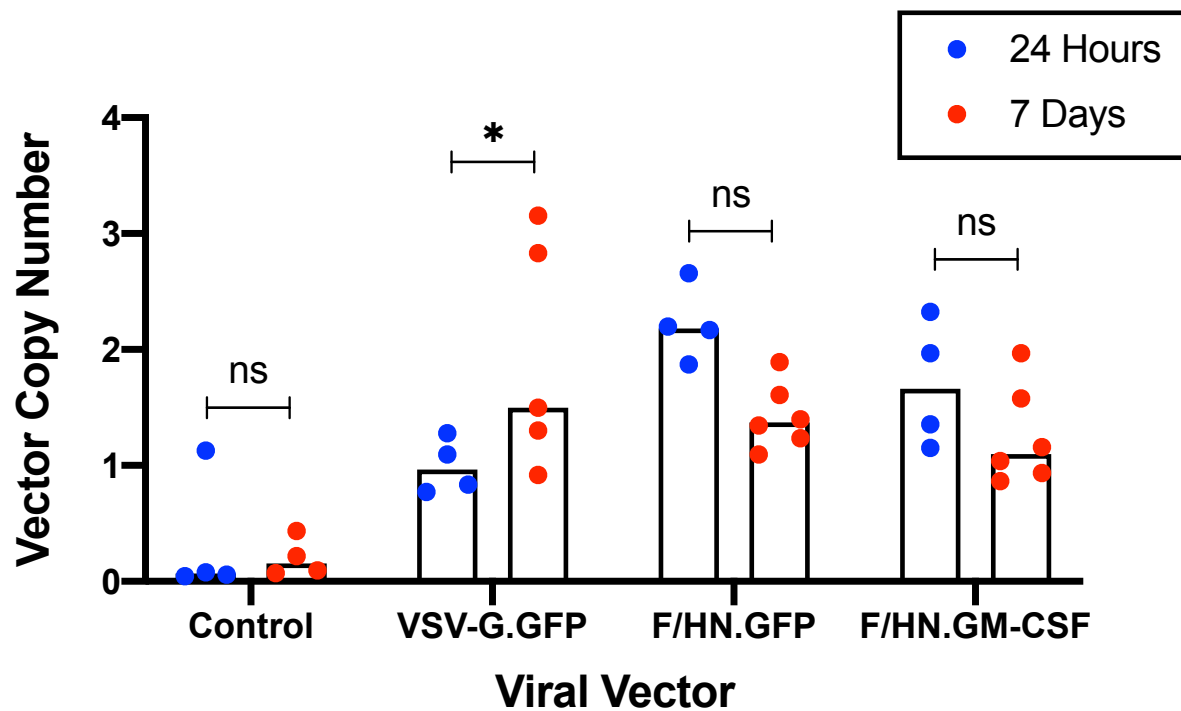


Figure 55 – Vector copy number (VCN) of rSIV in bone marrow-derived macrophages (BMDMs) 24 hours and 7 days post transduction. BMDMs were transduced with rSIV vectors pseudotyped with either F/HN or VSV-G and encoding EGFP or GM-CSF transgenes for 24 hours at MOI 46. Samples were either harvested at this 24-hour timepoint or 6 days later on day 7 (n=6/group). Untransduced cells served as control samples. Vector copy number (VCN) ddPCR analysis of reverse transcribed lentiviral genomes was performed from bulk extracted DNA samples and compared between each vector. Bars represent median. ns = not significant, $*=p<0.05$, 2-wayANOVA, Sidak's' multiple comparisons. Bars represent median.

5.3.10.3 No transgene-specific expression is detected 24-hours post-transduction

At 24 hours no significant difference could be seen between RRE and WPRE RNA levels, meaning no inferences on transgene specific levels of mRNA can be made as the ddPCR WPRE signal can all be attributed to the detection of viral genomes (Figure 56a). This is unsurprising as firstly; gene expression is limited at 24 hours as observed by a lack of EGFP expression at this time point. Secondly, virions are more likely to still be present in samples which would obscure mRNA specific signal. Indeed at 24 hours, all groups detected more virion specific RNA than on day 7 excluding un-transduced controls (Figure 56b):

- *Control*: median difference 31.5 copies/ng,
- *VSV-G.EGFP*: median difference 1780 copies/ng,
- *F/HN.EGFP*: median difference 9225 copies/ng RNA copies/ng,
- *F/HN.mGM-CSF*: median difference 34015 copies/ng ($p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

24 hours is likely insufficient to allow virus time to transduce cells and undergo reverse transcription meaning high levels of vector genome specific RNA is still present in samples (401). To overcome vector specific RNA interference, another probe specific to mRNA is being developed by colleagues in the Imperial College London Gene Therapy group, however at 24 hours mRNA levels are likely to be very low and therefore limited in use as a metric of transduction at 24 hours. The mGM-CSF virus showed much higher levels of RRE (and thus also WPRE) RNA copies at 24 hours than any other sample and this is likely explained by procedural error. Upon harvesting the cells in tissue culture, it was overlooked that these cells were washed one time less than EGFP counterparts, due to the extra step involved in preparing samples for flow cytometry, and as a result, more virus remained in the sample. Aside from this error, the number of media changes/washes was controlled for across each time point.

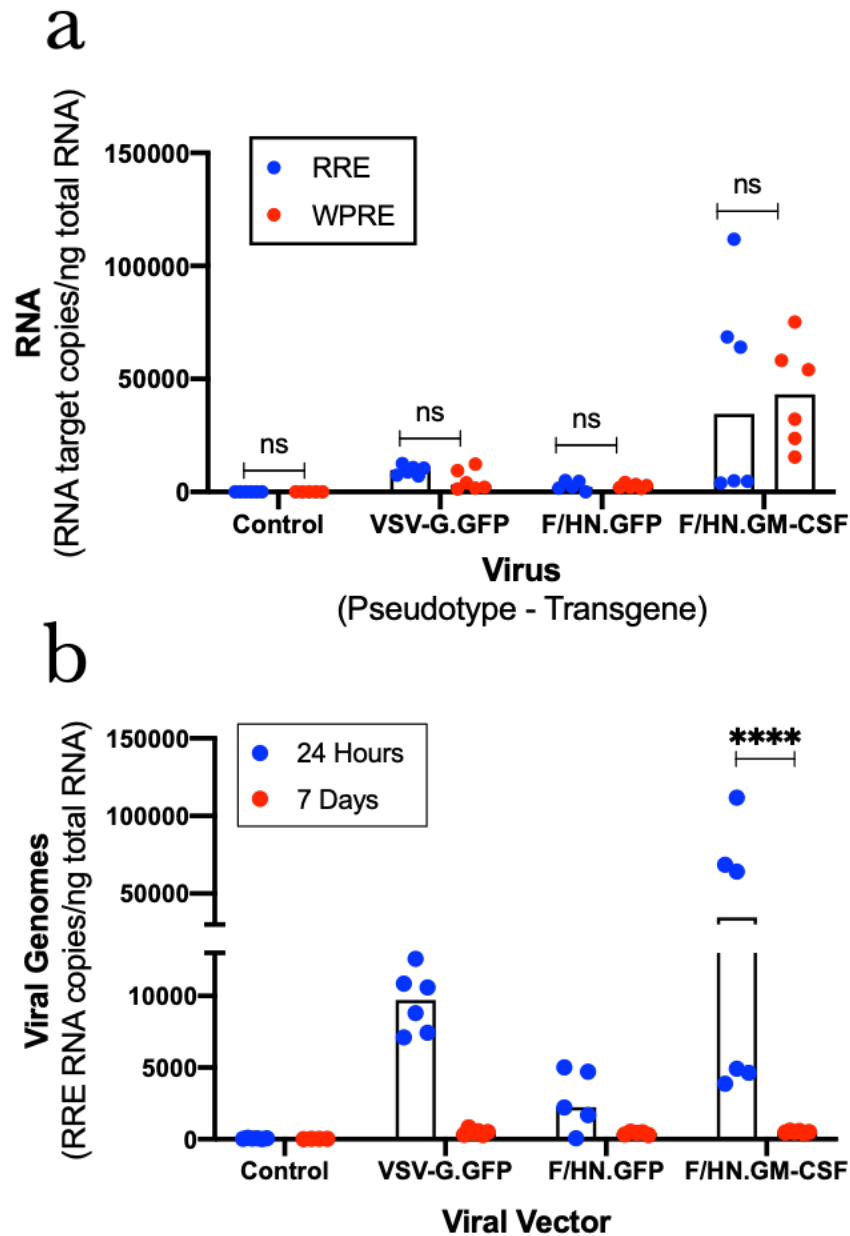


Figure 56 – Transgene mRNA and vector specific RNA comparisons from rSIV in bone marrow-derived macrophages (BMDMs) 24 hours and 7 days post transduction. BMDMs were transduced with rSIV vectors pseudotyped with either F/HN or VSV-G and encoding either EGFP or GM-CSF transgenes for 24 hours at MOI 46. **(a)** The concentration of RRE and WPRE positive RNA copies were quantified by ddPCR in BMDMs transduced by rSIV vectors with a VSV-G or F/HN pseudotype (MOI 46). Untransduced cells served as control. RNA analysis performed on bulk extracted samples of RNA, where RRE is specific to viral genomes, and WPRE is present on both viral genomes and transgene mRNA. **(b)** More vector specific RRE RNA is present in samples 24 hours after transduction than 7 days later. Bars represent median. ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

Barring VCN analysis, assays quantifying transduction and gene expression largely failed to provide information that might be used to compare inter-experimental variation in transduction when delivering gene-modified macrophages *in vivo*. This is likely due to the fact that lentiviral vectors require more time to stably integrate and start producing detectable levels of transgene mRNA or protein (395, 401, 525, 526). It is therefore unlikely that all vector specific DNA detected by VCN analysis has fully integrated, and some of the signal may be attributed to reverse transcribed, but still episomal DNA. Regardless, VCN analysis showed promisingly consistent data across time points but will require further characterisation to determine how sensitive it is in detecting differences in VCN at this early time point.

5.3.11 Transducing bone marrow during differentiation significantly increases genetically modified macrophage yields

I then investigated whether the time at which BMDMs are transduced has an effect on the yield of genetically modified macrophages. In the established protocol used thus far, macrophages are successfully transduced upon conclusion of the differentiation protocol, and studies have shown that lentiviral transduction is more successful in these differentiated macrophages than in their monocyte precursors during the process of differentiation (300, 301). However, this finding is not consistent across the literature, as others have shown greater success in transducing monocytes pre-differentiation and there is no consensus regarding the optimal time to transduce these cells (299). I therefore tested the transduction efficiency of the rSIV.F/HN lentivirus in monocytes to determine if this would increase the proportion of positively transduced macrophages at the conclusion of differentiation.

Fresh bone marrow isolates were transduced in the presence of M-CSF for 48 hours and analysed for EGFP expression on day 7, at the end of the differentiation protocol. This duration of viral incubation was chosen to enable comparisons to previous transduction experiments transducing differentiated macrophages. Transduction was halted after 48 hours by carefully removing viral media, washing, and re-plating any cells removed with the media. This protocol resulted in macrophage cultures with high proportions of genetically modified macrophages on day 7 indicating that viral gene transfer is highly efficient in immature macrophages/monocytes (Figure 57a). Increasing MOI saw a steady increase in transduction efficiency with increasing viral dose:

- *Control*: 0.4 (range 0.3–0.6) % transduction,
- *MOI 1*: 31.3 (range 27.7–33.9) % transduction, ($p=0.462$)
- *MOI 5*: 35.8 (range 25.0–42.1) % transduction, ($p=0.182$)
- *MOI 20*: 48.4 (range 41.5–55.8) % transduction, ($p<0.001$)
- *MOI 100*: 54.6 (range 48.9–58.7) % transduction, ($p<0.0001$, Kruskal-Wallis Dunn's multiple comparisons)

Transduction efficiencies at all MOIs (1, 5, 20 and 100) were significantly higher than previous experiments (Chapter 3.3.2) where differentiated macrophages were transduced on day 7 (across MOIs: $p<0.0001$, Kruskal Wallis test with Dunn's multiple comparisons) (Figure 57b). Strikingly, transduction with MOI 1 resulted in a similar transduction efficiency to that seen

previously at MOI 100 (median: 27.7, range 19.6–51.8%), requiring 2 logs less virus. Transducing monocytes during differentiation into macrophages may therefore be a more cost-effective way of producing genetically modified macrophages as much lower doses of costly virus can achieve similar transduction efficiencies. Importantly flow cytometry analysis of macrophage markers denoted highly homogenous populations of CD11b⁺ F4/80⁺ macrophages across all transduced and un-transduced groups (Figure 57c). Though the mean fluorescent intensity of CD11b was not affected, F4/80 expression dropped significantly between un-transduced controls and macrophages transduced at MOI 100, indicating that exposure to rSIV at high MOIs during viral incubation may have a slight effect on monocyte differentiation (Figure 57d-e). Having previously observed a similar, but more profound effect at MOI 100 when transducing differentiated macrophages, it is likely that at high viral doses rSIV is toxic to macrophages. I have shown evidence that the production of genetically modified macrophages for *ex vivo* cell therapy may be optimised by transducing BMDM progenitors without reducing macrophage differentiation, however the effect expression of a secreted transgene might have on differentiation needs to be evaluated, particularly GM-CSF which regulates macrophage differentiation and maturation (101, 102).

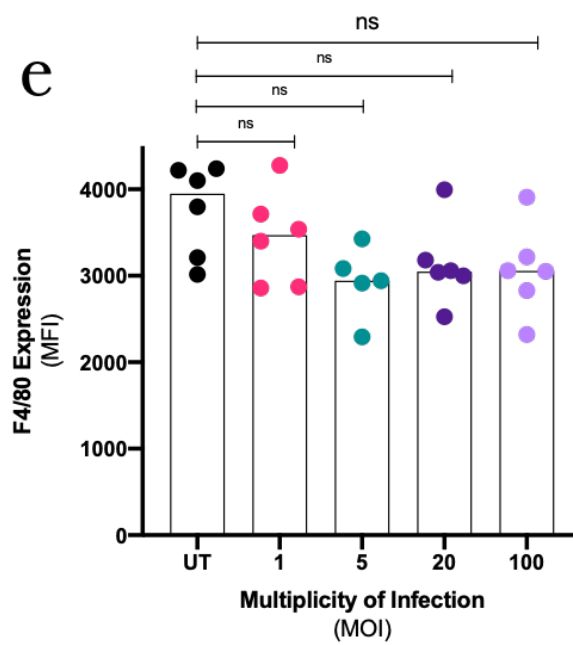
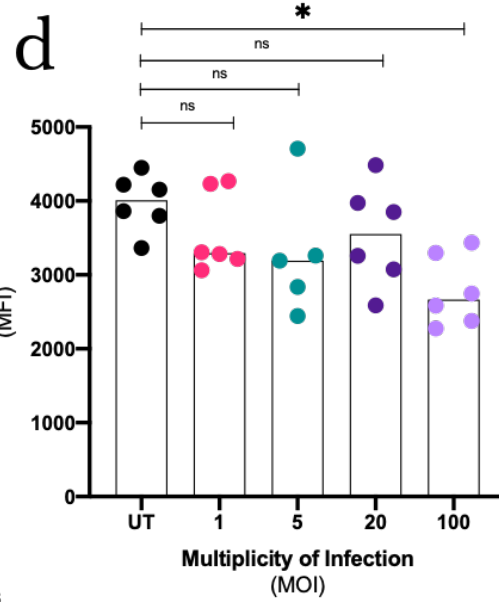
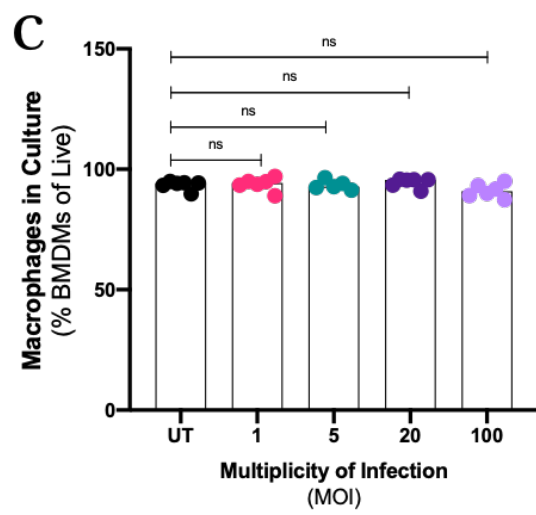
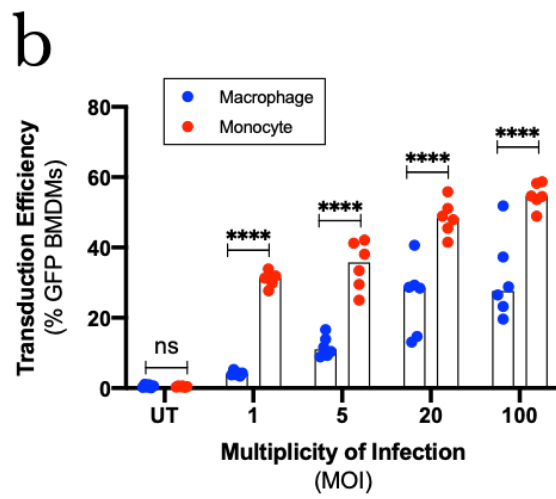
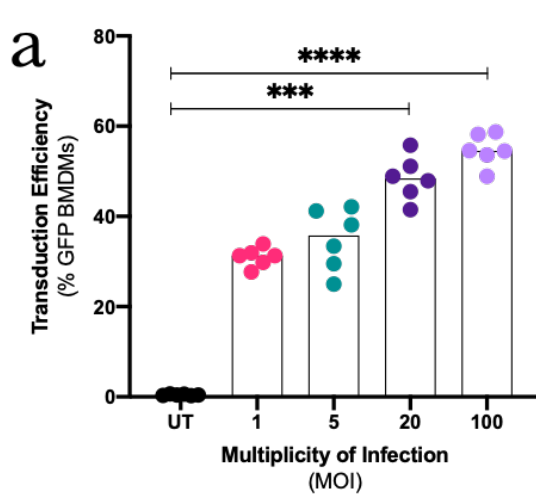


Figure 57 - Dose response in transduction efficiency following rSIV.F/HN lentivirus transduction of bone marrow monocytes during macrophage differentiation. (a) The transduction efficiency of SIV-F/HN expressing an EGFP transgene at increasing multiplicities of infection when transducing macrophages on days 0-2 of the differentiation procedure (n=6/MOI). Transduction efficiency was calculated using EGFP expression by flow cytometry on day 7. Untransduced cells served as controls. (b) Transducing macrophages during differentiation was more efficient than previous experiments (chapter 3.3.4) transducing BMDMs on days 7-9. (c) BMDMs were first gated based on expression of cell-surface phenotypic macrophage markers CD11b⁺, F4/80⁺ to calculate the percentage of macrophages in culture which were not affected by rSIV transduction. (d) CD11b and (e) F4/80 mean fluorescent intensities show only slight changes. Capture by BD LSRFortessa Cell Analyser. Each data point represents an individually transduced well of BMDMs. Bars represent median. (*=p<0.05, ***=p<0.001, ****=p<0.0001, Kruskal Wallis test with Dunn's multiple comparisons.)

5.3.12 Establishing a non-viral sleeping beauty transposon vector as an alternative to rSIV lentivirus mediated gene-transfer in macrophages

To compare the alternative non-viral gene transfer vector sleeping beauty (SB) to the lentiviral delivery system established here, I, along with master's student Martina Rossi, first characterised the SB transposition system and attempted to show proof-of-concept delivery in BMDMs.

5.3.12.1 EGFP expression in transiently transfected cells over 3 cell passages suggests successful transposition of the SB transposase leads to stable expression

Co-transfection with the SB transposase (SB100x) and EGFP transposon (PT2/HB-EGFP) expression plasmids in A549 cells showed first proof-of-concept of transposition, stable integration and expression of the transgene. Plasmids were transfected at a 2:1 ratio and analysed by flow cytometry for EGFP positive cells on day 3, 8 and 13, where in-between each time point cells underwent one passage. At each time point, there were more EGFP⁺ cells when transfected with both the transposase and transposon, than in transposon only controls (Figure 58):

- *Day 3:* 26.9% median difference in EGFP⁺ Cells, ($p < 0.0001$)
- *Day 8:* 17.4% median difference in EGFP⁺ Cells, ($p < 0.01$)
- *Day 13:* 17.1% median difference in EGFP⁺ Cells ($p < 0.0001$, 2way-ANOVA, Sidak's multiple comparisons)

Additionally, the percentage decrease in EGFP⁺ cells over time was significantly higher in the absence of the transposase, further suggesting it mediated successful integration of the EGFP transposon:

- *Transposon:* 90.5% median decrease in EGFP⁺ Cells
- *Transposon + transposase:* 64% median decrease in EGFP⁺ Cells ($p < 0.01$, Mann-Whitney test)

The median transposition efficiency in cells transfected with both transposase and transposon expression plasmids was 36% (range 34.5–41.4 %), however as not all cells were transfected, this equated to 19.5 % (range 19.2–25.6) EGFP⁺ cells in culture on day 13.

In this initial experiment, cells were only followed over 2 cell passages to day 13, at which point the single transfection controls (pIC064 and PT2/HB-EGFP) still had low levels of expression.

Cells transfected with only the transposon expression plasmid are important controls for background integration events, which on day 13 had a high transposition efficiency of 9.6% (range 6.6–23.43%), this high rate of transposition is probably due to the fact that after 13 days in culture and 2 passages, episomal driven EGFP expression has not been completely diluted out. Conclusive experiments will require longer periods in culture to establish clear and distinct proof of transposition, where in the literature transposition efficiency is calculated for cells 20–30 days post transfection (527). This test did however provide first indications of successful transposition following transfection.

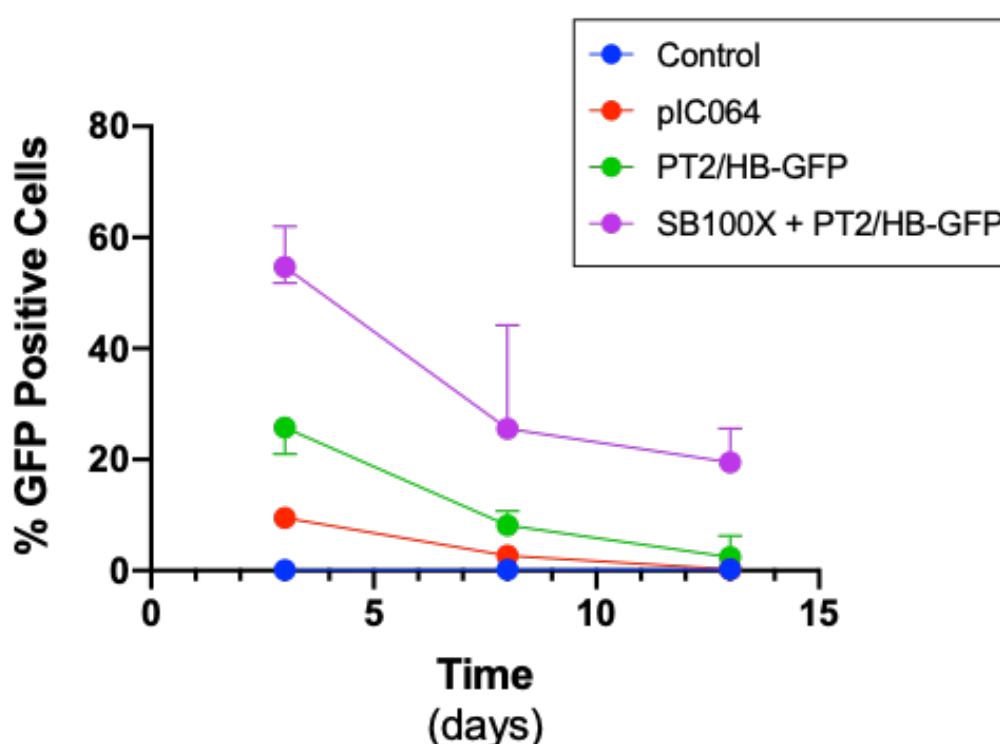


Figure 58 – EGFP expression in A549 cells following transfection of the Sleeping beauty (SB) transposon system. A549 cells were transfected using PEIPro® for 72 hours (day 0–3) in single or double transfections with plasmids encoding the two components of the SB system, the transposon (PT2/HB-GFP), and transposase (SB100X) (n=6/group). Positive controls received the non-viral EGFP expression plasmid (PIC064). Un-transduced cells served as negative controls (control). Cells were analysed for EGFP expression over time by flow cytometry on days 3, 8 and 13. Cells were passaged once between time points. Capture by BD Accuri™ C6 Plus Flow Cytometer. Points represent median, error bars represent range.

5.3.12.2 Transfecting BMDMs

BMDMs were then transfected under similar conditions with the PT2/HB-Glux transposon to determine if macrophages could be transfected with the SB transposase under the same protocol using the stable cationic polymer, PEIPro. Unfortunately, the transfection failed as Gaussia luciferase (Glux) expression was only observed in A549 controls and absent in BMDMs following a 72-hour transfection of the SB system or the pGM206 plasmid positive control (Figure 59). Macrophages are notoriously reticent to non-viral gene transfer vectors including liposomes and polyethyleneimine (287) and therefore future strategies will focus instead on electroporation which has been successful in introducing the SB system to HSCs. Though viral vectors could also be used to introduce the system to macrophages it eliminates most of the potential advantages of SB over our lentivirus. Currently I have been unable to successfully transfer the SB components into macrophages and therefore have not determined whether it offers a viable alternative to rSIV mediated gene transfer in primary macrophages.

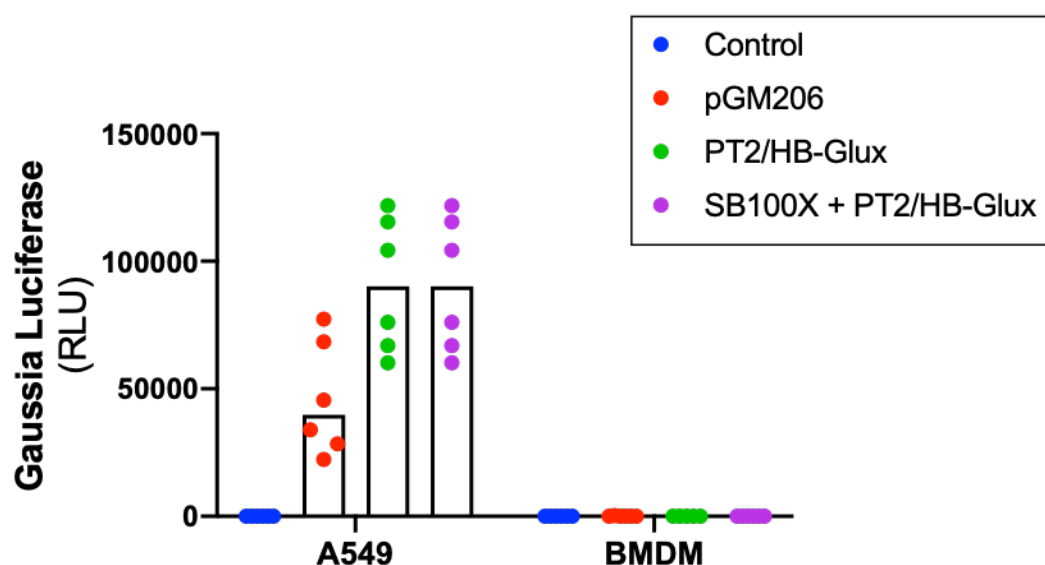


Figure 59 – Glux expression in A549 cells and BMDMs following transfection of the Sleeping beauty (SB) transposon system. A549 cells and BMDMs were transfected using PEIPro® for 72 hours in single or double transfections with plasmids encoding the two components of the SB system, the transposon (PT2/HB-Glux), and transposase (SB100X) (n=6/group). Positive controls received the non-viral Glux expression plasmid (pGM206). Un-transduced cells served as negative controls (control). Following the transfection, cells received fresh media which was taken for Glux expression analysis 24 hours later. RLU – relative light units. Bars represent median.

5.4 Discussion

In Chapter 3, I showed proof-of-concept for gene transfer to bone marrow-derived macrophages using a proprietary lentivirus developed by the UK Respiratory Gene Therapy Consortium, a simian immune deficiency virus (SIV) pseudotyped with the surface proteins F and HN from the Sendai virus (268). These findings were then applied to develop an *ex vivo* macrophage-based cell therapy approach to lung gene therapy and demonstrated proof-of-concept in a disease model of aPAP. Aiming to improve the overall efficacy of this platform technology, in this chapter I have further characterised the use of this virus in the generation of genetically modified bone marrow-derived macrophages (BMDMs) and compared the relative efficiency and activity of different pseudotypes and transgenes for optimal gene transfer and expression in these cells.

Using SIV lentivirus produced both in house and by the Oxford gene therapy group, I have shown (1) that at MOIs 50 and over, the VSV-G pseudotype may result in small increases to transduction efficiency over the F/HN pseudotype; (2) molecular analysis of SIV transduction efficiency is not transgene specific; (3) vector analysis and characterisations at early time points is not representative of stable gene expression, and (4) transducing monocytes during macrophage differentiation leads to higher yields of gene-modified BMDMs. Additionally, I have characterised the activity of different transcriptional regulation elements in SIV lentiviral vectors and shown that (4) in comparison to hCEF chimera and the EF1 α promoter, the CMV promoter/enhancer element drives higher levels of transgene expression at early time points; and lastly, (6) I have produced an alternative non-viral transposon-based gene transfer system for future comparison to the SIV lentivirus. Together this body of data suggests optimisations to the SIV vector to improve and optimise the production of genetically modified macrophages for more efficient macrophage *ex vivo* lung gene therapy.

5.4.1 Vector Production

Here small-scale lentiviral vectors were produced to limited success, where F/HN pseudotypes productions was generally less efficient than VSV-G. Increasing the amount of plasmid DNA required during the transient transfection to from 0.33 μ g to 1.5 μ g (per 1e6 cells in culture) did substantially increase titre yields in VSV-G viruses. Retrospectively, it was realised that the protocol under which F/HN small-batch virus was produced was established for large-scale

production in a WAVE bioreactor, where 0.33 µg of plasmid DNA were transfected per 1e6 cells in culture whereas small scale production in Erlenmeyer shake flasks also uses 1.5 µg (279, 517). This may explain why in my hands F/HN titres were so much lower than VSV-G. Nevertheless, Oxford collaborators also found that F/HN pseudotyped virus yielded lower titres than VSV-G. This may be due to decreased transfection efficiency with a 5-plasmid system required by F/HN as F and HN proteins are encoded on separate plasmids, while VSV-G production only requires transfection of 4 plasmids (528). However, overall titres achieved for both VSV-G and F/HN viruses were lower than vector yields in small-batch virus provided by Oxford collaborators or the reported yields in previous publications (268, 276, 279).

5.4.1.1 Determining Vector Titres

Here I used functional titring in the generally permissive HEK293T cells to determine vector yields. Titre was determined through transduction of known cell numbers in serial dilutions and analysis of EGFP expression by flow cytometry. However, the understanding that in BMDMs lentiviral integration can be silent, led to retrospective analysis of the discrepancy in vector titres achieved in house and in Oxford. I found that Oxford manufacturing facility titres vectors, including EGFP based virus, by molecular ddPCR analysis. As findings suggest that transgene expression-based analysis (GFP flow cytometry) underestimates vector insertion, it likely underestimates viral titres too. Indeed, studies have demonstrated that titres calculated by functional GFP expression assays are often one log lower than those obtained by molecular DNA analysis (529). This oversight in the discrepancy in titring methods likely explains how Oxford was seemingly able to produce higher titre viral vectors. It might be interesting to retrospectively re-titre EGFP virus with both methods to confirm this. Furthermore, it means that in the first pilot experiment, the large difference between VSV-G and F/HN virus at MOI 20 is likely due to poor dose control as a result of differences in vector titre, rather than increased efficiency of the VSV-G pseudotype. Therefore, the initial proof-of-concept experiment is unreliable and should be disregarded when comparing VSV-G and F/HN pseudotypes in macrophages.

5.4.1.2 Vector Concentration

Lentiviral vectors are routinely concentrated to increase viral titres and remove impurities which is critical for *in vivo* delivery in animal models and translation to the clinic, and also

enables more efficient transduction. This is usually achieved by ultrafiltration or ultracentrifugation and can produce final titres of $1e9$ - $1e10$ transduction units per mL (530, 531). At the GTC Core Facility in Oxford the rSIV.F/HN lentivirus is routinely anionexchange and hollow-fibre ultrafiltration/diafiltration size exclusion (276). Due to the cost of large-scale vector production, and the reduced need to purified vectors *ex vivo*, in initial studies comparing pseudotypes and candidate promoters, small-scale productions were used instead. Due to the low titres achieved in house, I attempted to concentrate these small-scale preparations, and due to the lack of access to an ultracentrifuge or ultrafiltration system, this was done by high-speed centrifugation which was a readily available technique. Though the standard procedure for concentrating lentivirus often employs ultracentrifugation, spinning samples at upwards of 90,000 *rcf* for 90 minutes (485, 530, 532), high-speed centrifugation at lower speeds, such as those employed here (26,000 *rcf* and 17,000 *rcf*) have also been employed successfully by others (533); one study demonstrating that at 10,000 *rcf* in a 10% sucrose gradient, VSV-G lentiviral particles can be successfully concentrated with an 80% recovery rate (534). Although the recovery rate achieved here was higher even in the absence of a sucrose gradient with 5.23% viral loss, this required aliquoting into 1ml samples which was highly time consuming and inefficient. In contrast this study achieved an 80% retention rate when centrifuging 50ml vector preparations (534). I did not spend a long-time optimising vector concentration and instead moved forward with the inefficient, but effective protocol developed here. In retrospect, obtaining access to an ultracentrifuge would have been the best way to move forward with in-house productions to more efficiently access concentrated virus, however instead I relied on virus produced by the GTC Core Facility. One of the limitations of this work is that the centrifugation process was only optimised in VSV-G pseudotypes. Here F/HN vectors were concentrated 52-, 15-, and 26- fold, while VSV-G vector concentrations were generally more successful at 96-, 144-, and 28- fold. As the stability of vectors during centrifugation varies between pseudotypes, another reason F/HN lentiviral production was reduced could be due to a reduced mechanical stability. Indeed, one study demonstrated that the stability of HIV vectors was lower with influenza virus WSN strain envelope proteins than VSV-G (535). As the final volumes of concentrated viruses were only recorded during optimisation, and not when concentrating the six candidate vectors, it is unclear if the relative loss due to viral instability was analogous across F/HN and VSV-G pseudotypes. Though it is important to note that VSV-G vector had more starting material to concentrate. Regardless, this method was only used briefly, and in subsequent experiments crude virus produce by the GTC Core Facility was used instead as they then appeared to achieve higher titres.

5.4.2 VSV-G vs F/HN Pseudotype

In Chapter 3, I showed for the first time that BMDMs can be transduced by SIV lentiviral vectors pseudotyped with F and HN envelope proteins. Here I assessed the relative efficacy of this pseudotype to that of VSV-G, which as the most prevalent pseudotype used in the literature could be considered the gold standard (176, 177, 298, 300, 301). The transduction efficiency of SIV virus enveloped with both F/HN and VSV-G proteins was comparable and showed some evidence that VSV-G might be more efficient at MOIs 50 and above. Statistically significant evidence of increased transduction with a VSV-G envelope was observed, however it is worth noting that as these differences were small and not highly reproducible, it may be within the margin of error associated with pipetting such highly concentrated viral preparations, where virus titres are $1e8$ - $1e10$ TU/ml. Further comparisons with a relevant secreted transgene and a dose escalation trial above MOI 50 are required to fully elucidate the potential a VSV-G envelope may have. However, should VSV-G show increased transduction at higher MOIs, when selecting the successful vector configuration with which to move forward, the cost-benefit analysis will need to consider the benefit provided by the increased effective dose and the cost of the additional virus required to achieve it. Nevertheless, as evidenced by the viral titres produced by Oxford in small batch experiments, VSV-G pseudotyped virus is produced at a higher titre and therefore, may, even without an increase in transduction efficiency, provide a more cost-effective alternative to F/HN.

That the F/HN pseudotype was successful in transducing macrophages is not unexpected as Sendai Virus entry is mediated by attachment of the HN proteins to ubiquitously expressed sialic acid containing ganglioside receptors (477-479, 533, 536). Sendai virus has also been shown to infect macrophages and support viral replication *in vitro*, where macrophage specific immunity to Sendai virus was only present in macrophages from immunized animals (537). As humans are not a natural host to the Sendai virus this complication is unlikely to arise if transducing primary cells from patients (538). However, it was somewhat surprising that the two envelopes demonstrated similar efficacy, as VSV-G is known for its broad tissue tropism and has been widely used in the literature to mediate gene transfer to macrophages *in vitro* (298-301), while F/HN shows preferential transduction of the lung epithelium (539). Even with pre-conditioning, VSV-G lentivirus expression in AMs is not ameliorated mice, marmosets and non-human primates (174). On the other hand, F/HN delivery to adult mice showed no evidence of macrophage transduction and instead transduced 15% of target airway epithelial

cells (302). Additionally, a GP64/Sendai chimeric envelope protein where the F2 domain of the Sendai virus fusion protein was fused to the amino terminus of GP64, reduced macrophage transduction *in vivo* and *in vitro* in comparison to the wildtype Gp64 envelope, though this was likely due to an increased hepatocyte tropism and reduced immune recognition (540). As we saw only minimal differences between pseudotypes in an *ex vivo* transduction setting, the lack of F/HN expression in macrophages following *in vivo* lung administration may just be due to increased tropism for the lung epithelium rather than reduced ability to transduce macrophages. This work therefore presents F/HN as a viable alternative lentiviral pseudotype to VSV-G with similar efficacy *in vitro*. Though evidence was provided for a potential increase in transduction efficiency with a VSV-G envelope at high multiplicities of infection, whether this would mediate significantly higher levels of protein production *in vivo* is currently unclear.

5.4.3 Transgene Specific Differences

Having used EGFP expression-based analysis to characterise and optimise SIV transduction in macrophages, I demonstrated that there is no evidence of transgene specific differences in macrophage transduction or gene expression. This was an important control to understand how applicable findings using EGFP are when transducing macrophages in therapeutic settings. Here lentivirus encoding EGFP, and mGM-CSF transgenes were compared as GM-CSF is the therapeutic protein suggested for treatment of aPAP. Transgenes would be more likely to exert influence over vector production than transduction, as for example, larger inserts are harder to package efficiently leading to a reduction in final titres (518). However, mGM-CSF and EGFP transgenes are similar sizes (EGFP: 720bp, GM-CSF: 426 bp) and titres were kept as consistent as possible during dosing. It is therefore not unsurprising that the transduction and expression profile of our lentivirus was consistent across the two tested transgenes. However, in *in vivo* mouse studies, a Glux transgene was used as a control for mGM-CSF treated mice and to determine duration of gene expression in WT mice. Therefore, further confirmation as to the lack of a transgene-specific effect on lentiviral transduction and gene expression is required with Glux and mGM-CSF transgene comparisons.

5.4.4 Vector Copy Number

Vector copy number was consistent across SIV vectors, however multiple vector insertions per cell were observed. A caveat is that these data were calculated from a bulk population of macrophages rather than single cell analysis, but it is in keeping with literature that suggests lentiviral vectors are often present at high VCNs (502, 520). HSCs are known to permit multiple integration events of HIV vectors, with up to 6 copies per cell observed in cells after engraftment (541). Increased VCN can increase the biological potency and transgene expression from a single cell, however it is countered by the increased risk of genotoxicity and potential silencing as a result of vector interference (542). This also raises questions as to the homogeneity of cultured BMDMs, and if there is a population of cells refractive to transduction, so that increasing MOI does not increase transduced cell numbers, but rather the number of insertions in those cells. Analysis of transduced and un-transduced cell populations, and cell sorting by flow cytometry would be useful in elucidating whether such a population exists and provide higher resolution VCN analysis. However, unpublished data produced by colleagues testing the SIV vector in air-liquid-interface cultures and other immortalised cell lines suggests multiple VCN is characteristic of the SIV lentivirus and not specific to macrophages. This suggested propensity for multiple VCNs in macrophages could be advantageous in a two-vector system where dual transduction is required and might explain the surprising success of the 2V GeneSwitch™ regulated expression system in Chapter 4.4.7.

5.4.5 Characterising Transduction and Expression at 24 Hours

In determining which molecular metrics could be used to generate a transduction profile of each cell batch delivered to mice for inter-experimental comparisons, attempts at characterising transduction at early (24 hour) time points largely failed, though in F/HN pseudotyped virus, VCN was representative of day 7 results.

The encapsulation assay could not be used to determine the percentage of cells with integrated/episomal viral cDNA as linkage analysis demonstrated significant dissociation of viral and host cellular DNA. This may be due to reduced cell viability but seems unlikely as, though low in number, samples were shown to have a high viability prior to processing. Additionally, this issue was only prevalent at 24 hours, and not 7 days post transduction, when the same downstream processing procedure was followed. Alternatively, the difference in

linkage between different vectors suggests that low linkage at 24 hours could be due to free floating viral DNA, as might occur due to plasmid contamination in viral preparations. F/HN.mGM-CSF treated samples had the lowest linkage overall, and upon harvesting the cells in tissue culture, it was overlooked that these cells were washed one time less than EGFP counterparts due to the extra step involved in preparing samples for flow cytometry. This resulted in significantly higher levels of virus-specific RNA in these samples at 24 hours. Therefore, any potential contaminant like plasmid DNA would likely also be higher in these samples and may explain the reduced linkage observed. Samples transduced with VSV-G had the highest linkage and were transduced with crude vector as opposed to samples transduced with one of the F/HN viruses which were both concentrated and purified. As differences in viral preparations led to different linkage outcomes it suggests that reduced linkage at 24 hours is due to influence of viral supernatant. Further examination of why linkage is so low is required, and future analysis could use an additional probe designed to specifically detect the plasmid encoding the viral genomes during production.

The kinetics of a lentiviral life cycle are species (viral), pseudotype, and host-cell specific, and has not been characterised for the rSIV.F/HN vector in primary macrophages (525, 526). A lack of transgenic protein expression and detectable levels of mRNA at 24 hours indicates that more time is required for cell entry, reverse transcription, integration and expression. This is in keeping with HIV transduction/infection experiments where in CD34⁺ cells HIV integration was completed after 3 days (395) and studies showing relatively slow kinetics of HIV vectors in non-dividing, cultured mononuclear phagocytes where between 36-48 hours are required for reverse transcription (401). Glux controls in inducible expression experiments in Chapter 4.4.7 demonstrated that with a constitutive promoter, at 24 hours transgene expression is minimal and largely attributable to pseudotransduction, and at least 6 days are required before peak gene expression. It is therefore surprising that at 24 hours VCN was representative of VCN at 7 days and further confirmation is required with dose escalation studies and assessment of potential plasmid contamination. The latter is of particular import as one might expect that VCN analysis at 24 hours would underestimate VCN results at 7 days, but here both F/HN viruses showed slightly increased VCNs at early time points (though not significant) which might be explained by plasmid contamination contributing to VCN calculations in bulk samples. Currently, however, these initial findings do suggest that VCN could be a viable read-out of transduction efficacy in gene-modified macrophages when delivered to mice. It may therefore be used as a metric by which to conduct cross-experimental comparisons of

transduction between *in vivo* experiments, having previously observed variation in the amount of GM-CSF protein present in the BALF of treated mice. It is important to note that these findings were only applicable to F/HN pseudotyped SIV, as VSV-G pseudotyped vector VCN was significantly different between time points. Pseudotype specific differences in viral entry kinetics have been noted and likely explain the discrepancy (525, 526).

It will be important to develop reliable methods to characterise cells delivered to mice as in Chapter 4, a degree of inter-experimental variation was observed in *in vivo* secreted protein production following macrophage transplantation. As across this body of work, a degree of variation was also observed in the success of *in vitro* lentiviral macrophage transduction, characterising the delivered cellular product is required to enable inter-experimental comparisons. Regardless of the applicability at 24 hours, the molecular assays used here to characterise transduction and gene expression *in vitro* could also be useful in tracking transduced macrophages and gene expression *in vivo*, for example mRNA expression in BALF cells or lung homogenate might provide a more sensitive assay for gene expression than currently offered by GM-CSF ELISA or dPLA and VCN analysis could provide a read out of macrophage retention which is currently not possible by flow cytometry in PAP mice.

5.4.6 Silent Integration

During vector optimisation and characterisation, transduction efficiencies are often calculated through flow cytometry quantification of EGFP transgene expression (298-301). With molecular analysis, here SIV was shown to transduce cells at a higher efficiency than that implied by EGFP analysis. While molecular analysis identifies all integration events, EGFP analysis only identifies insertions that lead to successful gene expression. This therefore suggests that in a significant proportion of cells, expression from the SIV provirus is silenced. This is in keeping with reports that lentiviral vectors can exhibit variegated expression and silencing affected by chromosomal positioning (522). Rather than focus on increasing the overall number of transduced cells, an alternative strategy to optimise *in vivo* therapeutic production could focus on increasing the number of transcriptionally active transduction events. This would make each viral dose more efficient and reduce vector costs.

The majority of retroviruses do not integrate randomly and exhibit genus specific bias. This bias is a result of the interaction enabled by viral integrase, between the retroviral pre-

integration complex (PIC) and host cellular proteins that localize to specific genomic sites (239, 242). Lentiviruses preferentially integrate within actively transcribed genes, and though biosafety concerns extol a more random integration pattern, it would suggest that it has an advantage in enabling more efficient viral gene expression (246). However, our results demonstrate that in approximately 70% of transduced cells, integrations are silent and that either the vector is subject to heterochromatin-mediated silencing, or it is prone to active silencing. Here, transduction comparisons between vectors encoding different internal enhancer/promoter sequences suggest EF1 α may minimise the effect of neighbouring chromatin on transgene expression as vectors reported higher transduction efficiencies, however this was not confirmed by molecular analysis. Therefore, using EF1 α promoter to drive transgene expression in macrophages may lead to higher levels of protein secretion *in vivo* than an equivalent cell and viral dose under CMV or hCEF transcriptional control. In addition to the use of alternative enhancer/promoter elements, modifications such as the inclusion of scaffold/matrix attachment regions, ubiquitous chromatin opening elements (UCOE) and chromatin domain insulators could be used to reduce position-effect-variegation and DNA methylation-mediated silencing (543, 544). Alternatively, others have also targeted integration to a safe harbour site (489). As all tests were performed in bone marrow-derived macrophages and this work represents the first comparisons between the encapsulation assay and transgene expression-based analysis of the UK Cystic Fibrosis Gene Therapy Consortium SIV vector, it is possible that this high degree of silent integrations is cell-specific or alternatively characteristic of the lentivirus which could have implications for the *in vivo* use of the vector when targeting the lung epithelium.

5.4.7 Promoter Differences

5.4.7.1 Choice of Candidate Promoters

With an improved understanding of vector biology and biosafety, lentivirus vector development has moved away from endogenous viral promoter driven expression and toward the inclusion of internal regulatory elements instead. The rSIV.F/HN-hCEF vector developed by the UK CF gene therapy consortium drives gene expression from a the CpG-free hCEF promoter/enhancer chimera composed of the EF1 α promoter coupled with the human CMV enhancer (280). This promoter was chosen as a lead candidate for its specific activity in relevant human lung models over other chimeras and the CMV and EF1 α native conformations (302).

I have demonstrated for the first time successful *in vitro* gene expression driven by the hCEF promoter in BMDMs, however as the developmental process of the consortium vector demonstrates, optimisation of the expression cassette is important and cell specific. I therefore compared the activity of hCEF to that of the CMV promoter/enhancer element, widely used in driving strong recombinant gene expression across a variety of cell types including macrophages (289, 545) and the EF1 α short promoter which has been used successfully to drive long-term transgene expression in macrophages following transplantation to the lung (176, 178). Utilizing macrophage specific promoters is an area of interest in *in vivo* macrophage gene transfer strategies, where transcriptional regulation is controlled to target expression to macrophages even though transduction is not limited to these cells. However, as the aim of this work is to develop an *ex vivo* cell therapy platform where virus is delivered *in vitro*, off target transduction not of major concern and little to no virus would be expected in clinical grade products. These promoters, though often specific to macrophages, generally exhibit a reduced strength of gene expression in comparison to common constitutive promoters like CMV (289, 546) and therefore were not chosen as potential candidates here.

5.4.7.2 Relative Transgene Expression

Though EF1 α may minimise the effect of neighbouring chromatin, comparisons also demonstrated that CMV drove higher levels of transgene expression on a per cell basis. To determine the relative effect of each, comparisons with a secreted transgene are needed. In contrast, in *in vivo* studies comparing these promoter/enhancers, hCEF showed the highest and most persistent level of expression in the murine nose and lung 130 days post administration, and *in vitro* in air liquid interface cultures (*in vitro* model of the lung epithelium) 5- and 14-days post transduction (302). Pulmonary delivery of plasmid DNA (pDNA)/cationic liposome complexes indicated that at early time frames hCEF expression was modest in comparison to CMV but was significantly higher in the lung at later time points (days 14 and 28), indicating the potential degree of inter-model/vector variability and cell-specific expression profiles (280). Indeed, studies have shown cell type variability in the relative effectiveness and persistence of the CMV promoter (547), in part due carrying degrees of methylation mediated silencing (548-550). In non-viral vectors expression was limited to approximately 2 weeks in clinical (515) and preclinical (280) settings due to posttranscriptional silencing. In BMDMs expression was quantified at an early 7-day time points, and though CMV showed the highest level of expression, this may not be stable over time. In order to determine if CMV is the better choice

for sustained high gene expression, long term comparisons following *in vivo* BMDM delivery and/or in immortalised macrophage cell lines are required. Furthermore, as previously discussed, the relative positional effects of neighbouring chromatin on gene expression may be more favourable in macrophages with the EF1 α internal promoter.

5.4.7.3 Moving to *in vivo* studies

It is important to note that in culture, M-CSF differentiated BMDMs exist in a unpolarized state (47). These cells are highly plastic and sensitive to conditioning by their environment (2). Following lung administration, BMDMs and iPSC-derived macrophages undergo changes in response to the lung milieu and resemble alveolar macrophages in their surface-receptor phenotype and functionality (176-178, 186, 420). As reduced gene expression can often be observed following cellular differentiation and proliferation (551), conclusions drawn from BMDMs in culture, need to be tested following *in vivo* administration as transcriptional regulation may be affected by the changing epigenetic landscape of transplanted cells. At this stage a definitive conclusion as to the most appropriate constitutive promoter cannot be made, however results indicate that *in vivo* transgene expression could be improved by replacing hCEF with CMV or EF1 α transcriptional control.

5.4.8 Timing of Transduction

Transducing macrophage precursors led to marked increases in transduction efficiency, and the final yield of genetically modified macrophages. The significant success seen even at low MOIs indicates that further optimisation of this adapted protocol could not only improve effective cell doses for *ex vivo* therapy but also reduce vector associated costs. This reason for this effect is currently unclear and requires further investigation, but may be due to increased transduction of monocytes, reduced vector silencing, or the proliferation of transduced cells during differentiation. This finding is inconsistent with *in vitro* studies showing that human primary monocytes are relatively refractory to HIV-1 infection (295). This is thought to occur independently of viral entry as reports by Zeng *et al* 2006 and Neil *et al* 2001 demonstrated that HIV-1 based vectors pseudotyped with VSV-G envelope proteins are increasingly more efficient at transducing bone marrow monocyte/macrophage (301) and peripheral blood mononuclear monocytes/macrophages (298, 300) at later time points during differentiation, reaching peak efficiency at the conclusion of the differentiation protocol. This is thought to

occur due to the anti-viral activity of the myeloid specific restriction factor SAM domain HD domain-containing protein 1 (SAMHD1). SAMHD1 possesses deoxynucleoside triphosphate triphosphohydrolase activity that prevents HIV-1 reverse transcription (552). It is antagonized by the viral accessory protein vpx encoded by HIV-2 and SIV and has demonstrated its essential role in macrophage infection *in vivo* and *in vitro*, as in vpx deficient macaque SIV and HIV-2, reverse transcription in monocytes is blocked (552-554). Expression of vpx can also rescue HIV-1 infection in monocytes and partially rescue transduction with HIV-1 based vectors (305, 555-557). It is important to note that the Consortium's rSIV lentiviral vector does not express vpx, and therefore the use of SIV versus HIV-1 in these contrasting studies does not explain the discrepancy in findings.

SAMHD1 activity is regulated by phosphorylation which abolishes the proteins anti-viral activity and restriction of reverse transcription, to enables HIV-1 infection (557, 558). Studies have shown that phosphorylation of SAMHD1 is higher in differentiated macrophages than undifferentiated monocytes (557, 559) and supports research that demonstrates transduction of macrophages is more efficient post-differentiation. However, these studies use human serum or GM-CSF to induce monocytes and drive macrophage differentiation and studies have demonstrated that GM-CSF can suppress HIV-1 replication in human monocyte-derived macrophages regardless of the stage of cellular maturation (560). Furthermore, monocytes induced with M-CSF, as they were here, are more susceptible to HIV-1 infection than GM-CSF induced monocytes (561, 562). Macrophages induced by human serum exhibit a GM-CSF phenotype (314), and this Serum/GM-CSF/M-CSF inducer difference likely accounts for much of the discrepancy in the effect of monocyte maturation shown by studies attempting lentiviral gene-transfer, as those demonstrating efficient monocyte transduction prior to differentiation relied on M-CSF induction (299). Therefore, studies showing that cellular differentiation and prolonged *in vitro* culture of monocytes makes macrophages more permissive to lentiviral transduction are likely seeing the effect of GM-CSF induced inhibition (295, 300, 301). Additionally, SAMHD1 is expressed in both dividing and non-dividing cells but is phosphorylated only in the former, and as a result its anti-viral properties are restricted to non-dividing cells (391, 563). As M-CSF induced monocytes have a higher rate of proliferation than GM-CSF induced monocytes (391), variable SAMHD1 inhibition may explain subsequent differences in lentiviral transduction. Correspondingly one study demonstrated SAMHD1 restriction was slightly lower in M-CSF induced than GM-CSF induced monocytes, though the exact mechanism underlying this difference was unclear as no significant difference in

SAMHD1 phosphorylation was observed (559). Though studies directly comparing the effect of time and maturation of monocytes on macrophage transduction are limited, the contrasting results are likely explained by the polarizing effect of different monocyte differentiation protocols. These results demonstrate that an M-CSF induced differentiation protocol is permissible to SIV.F/HN lentiviral transduction which is more efficient than in macrophages. However, it is also possible that during differentiation, as a result of changes to the epigenetic landscape, the degree of vector silencing in mature macrophages is reduced and therefore EGFP based findings here need confirmation with molecular assays of transduction efficiency. Alternatively, under this protocol virus was added to bone marrow cells upon initial plating and therefore the advanced efficiency may also be due in part to increased physical interactions between virus and cells as both are in suspension and spinoculation has shown enforcing closer contact between virus and cell through physical means has a concentrating effect on viral particles and can increase transduction (564, 565).

The prohibitive effects of GM-CSF transduction during macrophage differentiation however may have consequences transducing macrophages to express GM-CSF. GM-CSF expression during macrophage differentiation could alter the macrophage phenotype and affect not only transduction efficiency, but as a consequence of an altered phenotype transplanted cells may have altered activity and engraftment *in vivo*. In very general terms GM-CSF- and M-CSF-derived macrophages are differently polarized for inflammatory (M1, classically activated) and anti-inflammatory (M2, alternatively activated) phenotypes respectively (13, 18, 566). Though, in Chapter 4 I demonstrated preliminary evidence that GM-CSF expression *in vivo* increases the engraftment of transplanted macrophages, the effect of stimulation during differentiation is unclear. All current *in vivo* studies have used M-CSF induced macrophage differentiation and therefore could not be directly extrapolated to GM-CSF induced cells. The relative efficacy of monocyte and macrophage transduction requires transgene-specific confirmation to determine whether M-CSF induced monocytes can still be successfully transduced with a lentivirus encoding a mGM-CSF transgene. Though the efficacy of the regulated promoter and production of inducible macrophages with this alternative transduction protocol has not been demonstrated, macrophages transduced to express mGM-CSF under the regulated transcriptional control of the Geneswitch™ expression cassettes could solve any potential issues that may arise from GM-CSF production during differentiation.

5.4.9 Sleeping Beauty

Having demonstrated successful transduction of primary macrophages for *in vivo* protein production, an alternative non-viral transposon-based gene transfer platform was established to enable comparisons and potentially improve upon lentiviral based gene-transfer. The SB transposon system was initially chosen, as like retroviruses, it advantageously integrates into host chromosomal DNA to provide stable and long-lasting integration, though with a safer, more random integration profile (567, 568) and is more cost-effective to produce on a large scale than viral vectors (291, 569). Having faced delays in obtaining the system components and establishing relevant MTAs, this work was further delayed by successive failures to clone the relevant transgenes into the SB transposon. As a result, work has only nominally begun to characterise the use of this SB system in macrophages. An initial experiment with a EGFP transgene suggested successful transposition of the SB transposon in an immortalised cell line using a stable cationic polymer PEIPro, however longer-term characterisations are required and delivery to macrophages was unsuccessful. As macrophages are notoriously reticent to non-viral gene transfer vectors including liposomes and polyethyleneimine (287, 290, 568, 570) this was not unsurprising. The most likely successful route of SB delivery to macrophages will require electroporation/nucleofection which has successfully led to stable SB mediated Glux expression in M-CSF differentiated macrophages from hPBMCs (506), and a fluorescent reporter and selectable marker in a RAW 264.7 macrophage cell line (507), though these studies did not quantify electroporation or transposition efficacy. Additionally, transplantation of SB genetically modified cord blood-derived CD34⁺ progenitors produced gene-modified granulocyte/monocyte/macrophages with subsequent differentiation *in vivo* (571, 572). However, studies genetically modifying primary macrophages with SB are to my knowledge limited, and robust characterisation of electroporation and transposition success lacking. Whether gene modification of primary bone marrow-derived monocytes/macrophages through electroporation of the SB system will compare to success seen with our lentiviral system is yet to be determined. Electroporation of BMDMs will require extensive optimisation as studies have shown that the majority of electroporation/nucleofection conditions are toxic to macrophages (573). In human CD34⁺ cells, SB100X mediated stable transgenesis 35–50% of cells which is similar to the transduction efficiency of the lentiviral vector in monocytes (571). However, it is important to note that cell viability, transgene expression, and electroporation

success during SB delivery is cell-type specific and therefore makes extrapolations from non-macrophage results difficult (508).

5.4.10 Conclusion

The transduction efficacy of the rSIV.F/HN vector and strength of hCEF-driven gene expression in macrophages was previously sufficient to produce significant levels of secreted transgene expression *in vitro*. When transplanting these cells to the mouse lung, data suggests that resultant *in situ* expression leads to transgenic protein production *in vivo*, which in PAP mice may reach therapeutically relevant levels. In this chapter I have investigated how gene transfer and expression in macrophages may be improved to either drive higher levels of gene expression *in vivo* and/or decrease vector costs by improving efficiency. In doing so I have also further characterised lentiviral transduction in macrophages and discovered that likely most vector integrations are silent suggesting that one of the key targets for improved efficacy will be overcoming epigenetic remodelling and positional effects. It is currently unclear if the significant improvements observed in transduction efficiency when transducing bone marrow monocytes during differentiation is mediated at all by reduced vector silencing, however this work suggests this altered protocol could significantly reduce vector costs. Both CMV and EF1a were here suggested as candidates that could improve overall gene expression, but comparisons with secreted transgenes and duration studies are still required. Applying these findings to produce an optimised vector and transduction protocol has the potential to make macrophage cell therapy more efficient. Future work could also investigate the effect of polybrene and spinofection on transduction efficiency. Furthermore, future *in vivo* experiments could apply the molecular assays used here to a deeper insight into macrophage engraftment and retention, and strength of transgene expression.

Chapter 6 – Macrophage cell therapy for treatment of cystic fibrosis

6.1 Introduction

In addition to proof-of-concept studies of *ex vivo* macrophage cell therapy for autoimmune pulmonary alveolar proteinosis, I investigated whether macrophage-based cell therapy may be relevant in gene therapy treatment of cystic fibrosis.

6.1.1 Cystic Fibrosis

Cystic fibrosis (CF) is a multisystem, progressive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and it is currently the most common lethal genetic disease of Caucasian populations (574). It is an autosomal recessive disease that occurs in 1 in 2000 to 3000 live births world-wide and is characterised by pancreatic insufficiency and lung disease that ultimately leads to respiratory failure (575, 576).

Over 2000 different mutations to the CFTR gene have been described, of which 300 are known to cause disease, though all but 5 of these mutations occur at a frequency of less than 1.1% (577). The CFTR gene encodes a cAMP activated ABC transporter-class ion channel that is abundantly expressed on the apical membrane of many cell types including airway epithelium, gastrointestinal tract, pancreas, vas deferens, and skin (578). It facilitates chloride and bicarbonate transport across cell membranes and in the absence of functional protein patients with CF present with multiorgan disease manifestations (579).

Severe pulmonary disease is the hallmark of CF, and features mucus obstruction, neutrophil-dominated airway inflammation, and chronic bacterial infections, which all contribute to progressive pulmonary damage (433). Patient's lungs are colonized in infancy or early childhood by pathogenic bacteria like *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex species and consequent respiratory failure precipitates the majority of patient mortality (580, 581). Chronic infection with *P. aeruginosa* and *B. cepacia* perpetrates an accelerated decline in lung function and increased mortality (582, 583). Since its original description in 1938 (584), our understanding of the underlying pathophysiology and

improved standard of care has substantially improved patients' outcomes. Where previously CF was a fatal infantile disease, the median age of patients who died with CF in 2019 was 31, and the predicted survival for those born with CF today is 49 (585).

6.1.1.1 CF Pathophysiology

The airway epithelial cell layer plays key roles in the immune defence of the pulmonary system. These include its barrier function, ion and fluid transport regulation to control airway surface liquid (ASL) homeostasis, mucus secretion and clearance (586, 587). In healthy lungs the CFTR protein is expressed on the apical surface of these respiratory epithelial cells (588) and research has largely focused on how CFTR dysfunction subverts immune responses orchestrated by these cells.

CFTR protein is expressed on the apical surface of airway epithelial cells where it mediates the movement of anions, chloride and bicarbonate, out of the cell into the covering mucous layer (589). The secretion of chloride ions enables fluid secretion needed for airway mucus production (590). CFTR also regulates the ENaC channel (591), limiting Na⁺ reabsorption. In the absence of functional CFTR, ion concentrations in airway mucus are decreased, leading to water reabsorption and airway surface dehydration (592). This imbalance results in abnormal mucus secretions and a hypoxic mucus layer that is readily colonized by bacteria (105). Concomitant chronic lung infections occur as ciliary beating and mucus transport is impaired in dehydrated airways and bacteria bound mucous is not cleared (105). This is the primary mechanism believed to link CFTR mutations to the CF disease state.

CF patient phenotypes can vary significantly based on elemental influences, modifier genes and the different effects of CFTR mutations on the protein (593). CF causing mutations are traditionally classified into 6 groups according to their effect on CFTR protein function which have varying clinical outcomes, a summary of which can be seen in Figure 60. The first class (I) describes mutations that result in a lack of functional protein production, either due to the absence of mRNA production, or mRNA degradation caused by a premature stop-codon. The second class of mutations (II) is by far the most common and leads to errors in processing that impair trafficking to the apical membrane. The most common CFTR mutation, F508del, is a processing mutation, where a deletion in the codon encoding phenylalanine at position 508 on chromosome 7 results in retention of the CFTR protein in the endoplasmic reticulum and

subsequent proteolytic degradation (594). 50% of Caucasian CF patients are homozygous for this mutation, while 85% of CF patients carry at least one copy (595, 596). The third class (III) describes mutations that result in gating defects where non-functional CFTR is expressed on the apical membrane (597). CFTR protein with mutations in classes IV-VI often have residual function and patients present with milder phenotypes, and can be the result of reduced conductance, expression or stability of the protein (598).

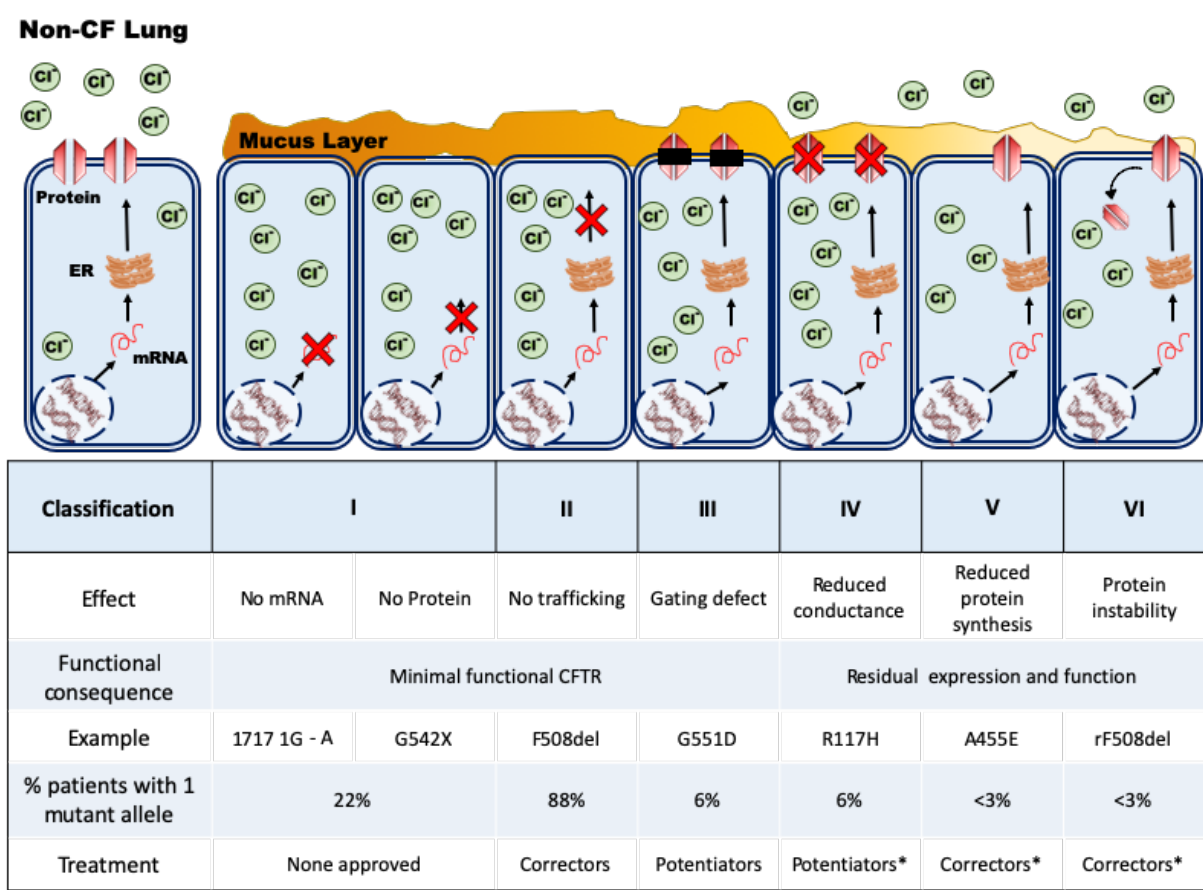


Figure 60 - Mutation classes in CF. Mutations have been classified according to effects on protein synthesis. Percentages of patients with mutations taken from US registry data. *Currently approved for some mutations. Produced by Nora Clarke and updated in collaboration with Aarash Saleh (599).

6.1.2 Alveolar Macrophages in CF

Alongside impairments in the pulmonary epithelial layer, recruited phagocytes are also abnormal in CF (600, 601). Alveolar macrophages dysfunction is thought to contribute to the chronic nature of bacterial infections observed in CF, as they exhibit a reduced capacity for phagocytosis and bacterial killing, and a hyper response to infection with an increased pro-inflammatory cytokine secretion profile (432).

As macrophages exhibit marked cellular plasticity, the chronic perturbation of the CF lung environment caused by dysfunctional CFTR protein in the airway epithelium leads to extrinsic alterations to AM function (524). One study demonstrated that the CF pro-inflammatory phenotype could be recapitulated in non-CF AMs through exposure to pulmonary mucopurulent material from CF patient (602). Increasingly studies are also demonstrating that changes to the AM functional phenotype in CF may also occur as a result of intrinsic effects of CFTR dysfunction and cannot be fully explained by lung conditioning (603-606).

6.1.2.1 *Intrinsic Consequences of CFTR Dysfunction*

Though the altered lung environment in CF modulates AM phenotypes, increasingly studies are also demonstrating that changes to the AM functional phenotype in CF may also occur as a result of intrinsic effects of CFTR dysfunction and cannot be fully explained by lung conditioning (603-606). These are largely performed on monocytes and PBMC-derived macrophages from CF patients and CF mouse models which have not been exposed to the lung environment and therefore should provide information as to the inherent effects of CFTR dysfunction. Indeed, one study demonstrated that monocytes and PBMC-derived macrophages from CF patients have altered gene expression profiles (429, 430). Furthermore, *in vivo* mouse studies suggest that in the absence of CFTR, AMs contribute the exaggerated inflammatory responses observed in the CF lung. Myeloid-specific depletion of CFTR and bone marrow transplants from CFTR knockout mice into WT mice partially recapitulated an exaggerated immune response to *P. aeruginosa* observed in mice lacking epithelial CFTR expression (435, 607, 608).

In addition to altered inflammatory responses, *in vitro* characterizations of human primary AM and PBMC-derived macrophages have suggested that owing to dysfunctional CFTR, AMs in CF have inherently reduced bactericidal activity that may contribute to patient's enhanced

susceptibility to infection (428). When comparing PBMC-derived macrophages from CF and non-CF donors, studies have shown that phagocytosis and subsequent intracellular killing or clearance of common CF pathogens *P. aeruginosa* and *B. cepacia*, is reduced (609, 610). This effect was recapitulated in non-CF macrophages by inhibiting CFTR function with CFTR(inh)-172 suggesting a causative role for CFTR dysfunction (430, 609, 611). Tarique *et al* similarly demonstrated decreased phagocytosis in M1 and M2 polarized PBMC-derived macrophages from CF donors, which was recapitulated in non-CF macrophages with CFTR(inh)-172 (430). In contrast another study only saw a reduction in intracellular killing of *P. aeruginosa* but no difference in initial phagocytosis (604). Similar studies in murine bone marrow-derived macrophages from CFTR knockout (KO) mice or mice homozygous for F508del mutations have also demonstrated a reduced ability to phagocytose and kill intracellular bacteria in the absence of functional CFTR (601, 603, 612). This growing body of data suggests that intrinsic defects in AMs may permit pathogen survival and chronic infections in the CF lung. However, there is no consensus as to the mechanism underlying this defect.

Di *et al* published a paper in 2006 claiming that AMs from CF patients have a decreased ability to clear phagocytized bacteria due directly to the absence of the CFTR protein (601). This study found that lysosomes failed to acidify in AMs from CF patients, and healthy volunteers treated with a CFTR inhibitor, resulting in intracellular bacterial survival. This paper sparked controversy as using alternative methods to measure acidification of lysosomal compartments, other papers found no difference in lysosomal, or phagolysosomal macrophage acidification between wildtype, CFTR KO and CFTRF508del murine AMs (613, 614). Alternatively, CFTR dysfunction has been proposed to reduce phagocytosis by increasing oxidative stress. One study of primary AMs from neonatal CFTR knockout mice rescued impaired phagocytosis of *S. aureus* by the addition of antioxidant glutathione which is decreased in the CF lung (615).

Defects in autophagy have also been linked to impaired bacterial killing. PBMC-derived macrophages from F508del patients were shown to have defective autophagy and one study demonstrated that stimulating autophagy with IFN- γ , rescued bacterial clearance in these cells (616). A study by Abdurahman *et al* in 2011 demonstrated that in bone marrow-derived macrophages (BMDMs) from F508del mice, phagosomes containing *B. cepacia* did not acquire autophagosome markers and as a result did not fuse with lysosomes, leading to increased intracellular bacterial survival (617). In a follow up study, they demonstrated that the essential autophagy molecule Beclin-1 is sequestered in F508del BMDMs by p62 aggregates which

prevents efficiency autophagosome formation, but that depletion of p62 not only rescued autophagosome formation, but also phagocytosis and clearance of *B. cepacia* (618). Interestingly, Gauthier *et al* demonstrated that while BMDMs from CFTR knock out mice and mice homozygous for the F508del mutation both exhibit reduced phagocytosis and bacterial clearance, this could be rescued in the F508 mice but not CFTR knockouts, by cysteamine mediated re-establishment of the autophagic pathway (615). As the addition of CFTR(inh)-172 reduced the therapeutic effect, it suggests that autophagy mediated rescue of bacterial clearance relies on the presence of reusable misfolded protein.

6.1.2.2 CFTR Expression in AMs

The argument for a cell intrinsic basis is supported by endogenous CFTR expression on macrophage plasma membranes and their internal vesicles (601). Flow cytometry analysis was able to detect expression on plasma membranes of human monocytes and lymphocytes, which was then used to distinguish between cells from non-CF healthy controls and CF patients (619). CFTR expression has also been confirmed via immunoblotting and immunofluorescent staining of human peripheral blood mononuclear cells and monocytes from healthy controls (123, 604), and was shown to be reduced on CF patient monocytes (123). Furthermore, the functionality of CFTR on human monocyte-derived macrophages has been demonstrated through whole-cell patch clamp assays and fluorescence-based chloride ion efflux measurements on CF and non-CF human macrophages (123, 604). Here only macrophages derived from healthy individuals were sensitive to inhibition by CFTR(inh)-172. These findings were similarly reported in studies using monocyte derived macrophages from various animal models (123, 604, 620, 621).

Evidence of AM expression in humans is less conclusive. Di *et al* reported plasma membrane localization of CFTR through immunoblotting and RT-PCR in human and murine AMs, though immunofluorescent staining reported expression in phagosomes (601). Similarly, another study reported CFTR expression in lysosomal membranes (603). In contrast, others failed to detect conclusive levels of CFTR mRNA in cultured primary human and murine AMs (435, 602).

6.1.3 Treatments for CF Lung Disease

6.1.3.1 Current Treatments

Patient care and improved understanding of pathophysiology has substantially improved patient outcomes since CF was first described. Treatments have historically largely targeted symptoms rather than the underlying CFTR dysfunction, focusing on controlling chronic infections, reducing airway inflammation, and improving mucus clearance and consistency.

Over the past decade the breakthrough of small-molecule drugs has shown substantial clinical success and efficacy in CF by modulating the CFTR protein (622). Delivered orally, these drugs are generally either CFTR potentiators or correctors which respectively increase CFTR channel conductance or improve membrane trafficking. These modulators, as they are known, are not suitable for all patients, as they are mutation specific. According to age and genotype current estimates say approximately 80% of CFTR patients could benefit from these treatments (580). Clinical trials have evidenced substantial increases in lung function as measured by FEV1 following treatment resulting in clinical approval. In patients with the class III gating mutation G551D, a potentiator ivacaftor led to upwards of a 10% gain in FEV1 in phase 3 clinical trials (623). Ivacaftor in combination with the corrector lumacaftor has also been approved, though limited FEV1 improvements were observed in patients with a homozygous F508del mutation (class II)(624-626). More recently, a combination of elexacaftor/tezacaftor/ivacaftor has shown impressive 15% improvements in FEV1 and has been approved by the FDA and EMA for patients with at least one F508del mutation (627, 628). These drugs represent a significant breakthrough in treatment for CF patients, but are only effective in patients with class II, III and IV mutations. Though, as these agents can show significant drug to drug interactions they are not always well tolerated, and a recent retrospective study demonstrated that out of 845 CF patients receiving a lumacaftor/ivacaftor, 22.8% halted treatment permanently or temporarily largely due to adverse respiratory events (629).

6.1.3.2 Gene Therapy for CF Lung Disease

Gene therapy could meet the unmet needs of patients for whom modulator therapy is not an option, and also has the potential to provide a universally corrective therapy to all CF patients as it is not mutation specific. As CF is a monogenic, loss-of-function, autosomal recessive

disease, it is an attractive target for gene therapy aiming to express functional CFTR protein in the lung airway epithelium. Indeed, four years after the cloning of the CFTR gene in 1989 the first clinical trial was conducted in CF patients with an adenoviral (AdV) based vector (630). In the following years, clinical trials strongly established proof-of-principle for CFTR gene transfer into human airways with non-viral, adenovirus (AdV), adeno-associated virus (AAV), and lentivirus gene transfer vectors (163).

The first clinical trials used AdV vectors, which are non-integrating viruses with a linear and double-stranded DNA genome and a natural tropism for the lungs. However, they have largely fallen out of favour for CF gene therapy due to their comparatively inefficient and transient gene transfer, and induction of immune responses that limit repeat administration (631, 632). These issues can be circumvented using non-viral gene transfer agents, which are engineered complexes or nanoparticles made of materials such as cationic lipids, peptides, and synthetic polymers that are generally less efficient than viral vectors but are less likely to trigger an immune response (633). In 2015 the first phase IIb clinical trial was published by the UK Respiratory Gene Therapy Consortium, it was a multi dose, double-blinded, placebo-controlled study that used a cationic lipid formulation (GL67A) with a codon-optimised CpG free plasmid (pGM169)(516). Here the treatment was well tolerated and more significantly the therapy stabilized the decline of lung function in CF patients, though the effect was moderate. This conservative but promising effect has led to the continued development of the product, and research into other viral vectors for improved clinical efficacy.

AAV vectors are non-pathogenic single stranded DNA viruses with a limited packaging capacity of 4.8 kb and engineering of these viruses has resulted in almost entirely nonintegrating vectors that, for the most part, remain episomal (634). They are less immunogenic than AdV vectors and until recently had an established safety record in clinical trials (252, 253). AAV vectors proved able to efficiently transduce airway epithelium cells *in vitro* and *in vivo* in animal models including in non-human primate models but did not demonstrate efficacy in clinical trials (635-637). In an initial randomized, double-blind, placebo-controlled phase II trial, AAV serotype 2 mediated delivery of wildtype CFTR showed promising improvements in FEV1 after a single dose, but the effects were transient and not maintained following repeat dosing (638). A subsequent phase IIb large multidose trial then did not meet its efficacy endpoint as there was no evidence of improved FEV1 (639). These trials failed largely owing to weak transgene promoters necessitated by vector packaging limits, the development of neutralizing

antibodies that limit repeat dosing, and inefficient transduction of the apical airway epithelial membrane (637, 640). Research into improving these vectors is ongoing and includes the use of CFTR minigenes and improved viral capsid engineering (641, 642). Furthermore, the safety profile of AAV vector administration, particularly at high doses, is under scrutiny. A first-in-human study of intravenously administered AT132, where a recombinant AAV8 vector is used to deliver a myotubulin gene to patients with X-linked Myotubular myopathy (XLMTM), has been put on hold for a second time following the death of a fourth child in September of 2021. The pathomechanism for this fourth patient who received a 1.3×10^{14} vg/kg dose has not yet been characterised. However, three previous patients who died in 2020 due to complications resulting from liver failure after receiving a dose of 3×10^{14} vg/kg are thought to have had an abnormal predilection to cholestasis that was exacerbated by the gene therapy (643). These deaths have ignited a discussion around safety concerns associated with dosing of gene therapies, especially at high doses (644). A more recent death in a phase I/II clinical trial delivering a mini-dystrophin gene using recombinant AAV9 vector to patients with Duchenne's muscular dystrophy at a dose of 2×10^{14} vg/kg in December of 2021 has further added to the emerging safety concerns surrounding AAV vector administration, including liver and immune toxicity as previous adverse events had been noted in this trial resulting from an immune response to the vector (645, 646).

Several groups are now investigating lentiviruses for pulmonary gene transfer, as they integrate into host genomes and transduce both dividing and non-dividing cells. The proprietary lentivirus developed by the UK Cystic Fibrosis Gene Therapy Consortium, SIV.F/HN, and used here for *ex vivo* macrophage gene-transfer, transduces airway epithelial cells efficiently *in vivo* (previous described in Chapter 2.1). Pre-clinical studies administering a single dose achieved sustained gene expression in the airway epithelium for the lifetime of the mouse, and repeat administration was possible without a loss of efficacy (268, 277, 647). To facilitate a first-in-man clinical trial delivering rSIV.FN with a CFTR construct to CF patients, the Consortium has formed a collaboration with the pharmaceutical company Boehringer Ingelheim and the biotechnology company Oxford BioMedica.

6.1.3.3 *Treating Macrophages in CF Lung Disease*

Though modulators have been shown to increase macrophage function *in vivo* no treatments specifically target AMs or immune cells in the CF lung (648-650). It has been suggested that

autologous cell strategies in the lung may provide immunological support to patients who are ineligible to receive CF modulators, and pre-clinical studies support the use of both macrophage and mesenchymal stem cell (MSC) strategies (435, 607). A recent comprehensive study by van Heeckeren *et al* nicely demonstrates how such an approach could minimize pulmonary damage caused by chronic infections and active immune responses (651). Bone marrow transplant studies from WT mice into CF mice improved survival and inflammation, while the opposite was true when transplanting CF bone marrow into WT mice. In a myeloid specific CFTR KO mouse model, animals demonstrated increased mortality and inefficient resolution of *P. aeruginosa* infection, while in CFTR KO mice, myeloid-specific correction of CFTR expression improved immune responses upon bacterial challenge. They then demonstrated proof-of-concept for pulmonary cell therapy, as transplantation of 1e6 WT BMDMs into CF mice previously infected with *P. aeruginosa* reduced white blood cell counts, and improved lung pathology and management of infection.

These findings further implicate intrinsic macrophage defects caused by CFTR loss-of-function in CF lung pathology. It highlights how the clinical efficacy of gene therapies that limit gene transfer to the lung epithelium may be reduced by not addressing these defects. In contrast, a study in G551D mice with specific epithelium expression of CFTR was sufficient to correct normal inflammatory responses and *P. aeruginosa* clearance (652). As the rSIV.F/HN lentivirus is highly efficient at transducing the airway epithelium but has demonstrated no evidence of transducing AMs *in vivo* it may benefit from, or even require, an adjuvant *ex vivo* cell therapy treatment to see improvements in CF lung function and disease phenotypes (302). The effect of CFTR transgene delivery in CF mice using rSIV.F/HN has not been tested and therefore, the subsequent effects on macrophage function are unknown.

6.1.4 Aims and Hypothesis

The aim of the work presented here is to determine if intrinsic defects in macrophage function owing to a loss of CFTR expression can be resolved by rSIV.F/HN gene transfer and provide proof-of-concept that *ex vivo* macrophage-based cell therapy could be useful in treating CF lung disease. Here I tested differences in bactericidal activity between BMDMs from CFTR KO mice and WT mice, hypothesising that if differences are intrinsic and not caused by lung conditioning, they should be observed in these primary macrophages and demonstrate rescue by gene transfer of wildtype CFTR. Here I present initial work attempting to develop assays

that can discern differences in bacterial phagocytosis and killing of internalised bacteria by BMDMs from WT and CFTR KO mice.

6.2 Methods

6.2.1 *Pseudomonas aeruginosa*

A PAO1 lab strain of *P. aeruginosa* was donated by Ronan Murphy in the Jane Davies group at Imperial College London. Bacteria were grown on selective cetrimide agar plates (Cat. #PO1168A, ThermoFischer Scientific) or in overnight suspension cultures in 5 ml LB broth at 37°C and 225 rpm. Overnight cultures were pelleted by centrifugation at 3,000 *rcf* for 20 minutes. For sterility, all procedures using *P. aeruginosa* were performed using flame sterilization.

6.2.2 Minimum inhibitory concentration assays

Pelleted overnight cultures of *P. aeruginosa* were resuspended in RPMI + 10% FCS media or LB broth according to the experimental design and diluted to two times the experimental optical density, where an OD of 0.5 equates to 1e8 colony forming units (CFU)/ml. The antibiotic was serially diluted to twice the working concentration and then plated in a 1:2 dilution with media (positive control) or the correct antibiotic dilution in a Nunc™ MicroWell™ 96-Well Microplate (ThermoFisher Scientific). The plate was placed in a FLOUstar® Omega plate reader (ThermoFischer Scientific) where it was incubated at 37°C with orbital shaking. Absorbance measures were automatically taken every 15 minutes at 600nm, for a minimum of 4 hours. The antibiotics used here were gentamicin (Cat. # 15710064, ThermoFisher Scientific) and colistin sulphate (Cat. #C4461, Sigma Aldrich).

6.2.3 Antibiotic protection assays

Specific detail about each experimental iteration is reported were relevant in the results in Chapter 6.3. Briefly, BMDMs were either passaged following differentiation and plated into a 24-well Nunc™ Non-Treated multidish at 100,000 cells/well or used directly after differentiation in the absence of passaging. Macrophages were gently washed three times with dPBS and *P. aeruginosa* suspended in RPMI + 10% FCS media was gently added to the

macrophages at an MOI of 50. To enable MOI calculation when using non-passaged cells, one representative well of BMDMs was first harvested and counted. Plates were incubated in a shaking incubator (225 rpm) at 37°C for the 30 minutes (unless otherwise stated), before washing wells three times with 500 µl of dPBS to remove extracellular bacteria. Either 32 µg/mL of gentamicin or colistin (Chapters 6.3.4 – 6.3.5) or 100 µg/ml of gentamicin (Chapters 6.3.6 – 6.3.9) diluted in RPMI media was added to each well. Cells were incubated at 37°C for 30 minutes or 1 hour to kill remaining extracellular bacteria and then washed four times with 500 µl of dPBS. Cells were then lysed with 300 µl of 0.1% Triton-X100 (Sigma Aldrich), pipetting up down multiple times to ensure lysis, at what is referred to time zero to measure phagocytosis. Cell lysate serially diluted three times, by 10-fold serial dilutions in dPBS. 10 µl of each dilution and neat lysate was then spread on a cetrimide agar plate and grown at room temperature overnight followed by 8 hours in a 37°C incubator. Colony forming units (CFU) were then counted to quantify the number of intracellular bacteria at the point of lysis.

To quantify killing of intracellular bacteria, other cells were not lysed after the antibiotic treatment at time zero, and instead kept in culture in RPMI + 10% FCS media for 1, 2 or 3 additional hours, at which point cells were washed 3 times in dPBS (1 less wash than time zero to account for the media change after antibiotic treatment) and lysed according to the same protocol followed at time zero. Where indicated, cells were incubated with an inhibitory concentration of 20ug/ml of gentamicin during this time period. Bacterial survival was then expressed as a median percentage change in bacterial number from time zero as an indicator of intracellular killing of bacteria by macrophages.

During optimisation a no-lysis controls were included where dPBS and not Triton-X100 was added to cells and then plated in serial dilutions. Additional controls also included a no antibiotic control where, following the initial co-culture, no antibiotic was added, and a no *P. aeruginosa* sterility control.

6.2.4 Vybrant™ phagocytosis assay

BMDM phagocytosis was also measured using the Vybrant™ Phagocytosis Assay Kit (Cat. #V6694, ThermoFischer Scientific) according to manufacturer's instructions. Briefly BMDMs plated in a Nunc™ MicroWell™ 96-Well Microplates (ThermoFisher Scientific) were incubated with 100 µl of green-fluorescent fluorescein-labelled *E. coli* BioParticles at MOI 300,

where for every cell, 300 bioparticles are added. The bioparticles were first resuspended in 500 µl of concentrated HBSS and 4.5 ml of ddH₂O and then diluted in RPMI + 10% FCS media. In lieu of sonication the suspension was vigorously vortexed and pipetted up and down for 2 minutes prior to dilution. An additional 100 µl of RPMI + 10% FCS media was added to each well for a final volume of 200 µl. If adding a phagocytosis effector this was diluted in this second 100 µl volume of media. Negative control wells received RPMI only and no *E. coli* bioparticles. Following a 2-hour incubation at 37°C and 5% CO₂, the bioparticle suspension was removed by pipetting and immediately 100 µl of trypan blue suspension was added to each well. Following a 1-minute incubation at room temperature, excess trypan blue was removed by pipetting and phagocytosis was quantified by measuring the intensity of fluorescence in each well using a FLUOstar® Omega microplate reader (480 nm excitation, 520 nm emission). Background signal was subtracted from control wells plated with *E. coli* bioparticles and trypan blue in the absence of BMDMs. Cells were then gently washed twice with 150 µl of pre-warmed RPMI + 10% FCS media. To normalise the fluorescent readings by the number of viable BMDMs present in each well, 100 µl of Invitrogen™ PrestoBlue™ Cell Viability Reagent (Cat. #A13261, ThermoFischer Scientific) diluted 10-fold in cRPMI media was added to each well. The PrestoReagent® is modified by the reducing environment in viable cells and becomes highly fluorescent. This was quantified following a 30- to 40-minute incubation at 37°C and 5% CO₂ using a FLUOstar Omega microplate reader (540-570 nm excitation, 5580-610 nm emission) and provided a reading of each sample's metabolic activity. The results of the phagocytosis assay were then divided by the results of the PrestoBlue® assay to calculate the phagocytic index, described as phagocytosis/metabolic activity.

Cytochalasin D (Cat. #PHZ1063, ThermoFischer Scientific) was used to inhibit phagocytosis and was added as an effector at the indicated concentration in the presence of the *E. coli* BioParticles. Cytochalasin D was solubilised in DMSO and diluted in RPMI + 10% FCS media to achieve the desired concentration. To investigate CFTR(inh)-172 (Cat. #C2992, Sigma-Aldrich) as an effector of phagocytosis, cells were treated 72 hours in advance, diluting the inhibitor in cRPMI, it was also added at the indicated concentration in the presence of the *E. coli* BioParticles during the phagocytosis assay. The effect of treatment was then calculated as the phagocytic index of treated cells as a percentage of untreated (positive) controls.

6.3 Results

6.3.1 Bone marrow derived macrophages from gut corrected CFTR knock-out mice are CD11b⁺, F4/80⁺

Gut corrected FABp-CF-knockout mice (CFTR KO) have been widely characterised as a model of CF (274). In these mice, regulation of human CFTR by the fatty acid-binding promoter (FABp)-leads to CFTR expression in the gut, but not the airways, to prevent the intestinal disease of the CF knockout mouse. In the literature BMDMs from these mice have been used to investigate the functional phenotypes of AM in CF, and studies have demonstrated a lack of functional CFTR in these cells through immunostaining (615) and whole-cell and single-channel voltage-clamp analyses of efflux function (601). I previously optimized a BMDM differentiation protocol wherein macrophages can be obtained from hematopoietic precursors in the bone marrow of C57BL/6J (wildtype) mice. Using this protocol, I demonstrated that BMDMs from CFTR KO mice (referred to as CF mice) give rise to similarly homogenous populations of macrophages. BMDMs from one mouse were differentiated in a 24-well plate and analysed independently by flow cytometry and reported $98.9 \pm 0.17\%$ (mean $\pm$ SEM) of cells in culture were F4/80⁺, CD11b⁺ cells (n=9 technical replicates) (Figure 61).

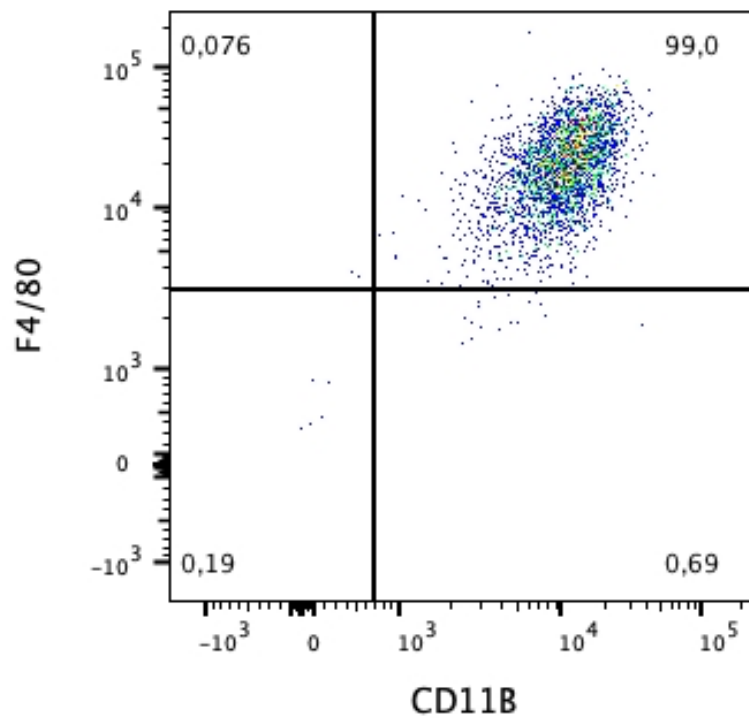


Figure 61 – Bone marrow derived macrophages (BMDMs) from CFTR KO have a characteristic macrophage phenotype. BMDMs from a CFTR KO mouse have a macrophage (CD11b⁺, F4/80⁺) phenotype following differentiation by the M-CSF induced protocol previously characterised in WT BMDMs as demonstrated here by a representative flow cytometry dot plot. Nine BMDM samples from one mouse were analysed and $98.9 \pm 0.17\%$ (mean $\pm$ SEM) cells in culture were CD11b⁺, F4/80⁺. Gates were drawn based off fluorescence minus one (FMO) controls. The percentage of each population is indicated in each quadrant. Captured by BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences).

6.3.2 Designing an antibiotic protection assay to quantify phagocytosis and killing of *Pseudomonas aeruginosa* by bone marrow derived macrophages.

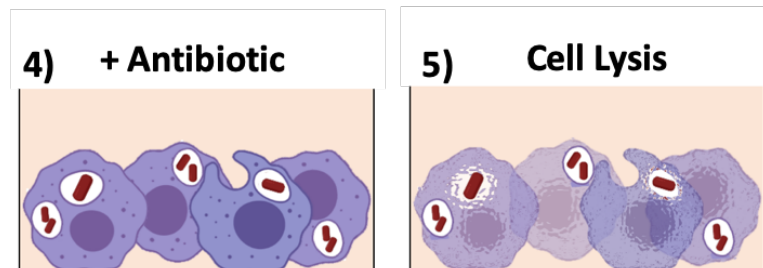
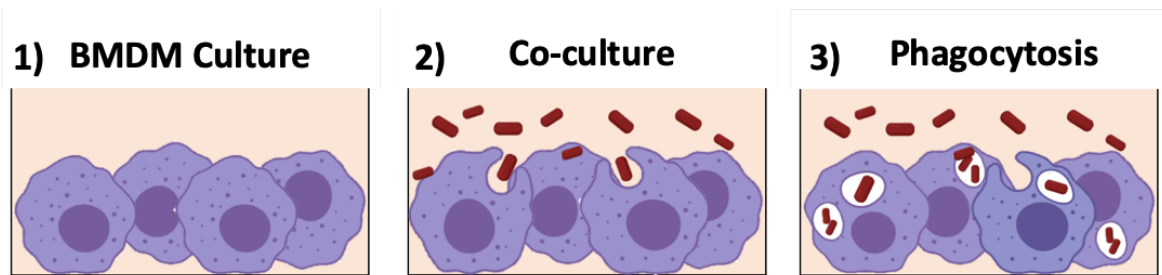
Aiming to determine whether there is a difference between the bactericidal capacity of macrophages from WT and CF mice an assay was designed which would quantify not only macrophage phagocytosis but also the fate of internalized bacteria. This was modelled on gentamicin protection assays, which are based on the premise that following co-culture of bacteria and macrophages, internalised bacteria are protected from gentamicin antibacterial activity (653).

In this antibiotic protection assay, bacteria and macrophages are first co-cultured to allow for phagocytosis to occur (Figure 62). Remaining extracellular bacteria are then washed off and remaining bacteria are killed by incubation with an antibiotic. Macrophages are lysed to release extracellular bacteria, which are quantified by plating serial dilutions of the lysis solution on selective agar. Following bacterial growth, the number of colony-forming units (CFU) per ml of each sample are calculated to provide a snapshot of how many live bacteria had to that point been phagocytosed. To gain an accurate image of phagocytosis this lysis step should occur at the earliest possible interval while still allowing sufficient time for phagocytosis and extracellular killing to occur.

In addition to measuring phagocytosis, this assay then tracks the fate of phagocytosed bacteria by keeping macrophages in culture after the antibiotic treatment and lysing them at regular intervals to measure the rate of intracellular bacterial killing. Successful removal of extracellular bacteria is therefore important to prevent continuous uptake of bacteria during this extended incubation time to ensure that only bacteria phagocytosed in the initial incubation period are being detected. Here I present extensive work attempting to optimise this assay to measure phagocytosis and killing of *Pseudomonas aeruginosa* by BMDMs. *P. aeruginosa* was chosen here as a clinically relevant pathogen to study, as it is found in 60% of adult CF patient's airways and infections are an indicator of advanced disease and predictor of patient morbidity and mortality (654).

In the literature similar antibiotic protection assays are widely used to characterize bacterial and macrophage interactions (655-659). A variety of bacterial species and macrophage cell

types are used, including immortalized cell lines and primary macrophages from various disease models in mice. There is no general consensus regarding the relative ratio of macrophages and bacteria (or MOI), the optimal incubation times or antibiotic killing times. Here the aim was to start with initial general values based off similar CF vs WT studies and then optimize these conditions for my specific purpose (660).



6) Quantification of Phagocytosed Bacteria

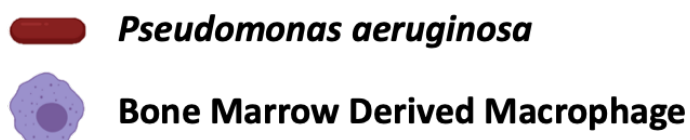
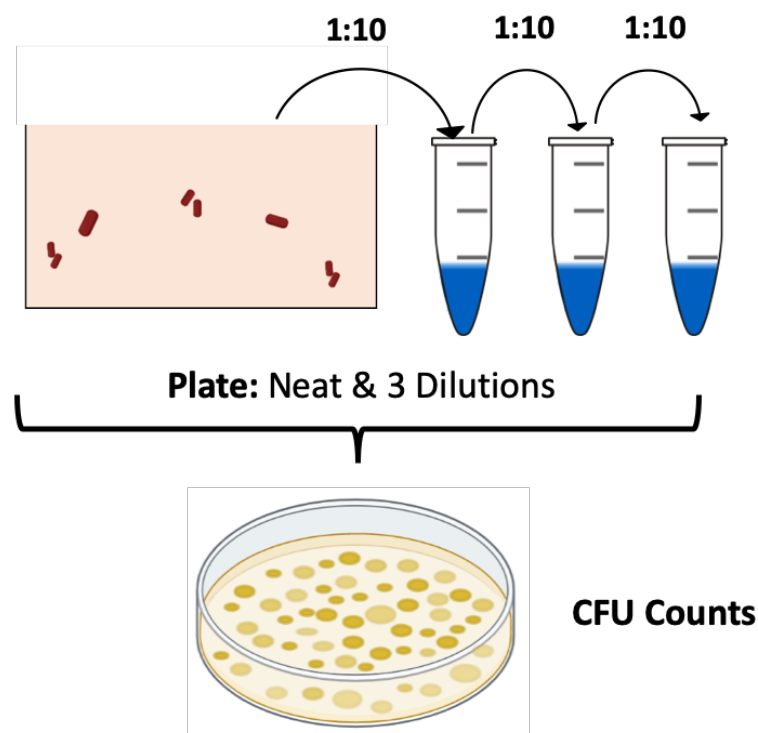


Figure 62: Schematic of an antibiotic protection assay to measure macrophage phagocytosis and clearance. Bone marrow derived macrophages (BMDMs) are co-cultured with bacteria, here *P. aeruginosa*, to allow phagocytosis to occur. Extra cellular bacteria are then washed off and an antibiotic is added to culture to kill remaining bacteria. As antibiotic entry into mammalian cells is minimal, intracellular bacteria can be quantified by lysing macrophages, and then plating the lysate in serial dilutions onto selective agar. Following overnight incubation, the number of phagocytosed bacteria can be quantified by counting the number of colony forming units (CFU). Figure produced by Nora Clarke, created with BioRender.

6.3.3 The minimum inhibitory concentration of gentamicin and colistin is 8 µg/ml

6.3.3.1 *Calculating the minimum inhibitory concentration of gentamicin*

The gentamicin protective assay requires efficient killing of extracellular bacteria before macrophage lysis to accurately determine the number of phagocytosed bacteria. Gentamicin is an aminoglycoside with effective bactericidal action against gram-negative bacteria, including *P. aeruginosa*. It works by binding the 16s rRNA at the 30s ribosomal subunit and disturbing mRNA translation (661). To determine the minimum inhibitory concentration (MIC) of gentamicin against *P. aeruginosa*, overnight cultures were grown with increasing antibiotic concentrations (32 µg/ml – 4096 µg/ml) in a FLUOstar® Omega Plate reader that measured cultures optical density every 15 minutes as a read out of bacterial growth. Two different starting optical densities (0.045 and 0.025) were tested which were chosen as they are candidate OD that would be used in the co-culture with BMDMs (n=2/condition). This assay as performed in RPMI media to replicate the experimental conditions under which bacteria would be growing in the gentamicin protection assay.

Over 4 hours and 15 minutes only minimal bacterial growth was observed at OD 0.045, after 2.5 hours with 32 µg/ml of gentamicin or positive (no antibiotic) controls. (Figure 63). These results suggest that within this time frame either RPMI media or the starting concentration of *P. aeruginosa* was insufficient to support growth and makes it difficult to determine effective concentrations of gentamicin.

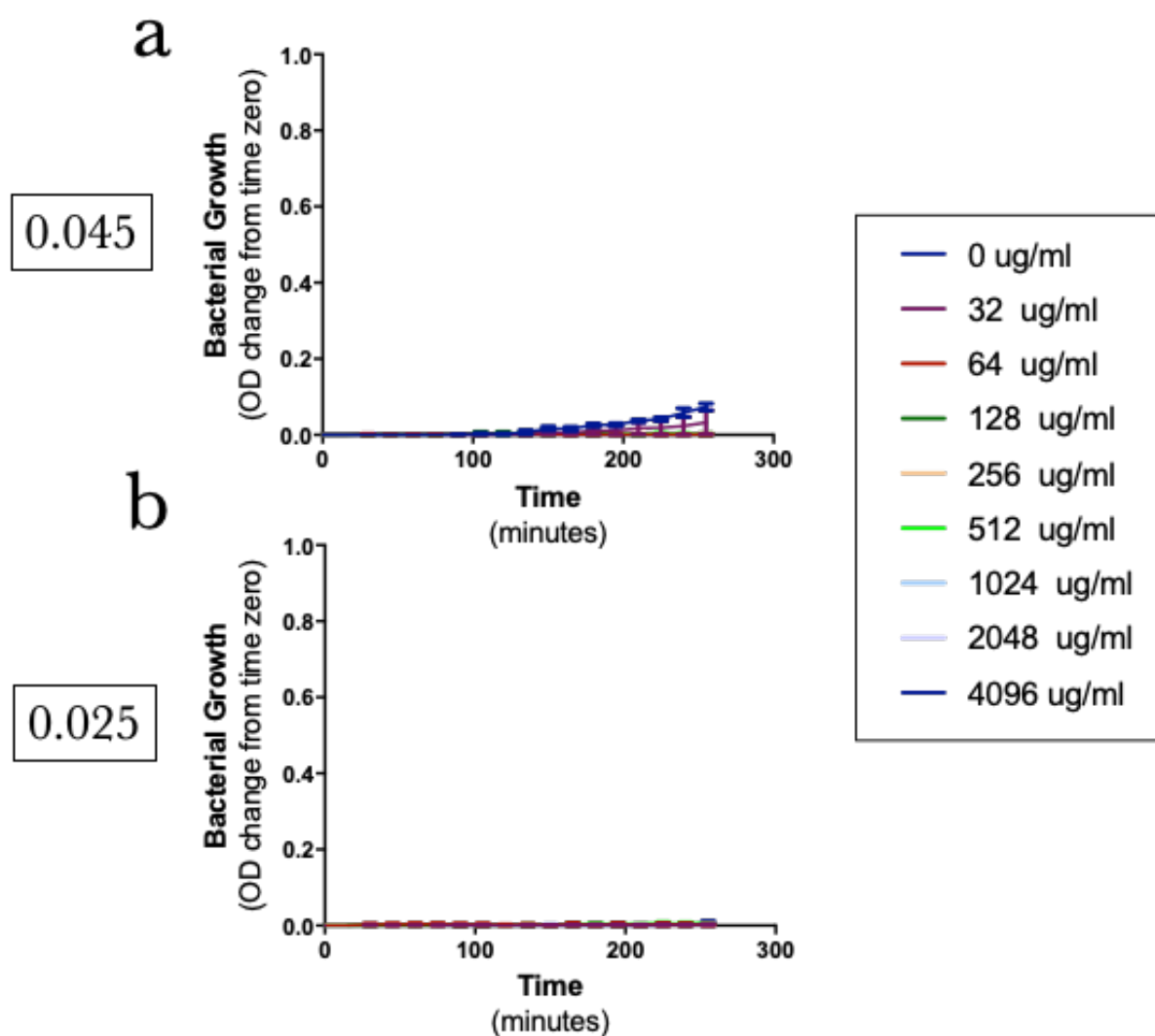


Figure 63 – Calculating the minimum inhibitory concentration of *Pseudomonas aeruginosa* at increasing concentrations of gentamicin in RPMI media. *P. aeruginosa* overnight cultures were re-suspended in RPMI 1640 medium and grown with increasing concentrations of gentamicin (32 $\mu\text{g}/\text{mL}$ - 4096 $\mu\text{g}/\text{mL}$) at final optical densities (ODs) of **a**) 0.025 and **b**) 0.045. Each condition was grown in duplicate. Positive controls grown in the absence of gentamicin (0 $\mu\text{g}/\text{mL}$). Suspensions were incubated at 37°C (225 rpm) for 4 hours and 15 minutes. OD measurements were taken every 15 minutes at 600nm. Duplicates of median $\pm$ range, are plotted as change in OD from time zero.

Therefore, the experiment was repeated at increasingly higher ODs (0.125, 0.06, and 0.03) in both RPMI and LB broth. Across all experimental conditions only LB controls with no gentamicin showed definitive growth, indicating that 8 µg/mL of gentamicin is sufficient to inhibit bacterial growth and that *P. aeruginosa* growth is not supported by RPMI media (Figure 64).

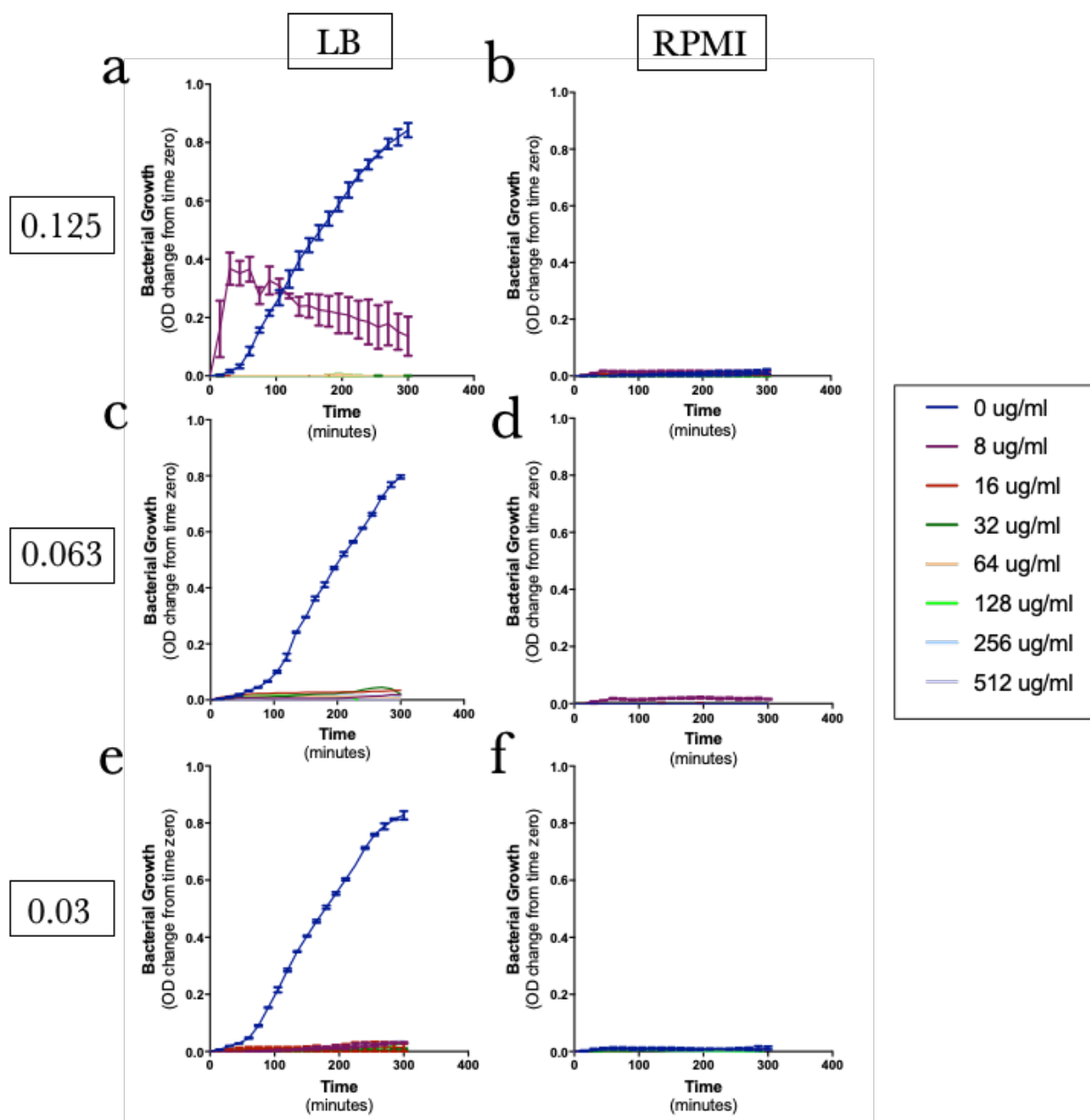


Figure 64 – Calculating the minimum inhibitory concentration of *Pseudomonas aeruginosa* at increasing concentrations of gentamicin in LB broth and RPMI media. *P. aeruginosa* overnight cultures were re-suspended in LB broth (a, c, e) or RPMI 1640 medium (b, d, e) and grown with increasing concentrations of gentamicin (8 $\mu\text{g}/\text{mL}$ - 512 $\mu\text{g}/\text{mL}$) at final optical densities (ODs) of a,b) 0.125, c,d) 0.063, and e,f) 0.03. Each condition was grown in duplicate. Positive controls grown in the absence of gentamicin (0 $\mu\text{g}/\text{mL}$). Suspensions were incubated at 37°C (225 rpm) for 5 hours and 15 minutes. OD measurements were taken every 15 minutes at 600nm. Duplicates of median $\pm$ range, are plotted as change in OD from time zero.

6.3.3.2 Calculating the minimum inhibitory concentration of colistin

Many papers investigating CF macrophages used gentamicin to clear extracellular bacteria, as gentamicin is a fast-acting antibiotic with low toxicity to mammalian cells (657). However, gentamicin shows reduced activity against non-dividing bacteria (656, 662). As previous results demonstrated that *P. aeruginosa* does not grow well in RPMI media this led to the hypothesis that gentamicin may not be an appropriate antibiotic to kill bacteria during co-culture with macrophages. Therefore, the polymyxin antibiotic colistin was also tested which is active against gram-negative bacteria and is used as a last resort in the clinic to treat *P. aeruginosa* infections (663). It exhibits bactericidal action through the disruption of the bacterial cell membrane via a detergent-like mechanism and its activity is not dependent on metabolic activity (664, 665). I reasoned therefore, that colistin should be more efficient at killing extracellular bacteria not actively dividing in RPMI media. The MIC of colistin against *P. aeruginosa* was tested under the same experimental conditions as previously used with gentamicin, where increasing concentrations of antibiotic (8 µg/ml – 512 µg/ml) were cultured with *P. aeruginosa* at increasing ODs (0.125, 0.06, and 0.03) in both RPMI and LB broth.

Growth was observed at an OD of 0.03 when grown in RPMI media at colistin concentrations of 8, 16, and 32 µg/mL (Figure 64). As positive controls did not grow and there was no growth at higher ODs, this is most likely due to contamination or incorrect bacterial plating in the experimental set up. At all ODs LB controls only showed growth in positive controls and in the presence of 8 µg/mL of colistin, suggesting a MIC of 16 µg/ml.

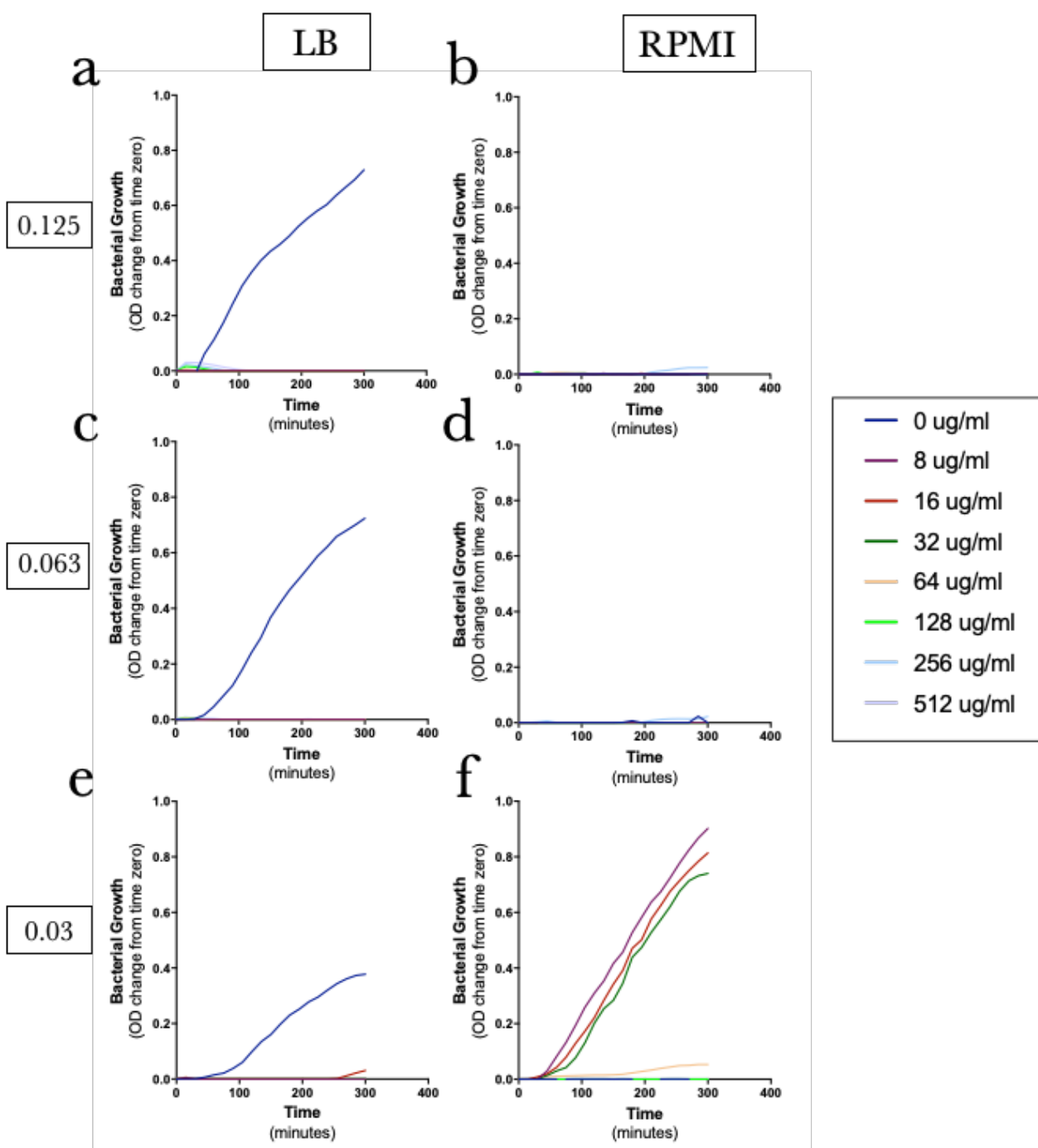


Figure 64 – Calculating the minimum inhibitory concentration of *Pseudomonas aeruginosa* at increasing concentrations of colistin in LB broth and RPMI media. *P. aeruginosa* overnight cultures were re-suspended in LB broth (**a**, **c**, **e**) or RPMI 1640 medium (**b**, **d**, **e**) and grown with increasing concentrations of colistin (8 µg/mL - 512 µg/mL) at final optical densities (ODs) of (**a**,**b**) 0.125, (**c**,**d**) 0.063, and (**e**,**f**) 0.03. Each condition was grown in duplicate. Positive controls grown in the absence of colistin (0 µg/ml). Suspensions were incubated at 37°C (225 rpm) for 5 hours and 15 minutes. Single OD measurements for each condition were taken every 15 minutes at 600nm.

6.3.4 Colistin is more effective against *Pseudomonas aeruginosa* than gentamicin

To determine if the macrophage bacterial killing assay could provide accurate measures of macrophage phagocytosis and killing of internalized bacteria, the assay was tested using WT BMDMs. In initial optimization experiments I included the controls where no antibiotic was used to measure the effect of extracellular antibiotic killing, others where no bacteria were added to macrophages as a sterility control, and thirdly where macrophages were not lysed to determine the amount of remaining extracellular bacteria at each time point. In these controls dPBS was added to macrophages instead of 0.1% Triton-X100 and serial dilutions (1:10) of the dPBS were plated instead.

In the first experiment I compared the efficacy of gentamicin and colistin antibiotics, which were used at a concentration of 32 µg/mL for 30 minutes as at this concentration both antibiotics inhibited bacterial growth. I used an MOI of 50, where for every macrophage in culture, there were 50 *P. aeruginosa* colony forming units (CFU). A 30-minute time point was chosen on the basis of similar assays in the literature, and as in MIC experiments, 30 minutes was when bacterial growth was first seen in LB positive controls. I also compared two different initial bacterial co-culture times of 15 and 30 minutes. Phagocytosis was measured at ‘time zero’ which occurred immediately after the 30-minute antibiotic treatment. A second time point of 2 hours was used in these initial tests to determine if an effect of intracellular killing could be observed. A schematic representation of this experiment can be seen in Figure 65.

Unfortunately, in initial comparisons when agar plates were incubated for 24 hours at 37°C, *P. aeruginosa* colonies had grown to such an extent that CFU counts, and subsequent quantitative analysis were not possible. Crude comparisons could be made however, and as colistin ‘No-Lysis’ plates only grew bacterial colonies when undiluted, while gentamicin equivalents grew bacteria in all dilutions (1:10 - 1:1000), it appears colistin is the more efficient treatment to eliminate extracellular bacteria. In subsequent experiments I therefore decided to focus on colistin as the antibiotic used. Additionally, the *P. aeruginosa* agar plates were grown overnight at room temperature and then incubated at 37°C under close observation to ensure colonies would not become over-grown.

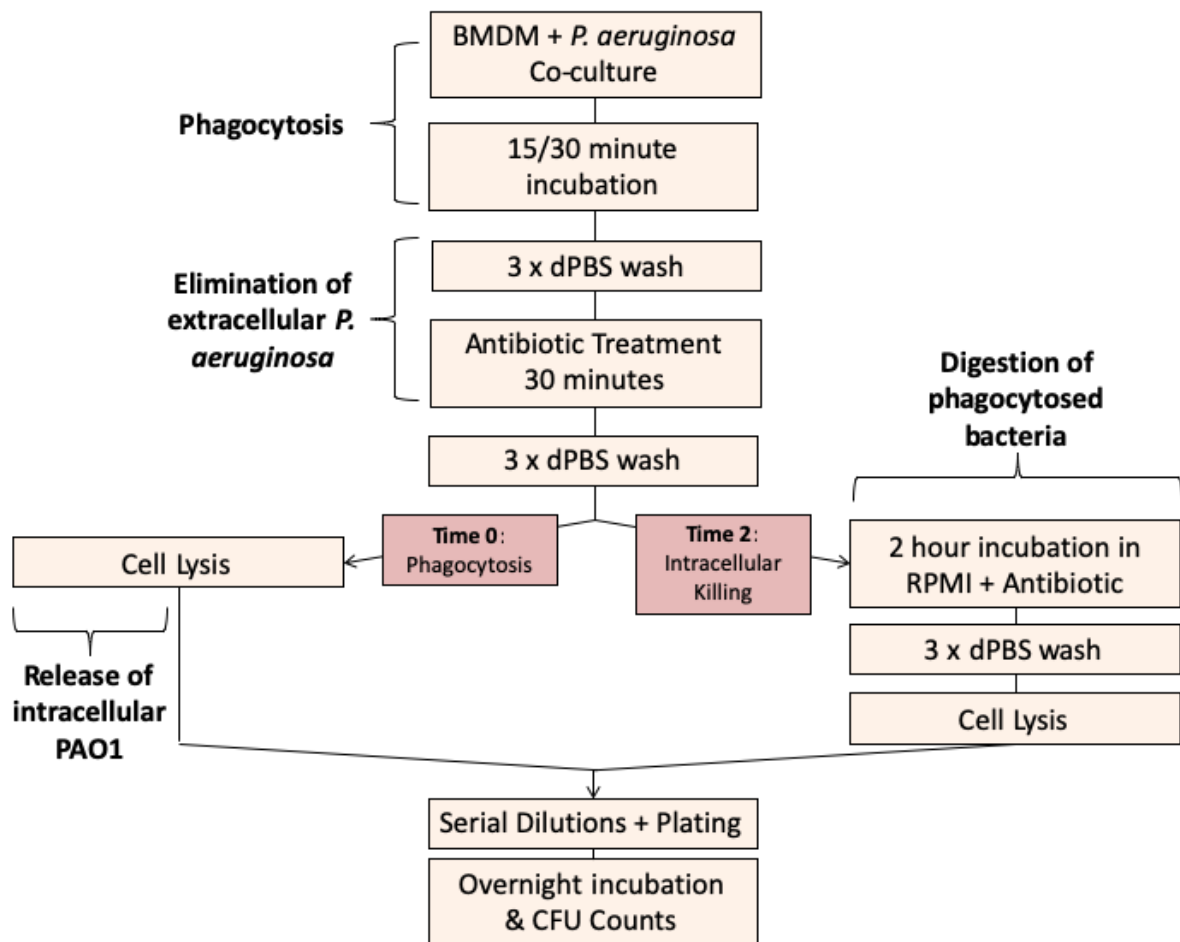


Figure 65: Schematic of the macrophage phagocytosis and clearance assay. Either gentamicin or colistin was used as the antibiotic. All incubation stages occurred at 37°C. Cells were either lysed at time zero to calculate phagocytosis, or further incubated to then measure the fate of phagocytosed bacteria over a 2-hour incubation.

6.3.5 Unsuccessful development of a colistin protection assay

6.3.5.1 *Reduced bacterial growth enables quantification of Pseudomonas aeruginosa*

As the assay is highly time consuming it also brought worries that with multiple conditions, timings would be inaccurate between different samples. Therefore, the assay was further simplified, and all samples used a 30-minute bacteria and macrophage co-culture time period. The aim of this experiment was to determine if across test conditions and controls, the correct pattern one would expect if the assay was accurately measuring phagocytosis and intracellular killing was observed. Again, an MOI of 50 and a 30-minute colistin treatment (32 µg/ml) was used, where bacteria CFU counts were calculated at 'time zero' and 2 hours later.

Upon repetition of the assay CFU counts were possible, however, in test conditions (with antibiotic, bacteria and lysed macrophages) CFU counts did not differ over time indicating intracellular killing was not observed (Figure 66):

- *Time Zero*: live bacteria 53 (range 26.7 – 146.7) CFU/ml
- *2 Hours*: live bacteria 185 (range 65 – 202) CFU/ml (p = ns, 2-wayANOVA, Sidak's multiple comparisons)

Additionally, remaining live bacteria in culture at both time points were lower than expected suggesting that either minimal phagocytosis had occurred, or the majority of intracellular bacterial killing had already occurred by time zero.

However, in comparison to lysed (test) samples, non-lysed controls which should only measure remaining extracellular bacteria, reported higher or similar CFU counts than test conditions at each time point:

- *Time Zero*: live bacteria 1.3e3 (range 983 – 3.1e3) CFU/ml (p<0.0001)
- *2 Hours*: live bacteria 83 (range 0 – 121.7) CFU/ml (p = ns, 2-wayANOVA, Sidak's multiple comparisons)

This is contrary to what would be expected as non lysed controls only measure remaining extracellular bacteria in comparison to lysed samples which should include all intracellular and extracellular bacteria. This suggests that either phagocytosis had not occurred or that the assay was unable to quantify it. As 0.1% Triton-X100 is commonly used across all gentamicin protection assays, and at the concentration used here is sufficient to selectively disrupt

mammalian cell membranes, it seems unlikely that this is an effect of the detergent on bacterial survival.

Here the assay was performed on macrophages that following differentiation, had been harvested on day 7 and seeded onto 24-well plates. As they had been recently passaged, and the protection assay requires numerous washing steps, I hypothesised that the reason for failure may have been due to macrophage loss during the assay.

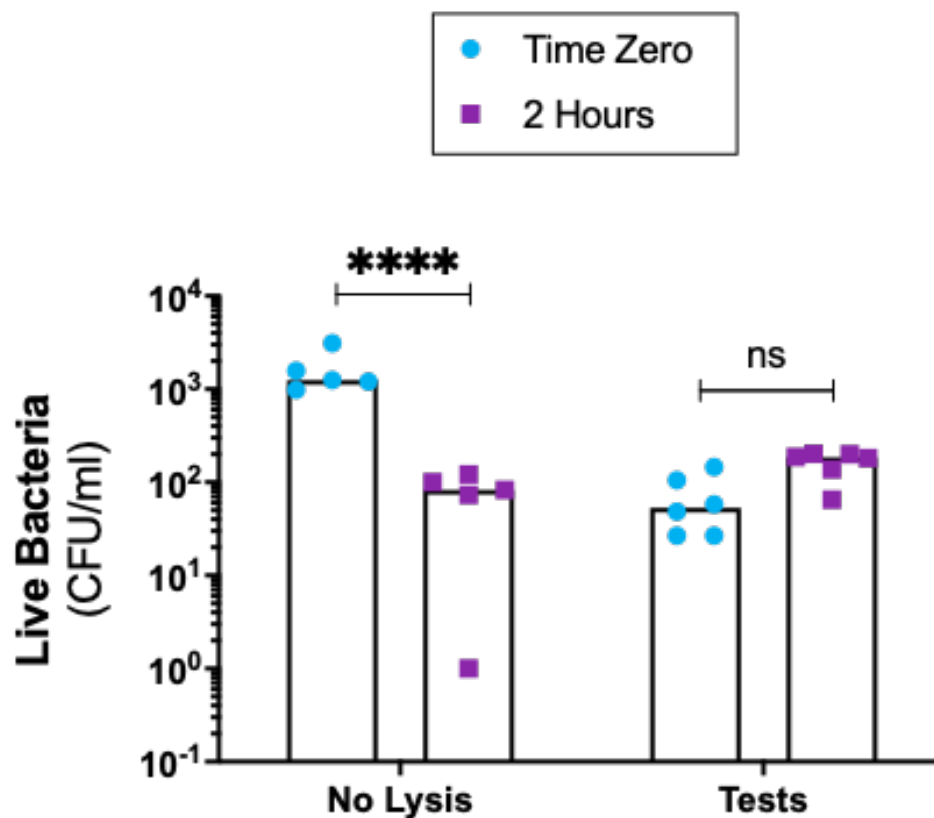


Figure 66 – Initial characterization of a colistin protection assay did not measure *P. aeruginosa* phagocytosis and bacterial killing by bone marrow derived macrophages (BMDMs). Comparison between the number of remaining intracellular bacteria present at time zero (phagocytosis) or following an additional 2 hours in culture in test samples where wildtype BMDMs were lysed with 0.1% Triton-X100 (n=6), and no lysis controls where cells were instead incubated with dPBS (n=5). Lysate and dPBS were plated in serial dilutions onto selective agar to quantify the number of remaining intracellular bacteria present at each time point as colony forming units per ml of lysate (CFU/ml). Here BMDMs were co-cultured with *P. aeruginosa* for 30 minutes at MOI 50 and extracellular

bacteria were then washed off and killed by colistin treatment (32 µg/ml) for half an hour. Bars represent median. ns = $p > 0.05$, ****= $p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

6.3.5.2 Increasing macrophage incubation time after passaging does not improve colistin protection assay outcomes

To determine if macrophage loss had resulted in a failure to quantify a phagocytic or bactericidal effect of macrophage co-culture on *P. aeruginosa*, I repeated the assay on cells seeded, 24, 48, or 72 hours prior to see if with increased time in culture, adherence would improve and rescue the ability to observe bacterial phagocytosis and killing. Though one of the limitations of this study is that adherence and macrophage viability between time points was not quantified.

Across experiments and all-time points, the expected pattern was not observed. After 24 hours, samples lysed after 2 hours contained more live bacteria than those at time zero (though due to low n numbers this difference was not significant) suggestive of bacterial growth over time rather than killing of intracellular bacteria (Figure 67a):

- *Time Zero*: live bacteria 83 (range 33.3 – 400) CFU/ml
- *2 Hours*: live bacteria 2.9e4 (range 65 – 202) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

This experiment was performed under the same protocol as the previous experiment and at time zero the number of live bacteria was comparable, with only low numbers of live bacteria detected reinforcing the idea that macrophage loss is occurring. However, in comparison to non-lysed controls, lysed (test) samples had lower or equivalent bacterial counts at time zero and 2 respectively:

- *Time Zero*: live bacteria 2.9e4 (range 1.1e4 – 6e4) CFU/ml ($p < 0.01$)
- *2 Hours*: live bacteria 2.9e4 (range 3.7e3– 3.3e4) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

This suggests that the bacteria measured at time 2 in lysed samples are largely remaining extracellular bacteria, and that the assay is therefore not providing an accurate measure of intracellular bacterial.

At 48 hours, there was no difference between time points in lysed controls, though consistently higher CFU counts were observed than in previous experiments (Figure 67b):

- *Time Zero*: live bacteria 1.4×10^4 (range $1 \times 10^4 - 3.3 \times 10^4$) CFU/ml
- *2 Hours*: live bacteria 3.7×10^4 (range $6.2 \times 10^3 - 5.3 \times 10^6$) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

As no difference was seen between these results and non-lysed controls at either time point it suggests that no phagocytosis or intracellular killing is occurring, or cannot be quantified due to high numbers of remaining live extracellular bacteria:

- *Time Zero*: live bacteria 3×10^4 (range $7 \times 10^3 - 6.7 \times 10^4$) CFU/ml ($p = \text{ns}$)
- *2 Hours*: live bacteria 1.1×10^5 (range $2 \times 10^4 - 1.7 \times 10^5$) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

Though no differences were observed, over time in both lysed and non-lysed samples, the number of surviving bacteria increased over time and is suggestive that perhaps a higher concentration of antibiotic is required.

At 72 hours, the same trend observed at 48 hours was seen where both lysed samples showed non-significant increases in bacterial number over time, indicating that over time the assay was not measuring bacterial killing and that bacteria may be surviving and proliferating (Figure 67c):

- *Time Zero*: live bacteria 3.5×10^3 (range $1 \times 10^3 - 9 \times 10^3$) CFU/ml
- *2 Hours*: live bacteria 5×10^4 (range $1 \times 10^4 - 4 \times 10^5$) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

This was also seen in non-lysed samples which reported corresponding numbers of live bacteria to lysed samples:

- *Time Zero*: live bacteria 1.5×10^4 (range $4 \times 10^3 - 2.5 \times 10^4$) CFU/ml ($p = \text{ns}$)
- *2 Hours*: live bacteria 4×10^4 (range $1 \times 10^4 - 6 \times 10^4$) CFU/ml ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

As previous data demonstrated that *P. aeruginosa* does not grow well in RPMI it was deemed unlikely that bacteria would be actively proliferating. Again, there was no difference between lysed and non-lysed controls within time points suggesting no measurable amount of phagocytosis had occurred.

Overall, these experiments did not indicate that the assay was successfully measuring macrophage phagocytosis or bacterial killing, regardless of the duration of macrophage culture. These findings coincided with initial transduction experiments in Chapter 3 which demonstrated that when passaging macrophages post-differentiation, cells were poorly adherent, and viability was low. This was rescued by avoiding cell passaging prior to testing and supports the theory that negligible levels of phagocytosis were occurring either as a result of cell loss caused by the number of washes performed in this assay and/or low cell viability of macrophages following passaging which could not be rescued by allowing macrophages more time in culture to adhere.

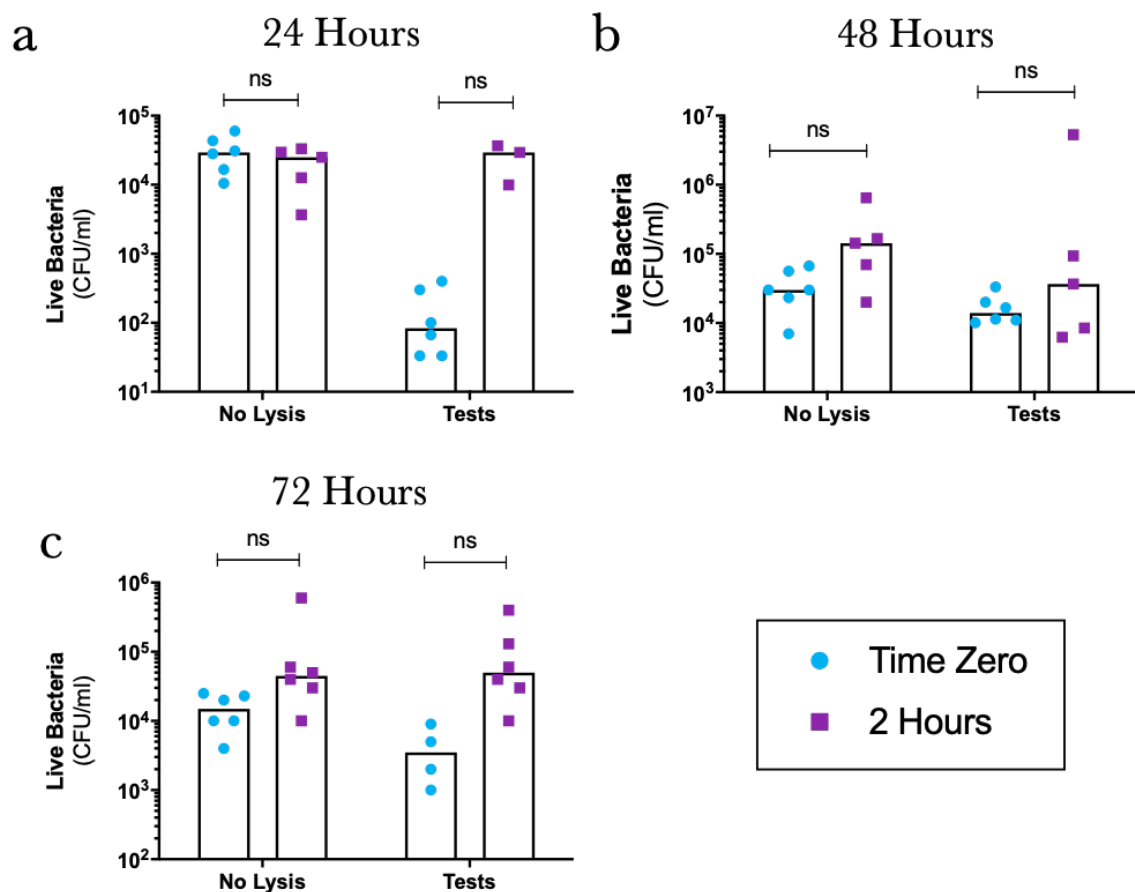


Figure 67 – Increasing bone marrow derived macrophages (BMDMs) time in culture prior to performing a colistin protection assay did not rescue quantification of *P. aeruginosa* phagocytosis and bacterial killing. Differentiated wildtype BMDMs were harvested and plated at 100,000 cells/well for (a) 24 hours, (b), 48 hours, or (c) 72 hours prior to performing a colistin protection assay to determine if at any time point the assay successfully quantified phagocytosis

or killing of intracellular *P. aeruginosa*. Comparison between the number of remaining intracellular bacteria present at time zero (phagocytosis) or following an additional 2 hours in culture in test samples where wildtype BMDMs were lysed with 0.1% Triton-X100 (n=6), and no lysis controls where cells were instead incubated with dPBS. In each condition an n=6 samples were tested, but due to error during processing or overgrowth, some samples were excluded. Lysate and dPBS were plated in serial dilutions onto selective agar to quantify the number of remaining intracellular bacteria present at each time point as colony forming units per ml of lysate (CFU/ml). Here BMDMs were co-cultured with *P. aeruginosa* for 30 minutes at MOI 50 and extracellular bacteria were then washed off and killed by colistin treatment (32 µg/ml) for half an hour. Bars represent median. ns = p>0.05, 2-wayANOVA, Sidak's multiple comparisons).

6.3.5.3 Using non-passaged, adherent BMDMs does not rescue inconsistent analysis of bacterial phagocytosis and killing

The assay was therefore repeated under the same conditions, but in BMDMs that had not undergone any passaging post-differentiation. One of the disadvantages of this approach is that there is more likely to be variation in macrophage cell numbers between wells. Visual monitoring of cells throughout the assay under an inverted light microscope confirmed that obvious macrophage loss was avoided throughout.

At the first attempt, the assay appeared to work as across treatment groups and controls the trends were suggestive of phagocytosis and killing of internalized bacteria (Figure 68a). Between time points the number of bacteria in lysed samples decreased over time indicative of intracellular killing:

- *Time Zero*: live bacteria 6.1e5 (range 2.3e5 – 8e5) CFU/ml
- *2 Hours*: live bacteria 1.1e5 (range 8.5e4 – 1.5e5) CFU/ml (p<0.0001, 2-wayANOVA, Sidak's multiple comparisons)

Additionally, non-lysed controls had lower numbers of live bacteria than lysed cells supporting the presence of bacteria within macrophages following a 30-minute co-culture, though at time 2 this was not significant:

- *Time Zero*: live bacteria 1.1e5 (range 2.3e4 – 1.8e5) CFU/ml (p<0.0001)
- *2 Hours*: live bacteria 2.9e4 (range 2.3e4 – 5.7e4) CFU/ml (p = ns, 2-wayANOVA, Sidak's multiple comparisons)

This suggested that failure to measure definitive phagocytosis or intracellular killing in previous attempts was due to low macrophage viability or adherence, and that active phagocytosis and intracellular killing occurs over a 2-hour time period.

However, upon two subsequent repeats I was unable to recapitulate these findings. In the first repeat under test conditions where cells were lysed, bacterial burdens increased dramatically over time (Figure 68b):

- *Time Zero*: live bacteria 675 (range 375 – 1350) CFU/ml
- *2 Hours*: live bacteria 8.9e4 (range 6.6e4 – 1.2e5) CFU/ml ($p < 0.0001$, 2-wayANOVA, Sidak's multiple comparisons)

Additionally, non-lysed cells showed significantly higher CFU counts than lysed cells:

- *Time Zero*: live bacteria 6.1e4 (range 4.5e3 – 1e5) CFU/ml ($p < 0.01$)
- *2 Hours*: live bacteria 3.2e4 (range 8.3e3 – 7.1e4) CFU/ml ($p < 0.01$, 2-wayANOVA, Sidak's multiple comparisons)

In the second repeat, most likely due to experimental error, no *P. aeruginosa* was quantified in lysed cells at time zero (Figure 68c). However, samples from the 2-hour time point showed similar levels of bacteria to non lysed controls:

Lysed:

- *2 Hours*: live bacteria 3.9e4 (range 2.9e4 – 4.1e4) CFU/ml

Non-lysed:

- *Time Zero*: live bacteria 2.9e4 (range 2.2e4 – 1.5e5) CFU/ml
- *2 Hours*: live bacteria 1.4e4 (range 86.2e3 – 2.3e4)

Despite the first experiment suggesting that with this protocol, phagocytosis and intracellular killing could be accurately measured over time, this finding was not recapitulated. The higher or equivalent numbers of live bacteria in non-lysed controls suggest that extracellular bacterial killing is insufficient, and that as a result these bacteria obscure specific quantification of intracellular bacteria.

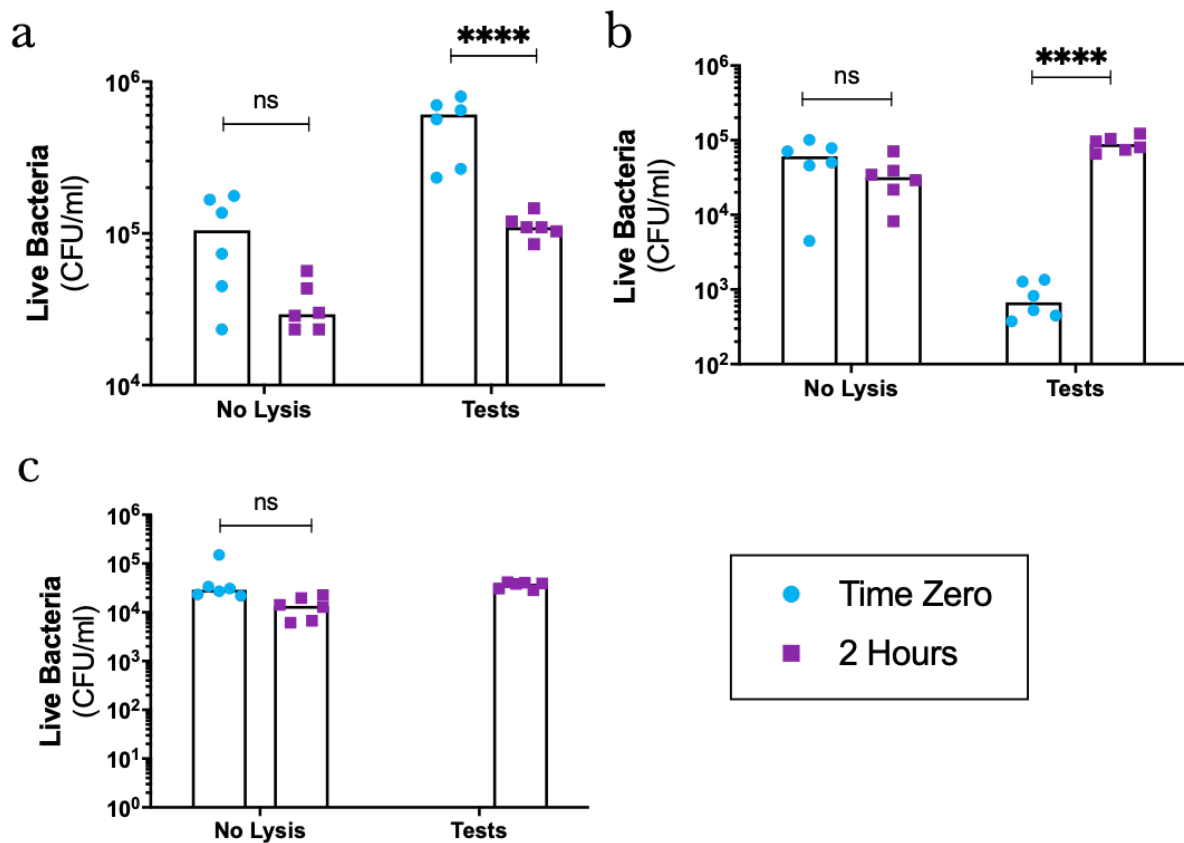


Figure 68 – A colistin protection assay does not consistently quantify of *P. aeruginosa* phagocytosis and bacterial killing by adherent bone marrow derived macrophages (BMDMs). Differentiated wildtype BMDMs were not passaged prior to performing a colistin protection assay to determine if at any time point the assay successfully quantified phagocytosis or killing of intracellular *P. aeruginosa*. Between separate experimental repeats (**a-c**) there was little consistency in bacterial numbers or trends between conditions. Comparisons were made between the number of remaining intracellular bacteria present at time zero (phagocytosis) or following an additional 2 hours in culture in test samples where wildtype BMDMs were lysed with 0.1% Triton-X100 (n=6), and no lysis controls where cells were instead incubated with dPBS. Lysate and dPBS were plated in serial dilutions onto selective agar to quantify the number of remaining intracellular bacteria present at each time point as colony forming units per ml of lysate (CFU/ml). In each condition an n=6 samples were tested, but due to error during processing or overgrowth, some samples were excluded. In the third experiment (**c**) no growth was observed in n=6 test samples at time zero likely due to error. Here BMDMs were co-cultured with *P. aeruginosa* for 30 minutes at MOI 50 and extracellular bacteria were then washed off and killed by colistin treatment (32 µg/ml) for half an hour. Bars represent median. ns = p>0.05, ****=p<0.0001, 2-wayANOVA, Sidak's multiple comparisons)

6.3.6 Successful quantification of bacterial phagocytosis and intracellular killing using a gentamicin protection assay

The colistin protection assay had largely failed to demonstrate a robust ability to quantify macrophage phagocytosis and intracellular killing. I hypothesised that this may be due to insufficient killing or inhibition of extracellular bacteria and may be rescued by higher antibiotic concentrations. As this point, I also reached out to Elli Mylona in the Gad Frankel lab at the MRC Centre for Molecular Bacteriology and Infection at Imperial College who has experience with the assay for advice. Rather than use a MIC of antibiotic, in her experiments a much higher dose of gentamicin (100-200 µg/ml) was used over a longer time period (1 hour instead of the half hour used here) supporting my hypothesis. Following this advice, I therefore attempted to characterise a protection assay, following the conditions of the established protocol used in the Frankel Lab.

The protocol was therefore adapted to use gentamicin instead of colistin. I increased the duration of the antibiotic incubation to 1 hour at an increased concentration of 100 µg/ml to ensure efficient killing. *P. aeruginosa* was still incubated with adherent BMDMs for half an hour at MOI 50. To measure the fate of internalised bacteria at greater detail, macrophages were lysed at hourly intervals for up to four hours following the antibiotic incubation, during which time cells were maintained in a low inhibitory concentration of gentamicin (20 µg/ml) (n=6/time point). This inhibitory antibiotic treatment was not included previously in colistin assays and may also explain why over-time no decrease in bacterial survival was observed, as the number of viable extracellular bacteria increased over time. Here I did not include no-lysis or no-antibiotic controls, instead, I relied on the trend over time. Importantly, the number of washes performed on each replicate was consistent across time points. To determine the degree of intracellular bacterial killing, I calculated the median number of surviving bacteria as a percentage of bacteria at time zero. With these adaptations, the assay reported a significant decrease in *P. aeruginosa* over a 3-hour window, suggestive of active bacterial killing by macrophages (Figure 69a):

- *Time 0*: median survival 100 % (range 31.6 – 189.5) CFU/ml
- *1 Hour*: median survival 19.5 % (range 12.6 – 31.6) CFU/ml (p<0.05)
- *2 Hours*: median survival 7.4 % (range 1.9 – 17.9) CFU/ml (p<0.05)
- *3 Hours*: median survival 0.8 % (range 0.3 – 1.8) CFU/ml (p< 0.0001, Kruskal-Wallis, Dunn's multiple comparisons)

This trend was confirmed over a second separate experimental repetition (Figure 69b):

- *Time 0*: median survival 100 % (range 58 – 113) CFU/ml
- *1 Hour*: median survival 23.2 % (range 17.4 – 29) CFU/ml ($p = \text{ns}$)
- *2 Hours*: median survival 7.7 % (range 6.7 – 10.4) CFU/ml ($p < 0.01$)
- *3 Hours*: median survival 3.2 % (range 2.5 – 14.1) CFU/ml ($p < 0.0001$, Kruskal-Wallis, Dunn's multiple comparisons)

Between the two experiments, there was no significant difference in bacterial survival at any time point suggesting it is a robust measure of the fate of phagocytosed *P. aeruginosa* in WT BMDMs:

- *1 hour*: median difference 3.72 % bacterial survival ($p = \text{ns}$)
- *2 hours*: median fold change 0.31 % bacterial survival ($p = \text{ns}$)
- *3 hours*: median fold change 2.34 % bacterial survival ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

Across both experiments, the majority of (~80%) bacterial killing occurred within the first hour, suggesting rapid bacterial killing. To confirm that this was not an effect of the inhibitory concentration of gentamicin on extracellular bacteria, I repeated the assay in the absence of this inhibitory antibiotic. A significant decrease in bacterial survival over time was still suggestive of intracellular killing (Figure 69c):

- *Time 0*: median survival 100 % (range 64.5 – 154.8) CFU/ml
- *1 Hour*: median survival 50 % (range 25.1 – 96.8) CFU/ml ($p = \text{ns}$)
- *2 Hours*: median survival 14.5 % (range 7.1 – 22.6) CFU/ml ($p < 0.05$)
- *3 Hours*: median survival 12.6 % (range 1.9 – 219.4) CFU/ml ($p = \text{ns}$, Kruskal-Wallis, Dunn's multiple comparisons)

As after 3 hours, some replicates showed indications of increased bacterial survival, these results support the use of an inhibitory concentration of gentamicin to reduce survival of remaining extracellular bacteria. In comparison to the previous experiments where gentamicin was added to culture after time zero, a less pronounced effect of bacterial killing was observed in the third experiment (Figure 69e). However, as a decrease in surviving bacteria was still seen over time it supports the hypothesis that this assay measures the fate of intracellular bacteria, rather than just seeing the effect of continuous killing of extracellular bacteria by the inhibitory concentration of gentamicin.

Between experiments, the number of phagocytosed bacteria measured at time zero was not consistent (Figure 69d) (Note that the only difference in protocols between the first two experiments and the third occurred after time zero when phagocytosis is measured):

- *Experiment 1*: phagocytosed bacteria $3.8e5$ (range $1.2e5 - 8.4e5$) CFU/ml
- *Experiment 2*: phagocytosed bacteria $1.4e5$ (range $8e4 - 1.6e5$) CFU/ml
- *Experiment 3*: phagocytosed bacteria $4.7e4$ (range $3e4 - 7.2e4$) CFU/ml

This suggests a degree of experimental variation that is not unexpected, as there may be differences in MOI between experiments as a result of human error during dilution of bacterial overnight cultures, and bacterial viability. As less bacteria were initially phagocytosed in experiment 3, this could also partially explain the reduced rate of intracellular killing over time. Overall, these three results suggest that this assay can be used to compare the rate of intracellular bacterial killing between experiments, but not phagocytosis, highlighting the requirement to compare treatments groups in the same experimental run.

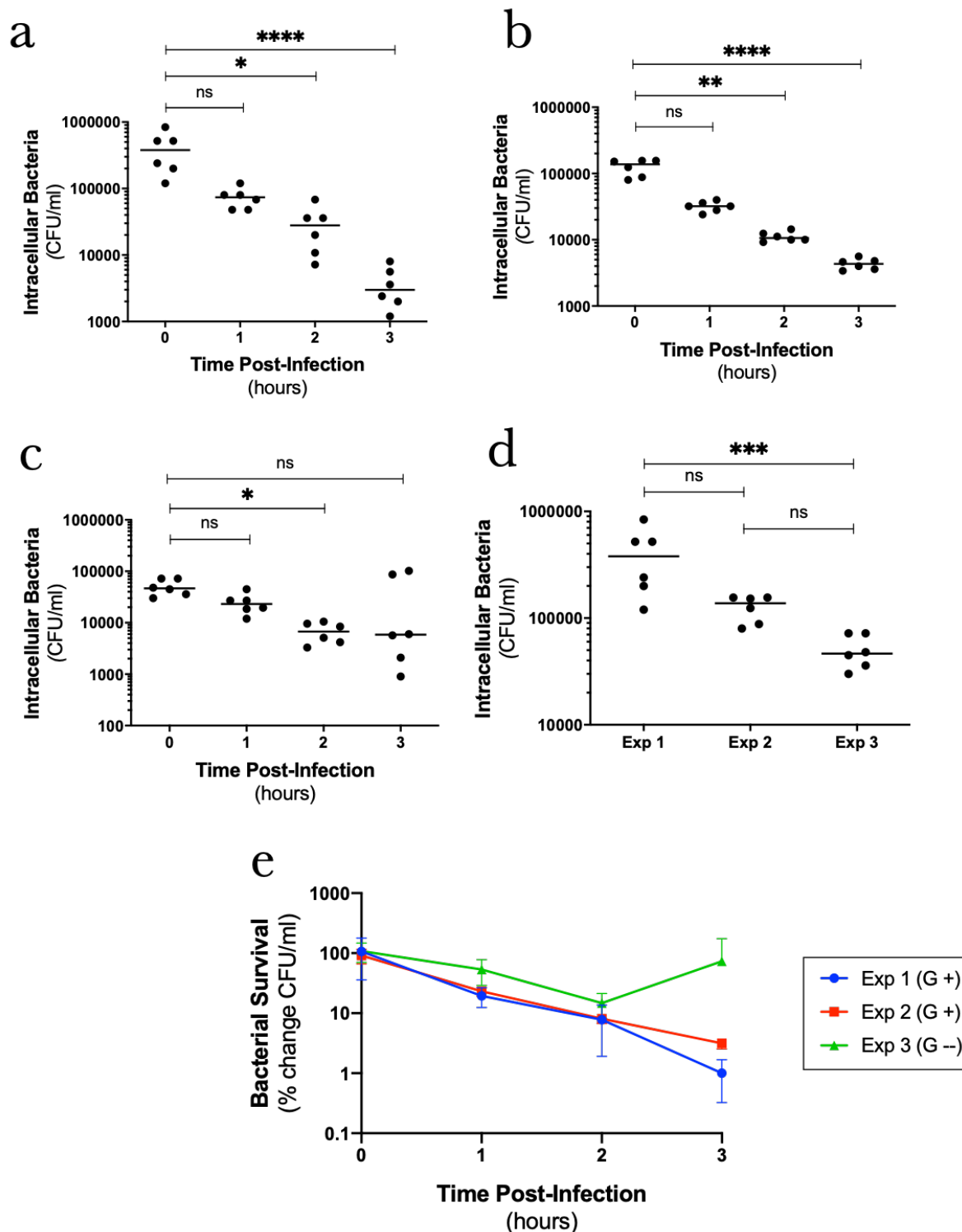


Figure 69 – Successful quantification of phagocytosis and intracellular killing of *P. aeruginosa* by adherent bone marrow derived macrophages (BMDMs) using a gentamicin protection assay. (a-b) Over two separate experiments (**a**: exp 1, **b**: exp 2) the percentage of live intracellular *P. aeruginosa* were quantified in BMDMs 1, 2, and 3 hours after phagocytosis, (n=6/condition). Here BMDMs were co-cultured with *P. aeruginosa* for 30 minutes at

MOI 50 and extracellular bacteria were then washed off and killed by gentamicin treatment (100 µg/ml) for one hour. Macrophages were then lysed with 0.1% Triton-X100 to quantify phagocytosis at time zero. Cells lysed at 1, 2, and 3 hours were cultured with an inhibitory 20 µg/ml of gentamicin before lysis (G+). **(c)** The assay was then repeated (exp 3) in the absence of this inhibitory gentamicin and cells were grown in antibiotic free RPMI media following time zero (G-). **(d)** The number of phagocytosed bacteria quantified at time zero between experiments was not consistent, but **(e)** the rate of intracellular killing of phagocytosed bacteria between experiments 1 and 2 was highly similar expressed here as a percentage of the number bacteria measured at time zero post-infection plotted as median ± range (statistical analysis not shown here). Intracellular bacterial were quantified by plating serial dilutions of cell lysate onto selective agar and following growth, counting the number of colony forming units per ml of lysate (CFU/ml). Bars represent median. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, Kruskal-Wallis t-test, Dunn's multiple comparisons)

6.3.7 A commercial assay using *E. coli* bioparticles successfully quantifies macrophage phagocytosis

In addition to the protection assay I also developed a protocol for a commercially available phagocytosis assay The Vybrant® Phagocytosis Assay Kit (Cat: V6694, ThermoFischer Scientific). This assay uses killed *E. coli* (K-12 strain) cells that have been labelled with the fluorescent dye fluorescein. It measures phagocytosis following a two-hour incubation with macrophages by selectively quenching extracellular fluorescein with trypan blue. Intracellular *E. coli* remain fluorescent however, as viable live cells exclude trypan blue. Here I calculated the phagocytic index of each sample by then correcting for macrophage cell number with a PrestoBlue Cell Viability Protocol which provides a fluorescent read out of metabolic activity.

I first confirmed the optimal ratio of *E. coli* to macrophages (or MOI) by co-culturing them at increasing MOIs (1-750). According to manufacturer's instructions the optimal MOI is 300. Here I observed a clear dose related response in phagocytosis output with increasing MOI and saw that above MOI 300 phagocytic index plateaued (Figure 70a). I then also confirmed that at this MOI, phagocytosis was occurring by incubating macrophages with cytochalasin-D, an inhibitor of phagocytosis. At increasing concentrations (0.063 - 1 µg/ml) cytochalasin-D reduced the phagocytic index reported by the assay in a dose related manner (Figure 70b). One caveat is that in retrospect I realised that a DMSO control was not included and therefore this reduction may not be specific to cytochalasin-D as it is solubilized in DMSO. Nevertheless, negative controls lacking macrophages reported no fluorescent signal. This indicates that all extracellular *E. coli* are successfully quenched, supporting the hypothesis that signal observed under test conditions is a result of phagocytosis by macrophages.

6.3.8 Initial phagocytic index comparisons between CF and WT mice BMDMs are inconclusive

In an initial experiment, I used the Vybrant® assay to compare phagocytosis of BMDMs from one WT (n=11 replicate samples) and one CF mouse (n=6 replicate samples) to determine if macrophages in CF have an inherent defect in bacterial phagocytosis as a result of CFTR loss-of-function. In this initial experiment, WT mice exhibited a significantly reduced phagocytic capacity (measured as *E.coli* relative light units (RLU) normalized to metabolic activity (RLU)) to CF mice (Figure 71a):

- *WT*: median phagocytic index 0.075 (range 0.025 – 0.18)
- *CF*: median phagocytic index 0.19 (range 0.15 – 0.22) (p<0.01, Mann-Whitney t-test)

However, upon repetition with samples from the same mice (n=10/mouse) the following day this effect was not recapitulated. No significant difference in phagocytic index was observed, and samples from the CF mouse displayed a large degree of variation (Figure 71b):

- *WT*: phagocytic index 1.35 ± 0.092 (mean $\pm$ SEM)
- *CF*: phagocytic index 1.87 ± 0.23 (mean $\pm$ SEM) (p=ns, Unpaired t-test)

This suggests that the reduced phagocytic index of WT mice observed in the first test is not robust.

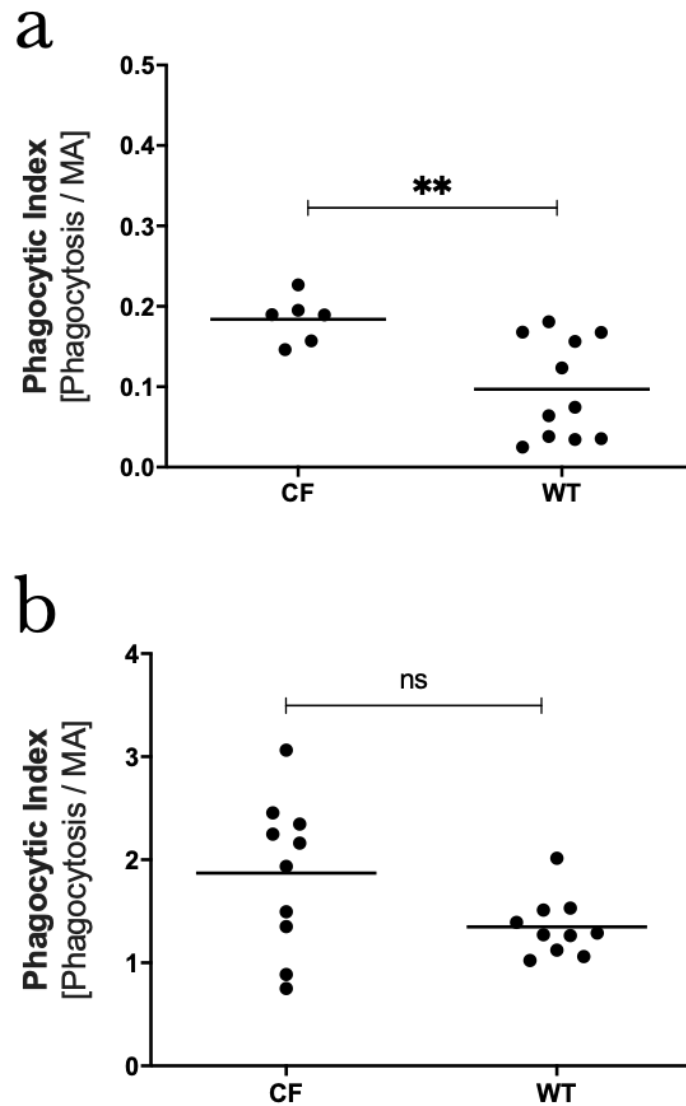


Figure 71 – Bone marrow derived macrophages (BMDMs) from wildtype (WT) and CFTR KO (CF) mice show no robust difference in phagocytic activity. Using the Vybrant® assay the number of *E. coli* bioparticles (MOI 300) phagocytosed macrophages from one CF and one WT mouse over a 2-hour incubation were compared on two separate days. **(a)** In the first experiment CF BMDMs (n=6 technical replicates) had a significantly higher phagocytic index than WT (n=10 technical replicates). **(b)** The experiment was repeated on the following day with replicate samples from the same CF and WT mice (n=10/mouse) and no significant difference was observed. The phagocytic index calculated by dividing the fluorescent read-out of internalised bioparticles in relative light units by the metabolic activity (MA) of each sample well measured by presto blue assay. Bars represent median. (Output is also relative light units). ns = $p > 0.05$, **= $p < 0.01$, Mann-Whitney t-test)

6.3.9 Macrophages from CF and WT mice exhibit no difference in phagocytosis or intracellular killing

To conclusively observe whether BMDMs from CF mice had a reduced ability to phagocytose or kill intracellular bacteria, I used both the gentamicin protection assay and the Vybrant® phagocytosis assay to compare BMDMs from a larger cohort of mice.

6.3.9.1 WT and CF have similar levels of phagocytosis: Vybrant® Assay

The Vybrant® assay was performed twice, each time comparing the phagocytic index of 5 CF and 5 WT mice, with 6 replicates per mouse. When comparing the median value for each mouse, no significant difference in *E. coli* phagocytosis was observed between CF and WT mice (Figure 72a):

- *WT*: median phagocytic index 12.33 (range 5.3 – 15.84)
- *CF*: median phagocytic index 11.5 (range 10.48 – 13.92) (p=ns, Mann-Whitney t-test)

In the second experiment, the same result was also observed, where CF and WT BMDMs reported similar phagocytic indexes (Figure 72b):

- *WT*: median phagocytic index 0.90 (range 0.59 – 1.44)
- *CF*: median phagocytic index 0.65 (range 0.59 – 0.76) (p=ns, Mann-Whitney t-test)

The findings of this assay are not suggestive of intrinsic CFTR expression affecting macrophage phagocytosis.

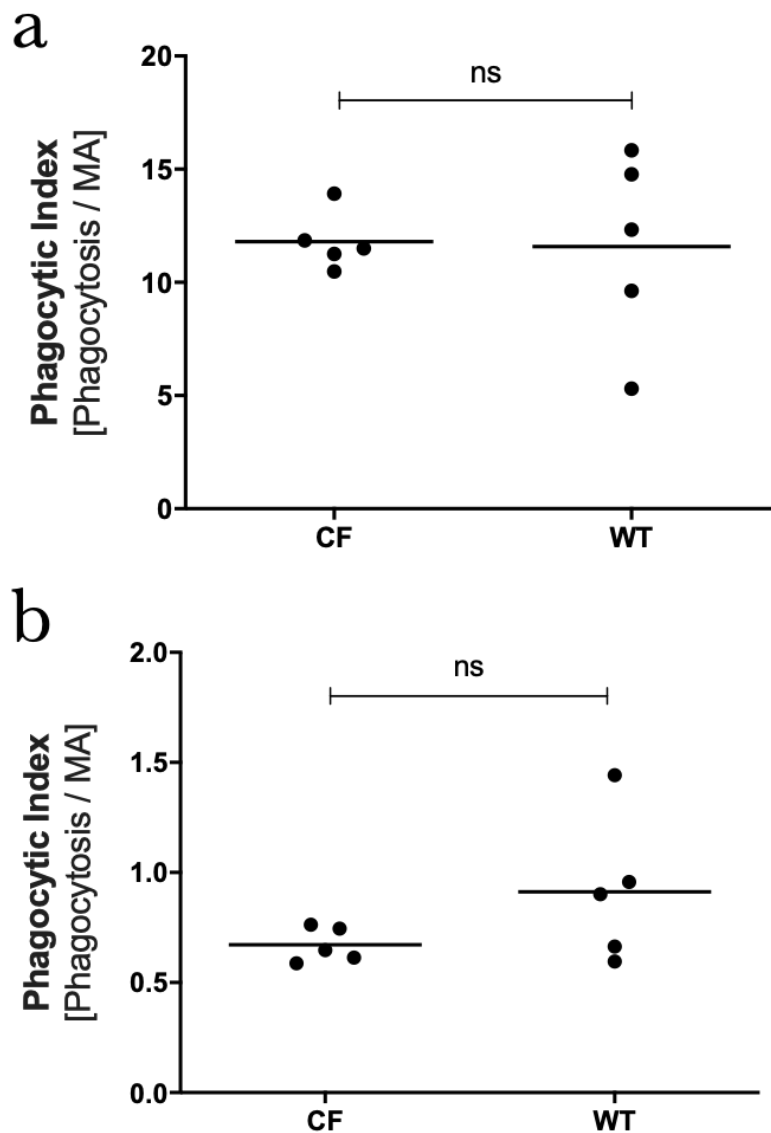


Figure 72 – Bone marrow derived macrophages (BMDMs) from wildtype (WT) and CFTR KO (CF) mice exhibit equivalent levels of phagocytic activity. Using the Vybrant® assay the number of *E. coli* bioparticles (MOI 300) phagocytosed macrophages from CF and WT mice (n=5/genotype) over a 2-hour incubation were compared. The experiment was repeated twice to compare 10 mice per genome total **(a)** mice 1-5, **(b)** mice 6-10. The phagocytic index calculated by dividing the fluorescent read-out of internalised bioparticles in relative light units by the metabolic activity (MA) of each sample well measured by presto blue assay (output is also relative light units). Bars represent median. ns = $p > 0.05$, Mann-Whitney t-test).

6.3.9.2 WT and CF have similar levels of phagocytosis and intracellular killing: Gentamicin Protection Assay

BMDMs from mice assayed with the Vybrant® assay were also compared with the gentamicin protection assay. The assay was performed multiple times where in each experiment either 2 or 3 CF and WT mice were compared (n=3 replicates/mouse/time point) to compare a total of 9 WT and 9 CF mice (referenced as mice 1-9) across 4 experiments.

To measure the relative rate of phagocytosis I compared the results at time zero between CF and WT BMDMs. Direct comparisons were only made between experiments run on the same day to control for experimental variation which may for example occur as a result of differences in timings, or bacterial MOI or fitness. With the exception of one experiment, differences in phagocytosed bacteria (CFU/ml) between CF and WT mice is not suggestive of a robust difference in phagocytic ability (Figure 73a):

- *Experiment 1 (mice 1-2):* median fold change 1.16 CFU/ml
- *Experiment 2 (mice 3-4):* median fold change 9.86 CFU/ml
- *Experiment 3 (mice 5-6):* median fold change 1.55 CFU/ml
- *Experiment 4 (mice 7-9):* median fold change 0.80 CFU/ml

Though the second iteration comparing CF and WT mice 3-4 did suggest a reduced phagocytic capacity by CF macrophages, as other experiments did not support this finding it is likely non-specific and may be due to differences in handling. Furthermore, when comparing the median phagocytic index reported by the Vybrant® assay for the same mice this effect was not observed where the median fold change in phagocytic index was 1.27.

Across WT and CF BMDMs significant bacterial killing was observed over time in comparison to time zero where survival was 100%. As no difference was observed between CF and WT macrophages a median percentage survival was calculated for all 18 mice:

- *1 Hour:* median survival 34.74 % (range 21.43 – 233.3) CFU/ml (p = ns)
- *2 Hours:* median survival 12.57 % (range 1.17 - 35) CFU/ml (p<0.0001)
- *3 Hours:* median survival 1.8 % (range 0.25 – 13.5) CFU/ml (p< 0.0001, Kruskal-Wallis, Dunn's multiple comparisons)

Here, ~65% of intracellular bacteria are cleared within the first hour, and after 3 hours bacterial survival was negligible.

In addition to no robust difference in phagocytosis, the gentamicin protection assay did not suggest a difference in the fate of phagocytosed bacteria. When comparing the median number of live bacteria present at each time point as a percentage of time zero per mouse, no significant difference was observed between CF and WT BMDMs at either time point (Figure 73b):

- *1 hour*: median difference 0.52 % bacterial survival ($p = \text{ns}$)
- *2 hours*: median fold change 0.86 % bacterial survival ($p = \text{ns}$)
- *3 hours*: median fold change 0.6 % bacterial survival ($p = \text{ns}$, 2-wayANOVA, Sidak's multiple comparisons)

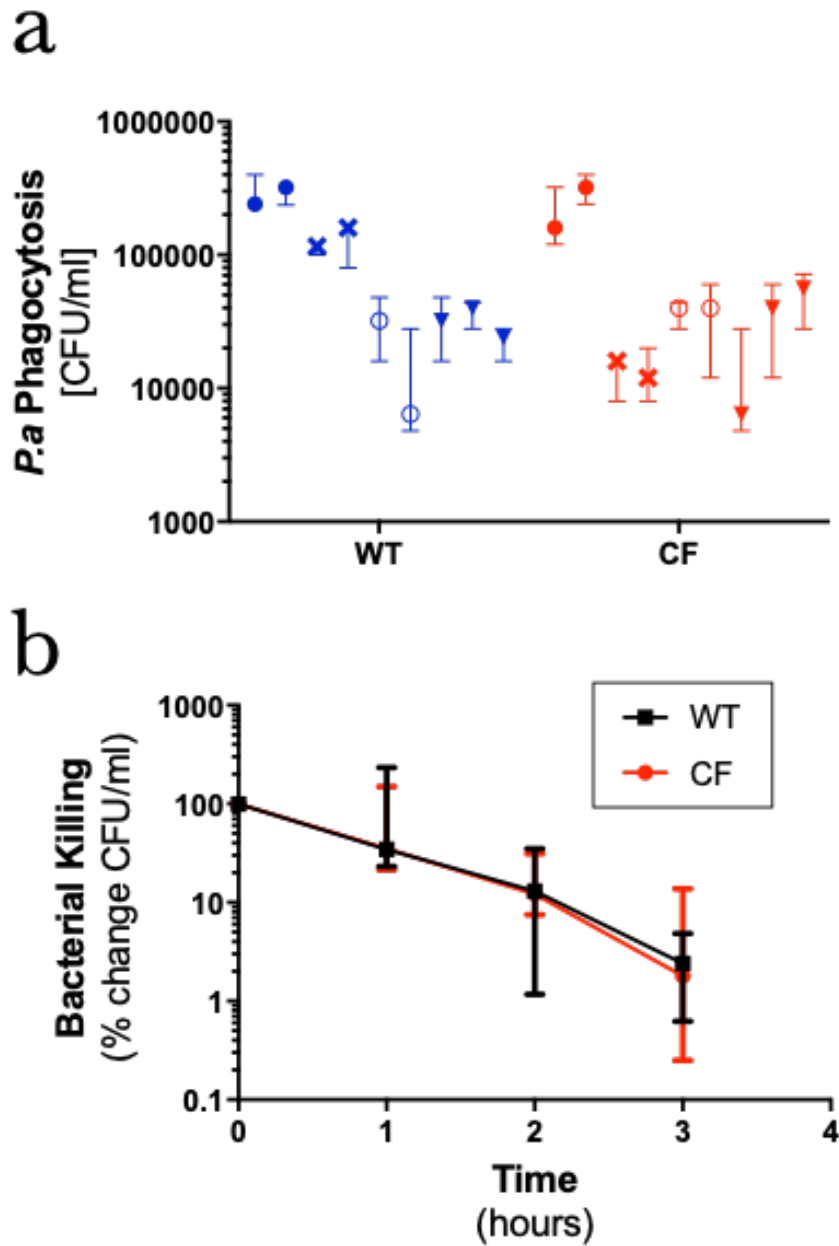


Figure 73 – There is no difference in bactericidal activity by bone marrow derived macrophages (BMDMs) from wildtype (WT) and CFTR KO (CF) mice. Using a gentamicin protection assay, the percentage of live intracellular *P. aeruginosa* were quantified in BMDMs from WT and CF mice at **(a)** time zero to measure phagocytosis. Across four experiments 9 mice from each genotype were compared in total (n=3 replicates/mouse/time point). Mice compared within the same experimental iteration are plotted with the same symbol (median $\pm$ range). **(b)** To measure intracellular killing of phagocytosed bacteria quantification was also performed 1, 2, and 3 hours after time zero. To calculate intracellular killing, the median number of surviving intracellular bacteria for each mouse was

calculated as a percentage of the median number bacteria measured at time zero. Each point represents the median value of 9 mice $\pm$ range. Here BMDMs were co-cultured with *P. aeruginosa* for 30 minutes at MOI 50 and extracellular bacteria were then washed off and killed by gentamicin treatment (100 μ g/ml) for one hour. Macrophages were then lysed with 0.1% Triton-X100 to quantify phagocytosis at time zero. Cells lysed at 1, 2, and 3 hours were cultured with an inhibitory 20 μ g/ml of gentamicin before lysis. Intracellular bacterial were quantified by plating serial dilutions of cell lysate onto selective agar and following growth, counting the number of colony forming units per ml of lysate.

6.3.10 CFTR inhibition has no effect on *E. coli* bioparticle phagocytosis by WT macrophages

To further examine whether there might be an effect of CFTR function on phagocytosis, BMDMs from WT mice were pre-treated with CFTR(inh)-172 for 72 hours at increasing concentrations (1-50 $\mu\text{g/ml}$). CFTR(inh)-172 results in rapid, reversible and voltage-independent inhibition of the CFTR chloride channel and has been used in the literature to recapitulate deficiencies in CF macrophage phagocytosis in WT mice (606). To control for the effect of DMSO in which the inhibitor is solubilized, BMDMs were also pre-treated with an equivalent dose of DMSO only. However due to availability, this was only controlled for at the maximum dose of CFTR(inh)-172 at 50 $\mu\text{g/ml}$.

The effect of CFTR(inh)-172 treatment was calculated as the amount of phagocytosis measured in treated samples relative to untreated controls. A significant effect of treatment was only observed at the highest concentration of 50 $\mu\text{g/ml}$, but as DMSO controls demonstrated an equivalent effect this is not likely specific to CFTR inhibition (Figure 74):

- 1 $\mu\text{g/ml}$: median effect of treatment 67.5 (24.8 – 191.2) % ($p = \text{ns}$)
- 5 $\mu\text{g/ml}$: median effect of treatment 88.7 (21.6 – 240.8) % ($p = \text{ns}$)
- 10 $\mu\text{g/ml}$: median effect of treatment 127.4 (32.9 – 399.6) % ($p = \text{ns}$)
- 50 $\mu\text{g/ml}$: median effect of treatment 9.2 (7.6 – 32.01) % ($p < 0.01$)
- DMSO control: median effect of treatment 13.0 (8.3 – 23.1) % ($p < 0.05$, Kruskal-Wallis t-test, Dunn's multiple comparisons)

These results therefore reflect previous findings with BMDMs from CF mice that there is no measurable effect of CFTR loss-of-function on BMDM phagocytosis.

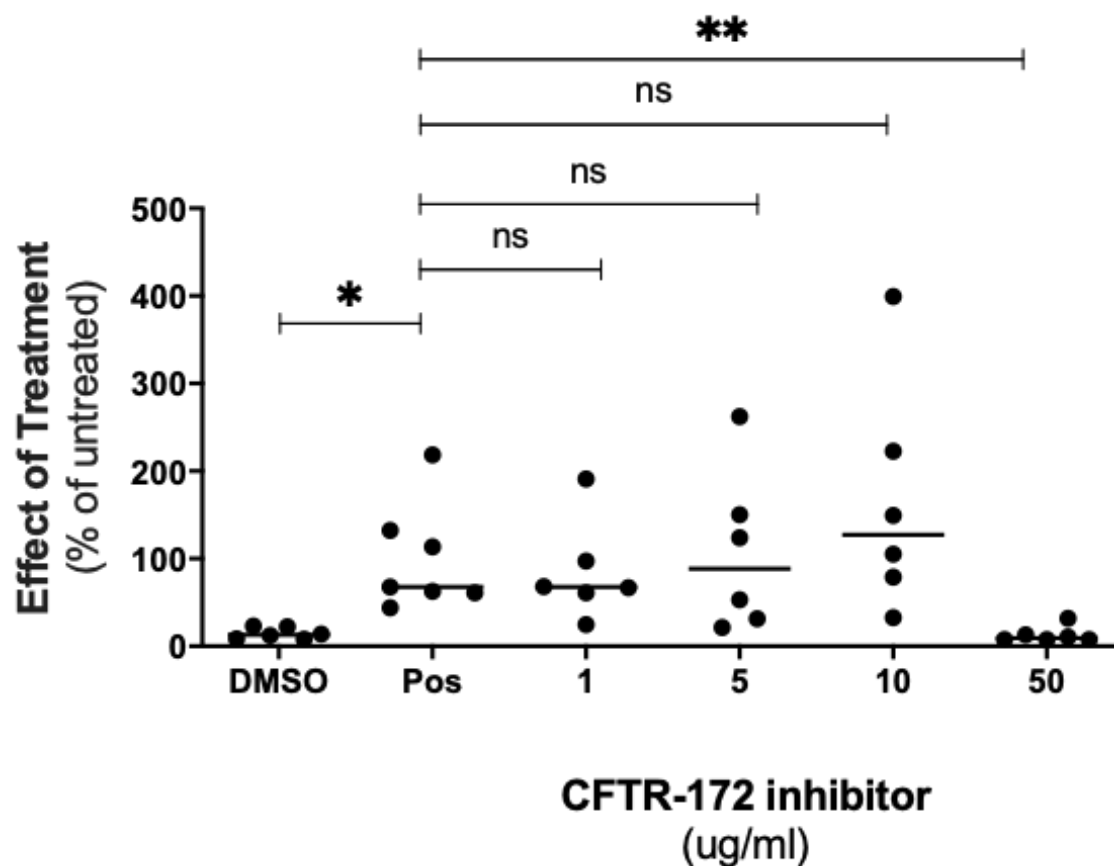


Figure 74 – CFTR inhibition has no measurable effect on phagocytosis of WT bone marrow derived macrophages (BMDMs). No effect of functional CFTR inhibition on phagocytosis in wildtype (WT) was observed following pre-treatment of cells with increasing concentrations of CFTR(inh)-172 (1-50 µg/ml) for 72 hours (n≥6/treatment group). Phagocytic index was measured using the Vybrant® assay normalised to metabolic activity using presto blue assay. To determine the effect of treatment, this was expressed as a percentage of the median index of positive (pos) untreated samples. DMSO controls were included for the highest CFTR(inh)-172 concentration (50 µg/ml). Bars represent median. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, Kruskal-Wallis t-test, Dunn's multiple comparisons)

6.4 Discussion

The work presented in this chapter represents initial work attempting to characterize the intrinsic effect of CFTR dysfunction on alveolar macrophages. Following extensive optimisation, two assays were produced to measure differences in bacterial phagocytosis and intracellular killing by bone marrow derived macrophages (BMDMs). With these assays no conclusive effect of CFTR expression or function was observed on macrophage activity against *P. aeruginosa*. As here no metric by which to demonstrate intrinsic AM dysfunction in the CF lung was developed, I have not yet been able to evaluate the potential improvement that might be seen in these cells following *ex vivo* lentiviral gene transfer of a wildtype CFTR gene.

6.4.1 Use of a Gentamicin Protection Assay

Antibiotic protection assays are widely used to measure macrophage phagocytosis and intracellular killing with various macrophage models, effectors, bacterial species and strains (653, 655-658, 666). To this end I developed a gentamicin protection assay that I used to compare bactericidal function of macrophages from wildtype and CF mice. When characterising the assay, I observed that in BMDMs from one WT mouse, up to 80% of intracellular bacteria are killed within the first hour. In the later comparison between WT and CF mice BMDMs, this was 64% and 65% respectively (median 9 mice). This is not dissimilar to the literature where a Zhang *et al* reported that 60% of *P. aeruginosa* is cleared by WT AMs in the first hour, though there is no consensus regarding what this figure is as Ferrari *et al* reported that 63% of *P. aeruginosa* is cleared by WT BMDMs in 3 hours (603, 612). Though the assay was relatively reproducible with regard to bacterial survival over time, additional controls such a non-lysis conditions and general inhibitors of phagocytosis could provide assurances of the accuracy of these findings. Future work could test the effect of cytochalasin-D, an inhibitor of phagocytosis which has been used in similar assays to inhibit AM phagocytosis *in vitro* (667). This was later used here in experiments to characterize the commercial Vybrant™ assay and demonstrated a dramatic effect on phagocytosis.

Extensive optimisation of the gentamicin assay was not performed prior to comparing CF and WT macrophages. Many studies that measured CFTR dependent differences in macrophage function used similar protection assays, a summary of which can be seen in Table 20, but there is little consensus in the exact parameters used in the protection protocol. The conditions used

here were similar to these other publications but testing of alternative conditions may be warranted, as for example the majority of studies summarised in Table 20 employ a longer bacterial infection time of 1 hour. To confirm results, the experiment could also be repeated using the exact protocol of one of these previous studies. It is worth noting that between experiments, the outcomes were not always consistent as two studies, one testing patient PBMC-derived macrophages (604) and another CFTR KO BMDMs (660), did not observe differences in phagocytosis but only intracellular killing. This suggests that even though these papers agree that there are intrinsic CFTR dependent effects on bactericidal activity in macrophages, there is no unanimity as to how these effects manifest (Table 20).

Table 20 – Summary of antibiotic protection assay protocols and results in studies investigating the effect of CFTR on macrophage function. The conclusion as to whether reduced phagocytosis (Ph) or killing of intracellular bacteria (K) was observed is indicated by yes/no (Y/N). hPBMCs: comparison of human peripheral blood mononuclear cells from CF and non-CF donors; AMs: alveolar macrophages, mBMDMs: murine bone marrow derived macrophages. F508del: mice homozygous for F508del mutation. CFTR KO: Gut corrected FABp-CF-knockout mice. WT: wildtype mice. M ϕ induction: inductor of macrophage differentiation. M-CSF: macrophage colony-stimulating factor. GM-CSF: Granulocyte macrophage colony-stimulating factor. MOI: Multiplicity of infection, where an MOI of 30 indicates that 30 bacterial cells were added for every 1 macrophage in culture. Gent: gentamicin. $\pm$ Indicates pathogens were first opsonized. *Macrophages and bacteria were first centrifuged.

Study	Cell Type	Mφ Induction	Bacterial Strain	Infection	Antibiotic Treatment	CF Defect (Y/N)
Del Porto <i>et al</i> (604)	hPBMCs	M-CSF	<i>P. aeruginosa</i>	MOI 30*; 1 hour	400 µg/ml; 1 hour; Gent.	Ph: N K: Y
Simonin-Le Jeune <i>et al</i> (611)	hPBMCs	GM-CSF	<i>P. aeruginosa</i>	MOI 100; 1 hour	150 µg/ml; 1 hour; Gent.	Ph: Y
Shrestha <i>et al</i> (610)	hPBMCs	GM-CSF	<i>B. cepacia</i> ±	MOI 10; 1 hour	50 µg/ml; 2 hour; Gent.	Ph: Y K: Y
Van De Weert-Van Leeuwen <i>et al</i> (609)	AMs	N/A	<i>P. aeruginosa</i> ±	MOI 100; 30 minutes	20 µg/ml; 20 minutes Gent.	Ph: Y K: Y
Ferrari <i>et al</i> (612)	F508del, CFTR KO, WT mBMDMs	M-CSF	<i>P. aeruginosa</i>	MOI 50; 10 minutes	100 µg/ml; 10 minutes; Gent.	Ph: Y K: Y
Gauthier <i>et al</i> (615)	Neonatal CFTR KO, WT mAMs	N/A	FITC labelled <i>S. aureus</i>	MOI 1; 2 hours	NA Fluorescent imaging fixed cells	Ph: Y
Li <i>et al</i> (660)	CFTR KO, WT mAMs	N/A	<i>S. aureus</i>	MOI 50; 2, 4, 8, 6, 12 hours	100 µg/ml; 1 hour Gent.	Ph: N K: Y
Zhang <i>et al</i> (603)	CFTR KO, WT mAMs	M-CSF	<i>P. aeruginosa</i> ±	MOI 1; 20 minutes	No Antibiotic	K: Y
This study	CFTR KO, WT mBMDMs	M-CSF	<i>P. aeruginosa</i>	MOI 50; 30 minutes	100 µg/ml; 1 hour Gent.	Ph: N K: N

6.4.2 Characterisation of a Colistin Protection Assay

Here initial attempts to develop a reproducible antibiotic protection assay using colistin failed. This was likely due initially to the effect of poor macrophage viability and adherence following passaging. In subsequent experiments in adherent cells, it is likely that due to low antibiotic concentrations, killing or inhibition of extracellular bacteria was not sufficient. This is supported by the overall higher concentrations of antibiotics used in the studies summaries in Table 6.2, though again they show little consistency. Furthermore, no inhibitory maintenance concentration of colistin was used following the initial incubation which follow-up studies suggested are necessary to maintain inhibition of extracellular bacterial growth. Similar incubations with gentamicin after an initial time point measuring phagocytosis were also employed by others (604, 660). Others have used colistin effectively to investigate intracellular survival of multidrug-resistant *Acinetobacter baumannii* in macrophage-like cell lines, where a concentration of 50 µg for 4 and 24 hours was effective at killing extracellular but not intracellular bacteria, indicating colistin is not transported into mammalian cells (523). Therefore, these studies suggest that colistin may still be an effective antibiotic to use but was here provided at sub-effective concentrations. With longer treatments of gentamicin at higher relative concentrations, the assay was effective, and the same result may also have been attained with colistin. However, this was not tested here. Instead, I decided to use gentamicin as not only is this the most widely used antibiotic in these settings, but it is also commonly used to investigate the effect of CFTR on macrophage function (see all but one of the studies in Table 20) and therefore provides a more controlled comparison to previous findings. Furthermore, by using gentamicin I was then able to follow an established protocol and advice from my collaborator Elli Mylona.

Initially colistin was used instead of gentamicin, based on the belief that due to the reduced growth of *P. aeruginosa* in RPMI gentamicin would be ineffective. In retrospect it is likely that these fears were unfounded. Though slow bacterial growth is associated with decreased bacterial susceptibility to many antibiotics including aminoglycosides, and gentamicin is more effective against actively dividing bacteria, it is still active against slow or non-dividing bacteria at increased concentrations (659, 662). Future experiments should however include an additional control group where bacteria alone are incubated with 100 µg/ml of gentamicin in the absence of macrophages and exposed to the same number of washes to confirm that no live bacteria remain.

6.4.3 BMDMs from CFTR KO Mice

Extensive investigations into the phenotype of BMDMs from these CFTR KO mice used here were not performed within this study. Flow cytometry analysis indicated that like WT BMDMs characterised in Chapter 3, BMDMs from CF mice are CD11b⁺, F4/80⁺ which was used to confirm the adequacy of the differentiation protocol. However, this comparison only used the cells of one CF mouse and direct phenotype comparisons were not made with WT mice. In chapter 5, section 5.3.4, the modality of BMDMs induced by M-CSF or GM-CSF were discussed with respect to anti-viral properties and inhibition of lentiviral transduction. M-CSF differentiated cells are more commonly used to study function and signalling of tissue resident macrophages and are considered to produce a more M2 (anti-inflammatory) cytokine profile (668-671). GM-CSF induced monocytes give rise to cells M1 like macrophages (pro-inflammatory) that while also expressing macrophage markers, with macrophage like transcriptomes, are more similar to dendritic cells (DCs) than M-CSF induced macrophages and are used as *in vitro* DC models (672, 673). Studies have suggested that these differences have functional consequences where M-CSF (M2-like) macrophages promote might bacterial tolerance (671), but at the same time also exhibit a greater capacity for immunoglobulin G Fc mediated phagocytosis (669). Owing to these differences, future work could also compare WT and CF BMDMs induced by GM-CSF, however as a model of AM function, M-CSF induced cells are likely a more suitable model. In the literature, M-CSF differentiated murine BMDMs are the more commonly used model to study CF and WT macrophage bactericidal activity, though studies of hPBMC from CF and non-CF donors have also used GM-CSF (Table 6.2). This supports the use of M-CSF induced BMDMs here and does not suggest that differences in macrophage differentiation explain why I was unable to recapitulate CFTR intrinsic effects on bactericidal macrophage function.

These intrinsic differences have been observed with murine BMDMs, and AMs, as well as human PBMCs and AMs. Here murine BMDMs were chosen as these cells should only demonstrate minimal to no effect of environmental conditioning that makes parsing extrinsic and intrinsic effects of CFTR dysfunction on AMs difficult. I did not however, confirm the absence of CFTR expression in these cells which future experiments could address. The CFTR KO mice used here, express a human CFTR gene under the control of the fatty acid-binding promoter (FABp) which in theory limits expression to the gut to prevent the intestinal disease of the CF knockout mouse (274). Other studies have investigated CFTR expression of BMDMs

from this mouse and through immunostaining (615) and whole-cell and single-channel voltage-clamp analyses of efflux function (601) seen no evidence of CFTR expression. Though BMDMs have distinct phenotypes to AMs and other tissue resident macrophage populations, they have been successfully used to observe reduced phagocytosis and bacterial killing in the absence of functional CFTR. As macrophages rely on local environmental conditioning to determine functional phenotypes (39), it is possible that these differences cannot be fully evaluated *in vitro*. *In vivo* studies such as those transplanting CF bone marrow transplants, or macrophages into infection models may be more useful models of macrophage function (608). These could be used in future work to determine the potential benefits of rSIV.F/HN mediated CFTR gene transfer in macrophage function.

One of the limitations of this study is that mice were not age-matched between WT and CF mice and the latter were an average of 13-14 months of age when bone marrow was harvested, opposed to WT mice who were 6-10 weeks of age. This may be significant as studies have shown that with increased age, phagocytic and opsonization capacities are impaired in peritoneal macrophages and wound macrophages (674, 675). Here, no reduced phagocytic or intracellular killing was observed in these aged CF BMDMs, and other studies demonstrated that phagocytosis of rat AMs (676) or mouse BMDMs (677) did not exhibit an age-related decline. These BMDM studies suggested that age related declines observed in peritoneal macrophages (675) and other tissue specific resident cells (678) are not due to intrinsic macrophage dysfunction, but rather the influence of an aged tissue-specific niche (677). Therefore, though this study was not properly controlled, the ensuing results and previous literature does not suggest age would have significantly altered results of CF vs WT investigations.

6.4.4 Effect of Functional CFTR in Macrophages

Here I was unable to recapitulate previous studies that measured significant reduction in phagocytosis or bacterial killing by CFTR deficient macrophages. I was unable to characterize a difference between WT and CF BMDMs with either a commercial kit based on the phagocytosis of fluorescent *E. coli* bioparticles, or a more clinically relevant model with the PAO1 lab strain of *Pseudomonas aeruginosa*. Though I have discussed how the gentamicin protection assay may be adapted and further characterised to confirm these results, the commercial Vybrant® assay was used according to manufacturer's instructions and Simonin-

Le Jeune *et al* used this assay to demonstrate that PBMC-derived macrophages from CF donors have a 67% reduced phagocytic function which was recapitulated in cells from non-CF donors by the addition of CFTR(inh)-172 (611). This could be due to differences in cell type, or the manner in which cells were plated. Here adherent cells were used which were plated as bone marrow and then differentiated in the 96-well plates used to perform the experiment. Though not directly stated, it is likely that Simonin-Le Jeune *et al* plated exact numbers of macrophages directly in advance of running the experiment. Though here the number of macrophages in representative wells were counted to estimate cell numbers, one might expect that here there were larger variations in cell counts between wells that may have obscured any slight differences between genotypes, though results were normalised for live cell numbers.

6.4.4.1 Macrophage Model Heterogeneity

Though adaptations the gentamicin protection assay may still be warranted, these initial findings do not support the theory that loss of CFTR function in macrophages causes intrinsic bactericidal deficiencies in AMs that contribute to CF lung disease. It is possible that this is due to the heterogenic ontogeny and genetics of *ex vivo* macrophage models. In comparison to human AMs or PBMCs *in vitro* it is possible that the extrinsic influences on murine BMDM function are different. CF is a multiorgan disease and cytokine levels are systemically altered in patients suggesting PBMCs may already be partially conditioned (524). Here BMDMs from CFTR KO mice were compared to WT mice, and as these CF mice do not develop spontaneous lung disease it suggests that effects of lung pre-conditioning would be diminished (274). It is of course also possible that due to differences between human and mouse models, these studies are not representative of the human condition and therefore CFTR specific defects were not observed.

Regardless, other studies have observed significant differences in macrophage phagocytosis and bacterial killing in murine AMs and BMDMs. Though some used CFTR KO AMs, which even though they don't fully recapitulate CF lung disease (274), are still harvested from a perturbed lung environment (615, 660). Some of the most convincing data has emerged from studies of functional CFTR inhibition in WT or non-CF donor macrophages and CRISPR/CAS9 mediated knockouts which have never been exposed to a CF environment (609, 611, 612, 679). This suggests that it is an experimental failure that has led to a failure to observe CFTR mediated bactericidal dysfunction.

6.4.4.2 Functional CFTR Inhibition

The effect of the CFTR(inh)-172 was only minimally investigated here, though across a range of increasing concentrations no inhibitory effect on phagocytosis was observed. Cells were pre-treated with the inhibitor for 72 hours prior to analysis and in retrospect, I realised that although different studies use different concentrations and incubation timings, those using a 72-hour time pre-treatment protocol, delivered daily administration of the inhibitor, whereas here the inhibitor was only added once (611). As CFTR(inh)-172 reversibly inhibits CFTR function it may be that by the time the assay was performed the effect had worn off. For example, Ferrari *et al* pre-treated cells only 18 hours in advance with 20 μ M of CFTR(inh)-172 (612). As a result, at this stage it would likely be premature to make robust conclusions as the effect of functional CFTR inhibition in WT BMDMs on phagocytosis. Future work should optimise the appropriate timing of inhibitor addition.

Additionally, these studies could be expanded by investigating alternative bacterial species such as *S. aureus* and *B. cepacia*, though as many studies using live bacterial strains have used *P. aeruginosa* this is also unlikely to be a confounding factor here (Table 20). Further comparisons could be made with alternative bacterial strains.

6.4.4.3 Opsonisation

An interesting discrepancy in the literature as to the necessity and effect of opsonization prior to macrophage infections could also explain why here CFTR KO and WT BMDMs were not functionally different. Though numerous studies, including those testing *P. aeruginosa* in CFTR KO and WT BMDMs did not report opsonizing bacteria or bioparticles prior to use (604, 612, 615, 660), many others did (601, 603, 609-611). Opsonins are extracellular proteins that induce phagocytosis of bound material, including apoptotic cells and pathogens, by binding phagocytic receptors on phagocytes (680). By flow cytometry analysis of EGFP labelled *P. aeruginosa* phagocytosis in primary hAMs Van De Weert-Van Leeuwen *et al* interestingly demonstrated that blocking C3, an opsonin of the complement system, phagocytosis was reduced by 50-60% (609). They also demonstrated that between PBMC-derived macrophages from CF and non-CF donors, observations of a CF specific reduction in phagocytosis and intracellular killing were dependent on an active complement system and heat-inactivation of the serum used to

opsonize pathogens eliminated the effect of CFTR(inh)-172 on non-CF donor cells. CD11b is a subunit of the complement C3 receptor (681) and here they also showed that in CF patient AMs, expression was reduced (609). Their hypothesis that loss of functional CFTR produces a decrease in complement dependent phagocytosis as a result reduced CD11b, was also supported by Simonin-Le Jeune *et al* where PBMC-derived macrophages from CF patients showed a significant decrease in CD11b expression over non-CF macrophages (611). As these studies were both in human macrophage models from CF patients, it is possible that reduced CD11b expression is an extrinsic effect of macrophage conditioning. It may therefore be worth investigating whether opsonisation of *P. aeruginosa* could change the outcomes presented here. Nevertheless, as non-opsonized phagocytosis of *P. aeruginosa* is also mediated by C3 receptor (CD11b/CD18) receptor, in murine AMs it suggests that the effect of reduced CD11b should also be observed in the absence of opsonin proteins (682). Though here characterisation of CFTR KO BMDMs did not indicate reduced expression of CD11b, this experiment did not directly compare these cells to WT BMDMs and to determine if intrinsic CFTR dysfunction in BMDMs reduces CD11b expression a larger experiment is required.

6.4.4.4 Alternative Effects of CFTR Dysfunction

In this initial work I compared the effect of CFTR dysfunction on macrophage phagocytosis and killing of intracellular bacteria, but research has also suggested that macrophages exhibit other intrinsic effects. In CF AMs, exhibit a hyper response to infection with an increased pro-inflammatory cytokine secretion profile (432). Inhibition or mutation of CFTR in AMs, hPBMCs and BMDMs has been shown to promote a pro-inflammatory phenotype suggesting the chronic inflammation observed in the CF lung may be in part due to intrinsic macrophage dysfunction (683-685). CFTR has also been implicated in the production of reactive oxygen species (ROS). Macrophages can produce ROS in response to various stimuli to regulate inflammation and as part of an antimicrobial response which is highly effective against gram-negative and gram-positive bacteria, viruses and fungi (686, 687). Assani *et al* compared PBMC-derived macrophages from CF and non-CF patients and observed a 60% reduction in ROS production following stimulation in CF macrophages due to decreased phosphorylation of NADPH oxidase complex (616), and a similar result was also observed in CRISPR/CAS 9 CFTR KO macrophages (679). However, in contrast Cifani *et al* demonstrated that the contribution of ROS to *P. aeruginosa* killing is not affected by CFTR mutations in human AMs and PBMCs (688). As gene transfer by rSIV/F/HN could also mediate these other potential

pathological contributions of AMs in the CF lung future research could explore these mechanisms in addition to the direct bactericidal effects of CFTR loss-of-function.

6.4.5 Conclusion

This work was unable to observe differences in macrophage bactericidal activity as a consequence of CFTR loss-of-function. As a result, I did not explore gene transfer of wildtype CFTR into macrophages from CFTR KO mice. The lack of consensus regarding methodology and reported outcomes between studies investigating the effect of CFTR expression in macrophages, makes evaluating these results difficult. This work also highlights the importance of publishing negative results, as it is possible that others were also unable to recapitulate these findings but chalked it up to procedural error. It is also possible, that even if CF macrophages have intrinsic faults that contribute to the sustained pro-inflammatory environment in the lung and increased susceptibility to bacterial infection, that their overall contribution to CF pathology is not clinically significant. Upcoming clinical trials delivering wildtype CFTR to the lung epithelium may effectively reduce disease mortality and morbidity without directly addressing AM dysfunction. If this is not the case, an argument could be made for macrophage cell therapy to replace dysfunctional endogenous AMs.

Summary and Future Directions

Lung gene therapy has been investigated for pulmonary diseases like cystic fibrosis (CF), alpha-1 antitrypsin deficiency (AATD) and autoimmune pulmonary alveolar proteinosis (PAP), which are rare or ultra-rare diseases where patients often have unmet needs and lack efficacious treatment options (163). Here I have presented an alternative approach to *in vivo* gene therapy that could avoid potential safety concerns associated with *in vivo* vector delivery, reduce immune recognition, and reduce the required viral vector dosage.

In this thesis I have developed a gene-modified macrophage cell-therapy strategy that future work will aim to expand into a platform technology for a variety of respiratory diseases. Macrophages were chosen as the candidate cellular vehicles for secreted pulmonary gene expression as owing to their plasticity, previous studies had suggested they might have the potential to function as long-term pulmonary delivery systems of therapeutic proteins (39, 176, 177). Other cell types have also been investigated for lung cell therapy, including mesenchymal stem cells (MSC), which have been widely investigated in transplantation strategies to modulate inflammatory responses in lung disease (689, 690). They therefore could represent an alternative cell type for secreted protein delivery. However, studies suggest that in comparison to macrophages, their engraftment and duration of retention is limited (689, 691-693).

Here I used lentiviral vectors to mediate gene transfer in bone marrow-derived macrophages (BMDMs) that when transplanted to the lung can secrete therapeutic proteins and act as protein secretion ‘factories’. Studies investigating similar approaches are limited in number and have largely relied on transplantation in the absence of a mature alveolar macrophage (AM) population (176-178, 186). Here, duration studies in wildtype mice led to sustained expression of the secreted reporter gene Gaussia luciferase (Gluc) for up to 16 weeks. As there was no evidence to indicate a drop in gene expression over time, it suggests for the first time that even in the presence of a mature AM niche, transplanted macrophages can be retained long-term in the lungs. *In vivo* rSIV.F/HN lentiviral administration can stably transduce the lung epithelium and results in long-term gene expression of 15+ months (268, 302). In comparison it is unclear how long macrophages will be retained in wildtype lungs as longer duration studies were not within the scope of this thesis. In the absence of endogenous AMs, macrophage engraftment was sustained for the lifetime of the mouse (176, 177, 186). However, as the fate of transplanted cells is likely dictated by the state of the AM niche, it cautions extrapolations to mouse models

or patients with endogenous mature AMs. Future work should therefore investigate extended duration studies to determine how long a single dose of modified macrophages can last. One of the advantages of *ex vivo* therapy with autologous cells is the established safety record of repeat administration in clinical trials (61-64, 88-92). As potential target diseases like CF, aPAP and AATD are chronic in nature, repeat dosing could provide long-term sustained therapeutic benefits even if duration is sub-optimal.

Though I have shown evidence suggesting macrophages are retained and express transgenes *in vivo*, I did not characterise macrophage phenotypes. Here I hypothesise that macrophages transplanted into wildtype mice with established AM compartments are maintained in an immature state alongside endogenous AM as suggested by retention of circulating monocytes following mild lung inflammation (419). As this has not been confirmed by phenotypic or functional analysis, this will need to be addressed to understand the mechanism underlying the macrophage retention and sustained transgene expression and inform longer term outcomes and extrapolations to patients.

Donor-derived macrophages were detected in bronchoalveolar lavage fluid (BALF) of wildtype mice up to 16 weeks post-delivery, as would be predicted by the sustained gene expression observed. Moving forward, alternative or additional assays to track engraftment and donor-derived cells will be required. Likely due to variations in handling and sampling, the BALF flow-cytometry protocol used here was inconsistent when quantifying macrophage retention in the long-term duration study. Furthermore, the significant build-up of surfactant and debris found in the BALF of GM-CSF KO mice precluded analysis by flow cytometry. Alternative approaches include lung histological analysis or by applying the molecular assays used to characterise *in vitro* transductions. Quantifying VCN in BALF cells or lung homogenate might provide insight into the number of transduced donor-derived cells retained in the lung over time. Additionally, transgene mRNA quantification could be useful to compare relative expression of therapeutic and control transgenes. The existing flow cytometry protocol could also be adapted to match previous studies and enable more accurate comparisons to published data (176, 186, 321).

One of the limitations of this work that needs to be addressed is that transplanted macrophages have not been definitively linked to *in vivo* transgene production. Future work will need to address the possibility of vector carry-over to confirm that the effects of treatment observed

here are not due to *in vivo* transduction. This might involve histological analysis to visualise transplanted cells *in situ* with a non-secreted reporter gene or testing of cell suspension supernatants.

Gene modified macrophage transplantation in GM-CSF KO mice, reduced biomarkers of disease 4 weeks after treatment. As this was only an initial proof-of-concept test in a therapeutic model, only two time points at 2- and 4 weeks were investigated. A more substantial effect of treatment might be observed after longer exposure to GM-CSF, as in clinical trials inhaled GM-CSF therapies are often administered daily over a 24-week time period or longer (161, 380-382). Here only BALF turbidity and concentration of surfactant protein-D were tested as disease biomarkers, while assessment of surfactant deposition by histological analysis of periodic acid-Schiff (PAS) is ongoing. To determine whether macrophage therapy represents a viable alternative treatment for these patients, the number of biomarkers assessed should ideally be expanded to include more clinically relevant outcome measures. Tazawa *et al* contended that the degree of lung function impairment in PAP patients is not reflected in the degree of surfactant deposition (399), whilst previous work by Helena Lund-Palau (unpublished) found that clinical endpoint measures used in human trials, including AaO₂, PaO₂, and radiological assessment could not be powered to measure the effect of treatment in GM-CSF KO mice.

The degree of engraftment observed in the absence of endogenous AM depletion will likely show a degree of disease specificity. GM-CSF KO mice are a prime example, where the lack of GM-CSF expression prevents AM maturation (39). This also highlights that more accurate autoimmune animal models of aPAP should be tested (694). It will not only be important to determine if vector-derived GM-CSF can increase bioavailability in the presence of autoantibodies, but as patients do produce GM-CSF, the state of the AM niche will not likely reflect that of GM-CSF KO mice. This is reflected by the variation in presentation between patients with hereditary and autoimmune PAP, where the former present at a much younger age (185, 339).

When extrapolating to other diseases, as macrophages are highly plastic cells, disease-specific perturbations of the lung environment will likely influence macrophage behaviour and engraftment (2). Therefore, although the wildtype studies are useful in establishing a general idea of the duration and strength of macrophage driven gene expression, they are unlikely accurately to model disease outcomes which will need to be assessed on a case-by-case basis. In

addition, the protocol used in each disease will also require specific adaptations. When aiming to express secreted proteins in the lung, wildtype results seen here suggest prior ablation of the AM niche is unnecessary to produce sustained expression or the production of therapeutic levels of GM-CSF in PAP mice. However, other diseases might require higher concentrations and necessitate a higher degree of engraftment that might be facilitated by depletion of endogenous AMs. Janosz *et al* found that even in the absence of a populated AM compartment in *Csf2rb*^{-/-} deficient mice, macrophages produced alpha-1 antitrypsin far below the postulated therapeutic threshold in humans, or physiological levels in mice (178).

Here I have also identified various mechanisms by which transduction of macrophages and subsequent gene expression *in vivo* might be increased or made more efficient. Key findings suggest that regulation of gene expression by EF1a or CMV might be more efficient than hCEF, VSV-G pseudotypes may have a slight advantage over F/HN vectors at higher MOIs, and that transduction of monocytes is more efficient than differentiated macrophages. Subsequent studies should investigate the effect of these changes in unison and using secreted transgenes. By adopting these changes, a more efficient vector may be used that could drive increased transgene expression. However, the evidence of GM-CSF mediated toxicity in PAP patients and animal models demonstrates that sustained levels of protein expression can have adverse consequences (384). To address any potential toxicity that might occur, I have also shown proof-of-concept for a regulated system of gene expression in macrophages that could overcome potential side effects of long-term, constitutive transgene expression.

Where significant increases of gene expression may be required, as appears likely in AATD, depletion of endogenous macrophages prior to administration may be the most efficient way to increase *in vivo* expression. However, ablating AMs in patients with already compromised lungs may not be well tolerated. Studies have demonstrated the efficiency of AM depletion following intratracheally inhaled aerosols of liposome-encapsulated clodronate, but thus far subsequent pulmonary macrophage transplantation therapies have not been investigated (427).

I also attempted to show proof-of-concept for macrophage cell therapy targeting inherent AM dysfunction by characterising alveolar macrophages in CF. As I was unable to recapitulate studies demonstrating that lack of functional CFTR inhibits either bacterial phagocytosis or killing of internalized bacteria testing the potential therapeutic benefits of lentiviral gene transfer of wildtype CFTR fell outside the scope of this project (604, 606, 688). Targeting

intrinsic AM dysfunction, as might be required in CF, would necessitate prior AM depletion, as transplanted macrophages would be required to adopt AM functional phenotypes. Therefore, to develop a therapeutic approach with an expanded disease repertoire, macrophage transplantation needs to be further characterised in animal models with existing and ablated AM niches.

This data represents initial work towards developing a macrophage cell therapy as a platform technology that could be used to target a variety of lung diseases. This approach could be useful in addressing AM dysfunction in CF, hereditary PAP, or diseases caused by a lack of secreted pulmonary proteins as is the case in AATD, aPAP and surfactant protein B deficiencies.

Conclusions

The results of this thesis support the following conclusions:

- Bone marrow derived macrophages (BMDMs) can be transduced in a dose-dependent manner by the F/HN.SIV vector to express secreted and intracellular proteins from the hCEF promoter.
- Following transplantation to the lungs of wildtype mice, donor-specific macrophages from Ly5.1 mice collected by broncho alveolar lavage can be identified by flow cytometry analysis of CD45.1 expression.
- Transplantation of donor derived-macrophages to the lung leads to dose-dependent cell retention and expression of a transgenic proteins one week post-delivery.
- Initial evidence suggests that transplanted macrophages can be retained in the lungs of wildtype mice for up to 16 weeks post-transplant, during which time transgene expression is sustained.
- Four-weeks post-transplantation to the lungs of GM-CSF knockout mice, macrophages expressing a secreted GM-CSF transgene can initiate alveolar macrophage maturation to stimulate breakdown of built-up surfactant and begin amelioration of a PAP-like phenotype.
- Further confirmation of macrophage phenotype following transplant and macrophage-specific transgene expression are required to support conclusions that transplanted macrophages can engraft and act as protein secretion factories in the lungs.
- Regulated transgene expression in macrophages was achieved using the commercial GeneSwitch™ system in a lentiviral vector expression cassette, where a dual vector configuration was more successful than a single vector counterpart.
- Lentiviral vectors with F/HN and VSV-G pseudotypes reported similar transduction efficiencies in BMDMs.
- CMV and EF1a promoter configurations drove higher levels of transgene expression (EGFP) in macrophages than hCEF.
- Transducing monocyte precursors during macrophage differentiation significantly increased transduction efficiency across multiplicities of infection.
- Further experiments are required to determine whether a lack of CFTR expression in macrophages affects their bactericidal activity and whether this could be corrected for by lentiviral gene transfer.

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