Mechanisms of pulmonary inflammation in ageing and chronic lung disease

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Abstract

The human respiratory tract is exposed to copious antigen over the course of the lifespan by virtue of its free communication with the external environment through the process of ventilation. The host immune system must therefore distinguish innocuous inhaled antigen in the respiratory tract from antigen potentially associated with infection, produce an inflammatory response with minimal collateral host damage if required, and allow a return to homeostasis once infection is cleared. Inadequacies of these processes can result in a predisposition to respiratory illnesses. Respiratory diseases are leading causes of morbidity and mortality worldwide. It is notable that the burden of respiratory disease disproportionately falls upon older adults, and disorders of inflammation arising with ageing may contribute to this disparity.

The work in this thesis describes a viable platform for the assessment of pulmonary inflammation that could be adapted to facilitate experimental medicine studies characterising inflammation in advanced age or early phase trials of immunomodulatory drugs that might alter the course and resolution of inflammation. This thesis also describes a method to identify candidate immunomodulatory drugs using connectivity maps and puts forward Del-1 as a target for drugs that enhance the resolution of inflammation.

In considering the role of inflammation in chronic lung disease, this thesis also presents an exploration of the mechanisms of inflammation in chronic hypersensitivity pneumonitis, using a systems biology approach to implicate tissue-resident memory T lymphocytes in the pathogenesis of the disease. This opens up the possibility of IL-15 signalling as a potential target for treatment of the disease where few treatments are currently proven to be effective.

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Chapter 1. Introduction

Inflammation is the set of processes that constitute the immune system's initial response to tissue injury that might be associated with an invading pathogen. Sterile and non-sterile tissue injury both result in acute inflammation, and the cellular responses that are initiated downstream of the initiating event are geared towards limiting microbial infection and repairing damaged tissues.

Inflammation itself can, however, be detrimental to the host. The cellular responses that are toxic to pathogens may also be toxic to host cells, and poorly controlled inflammation can increase the risk of this collateral damage. In addition, inflammation can be triggered inappropriately (as in allergy, for example), or persist in the absence of antigen (as can be the case in ageing).

The human respiratory tract is exposed to copious antigen over the course of the lifespan by virtue of its free communication with the external environment through the process of ventilation. The host immune system must therefore distinguish innocuous inhaled antigen in the respiratory tract from antigen potentially associated with infection, produce an inflammatory response with minimal collateral host damage if required, and allow a return to homeostasis once infection is cleared. Inadequacies of these processes can result in a predisposition to respiratory illnesses. Respiratory diseases are leading causes of morbidity and mortality worldwide even without considering COVID-19, the pandemic illness that has claimed millions of lives since its emergence in late 2019. It is notable that the burden of respiratory disease disproportionately falls upon older adults, and disorders of inflammation arising with ageing may contribute to this disparity.

As well as infectious diseases of the respiratory system, ageing appears to be associated with adverse outcomes in interstitial lung diseases, which often manifest as conditions characterised by disordered lung inflammation that can progress to fibrosis. Hypersensitivity pneumonitis is an interstitial lung disease in which the inflammation is driven by exposure to inhaled antigens in susceptible individuals, and once again, an increased burden of disease is seen in advanced age.

The work in this thesis aims to employ experimental medicine approaches to describe the cellular immune responses to inhaled antigens such as lipopolysaccharide (LPS) that contribute to inflammatory and infective respiratory disease as humans age. The thesis also aims to explore how inflammation and its resolution could be modulated by drugs to reduce the severity and impact of inflammation in respiratory disease.

1.1 The initiation of inflammation

The initial tissue response to infection or injury (*i.e.* inflammation) is characterised by increased trafficking of immune cells and blood components to the region of interest to promote microbial clearance and tissue repair (Medzhitov, 2008). The inflammatory response is triggered by cells at the site of the insult following their recognition of molecular motifs that might suggest invading pathogens (pathogen-associated molecular patterns, PAMPs) or damage to host cells (damage-associated molecular patterns, DAMPs). Various cell types express pattern recognition receptors (PRRs) that allow them to initiate the inflammatory response upon recognition of DAMPs or PAMPs (Takeuchi and Akira, 2010).

A cell on which PRRs are ligated typically responds by activating intracellular signalling pathways that promote the expression of products that perform antimicrobial functions and amplify the inflammatory response (Brubaker *et al.*, 2015). PRR ligation may also induce other changes in the cell such as the initiation of controlled cell suicide programs such as apoptosis and pyroptosis, which can contain the spread of infection by hampering the intracellular replication of microbes (Amarante-Mendes *et al.*, 2018).

1.1.1 Pattern recognition receptors (PRRs)

Immunity is often considered within a paradigm that separates it into *adaptive immunity* and *innate immunity*. The adaptive immune system relies upon genetic recombination to generate a massively diverse repertoire of lymphocyte receptors, and a lymphocyte expressing receptors that are circumstantially specific to a pathogen expands clonally upon encounter with a processed version of that pathogen-associated antigen, resulting in a form of immunological memory (Chaplin, 2010).

Pathogen recognition by the innate immune system, by contrast, can be initiated by germline-encoded receptors specific for molecules conserved over a range of microorganisms such as

the bacterial product lipopolysaccharide, which is recognised by the PRR Toll-like receptor 4 (TLR4) (Akira, Uematsu and Takeuchi, 2006). Although the resulting immune response is therefore less specific than that of the adaptive immune system, a rapid response is initiated at the time of the insult without the requirement for prior encounter with the antigen. Cell injury may also be sensed by PRRs that can recognise host cell components normally confined to the interior environments of neighbour cells. Damaged cells may leak heat shock proteins, for example, and heat shock proteins of 60 kilodalton molecular weight (hsp60) are recognised by TLR4 in a manner that stimulates inflammation (Ohashi *et al.*, 2000).

Four classes of PRR have been described: NOD-like receptors (NLRs), which are primarily intracellular sensors of bacterial components; retinoic acid-inducible gene-I-like receptors (RLRs), which are predominantly intracellular sensors of viral nucleic acids; C-type lectin receptors (CLRs), which are mostly transmembrane sensors of fungal PAMPs such as β-glucan; and the aforementioned Toll-like receptors (TLRs), which are perhaps the best-characterised PRRs. TLRs comprise a family of transmembrane receptors sensitive to a number of microbial- and self-derived ligands (Takeuchi and Akira, 2010), and are perhaps the most relevant PRRs to the work in this thesis.

1.1.2 Toll-like receptors

Toll-like receptors (TLRs) are so named due to their similarities in structure and function to the Toll family of proteins, which are transmembrane receptors that can act as mediators of innate immunity in *Drosophila* (Rosetto *et al.*, 1995; Lemaitre *et al.*, 1996). The likeness of Toll receptors in fruit flies and TLRs in mammals suggests that they provide for the same evolutionarily conserved immune mechanism to protect insects and mammals alike against invasion by microbes (Hultmark, 1994). The identification of a gene in plants with homologous sequences to Toll and a role in host defence may even suggest that such innate immune mechanisms arose as far back in phylogeny as the common ancestry of eukaryotic life two billion years ago (Whitham *et al.*, 1994; Kimbrell and Beutler, 2001).

TLRs are expressed in a wide range of human cells but are preferentially expressed in cells of immunological importance such as tissue-resident and peripheral blood leukocytes (due to their roles in immune surveillance) and the lung epithelium (due to its exposure to the external environment) (Zarember and Godowski, 2002). The first TLR to be characterised

functionally was human Toll, now known as TLR4 (Medzhitov, Preston-Hurlburt and Janeway, 1997). Like all TLRs, TLR4 is a transmembrane protein comprising an extracytoplasmic domain formed of a leucine-rich repeat (LRR) structure and a cytoplasmic domain that has homologous structure to Toll and the interleukin (IL)-1 receptor family (therefore termed the TIR domain) (Medzhitov, 2001). The shape formed by the LRR domains of two TLRs in a homologous or heterologous dimer allows for either direct interaction with a DAMP/PAMP or indirect DAMP/PAMP recognition in conjunction with accessory molecules (Bell *et al.*, 2003; Botos, Segal and Davies, 2011). Dimerised TLRs with a DAMP/PAMP bound to their LRR domains are able to recruit adaptor proteins such as myeloid differentiator primary response 88 (MyD88) to their TIR domains in the cytoplasm (Brubaker *et al.*, 2015). These adaptors subsequently initiate a cascade of intracellular signalling that results in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor that regulates the expression of genes encoding numerous pro-inflammatory cytokines (Kawai and Akira, 2007).

1.1.3 Cytoplasmic TLR signalling in the initiation of inflammation

The dimerisation of homologous TLRs (a pair of TLR4 molecules, for instance) or heterologous TLRs (perhaps TLR1 and TLR2 in complex) is not sufficient to initiate cytoplasmic signalling without the binding of a DAMP or PAMP to the extracytoplasmic domain of the dimer. It is likely that ligand binding on the ectodomains of a TLR dimer induces a conformational and/or positional change of the receptors that associates the TIR domains, forming a scaffold in the cytoplasm to which adaptor proteins can be recruited (Latz *et al.*, 2007; Miguel *et al.*, 2007).

The recruitment of adaptor proteins to the cytoplasmic domains of TLR dimers is the first step in the signalling that eventually allows NF-κB to translocate to the nucleus and facilitate the transcription of inflammation-associated genes. Although all known TLRs facilitate NF-κB activation, particular TLRs may also stimulate more specific responses in addition to the NF-κB response through the recruitment of different combinations of adaptor proteins following ligation (Kawai and Akira, 2010). TLR3, for example, senses double-stranded RNA (that is likely to be associated with viruses) in endosomes and recruits the TIR-domain-containing adaptor inducing interferon β (TRIF, also known as TICAM-1) adaptor to its dimerised TIR domains (Oshiumi *et al.*, 2003). As is implied by its name, signalling downstream of TRIF results in the

induction of cytokines including interferon β, which enables the cell to perform antiviral functions.

All TLRs except TLR3 can recruit the MyD88 adaptor protein to trigger the pathway that activates NF-κB, resulting in the archetypal cellular inflammatory response (O'Neill and Bowie, 2007). (TLR3 activates NF-κB through TRIF in a MyD88-independent pathway [Yamamoto *et al.*, 2003].) Once recruited to the TIR domains of ligated TLRs, MyD88 forms a complex with members of the IL-1 receptor-associated kinase (IRAK) family. The structure of these kinases in complex allows them to phosphorylate one another, after which they can recruit tumour necrosis factor receptor-associated factor 6 (TRAF6) to the complex (Cao *et al.*, 1996). Once recruited, TRAF6, a ubiquitin ligase, becomes active and synthesises polyubiquitin chains that bind to transforming growth factor β activated kinase-1 (TAK1), a kinase complex belonging to the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) family (Adhikari, Xu and Chen, 2007). TAK1 in turn becomes activated by this polyubiquitination and continues the cascade by phosphorylating and activating the inhibitor of NF-κB (IκB) kinase (IKK) complex, which goes on to phosphorylate the IκB proteins that keep NF-κB sequestered in the cytoplasm. This phosphorylation allows IκB to be polyubiquitinated to tag it for degradation by the 26S proteasome (DiDonato *et al.*, 1996). Freed from IκB, NF-κB may then enter the nucleus to facilitate the transcription of inflammatory cytokine genes (**Figure 1.1**) (Kawai and Akira, 2007). NF-κB can also be activated through a noncanonical pathway that involves the degradation of an IκB-like structure on an NF-κB protein precursor that subsequently translocates to the nucleus (Liu *et al.*, 2017).

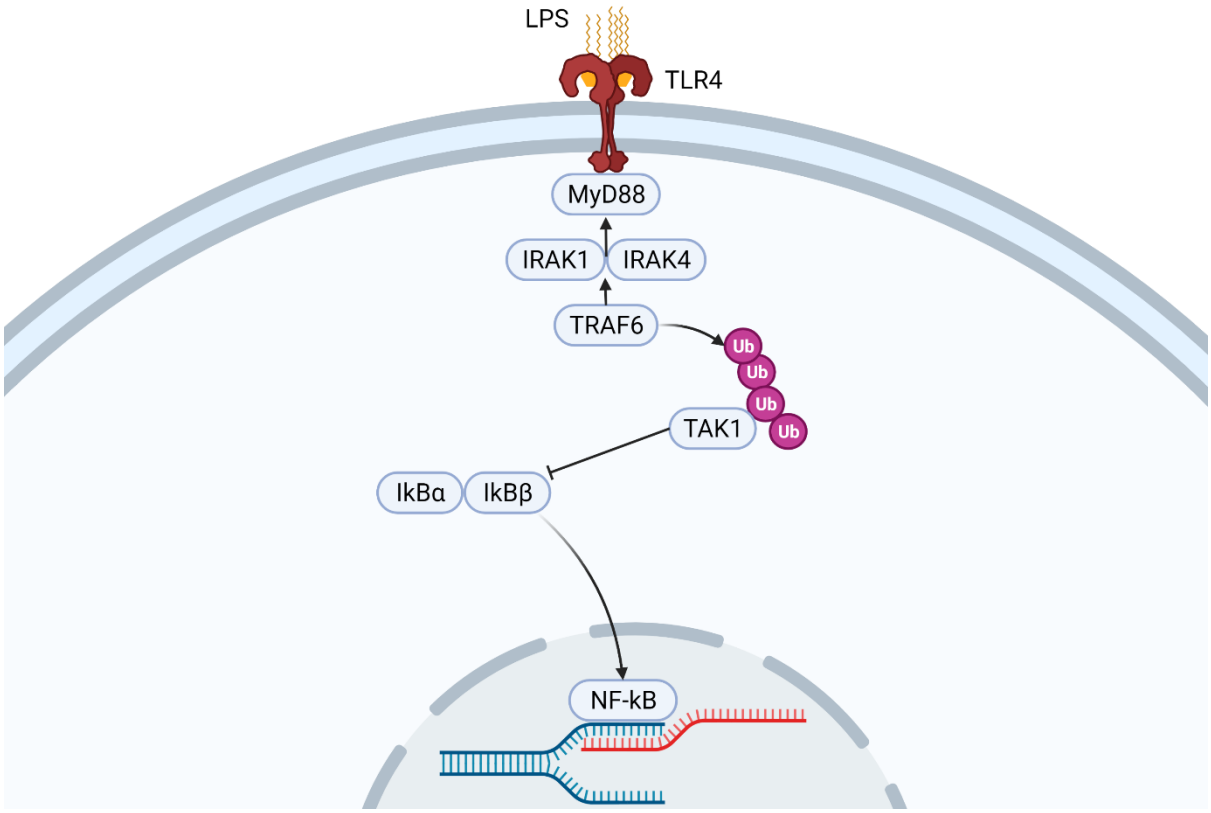

Figure 1.1*. TLR4 signalling through MyD88. Ligation of TLR4 by LPS recruits MyD88 to the TLR, which recruits IRAK proteins and then TRAF6 in turn. TRAF6 activates TAK1, which degrades IκB to set NF-κB free.*

1.1.4 NF-κB and target genes

Although often considered in the singular, NF-κB is a family of related transcription factors that form homo- and heterodimers and bind specific regions of DNA to promote the transcription of genes with a role in effecting inflammatory, immune and cell stress responses (Oeckinghaus and Ghosh, 2009). The five proteins comprising the NF-κB family (RelA, RelB, c-Rel, NF-κB1 and NF-κB2) have in common a domain of 300 amino acids termed the Rel homology domain (RHD) containing structures that enable the proteins to dimerise and bind to promoter/enhancer regions of NF-κB target genes (Barnes, 1997).

After being freed from IκB in the cytoplasm, NF-κB translocates to the nucleus to bind DNA segments with a consensus sequence of 5'-GGGRNWYYCC-3' (where R is any purine, N is any base, W is adenine or thymine and Y is any pyrimidine) known as κB elements (Wang *et al.*, 2012). κB elements are found in the promoter and enhancer regions of numerous genes including those encoding the cytokines IL-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α), which are important mediators of inflammation (Pahl, 1999). Once bound to κB elements, NF-κB facilitates the recruitment of co-transcriptional regulators and transcription apparatus to

the target, allowing transcription of the associated genes by RNA polymerases (O'Shea and Perkins, 2008).

1.1.5 NF-κB-associated cytokines and the recruitment of inflammatory cells

The mRNA transcribed from inflammatory cytokine genes after NF-κB nuclear translocation is translated into protein, then secreted to permit cytokine effector functions. As stated previously, an outcome of inflammation is increased delivery of blood components (including immune cells such as neutrophils) to the site of tissue injury/infection. As such, inflammatory cytokines act on cells in the vicinity to facilitate this: some cytokines may act on endothelial cells to increase capillary permeability; some cytokines may act as chemoattractants to draw immune cells out of the circulation down a concentration gradient; some cytokines may stimulate NF-κB activation in other cells to amplify inflammation in a positive feedback loop (Medzhitov, 2008; Dinarello, 2009). I consider a selection of the NF-κB-associated cytokines below.

1.1.5.1 Interleukin-1α and interleukin-1β

Of the multitude of pro-inflammatory gene products induced after NF-κB activation, perhaps the best understood are IL-1α and IL-1β. IL-1 cytokines were likely the molecules described when researchers first identified two proteins secreted by stimulated human leukocytes that were able to induce fevers in rabbits (Dinarello, Goldin and Wolff, 1974).

Despite being structurally distinct, both IL-1α and IL-1β are agonists of IL-1 receptors. IL-1 receptors have been detected in diverse tissues, but expression of the receptors on innate immune cells in particular enables the participation of these cells in the early stages of inflammation under the influence of IL-1α and IL-1β (Song *et al.*, 2018).

One of the mechanisms by which IL-1 cytokines can promote the influx of immune cells to the site of inflammation is by stimulating the release of chemokines such as IL-8 (also known as C-X-C motif chemokine ligand 8 [CXCL8]) from neighbouring cells (Cromwell *et al.*, 1992; Kida *et al.*, 2005). IL-8 is a potent mediator of neutrophil recruitment, and release of IL-8 at and around the site of infection/injury generates a concentration gradient down which neutrophils migrate before arriving to carry out their effector functions (Baggiolini and Clark-Lewis, 1992; Das *et al.*, 2010). IL-1 may also enhance the effector function of the arriving

neutrophils: exposure to IL-1β appears to potentiate the IL-8-induced release of microbicidal granules by neutrophils (Brandolini *et al.*, 1996).

IL-1 also facilitates the arrival of immune cells at an inflamed site by causing the upregulation of adhesion molecules on capillary endothelium, allowing circulating leukocytes to attach to capillary walls to begin the process of transmigrating into the tissue (Bevilacqua *et al.*, 1985; Schleimer and Rutledge, 1986). IL-1β may also affect the integrity of endothelial cell junctions, thereby increasing microvascular wall permeability to permit greater immune cell efflux during inflammation. In experiments involving mice injected with LPS to induce systemic inflammation, pulmonary vascular permeability was increased in a manner that suggested IL-1β impaired the production of vascular endothelial cadherin, a mediator of cell-cell adhesion (Xiong *et al.*, 2020).

Another important function of IL-1 cytokines is to sustain inflammation through a positive feedback loop in which IL-1 induces more IL-1. In diverse cell types in culture, treatment with recombinant forms of IL-1α and IL-1β stimulates the production of further IL-1α and pro-IL-1β mRNA (Dinarello *et al.*, 1987; Warner, Auger and Libby, 1987). Pro-IL-1β is an inactive form of the cytokine, which becomes active once cleaved into IL-1β by the intracellular protease caspase-1. Caspase-1 is activated by a group of protein complexes known as inflammasomes, which are discussed below.

1.1.5.1.1 The inflammasome

The pro-inflammatory cytokine IL-1β is notable for its requirement for proteolytic cleavage by proteases such as caspase-1 in order to become bioactive. The inflammasome, which activates caspase-1, functions as an intracellular protein platform to facilitate activation of IL-1β in this way, and seeing as inflammasomes are activated by signals including IL-1 family cytokines, they also function as part of the positive feedback loop of IL-1 induction (mentioned above) that amplifies inflammation (Chen *et al.*, 2020; Romero *et al.*, 2022).

Four inflammasomes have been described, and although these differ in structure, the typical composition is of an NLR (the NOD-like pattern recognition receptors mentioned earlier), the ASC (apoptosis-associated Speck-like protein containing a caspase activation and recruitment domain) adaptor protein, and caspase-1 (van de Veerdonk *et al.*, 2011).

The inflammasome perhaps best described is the NLRP3 inflammasome (named for the NLR in its structure). Expression of the NLRP3 protein is induced by NF-κB-activating substances including IL-1β and TLR ligands (including LPS), although this is insufficient for the activation of the inflammasome (Bauernfeind *et al.*, 2009). The mechanism of subsequent activation of the inflammasome is unclear, although it has been suggested that events associated with antimicrobial defence (such as damage to cell lysosomes caused by engulfment of DAMPs, or the formation of microbicidal superoxide anions — discussed in section 1.2.2) may stimulate inflammasome activation (van de Veerdonk *et al.*, 2011). The activated inflammasome allows the activity of caspase-1, which is an important converter of pro-IL-1β to its bioactive form, meaning the inflammasome is an important regulator of inflammation as driven by the IL-1 family signalling pathway. IL-18 is another pro-inflammatory cytokine that is activated by caspase-1 in this manner (Gross *et al.*, 2011).

1.1.5.2 Interleukin-6

IL-6 is an NF-κB-associated cytokine that appears to have both pro- and anti-inflammatory properties depending on context and the expression of its receptor components by target cells (Scheller *et al.*, 2011). The classical model of IL-6 receptor activation is one in which the IL-6 receptor (IL-6R) and its signal-transducing subunit gp130 are bound in a plasma membrane, and free IL-6 ligates the receptor. Alternatively, cleaved, soluble IL-6R (sIL-6R) may form a complex with free IL-6, then interact with membrane-bound gp130: this is known as IL-6 *trans*-signalling, and allows for communication with cells lacking IL-6R (Hurst *et al.*, 2001). It is also possible for IL-6 in complex with sIL-6R to bind a soluble form of gp130 (sgp130), forming a structure that is unable to interact with membrane-bound IL-6R or gp130, thereby abrogating signalling (**Figure 1.2**) (Garbers, Aparicio-Siegmund and Rose-John, 2015; Kaur *et al.*, 2020).

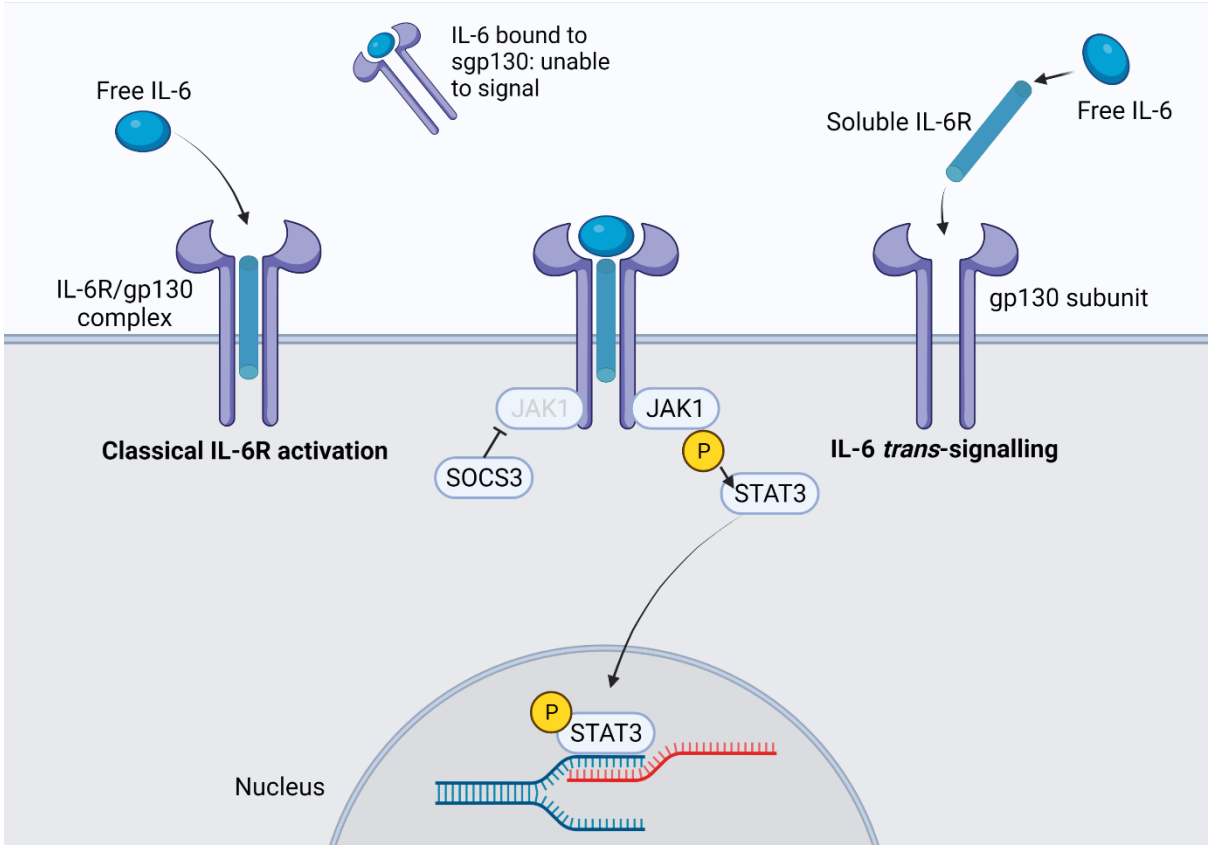

***Figure 1.2**. IL-6 signalling. Once activated either through classical signalling with free IL-6 or trans-signalling by sIL-6R, JAK1 phosphorylates STAT3, which translocates to the nucleus to promote transcription of its gene targets. SOCS3 inhibits JAK1 as an autoregulatory mechanism.*

Intracellular signalling distal to the IL-6 receptor complex is facilitated by Janus kinase (JAK) enzymes that phosphorylate portions of the cytoplasmic part of gp130 upon receptor ligation (Eulenfeld *et al.*, 2012). This phosphorylation enables the recruitment of signal transducer and activator of transcription 3 (STAT3, a transcription factor), which is phosphorylated by gp130-associated JAK1 before translocating to the nucleus to promote the transcription of its gene targets (Heinrich *et al.*, 1998). JAKs also prepare sites on gp130 that enable it to recruit molecules that *inhibit* STAT3-mediated signalling to provide a mechanism for autoregulation. One of the inhibitory molecules is the enzyme Src homology 2 domain-containing phosphatase 2 (SHP2), which dephosphorylates portions of gp130 that are phosphorylated by JAKs (Schaper and Rose-John, 2015). Another is the protein suppressor of cytokine signalling 3 (SOCS3), which inhibits the activity of JAK1 (Garbers, Aparicio-Siegmund and Rose-John, 2015).

An important function of IL-6-activated STAT3 in immunity is to imprint naïve T lymphocytes with a phenotype — the T helper 17 (Th17) phenotype — that renders them specialists in the

production of pro-inflammatory IL-17 family cytokines (McGeachy *et al.*, 2007; Yang *et al.*, 2007; Tripathi *et al.*, 2017). These cytokines can stimulate the production of antimicrobial peptides such as defensins at the epithelial barrier to augment host defences (Li, Casanova and Puel, 2018). The IL-17 produced by Th17 cells is also able to mediate further immune cell recruitment to the site of invasion by recapitulating NF-κB activation downstream of the IL-17 receptor (Chang, Park and Dong, 2006).

A potential mechanism for the paradoxical anti-inflammatory effect of IL-6 could be evident in the observation that Th17 cells produce the anti-inflammatory cytokine IL-10 under the influence of IL-6 when the cytokine transforming growth factor beta (TGF-β) is also present (McGeachy *et al.*, 2007). TGF-β is produced by cells during the resolution of inflammation as redundant inflammatory cells are cleared (Fadok *et al.*, 1998).

1.1.5.3 Interleukin-8

The NF-κB target IL-8 (also known as CXCL8) belongs to the family of C-X-C chemokines, a group of proteins named for their shared structural motif of two cysteine (C) residues sandwiching another amino acid (X) residue near the N-terminus (Baggiolini, Dewald and Moser, 1993). A potent mediator of the initial influx of neutrophils in inflammation, IL-8 was first identified as a factor produced by LPS-stimulated human monocytes that was able to attract neutrophils *in vitro* (Yoshimura *et al.*, 1987; Matsushima *et al.*, 1988).

Neutrophils sense IL-8 with CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2), which signal to the Rac and Rho hydrolase enzymes that facilitate cytoskeletal rearrangement in order to effect chemotaxis (Rajarathnam *et al.*, 2019). Neutrophils thus migrate down an IL-8 concentration gradient, and at the onset of inflammation the concentration is likely to be greatest at the site of PAMP/DAMP encounter due to TLR-mediated NF-κB activation.

CXCR1 also signals through parallel intracellular pathways to enhance the microbicidal functions of neutrophils exposed to IL-8. As mentioned above, IL-8 stimulates the release of antimicrobial granules from neutrophils, and also activates the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates the superoxide free radicals that form part of a neutrophil's antimicrobial arsenal (Jones *et al.*, 1996).

1.1.5.4 Tumour necrosis factor α

TNF-α was first identified in the 1970s as a host-derived factor with anti-tumour properties that appeared in the serum of mice injected with LPS (Carswell *et al.*, 1975). TNF-α (encoded by the gene *TNF*) is transcribed under the control of NF-κB as a transmembrane molecule that can then be cleaved into soluble form by TNF-α-converting enzyme (TACE). Both forms of the molecule may interact with its cognate receptors TNF receptor 1 (TNFR1) or TNF receptor 2 (TNFR2) to produce its effects on inflammation and cell survival (Kalliolias and Ivashkiv, 2016).

Activation of TNF receptors may amplify the NF-κB-related inflammatory response by signalling through TAK1, which results in the degradation of IκB (as with TLR signalling described above) to allow further NF-κB to translocate to promote transcription of its target genes, including *TNF* itself (Brenner, Blaser and Mak, 2015).

Aside from reproducing the NF-κB response, TNF-α also acts directly to support inflammation. TNF receptor signalling induces the surface expression of adhesion molecules on endothelial cells, allowing the capture and extravasation of circulating leukocytes once inflammation has been initiated (Chandrasekharan *et al.*, 2007).

Immune cells arriving at the site of inflammation are supported to carry out their functions through signalling that prolongs the survival of the cells, and TNF-α (as a consequence of NF-κB activation) contributes to this by inducing proteins that increase the threshold for initiation of immune cell suicide programs such as apoptosis (Wang *et al.*, 1998). This anti-apoptotic effect of TNF-α appears to be conditional on the adaptor protein complexes recruited to the cytoplasmic portion of TNFR1 immediately after ligand binding. If the adaptor complex not containing the ubiquitin ligase RNF8 (termed TNFR1 complex 1) is recruited, the receptor signals to activate NF-κB, leading to the subsequent transcription of anti-apoptotic gene products. If RNF8 is recruited, however, the ligase ubiquitinates TNFR1, allowing the receptor to be internalised in an endosome. The adaptor complex that then forms at the cytoplasmic portion of the endosomal TNFR1 (TNFR1 complex 2) allows the activation of caspase 8, a mediator of apoptosis, which leads to cell death (Fritsch *et al.*, 2014, 2017). With RNF8 acting as a cell fate checkpoint in this manner, TNF-α demonstrates pro- or anti-apoptotic properties depending on additional environmental signals.

1.2 Effector cells of inflammation

Although cells of non-haematopoietic origin play an important role in initiating inflammation — in the lung, for example, the sensing and signalling functions of the respiratory epithelium and other airway structural cells contribute to defence against microbes (Parker and Prince, 2011; Perros, Lambrecht and Hammad, 2011) — the immune cells of haematopoietic origin are usually considered the effector cells of innate immunity and inflammation. The cell types with the greatest relevance to the work in this thesis are considered below.

1.2.1 Macrophages

As sentinel cells stationed in most tissues, macrophages are among the earliest immune cells to encounter interloping antigen. Macrophages take environmental cues to adopt specialised phenotypes specific to their tissue of residency (Gosselin *et al.*, 2014), but between tissues the cells perform broadly similar roles in immune surveillance and defence, tissue development, tissue homeostasis and tissue repair (Okabe and Medzhitov, 2016).

Although macrophages were previously considered to be derived exclusively from circulating blood monocytes (van Furth and Cohn, 1968), more recent investigation has demonstrated that tissue macrophages may also originate in variable proportions from erythro-myeloid progenitor cells in the extra-embryonic yolk sac and the foetal liver (Ginhoux and Guilliams, 2016). Once engrafted in their tissue niche, macrophages develop the capability to proliferate and self-maintain *in situ* over the course of the lifespan without necessarily requiring ongoing contribution from circulating monocytes (Hashimoto *et al.*, 2013; van de Laar *et al.*, 2016). Largely due to ease of access, scientific investigation of macrophage function frequently employs blood monocytes conditioned into a macrophage-like phenotype. This might be a good model of macrophage function but it is not a perfect one: monocyte-derived macrophages remain transcriptionally and phenotypically distinct from tissue macrophages even after differentiation (Gundra *et al.*, 2014; Gibbings *et al.*, 2015).

By virtue of their presence within tissues, their strong expression of pattern recognition receptors and their capacity for signalling, macrophages function as an early warning system for pathogenic invasion or tissue damage. Using pathways downstream of pattern recognition receptors as described above, macrophages may signal for backup from circulating immune cells when DAMPs or PAMPs are encountered (Okabe and Medzhitov, 2016). In addition,

macrophages provide a valuable link to the adaptive immune system by phagocytosing microbes and presenting microbial-derived peptides to lymphocytes. Macrophage populations with phenotypes optimised for antigen presentation such as skin Langerhans cells may preferentially do this directly (Doebel, Voisin and Nagao, 2017), but certain macrophage types may pass antigen onward to dendritic cells — specialists in antigen presentation — to be presented to lymphocytes (Backer *et al.*, 2010).

As part of their role in maintaining and restoring tissue homeostasis, macrophages are also the primary contributors to the clearance of corpses of infiltrating immune cells that have served their function and since become apoptotic (Poon *et al.*, 2014). This process, a particular form of phagocytosis termed “efferocytosis”, is a pivotal process in the resolution of inflammation, and will be discussed later in this section.

1.2.2 Neutrophils

Neutrophils, phagocytic leukocytes derived from myeloid progenitor cells in the bone marrow, make up the largest fraction of leukocytes in human blood (Ley *et al.*, 2018). While macrophages are perhaps more suited to surveillance and signalling, neutrophils specialise in killing the microbes they ingest, containing granules harbouring antimicrobial compounds that fuse with phagosomes to kill the phagocytosed pathogens (Amulic *et al.*, 2012). The phagosomal membrane also contains the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyses the formation of superoxide anions and hydrogen peroxide. The enzyme myeloperoxidase (contained within granules entering the phagosome) catalyses the formation of hypochlorous acid from hydrogen peroxide, superoxide and chloride. Hypochlorous acid has a microbicidal action in the phagosome (Nauseef, 2014). Neutrophils may also release microbicidal granules into the microenvironment under the influence of secretagogue molecules such as the PAMP N-formyl-methionyl-leucyl-phenylalanine (fMLF) in a process known as degranulation (Lacy, 2006).

Although in inflammation it is often advantageous for neutrophils to arrive carrying their toxic cargo ready to phagocytose and destroy microbes, it is also the case that the granule contents — including serine proteases such as neutrophil elastase — may be harmful to host cells. The injury caused to host cells by neutrophil products at a site of inflammation can be enough to

create a feedback loop that recruits further neutrophils in response to the collateral tissue damage rather than the pathogen, forming an inflammatory circuit that may even be lethal (Brandes *et al.*, 2013).

The homeostatic functions of macrophages may be important in mitigating the deleterious effects of excessive neutrophil infiltration in inflammation. In a murine model of inflammation, macrophages have been observed to migrate to shield tissue microlesions from activated neutrophils, although this mechanism can be overwhelmed with enough prior tissue injury relative to the number of available macrophages in the region (Uderhardt *et al.*, 2019). Efferocytosis, as mentioned above, is another important macrophage function that limits collateral tissue damage by neutrophils: neutrophils may become apoptotic at an inflamed site, which allows them to be efferocytosed by macrophages before they would otherwise become necrotic and leak their histotoxic contents into the microenvironment (Poon *et al.*, 2014).

1.2.3 Helper T lymphocytes

Antigen naïve T lymphocytes expressing the surface protein CD4 (naïve $CD4^+$ T cells) are able to produce inflammatory effects once differentiated into T helper cells, at which point they also begin to expand clonally, amplifying their effector functions (Bhattacharyya and Feng, 2020). When presented with its cognate antigen by an antigen-presenting cell in the presence of particular signals in the microenvironment, a naïve T cell may differentiate into a T helper cell specialised in the production of combinations of inflammatory cytokines associated with antimicrobial defence (Zhu and Paul, 2008). The canonical paradigm of T helper cell differentiation is of rigid differentiation into subtypes (for example Th1 cells specialised in producing cytokines such as interferon gamma that augment defence against intracellular pathogens, and Th2 cells specialised in secreting cytokines including IL-5 that aid defence against extracellular parasites) but it is now appreciated that T helper cells are more fluid in their differentiation than that paradigm suggests, with plasticity between subtypes in appropriate circumstances (O'Shea and Paul, 2010). Nonetheless, the nomenclature of subtypes is helpful, and the described subtypes also include the Th17 cells mentioned previously that differentiate from naïve T cells in the presence of IL-6 and secrete pro-inflammatory IL-17 family cytokines involved in defence against extracellular bacterial pathogens.

Once the initial response to pathogen subsides the majority of T cell clones will die, but a proportion will survive as memory cells that can provide a rapid response to a repeat encounter with their cognate antigen (MacLeod *et al.*, 2009).

1.2.4 Memory T lymphocytes

Once T lymphocytes specific to a foreign antigen are differentiated into memory T cells, they can play a powerful role in rapid host defence when an antigen is reintroduced, particularly if they are stationed at epithelial barrier sites such as the lung airway mucosa.

Tissue-resident memory T cells (T_{RM} cells) are a recently identified subgroup of memory T lymphocytes with a phenotype that enables them to become durably resident at epithelial barrier sites, poised for encounter with their cognate antigen (Sasson *et al.*, 2020). Although the mechanism of their reactivation has not been described completely, reactivated T_{RM} cells can proliferate *in situ* and contribute to antimicrobial defence by secreting pro-inflammatory cytokines and performing cytolytic functions depending on the target pathogen (Paik and Farber, 2021).

1.2.5 Regulatory T lymphocytes

Regulatory T lymphocytes (Treg cells) are a subgroup of T cells that provide an autoregulatory, immunosuppressive function to prevent unrestrained inflammation and autoimmunity. Treg cells are distinguished by their expression of the transcription factor forkhead box P3 (FOXP3), and once activated, diminish the effector capabilities of other T cells at the site of inflammation (Sakaguchi *et al.*, 2008). A number of mechanisms of immunoregulation by Tregs have been suggested, including the secretion of the immunosuppressive cytokine IL-10, and the capture and sequestration of IL-2, a cytokine important for the activity of effector T cells (Li *et al.*, 2015).

1.3 The resolution of inflammation

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The resolution of inflammation is an active rather than a passive process, with the return to homeostasis facilitated by the clearance of extravasated immune cells from the inflamed site by efferocytosis as described previously. As well as providing a safe disposal mechanism for

the potentially toxic corpses of neutrophils, efferocytosis results in the upregulation of signalling by the efferocytosing cells that limits further leukocyte recruitment to the inflamed tissue (**Figure 1.3**). This signalling, as well as the downregulation of pro-inflammatory signalling, supports a return to quiescence when inflammation is no longer desirable (Serhan and Savill, 2005).

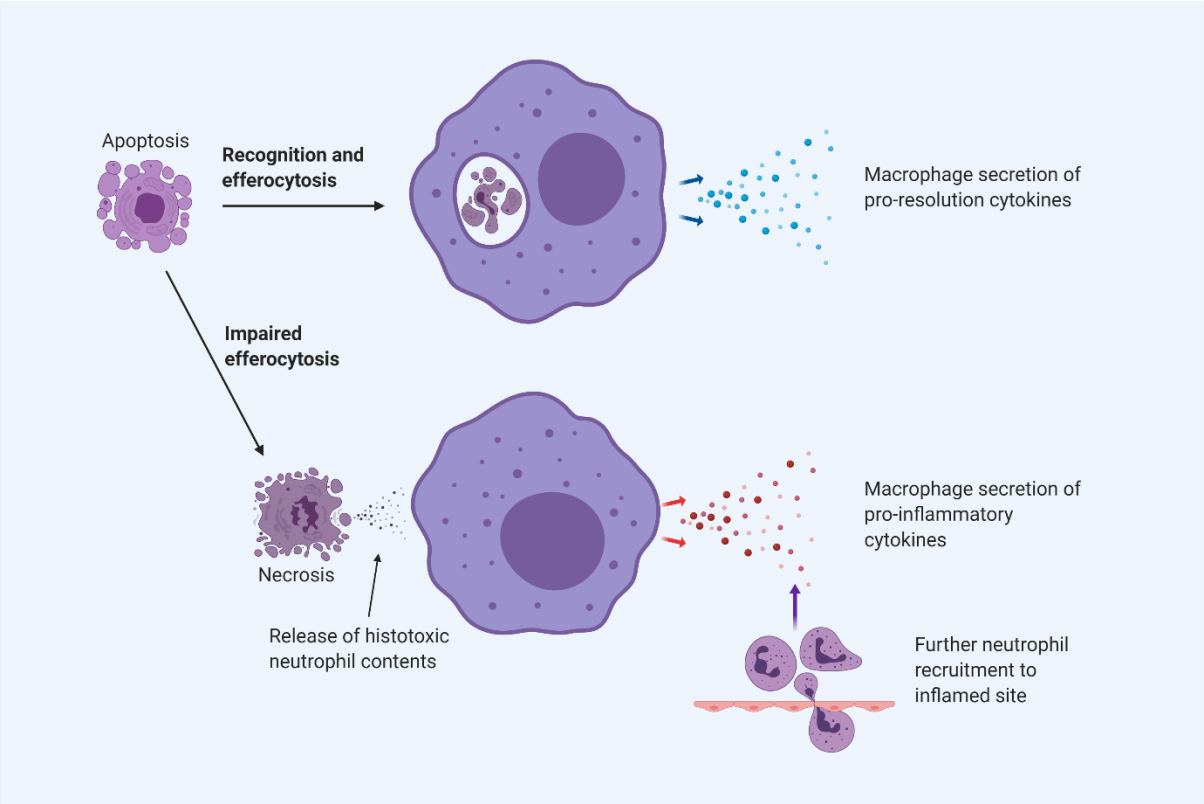

***Figure 1.3**. Macrophage efferocytosis. An apoptotic neutrophil at an inflamed site is recognised by a macrophage and phagocytosed. The interaction of the macrophage with the apoptotic cell results in macrophage secretion of pro-resolution cytokines and downregulation of pro-inflammatory cytokines. If efferocytosis is impaired (as in ageing), the apoptotic neutrophil may progress to secondary necrosis, allowing leakage of histotoxic contents, resulting in tissue injury, further pro-inflammatory signalling and chronic inflammation.*

Central to the process of efferocytosis is the means by which cells undertaking efferocytosis — most commonly macrophages, but other cells including epithelial cells and fibroblasts may contribute, to varying degrees (Arandjelovic and Ravichandran, 2015) — recognise that a target has outlived its usefulness and is ready to be phagocytosed. Cells are flagged for efferocytosis when they enter apoptosis, a state of programmed cell death characterised by a structural rearrangement of the plasma membrane and nuclear material (Kerr, Wyllie and Currie, 1972; Savill and Haslett, 1995).

1.3.1 Apoptosis in the resolution of inflammation

In experiments involving the co-culture of human monocyte-derived macrophages with isolated neutrophils, Savill and colleagues demonstrated that the proportion of neutrophils ingested by the macrophages was directly linked to the proportion of neutrophils that were apoptotic (Savill *et al.*, 1989). It was therefore concluded that apoptosis acts as a flag by which macrophages preferentially recognise redundant neutrophils for clearance, suggesting that the process plays a central role in the resolution of inflammation.

During apoptosis, cells undergo morphological changes that result in condensed nuclei, closely packed organelles and lucent cytoplasmic vacuoles (Kerr, Wyllie and Currie, 1972). Another crucial structural change in apoptotic cells is the oxidation and relocation of the membrane lipid phosphatidylserine, which moves from the inner plasma membrane leaflet to the outer plasma membrane leaflet. The increased outward exposure of oxidised phosphatidylserine by apoptotic cells appears to be a signal by which macrophages recognise them for engulfment in preference to non-apoptotic cells (Fadok *et al.*, 1992; Greenberg *et al.*, 2006).

Apoptosis is usually initiated either in response to an extracellular pro-apoptotic stimulus or a perturbation of the intracellular environment; these apoptotic pathways are referred to as the extrinsic and intrinsic apoptotic pathways respectively (Galluzzi *et al.*, 2018). Both pathways lead to the activation of intracellular endoproteases known as caspases that facilitate a cascade of further protease and DNase activity, eventually leading to the structural and functional changes that are characteristic of apoptosis (McIlwain, Berger and Mak, 2013).

The extrinsic apoptotic pathway is activated by the engagement of a death receptor on the cell surface by its ligand. The best described death receptors are members of the tumour necrosis factor receptor superfamily including Fas, TNFR1, death receptor (DR) 4 and DR5 (Ashkenazi and Dixit, 1998). Ligation of a death receptor by a molecule such as Fas ligand or TNF-α (ligands of Fas and TNFR1 respectively) allows the formation of the death-inducing signalling complex (DISC), a protein complex that dimerises the inactive procaspase-8 into active caspase-8 (Scott *et al.*, 2009). Caspase-8 can then either trigger the common terminal portion of the apoptotic signalling cascade by activating the executioner caspases -3, -6 and -7, or can secondarily activate the intrinsic apoptotic pathway by cleaving the BH3-interacting domain death agonist (BID) protein into its active form, truncated BID (tBID) (**Figure 1.4**).

Active tBID plays a role in permeabilising the outer mitochondrial membrane, which is a defining feature of the intrinsic apoptotic pathway (McIlwain, Berger and Mak, 2013; Kalkavan and Green, 2018).

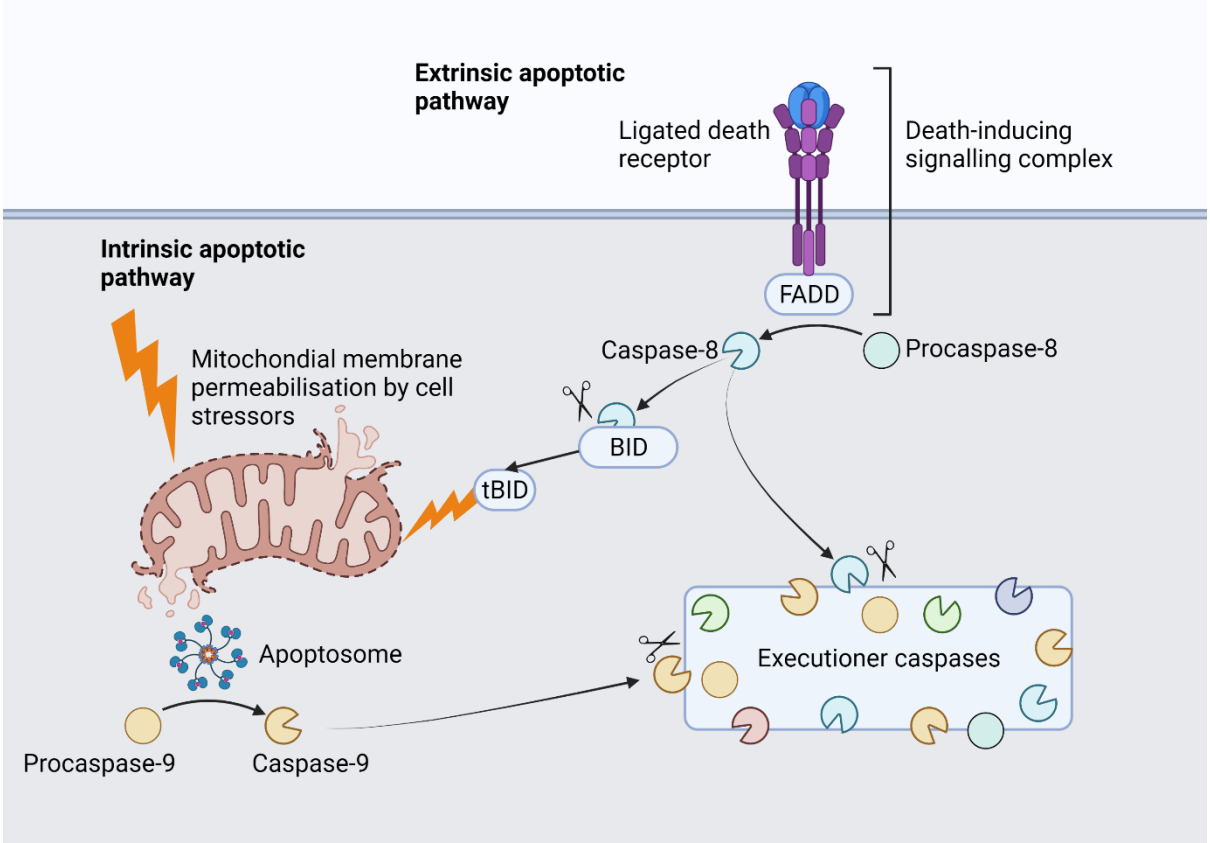

***Figure 1.4**. Initiation of apoptosis. Apoptosis can be initiated by the extrinsic pathway (ligation of a death receptor on the cell surface) or by the intrinsic pathway (disturbance of intrinsic cell homeostasis leading to mitochondrial outer membrane permeabilisation). BID truncated to tBID by caspase-8 provides crosstalk between the two pathways.*

As opposed to indirect stimulation by crosstalk from the extrinsic pathway via tBID, direct initiation of the intrinsic apoptotic pathway occurs when DNA damage or other disturbances in the intracellular environment are detected (Wu and Bratton, 2013; Kalkavan and Green, 2018). These disturbances inactivate cell survival-promoting members of the Bcl-2 protein family, which results in mitochondrial outer membrane permeabilisation. The permeabilised mitochondria release cytochrome *c* into the cytosol which can then interface with apoptotic protease-activating factor 1 (Apaf-1) to form an annular protein complex known as the apoptosome (Bratton and Salvesen, 2010). The cytochrome *c*-Apaf-1 apoptosome activates procaspase-9, and active caspase-9 can then proceed to activate the executioner caspases (**Figure 1.4**) (Youle and Strasser, 2008; Kalkavan and Green, 2018).

The executioner caspases are important actors in the exposure of phosphatidylserine on the cell surface during apoptosis. As mentioned above, phosphatidylserine is principally confined to the inner leaflet of the plasma membrane of a viable cell. This sequestration occurs against a charge gradient and is therefore an active process, requiring ATP-dependent lipid transport enzymes known as aminophospholipid translocases or flippases (Zachowski, 1993; Tang *et al.*, 1996). Caspase-3 activity during apoptosis is associated with aminophospholipid translocase inactivation, allowing phosphatidylserine to accumulate on the outer surface of the cell (Mandal *et al.*, 2002). In addition, caspases -3 and -6 activate Xk-related protein 8, a scramblase enzyme that, when active, facilitates movement of phosphatidylserine from inner to outer plasma membrane leaflets (Suzuki *et al.*, 2013).

While the majority of membrane phosphatidylserine is localised to the inner membrane leaflet in a viable cell, this localisation is by no means absolute. Indeed, phosphatidylserine can be observed on the outer surface of non-apoptotic cells (Elliott *et al.*, 2005). As such, macrophages involved in clearing apoptotic material should ideally be able to distinguish between cells that are truly apoptotic and those that are externalising phosphatidylserine while they are viable. Borisenko and colleagues demonstrated that the proportion of Jurkat T cells ingested by macrophages in an efferocytosis assay increased with greater incorporation of phosphatidylserine liposomes into the Jurkat cell membranes, but only after reaching a threshold phosphatidylserine concentration (Borisenko *et al.*, 2003). This suggests that macrophages might ignore cells externalising phosphatidylserine below a critical level required for recognition for efferocytosis.

Although it had been assumed previously that phosphatidylserine exposure in and of itself was sufficient for macrophage recognition of an apoptotic cell, Greenberg *et al.* demonstrated that the interaction between the efferocytosing cell and its apoptotic prey is also dependent on the oxidation of phosphatidylserine prior to its exposure. In their experiments, cells with oxidised phosphatidylserine incorporated into their membranes were more readily phagocytosed by macrophages than cells with membranes enriched with non-oxidised phosphatidylserine (Greenberg *et al.*, 2006).

Oxidation of phosphatidylserine during apoptosis can occur as a consequence of increased reactive oxygen species (ROS) production by NADPH oxidase within the cell, and this oxidised phosphatidylserine can then be exposed on the outer surface of the cell when flippases are

inactivated and scramblases are activated (Arroyo *et al.*, 2002). An apoptotic neutrophil with oxidised phosphatidylserine exposed on its surface is then readily recognised by macrophages for efferocytosis, a pivotal step in the resolution of neutrophilic inflammation.

1.3.2 Chemotaxis of macrophages towards apoptotic cells

For efferocytosis to take place it is often necessary for macrophages to migrate towards regions of an inflammatory milieu containing greater numbers of apoptotic cells. To promote this, apoptotic cells release chemotactic mediators that serve to draw macrophages towards them. Lauber *et al.* found that culture supernatants of varieties of cells irradiated to induce apoptosis were able to stimulate migration of macrophages and monocytes. Further testing established one of the chemotactic factors to be the phospholipid lysophosphatidylcholine (LPC), which was released by the apoptotic cells after calcium-independent phospholipase A_2 was activated in the cytosol by caspase-3 (Lauber *et al.*, 2003). Phospholipase A_2 hydrolyses phosphatidylcholine in the cell membrane to arachidonic acid and LPC.

LPC is a ligand of the G-protein coupled receptor G2A, which is expressed on macrophages. In experiments to define the nature of the interaction between LPC and the receptor, Li Yang and colleagues showed that wild type mouse peritoneal macrophages more readily migrated towards LPC in a Transwell assay than G2A-deficient macrophages. Migration of both sets of macrophages towards the chemotactic agent complement component 5a (C5a) was similar, suggesting unimpaired general chemotactic ability in the G2A-deficient cells (Yang *et al.*, 2005).

Sphingosine-1-phosphate (S1P) is another lipid mediator that has been identified as a chemoattractant signal from apoptotic cells to scavenging macrophages. Gude *et al.* found that apoptotic cells of lymphoid and myeloid lineage upregulated their expression of the enzyme sphingosine kinase, which phosphorylates the cell membrane lipid sphingosine to S1P. The S1P secreted by the apoptotic cells was found to be a potent attractant of monocytic cells in cell migration assays (Gude *et al.*, 2008). Extracellular S1P is recognised by S1P receptors on macrophages, and in particular the S1P receptor isoforms $S1PR_1$ and $S1PR_3$ appear to be involved in macrophage migration towards high concentrations of S1P (Keul *et al.*, 2011; Weichand *et al.*, 2013).

Non-lipid chemotactic mediators of macrophage migration towards apoptotic cells have also been described. The nucleotides ATP and UDP, found in greater concentrations in the supernatants of apoptotic primary thymocytes and Jurkat T cells by Elliott *et al.*, were shown to have monocyte chemoattractant ability *in vitro* and *in vivo* through their interactions with P2Y purinergic receptors on monocytic cells (Elliott *et al.*, 2009). Fractalkine (CX3CL1) has also been shown to induce chemokinesis of macrophages towards apoptotic cells. Truman and colleagues were able to demonstrate that the transmembrane protein is cleaved into its active, soluble form at the time of apoptosis either by direct or indirect action of caspases, and active fractalkine then acts to attract macrophages after its secretion through interaction with the receptor protein CX3CR1 on the scavenging cells (Truman *et al.*, 2008).

Fractalkine has the additional effect of stimulating the secretion of the milk fat globule-epidermal growth factor-factor 8 (MFG-E8) glycoprotein from macrophages. MFG-E8 acts as an opsonin; after its secretion it forms a bridge between oxidised phosphatidylserine on the surfaces of apoptotic cells and integrins on macrophage surfaces, thereby allowing macrophage recognition of the apoptotic cell and the initiation of efferocytosis (Hanayama *et al.*, 2002; Miksa *et al.*, 2007).

1.3.3 Macrophage recognition of apoptotic cells for engulfment

Although by no means an exclusive flag for macrophage engulfment, oxidised phosphatidylserine on the apoptotic cell surface can engage with a macrophage to stimulate phagocytosis in a variety of ways (**Figure 1.5**). Some macrophage membrane receptors such as brain-specific angiogenesis inhibitor 1 (BAI1) and T cell membrane protein 4 (TIM4) have been shown to engage directly with phosphatidylserine, but engagement can also occur via bridging molecules such as MFG-E8, which serves as a link between phosphatidylserine and macrophage αvβ3 or αvβ5 integrins as described above (Miksa *et al.*, 2007; Poon *et al.*, 2014). Thrombospondin 1 (TSP1) is another bridging molecule that has been shown to tether apoptotic neutrophils to macrophages by binding CD36 and αvβ3 integrin cooperatively on the macrophage surface (Savill *et al.*, 1992).

Engagement of either BAI1 or αvβ3/5 integrins initiates intracellular signalling that acts through a complex of the DOCK180 and ELMO1 adaptor proteins to activate Rac1, a protein

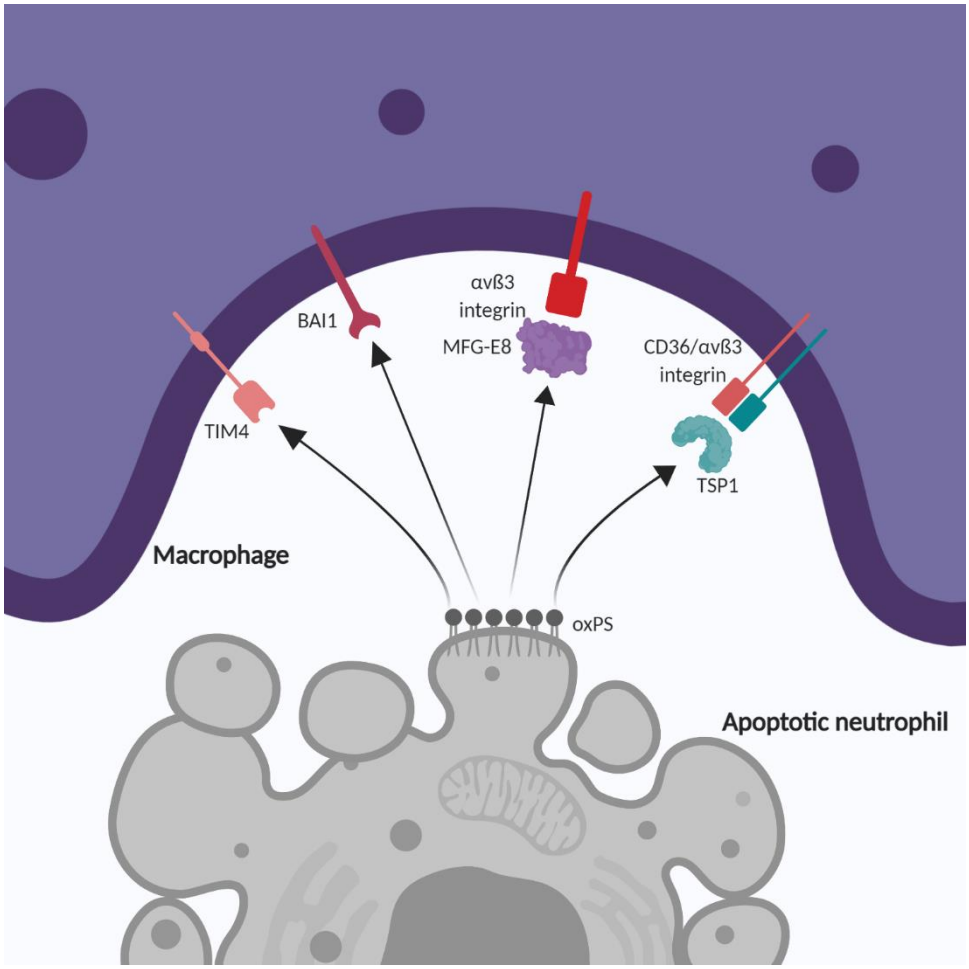

***Figure 1.5**. Macrophage recognition of phosphatidylserine on the apoptotic cell surface. BAI1 = brain-specific angiogenesis inhibitor; MFG-E8 = milk fat globule-epidermal growth factor 8; TIM4 = T cell membrane protein 4; TSP1 = thrombospondin 1; oxPS = oxidised phosphatidylserine*

that regulates cytoskeletal rearrangement to allow engulfment of the target (Martinez, 2015). It is less clear, however, how TIM4 signalling causes phagocyte shape alteration, given that cells transfected with inactive mutant forms of DOCK180, ELMO1 or Rac1 are still able to engulf apoptotic cells in a TIM4-mediated manner (Park, Hochreiter-Hufford and Ravichandran, 2009).

Calreticulin is another important “eat-me” signal expressed on the outer surface of apoptotic cells. In a live cell, calreticulin is usually present in its greatest concentrations in the endoplasmic reticulum. During apoptosis, calreticulin is expressed more strongly on the cell surface and appears to co-localise with exposed phosphatidylserine. Once on the cell surface, it interacts with low-density lipoprotein receptor-related protein (also known as LRP or CD91) on the macrophage surface to stimulate efferocytosis, again likely through Rac1-mediated cytoskeletal rearrangement (Gardai *et al.*, 2005).

1.3.4 Pro-resolution signalling by macrophages after efferocytosis

As well as being a means to clear apoptotic cells before they become secondarily necrotic, efferocytosis also causes a shift in cytokine signalling by macrophages to a pattern that favours the resolution of inflammation. By feeding apoptotic neutrophils to monocyte-derived macrophages cultured in monolayers, Fadok and colleagues found that efferocytosis (compared to conventional phagocytosis mediated by Fc receptors) resulted in lower concentrations of pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α in the culture supernatant. At the same time, elevated supernatant concentrations of the pro-resolution cytokine TGF-β1 were noted (Fadok *et al.*, 1998). Further work by the same research group suggested that this shift of macrophages to a pro-resolution phenotype was dependent on whether their targets displayed phosphatidylserine on their external surfaces, as the effect was not replicated with cells that did not constitutively expose phosphatidylserine in apoptosis unless the cells were enriched with phosphatidylserine liposomes. Direct instillation of phosphatidylserine liposomes into lipopolysaccharide-stimulated mouse lungs could also stimulate pro-resolution signalling, though not to the same extent as whole apoptotic cells (Huynh, Fadok and Henson, 2002).

Macrophages that perform efferocytosis may also polarise neighbouring cells to a pro-resolution phenotype by secreting specialised pro-resolving mediators (SPMs), a superfamily of bioactive substances synthesised from essential fatty acids by leukocytes (Serhan and Levy, 2018). SPMs include the lipoxins, resolvins, protectins and maresins, which serve to resolve inflammation actively by mechanisms including enhanced macrophage efferocytosis and the inhibition of further neutrophil extravasation (Serhan, 2014). In a model employing intraperitoneal zymosan challenge to induce a self-resolving peritonitis in mice, Arnardottir and colleagues found that human monocytes reprogrammed by incubation with SPMs took on a phenotype similar to M2 macrophages and were able to accelerate resolution when injected into inflamed mouse peritoneal cavities (Arnardottir *et al.*, 2014).

1.4 Disordered inflammation resolution in ageing

It is recognised that with age the mechanisms regulating inflammation become impaired in a manner that might contribute to a susceptibility of older people to infection and age-related chronic diseases (Pawelec, Goldeck and Derhovanessian, 2014). This dysregulation has been

inferred in part from an observation of increased pro-inflammatory cytokines in the tissues and circulation in advanced age even in the absence of infection, suggesting a low-level chronic inflammation of ageing known as “inflammaging” (Ferrucci *et al.*, 2005; Bartlett *et al.*, 2012; Pinti *et al.*, 2014). In the human lung, for instance, advanced age is associated with increased IL-6, IL-8 and neutrophils in bronchoalveolar lavage fluid from non-smoking, healthy individuals (Meyer *et al.*, 1996, 1998). This inappropriate persistence of inflammation with age may result in tissue damage which results in further inflammation and more tissue damage, leading to the “cycle of inflammaging” and predisposing to age-related diseases such as chronic obstructive pulmonary disease (Aoshiba and Nagai, 2009). In addition, it has been suggested that inflammaging can impair both innate and adaptive immune responses, which could contribute to the vulnerability of older people to infectious diseases such as pneumonia (Boyd and Orihuela, 2011; Frasca and Blomberg, 2016).

The mechanisms driving the age-related loss of control over the initiation of inflammation have been investigated, and the replicative senescence of cells (due to DNA damage over time, among other factors), altered body fat composition and an altered gut microbiome have all been implicated (Leonardi *et al.*, 2018). There has been less focus, however, on how ageing might affect the processes governing the *resolution* of inflammation, including apoptosis of immune cells, their efferocytosis by macrophages and associated pro-resolution signalling in humans of advanced age. A portion of the work in this thesis aims to explore these mechanisms, and some of the prior literature on this topic is reviewed below.

1.4.1 Ageing and neutrophil apoptosis

Ageing does not appear to affect the rate at which unstimulated neutrophils undergo spontaneous apoptosis in culture, although this tells us little about how the cells will behave in conditions of inflammation (Fülöp Jr *et al.*, 1997). When in inflamed environments it is often the case that neutrophils are exposed to anti-apoptotic signals such that the neutrophils are able to perform their antimicrobial functions for a longer period prior to their death and clearance. Ageing seems to reduce the responsiveness of neutrophils to these anti-apoptotic signals, which include granulocyte-macrophage colony stimulating factor (GM-CSF), LPS and IL-2. In experiments using isolated neutrophils from the venous blood of healthy younger (aged 20-25) and older human volunteers (aged 65-85), Fülöp *et al.* found that although GM-CSF, LPS and IL-2 delayed apoptosis in neutrophils from both sets of volunteers, the effects of

these compounds on the neutrophils from the older volunteers were weaker (Fülöp Jr *et al.*, 1997).

Neutrophils are also subject to extrinsic pro-apoptotic stimuli in circumstances of inflammation, but initiation of the extrinsic apoptotic pathway, at least via the Fas receptor, appears unaffected by age. Tortorella *et al.* identified similar neutrophil surface expression of Fas in neutrophils from younger and older human volunteers, and ligation of the Fas receptor by monoclonal anti-Fas IgM resulted in similar rates of apoptosis in both sets of neutrophils. Much like in the Fülöp study, however, Tortorella and colleagues found a reduced sensitivity of Fas-ligated neutrophils to anti-apoptotic factors such as GM-CSF and LPS with age (Tortorella *et al.*, 1998).

There are scant data on the impact of ageing on phosphatidylserine externalisation during apoptosis. Espino and colleagues provided some useful results within a study investigating the modulatory effect of melatonin on intrinsic apoptosis in leukocytes. The findings suggest that neutrophils isolated from the peripheral blood of healthy older human volunteers (aged 65-75) externalised greater amounts of phosphatidylserine than neutrophils from healthy younger volunteers (aged 20-30) when stimulated to undergo apoptosis *in vitro* with the endoplasmic reticulum stressor thapsigargin or the ROS-inducing PAMP fMLF (Espino *et al.*, 2011). The results must be accepted with caution as the externalisation of phosphatidylserine was quantified using a fluorophore-conjugated annexin V binding assay, and some authors have expressed reservations about the sensitivity of fluorometric assays for the assessment of absolute numbers of externalised phosphatidylserine molecules rather than the assessment of the loss of membrane lipid asymmetry (Morita *et al.*, 2011; Fabisiak, Borisenko and Kagan, 2014).

If a neutrophil's abilities to initiate apoptosis and externalise phosphatidylserine are not diminished with ageing, could a failure of phosphatidylserine oxidation prior to its exposure contribute to impaired neutrophil clearance in older people? Again, little data exist providing direct evidence to this effect. An indirect answer might be discerned from the measurement of an age-related diminution in ROS generation, but researchers have variously reported increased and reduced ROS generation by human neutrophils with increasing age (Wenisch *et al.*, 2000; Butcher *et al.*, 2001; Chaves *et al.*, 2009; Sauce *et al.*, 2017).

1.4.2 Ageing and tissue macrophage chemotaxis

Macrophage chemotaxis in ageing is poorly investigated, especially where human macrophages are concerned. It is often the case that conclusions are drawn about macrophage function from studies of circulating monocyte function, but in varying tissues resident macrophages may be predominantly of distinct lineage to circulating monocytes. Using mouse models, Hashimoto *et al.* were able to show that circulating monocytes contributed minimally to populations of tissue macrophages at steady state, even during repopulation after cytoablation (Hashimoto *et al.*, 2013). This is probably not the case during inflammation, however. Peripheral blood monocytes have been observed to enter the human lung during experimental inflammation and subsequently adopt gene expression profiles that suggest an ability to regulate the immune response in a similar manner to alveolar macrophages (Jardine *et al.*, 2019). It is not known how long these monocyte-macrophages stay in the tissues after infiltration and whether (and if so, to what extent) they play a role in the clearance of apoptotic cells.

In any case, there is little evidence to suggest peripheral monocyte chemotactic ability is impaired with age. Two studies have suggested no effect of age on general chemotaxis, although in addition to the caveat regarding the use of monocytes as a model for macrophages, it is notable that the monocytes were not tested for chemotaxis towards chemoattractants typically secreted by apoptotic cells such as LPC and S1P (Gardner, Lim and Lawton, 1981; Nielsen, Blom and Larsen, 1984).

Animal models have been employed to study tissue macrophage chemotaxis in ageing but show varying results. Forner *et al.* observed an age-related decline in chemotaxis towards casein in peritoneal macrophages from mice and guinea pigs (Forner *et al.*, 1994). Wustrow et al. noted increasing chemotaxis with age in peritoneal macrophages from C57BL/6 mice (Wustrow *et al.*, 1982). It is of note that the chemoattractant in the latter study was serum incubated with a *Salmonella* species to activate complement protein C5. Given that it is possible to impair chemotaxis specifically towards chemoattractants known to be secreted by apoptotic cells and leave general chemotaxis intact (Yang *et al.*, 2005), evidence regarding age-related change in macrophage movement towards efferocytosis-associated chemotactic factors in particular would be welcomed.

1.4.3 Ageing and efferocytosis

Despite few studies of the mechanisms, it does appear that macrophage clearance of apoptotic cells is diminished with age. In a mouse model, *in vivo* peritoneal macrophages in aged (24-month-old) mice showed impaired efferocytosis of intraperitoneally injected apoptotic Jurkat cells compared to younger (2-month-old) mice (Aprahamian *et al.*, 2008). A similar observation was made by Arnardottir *et al.* using *in vitro* mouse bone marrow-derived macrophages in culture with apoptotic neutrophils isolated from human peripheral blood. Again, the macrophages from aged (20-month-old) mice were less able to clear the neutrophils than macrophages from 2-month-old mice. It was postulated that this contributed to an overall impairment of the resolution of inflammation with increasing age (Arnardottir *et al.*, 2014).

It is possible that this defect is mediated by an impaired recognition of apoptotic cells by macrophages, for instance as a consequence of attenuated expression of phosphatidylserine receptors such as TIM4. Using a human skin blistering model, de Maeyer and colleagues observed reduced TIM4 expression on macrophages isolated from the blister fluid of older volunteers, and monocytes from the peripheral blood of the older volunteers also demonstrated reduced efferocytosis (De Maeyer *et al.*, 2020). TIM4 expression on macrophages could be restored in the older volunteers by administration of the MAPK inhibitor losmapimod, and this also appeared to reduce the numbers of neutrophils in an early state of apoptosis in the blister fluid (De Maeyer *et al.*, 2020). It is uncertain whether other phosphatidylserine receptors may also be implicated, and it is additionally unclear whether this finding holds in different human tissues with distinct microenvironments, where tissue and inflammatory monocyte/macrophage populations may be of haematopoietic origins in different proportions to the skin.

1.4.4 Ageing and pro-resolution signalling

It is possible that the features of inflammaging arise due to a failure of macrophages to polarise to a phenotype that favours the resolution of inflammation. A pro-resolution pattern of macrophage behaviour is often referred to as alternative activation or M2 activation. It is important to note that while the commonly used nomenclature of “M1” and “M2” activation implies a linear spectrum or dichotomy of anti- or pro-inflammatory polarisation, a more

accurate model is probably that of multidimensional polarisation in response to variations in the macrophage microenvironment (Byrne, Maher and Lloyd, 2016).

The mechanisms that govern the change in macrophage behaviour at the time of efferocytosis are not fully understood. The interaction between phosphatidylserine on the outer surface of an apoptotic cell and direct or indirect phosphatidylserine receptors on the macrophage surface appears crucial, however, and the alteration in signalling profile looks to take place prior to target engulfment or internalisation. When actin polymerisation is inhibited with cytochalasin D to limit macrophage engulfment of apoptotic cells, contact between apoptotic cells and macrophages is still enough to produce a diminution of NF-κB-dependent transcription of genes coding for pro-inflammatory cytokines (Cvetanovic and Ucker, 2004).

It is not clear to what extent the polarisation of macrophages by apoptotic material shares the mechanisms of polarisation in response to other stimuli such as IL-4, IL-10 or corticosteroids. If the mechanisms are similar, then there is potentially relevant evidence that ageing might impair polarisation. In a study comparing mice aged 18-20 months to younger mice aged 10-12 weeks, splenic macrophages from the older mice showed a reduction in mRNA expression of M2-associated genes such as *Arg1*, *Fizz1* and *Ym1* compared to macrophages from the younger mice when pre-stimulated with IL-4 (Mahbub, Deburghgraeve and Kovacs, 2012). Bone marrow-derived macrophages differentiated *ex vivo* did not display the same age-associated impairment of polarisation, leading the researchers to conclude that the defect might arise due to an alteration of the tissue microenvironment with ageing rather than some intrinsic change in macrophage function (Mahbub, Deburghgraeve and Kovacs, 2012).

It is also possible that ageing impairs the biosynthesis of specialised pro-resolving mediators. Halade *et al.* reported similar levels of arachidonic acid-derived eicosanoids (including the SPM lipoxin B_4) in the spleens of young (6 months old) and aged (18 months old) mice up until the induction of myocardial infarction by coronary artery ligation, at which point splenic levels of eicosanoids decreased to a greater extent in aged mice than young mice. Ageing was associated with reduced mRNA expression of genes encoding lipoxygenase enzymes, which are essential in the derivation of eicosanoids from arachidonic acid (Halade *et al.*, 2016). The conclusion that ageing might impair production of pro-resolution eicosanoids is shared by

Gangemi and colleagues, who found that ageing was associated with reduced urinary lipoxin A_4 in healthy human volunteers (Gangemi *et al.*, 2005).

1.5 Disordered inflammation in chronic lung disease

Some of the work in this thesis aims to explore the mechanisms of disordered inflammation in the context of chronic lung disease. Several chronic lung diseases associated with disordered inflammation such as chronic obstructive pulmonary disease (COPD) and bronchiectasis are more common with age and are associated with periodic deteriorations in clinical status known as acute exacerbations, most often due to acute-on-chronic inflammation (Papi *et al.*, 2006).

The model disease used to explore disordered lung inflammation in this thesis is hypersensitivity pneumonitis (HP), a condition characterised by an excessive pulmonary immune response to inhaled material containing small diameter (<5 μm) organic particles. HP frequently takes the form of inflammation of the lung parenchyma with lymphocyte-rich infiltrates and granuloma formation, and this may overlap with a fibrotic interstitial pneumonia similar to idiopathic pulmonary fibrosis (an age-related, progressive lung disease) in a syndrome commonly known as chronic hypersensitivity pneumonitis (CHP) (Vasakova *et al.*, 2019). When fibrosis does occur, the disease is associated with a progression to respiratory failure and death within 5 to 8 years (De Sadeleer *et al.*, 2018).

The development of fibrosis in chronic lung diseases such as idiopathic pulmonary fibrosis and CHP is associated with the observation of pronounced cellular changes of ageing such as reduction in the lengths of telomeres, the repetitive sequences of DNA that cap chromosomes to provide some protection from degradation. Telomere attrition is seen in dividing cells in normal ageing, and shorter telomeres are associated with a phenotype of accelerated ageing and increased mortality risk (Cawthon *et al.*, 2003; López-Otín *et al.*, 2013). In that light, it may be possible to consider the fibrotic phenotype in CHP as a manifestation of accelerated ageing, with shorter relative telomere length observed in CHP compared to age-matched normal lungs (Everaerts *et al.*, 2018; de Sadeleer *et al.*, 2022).

The pathophysiology of HP is unclear. It has been suggested that in HP dysfunctional regulatory T cells allow the proliferation and pro-inflammatory activity of effector T lymphocytes specific to inhaled culprit antigens, and this may act in concert with

inflammation caused by accumulating antibody-antigen immune complexes to result in the clinical syndrome of respiratory symptoms and impaired lung function (Barrera *et al.*, 2008; Vasakova *et al.*, 2019). An incomplete understanding of the mechanisms of inflammation in HP means that options for pharmacological management are limited (Vasakova *et al.*, 2017), but the therapies that are used in clinical practice are used with the aim of restraining inflammation and chronic disease progression.

1.5.1 Immunopathology of hypersensitivity pneumonitis

The aberrant pulmonary immune response in acute and subacute HP begins after the inhalation of aerosols containing particulate antigens small enough to reach the narrowest airways of the respiratory tree, usually particles with diameters of less than five microns. In most cases of HP these are antigens of microbial or animal origin, but they may also be non-organic substances such as isocyanates that may become antigenic when conjugated with host proteins (Selman, Pardo and King, 2012).

In contrast to the predominantly neutrophilic inflammation induced by LPS inhalation, the inflammation in hypersensitivity pneumonitis is described as infiltration of the lung parenchyma with lymphocytes, plasma cells and macrophages that occasionally coalesce into granulomata (Greenberger, 2019). The difference in the character of the inflammation may be influenced by the pattern recognition receptors engaged by the culprit antigens, with TLR6, TLR9 and dectin-1 implicated as drivers of inflammation in murine models of HP (Fong *et al.*, 2010; Bhan *et al.*, 2013; Higashino-Kameda *et al.*, 2016). The antigens are also processed by antigen-presenting cells (APCs) and presented to $CD4^+$ and $CD8^+$ lymphocytes, which facilitates the development of immune memory of the antigens (Vasakova *et al.*, 2019).

PRR engagement and T lymphocyte recognition of previously encountered antigens may not be sufficient for the pathogenic inflammation seen in the clinical syndrome of HP. Indeed, HP can manifest in some individuals with exposure to antigens that do not induce HP in others. It has therefore been postulated that dysregulation of an immune tolerance mechanism such as the immunosuppressive actions of regulatory T cells may contribute to the development of HP. In a study comparing the function of regulatory T cells from the blood and bronchoalveolar lavage (BAL) fluid of non-smoking patients with HP with those

from healthy volunteers, Girard and colleagues found an impaired ability of regulatory T cells from HP patients to suppress *ex vivo* lymphocyte proliferation (Girard, Israel-Assayag and Cormier, 2011). Depletion of regulatory T cells in mice leads to an exaggerated phenotype of HP in a model of the disease induced by repeated exposure to *Saccharopolyspora rectivirgula*, and furthermore, the regulatory T cells appear to attenuate the disease through direct suppression of interferon gamma (IFN-γ) production by $CD4^+$ and $CD8^+$ lymphocytes (Park, Oh and Chung, 2009).

IFN-γ produced by macrophages and lymphocytes appears to propagate the inflammation of HP by stimulating the secretion of chemokines such as CXCL9 and CXCL10 (previously known as MIG and IP-10 respectively) that serve to attract more lymphocytes to the inflamed region (Nance, Cross and Fitzpatrick, 2004; Agostini, Calabrese, *et al.*, 2005). IL-17 family cytokines also appear to drive inflammation in HP, or at least in the murine *Saccharopolyspora rectivirgula* model of HP. Hassan and colleagues described a reduction in BAL inflammatory exudate in the sensitised mice if IL-17A was blocked with antibody or neutrophils (the primary secreting cells of IL-17A in the model alongside monocytes) were depleted (Hasan *et al.*, 2013).

Although it is sometimes possible to detect precipitating antibodies to potential causative antigens in the serum of HP patients, there is perhaps insufficient evidence to suggest that the inflammation in most forms of HP is due to the local deposition of antigen-antibody immune complexes. The alveolitis associated with immune complex deposition is often a haemorrhagic neutrophilic alveolitis (Johnson and Ward, 1974), a pattern that differs from the lymphocytic infiltration typically described in HP.

The mechanism behind the development of fibrosis in CHP is unclear. Fibrocytes are recruited and activate local fibroblasts, leading to the deposition of extracellular matrix proteins in an uncontrolled fashion (García de Alba *et al.*, 2015; Vasakova *et al.*, 2019).

1.5.2 Anti-inflammatory therapy for hypersensitivity pneumonitis

In light of the possibility for HP to progress to fibrotic disease and the poor prognosis that fibrosis confers, part of the therapeutic strategy (alongside aiming to identify and prevent exposure to the trigger antigen as far as is practicable) is targeted at limiting inflammation with drugs such as corticosteroids. Corticosteroids produce anti-inflammatory effects through

multiple mechanisms mediated by the transcription factor glucocorticoid receptor (GR), including the blockade of NF-κB binding on κB elements and the upregulated transcription of NF-κB activity inhibitors such as IκB (Oh *et al.*, 2017).

Due to uncertainty about the position of fibrosis within the natural history of HP (and, quite crucially, the absence of randomised controlled trials of treatments for HP), it is unclear whether anti-inflammatory therapy with corticosteroids prevents the appearance of fibrosis in HP or has any effect on disease progression when fibrosis is present. In a retrospective, single centre cohort study of patients with HP stratified into those with fibrosis and those without, corticosteroids appeared to reverse lung function decline in non-fibrotic HP while having no effect on fibrotic HP (De Sadeleer *et al.*, 2018). There was no effect on survival in either group.

Other immunomodulatory agents have been used to treat HP, and in the absence of data from HP-specific clinical trials these drugs are often used on the strength of extrapolated data gleaned from their use in other chronic inflammatory lung diseases. Mycophenolate mofetil (MMF) and azathioprine are immunomodulatory agents that may be used in conjunction with corticosteroids, sometimes with the aim of reducing the dose of corticosteroid required for effective inhibition of inflammation in HP.

MMF is a prodrug of mycophenolic acid, a potent inhibitor of the type 2 isoform of the enzyme inosine 5'-monophosphate dehydrogenase (IMPDH). IMPDH plays a critical role in *de novo* purine synthesis — a process essential in DNA/RNA production and cell proliferation — and the type 2 isoform of IMPDH is expressed more strongly in activated lymphocytes than other cells. As such, MMF impairs lymphocyte proliferation and can also induce lymphocyte apoptosis (Allison and Eugui, 2000). In addition, MMF inhibits the expression of cell surface adhesion molecules. This results in impaired interactions between immune cells and endothelium, thereby reducing extravasation of circulating cells to sites of inflammation (Glomsda, Blaheta and Hailer, 2003).

Azathioprine is a prodrug of 6-mercaptopurine (6-MP) that induces apoptosis in stimulated T lymphocytes through a mechanism that likely involves the disruption of signalling downstream of Rac1. As well as mediating cytoskeletal rearrangement as discussed earlier, Rac1 acts as an activator of NF-κB (via the MAPKKK pathway) and STAT3, both of which

upregulate the transcription of members of the anti-apoptotic Bcl-2 protein family. A metabolite of 6-MP, 6-thioguanosine 5'-triphosphate (6-thio-GTP), binds to Rac1 and blocks its activation in a stimulated T cell, depleting Bcl proteins and initiating the intrinsic apoptotic pathway (Tiede *et al.*, 2003).

The cytotoxic drug cyclophosphamide may also be used as an immunosuppressive agent in the treatment of HP. Cyclophosphamide is metabolised to 4-hydroxycyclophosphamide and aldophosphamide in the liver. These metabolites enter cells and degrade to acrolein and phosphoramide mustard, the latter of which alkylates guanine nucleotides in DNA, forming abnormal covalent bonds between and within DNA strands (Hall and Tilby, 1992; Hughes *et al.*, 2018). This DNA damage results in cell death preferentially in cells containing low levels of aldehyde dehydrogenase such as lymphocytes, as aldehyde dehydrogenase converts 4-hydroxycyclophosphamide to inactive carboxyphosphamide (Kastan *et al.*, 1990).

As above, the true effect on HP disease severity and progression with drugs targeted at immunomodulators such as these is uncertain, and further evaluation is required to elucidate the mechanisms of HP pathogenesis in order to establish the role for these drugs in treatment.

1.6 Summary

Chronic inflammation in the human lung may arise in the contexts of ageing and disease. This can result in significant morbidity and mortality, often through a susceptibility to infection or the manifestation of progressive diseases such as hypersensitivity pneumonitis. When treating chronic inflammatory lung conditions in clinical practice it is common to deploy strategies that target the initiation of inflammation, but this may be of uncertain benefit in some circumstances, particularly where the mechanisms of the diseases are incompletely understood.

Considering chronic inflammation as a failure of inflammation resolution mechanisms (rather than persistent inflammation initiation) might lead to new insights in its treatment in ageing and disease. The work in this thesis therefore seeks to consider the role of these resolution mechanisms in the development of the persistent inflammation in ageing and chronic lung disease, using hypersensitivity pneumonitis as a model of a chronic lung disease characterised by disordered inflammation whose progression is influenced by ageing.

1.7 Aims and objectives

The main hypotheses the work in this thesis aimed to test are:

- ageing is associated with a failure of inflammation resolution processes in the immune cells of the human lung;
- existing drug treatments can be identified and repurposed to augment the processes governing the resolution of inflammation;
- drug treatments currently in use targeted at disrupting the *initiation* of inflammation are ineffective in slowing the progression of the chronic inflammatory lung disease hypersensitivity pneumonitis; *and*
- hypersensitivity pneumonitis is associated with a failure of inflammation resolution processes in the immune cells of the human lung.

To test the first hypothesis I aimed to complete an experimental medicine study comparing the cellular responses of lung immune cells from healthy older volunteers and healthy younger volunteers to an inhaled pro-inflammatory stimulus (or control), seeking to discern differences in the initiation and resolution of inflammation with age by observing surrogates of those processes. The preparatory work is described in Chapter 3, but the study was unfortunately subject to interruption by stay-at-home orders imposed during the initial wave of the COVID-19 pandemic in the United Kingdom. As such, the objective was not met, but it is hoped that the work that could be completed provides a sound basis for future investigation of the nature of immune ageing in the lung and its modulation by drugs.

The work to test the second hypothesis — that existing drug treatments can be repurposed to augment inflammation resolution — had been due to follow the work presented in Chapter 3. In order to test this hypothesis, I aimed to perform *in silico* drug screening for compounds with the potential to modulate efferocytosis, and if the screens yielded drug candidates, assess their ability to do so by treating lung immune cells donated by participants from the study from Chapter 3. As COVID-19 interrupted recruitment for that study, cells derived from the peripheral blood of healthy volunteers were used for *ex vivo* assays instead of primary lung immune cells, and little time was left for optimisation of the efferocytosis assays. That said, some results are gleaned, and are presented in Chapter 4.

The rationale for the current use of immunosuppressive drugs in the management of chronic hypersensitivity pneumonitis is that disrupting the initiation of inflammation interferes with the progression of the disease. There have, however, been no randomised controlled trials of treatments in chronic hypersensitivity pneumonitis, and it is unclear whether treatments currently used for the disease are effective. The work presented in Chapter 5 aimed to test the hypothesis that treatments currently in use do not have an effect on disease progression, and takes the form of a retrospective case series evaluation of CHP patients treated by the Newcastle Interstitial Lung Disease service to discern differences in trajectories of lung function decline before and after treatment.

A lack of progress on effective treatments for CHP is perhaps due to an incomplete understanding of the mechanisms of the disease, and in light of the apparent overlap of the progression to fibrosis in CHP with a phenotype of accelerated cellular ageing, I sought to use public transcriptomic data to determine the nature of inflammation in CHP as an exploratory analysis, but with the aim of working towards testing the hypothesis that inflammation resolution processes are defective in CHP. The results of the exploratory analysis are presented in Chapter 6, and although inflammation resolution processes are not implicated, the results provide a plausible drug target and a basis for future work to develop effective treatments for CHP.

Chapter 2. Methods and resources

2.1 Ageing and pulmonary inflammation resolution

This work partly concerning the inhalation of LPS to model pulmonary inflammation is presented in Chapter 3. The study was interrupted by COVID-19, and some of the methods are presented as plans rather than retrospective methods.

2.1.1 Participant screening and recruitment

Poster advertisements were placed on notice boards in Newcastle University seeking volunteers between the ages of 16 and 30 who considered themselves healthy. Advertisements were also posted in general practitioners' surgeries in the Newcastle region seeking volunteers above the age of 65 who considered themselves healthy, with the aim of reaching people who were attending for influenza vaccination (**Appendix A**).

Interested people were encouraged to contact the research team by email or by telephone. They were then sent participant information documentation detailing the study and procedures. If they remained interested following appraisal of the participant information documents, they were invited to attend a screening visit in the pre-assessment clinic at Sunderland Royal Hospital during which a clinician administered a questionnaire regarding the volunteer's medical history (**Appendix A, page 181**) and performed a cardiorespiratory examination, electrocardiogram, lung function tests and blood tests to determine whether a potential participant was suitable for inclusion in the study.

2.1.1.1 Inclusion and exclusion criteria

The inclusion criteria were:

- The individual considered themselves to be healthy
- Age over 65 (older group) or age 18-30 (younger group)

The exclusion criteria were:

- Current smoker
- Ex-smoker (unless smoking stopped more than 5 years previously and smoking history was no more than 5 pack-years)

- Current diagnosis of asthma, chronic obstructive pulmonary disease, bronchiectasis, pulmonary fibrosis, cardiac failure, ischaemic heart disease, diabetes mellitus, rheumatoid arthritis, or any other chronic medical condition considered to compromise safety (at bronchoscopy) or scientific quality of the study, as judged by the chief investigator
- Current use of medication known to have immunosuppressive or stimulatory effect
- HIV infection
- Needle phobia
- Pregnancy or breastfeeding
- Abnormal physical signs detected at cardiorespiratory examination
- Clinical evidence of current respiratory tract infection
- Body temperature > 37.3 °C
- Oxygen saturation < 95% breathing room air
- Blood haemoglobin concentration outside the laboratory reference range
- Platelet count less than 100×10^9/L or greater than 650×10^9/L
- Total white cell count outside the laboratory reference range
- Any deviation of greater than 20% from normal limits in the differential white cell count.
- Serum sodium, potassium or creatinine concentrations outside the laboratory reference range
- Blood urea concentration greater than 10 mg/dL
- Serum bilirubin concentration greater than 30 µmol/L
- Alanine aminotransferase greater than twice the upper limit of the laboratory reference range
- Current participation in a clinical trial that might interfere with the results of the study as judged by the chief investigator

Current smokers or recent ex-smokers were excluded on the rationale that exposure to cigarette smoke alters pulmonary immunity, and in particular there is evidence from murine and human experimental models that suggests that cigarette smoke has lasting deleterious effects on alveolar macrophage phagocytic function (Hodge *et al.*, 2007; Wang *et al.*, 2017).

Pregnancy was an exclusion criterion on the rationale that lipopolysaccharide has been associated with fetal toxicity in animal models, with reports of reduced fetal weight, impaired skeletal development and increased rates of fetal mortality in pregnant mice exposed to intraperitoneal lipopolysaccharide (Xu *et al.*, 2006).

LPS is appreciated as a cause of disruption of the milk-blood barrier in models of mastitis in lactating animals (Kobayashi *et al.*, 2013), but there are scant data on whether this effect can be observed where the LPS is administered at a distant site rather than directly injected into mammary glands. In the absence of data that could be extrapolated to provide reasonable reassurance about safety during human lactation, breastfeeding was also an exclusion criterion.

With participants due to require repeated blood testing over the course of their involvement in the study, people with needle phobia were excluded.

The remainder of the exclusion criteria were primarily aimed at excluding volunteers with altered immunity or with conditions that could potentially compromise the safety of bronchoscopy. Volunteers found to be ineligible for inclusion at screening had the reasons for their exclusion documented in the study database.

2.1.2 Study clinical procedures

Eligible participants were invited to attend the main study day on the Integrated Critical Care Unit (ICCU) at Sunderland Royal Hospital. The purpose of the study day was to facilitate the safe inhalation of LPS or saline control by volunteers, take a time series of blood samples and obtain a single BAL sample. The clinical procedures that are described below were performed according to a protocol (**Appendix A, page 182**), and the procedures are described here as undergone by the volunteer who was able to attend before study interruption.

The participant was asked to arrive at 08:00 hours. After confirming no major changes to health status since the screening visit, the volunteer signed a form indicating consent to undergo the clinical procedures.

Female volunteers of reproductive age were to be asked at this point to complete a urinary pregnancy test. A positive test would exclude the volunteer from the rest of the study.

Baseline clinical observations (blood pressure, heart rate, respiratory rate, oxygen saturations and temperature) were recorded by clinical staff, and an open question about constitutional symptoms was asked of the volunteer. Baseline spirometry was also recorded.

An intravenous cannula was inserted to facilitate repeated blood sampling over the course of the day. The chamber of the Akita Jet nebuliser (Vectura, UK) was filled with saline for practice inhalations. The device provided visual and audio feedback to facilitate an optimal inhalation for drug delivery to the lower airways, ensuring the volunteer performed a maximal expiration prior to a slow inhalation with a breath hold at maximum inspiration.

After the practice breaths, the chamber was filled with either saline or LPS solution. The volunteer was blinded to the identity of the contents of the nebuliser, but for the sake of safety the supervising clinical researcher was not. (This volunteer inhaled saline.) The participant performed the inhalations as practiced, starting the study day clock.

Clinical observations and reports of symptoms were recorded half-hourly for the first four hours, then hourly up until hour eight. Blood sampling was performed at 1 hour, 2 hours, 4 hours and 8 hours post-inhalation.

Following the blood sampling at the 8-hour time point, spirometry was repeated. If the volume of air expired in the first second of the spirometry procedure (FEV1) had fallen by 15% or more compared to the baseline reading, then this would have suggested bronchospasm and the volunteer would be deemed ineligible for bronchoscopy. After ensuring safety, the volunteer underwent bronchoscopy and bronchoalveolar lavage as detailed in the methods chapter.

The blood and BAL samples were then transported on ice to Newcastle for analysis (also detailed in the methods chapter), and the volunteer was observed for a further hour before being allowed home with a telephone number on which to contact the research team if they experienced any symptoms in the period after the procedure. The volunteer then returned to Sunderland Royal Hospital the next morning for a post-procedure follow-up visit and blood sampling 24 hours post-inhalation. This completed their involvement in the study.

2.1.3 Nebuliser calibration

To ensure an accurate delivery of LPS dose during the study presented in Chapter 3 it was necessary to determine what volume of solution was nebulised upon each actuation. The nebuliser was calibrated by measuring weight change before and after nebulisation. It is appreciated that gravimetric calibration of nebulisers in this fashion may overestimate the delivered dose due to changes in the proportions of vapour and aerosol in the output caused by the cooling effects of jet nebulisation (Dennis *et al.*, 1990). Absent the availability of more sophisticated calibration techniques — measurements of a tracer substance retained upon filter paper after actuation of the nebuliser by a vacuum system, for example (Ward *et al.*, 1998) — gravimetric calibration was chosen as the most practical.

The duration of nebuliser actuation during the user's inspiration can be selected on the Akita Jet control unit. For the calibration procedure the 5 s duration was selected. The nebuliser chamber was weighed using a laboratory balance (Adam Equipment, UK) without and with 5 mL of saline, then weighed after every ten inhalations by a test participant. Over 40 test actuations the nebuliser was determined to dispense a mean of 22 µL (standard deviation 2.2 µL) per actuation.

2.1.4 Nebulised drug administration

10 µg vials of Clinical Center Reference Endotoxin (CCRE; lipopolysaccharide from Escherichia coli O113) were obtained from Dr Anthony Suffredini at the National Institutes of Health (NIH), USA. CCRE is available from the NIH for investigational studies upon request and provision of study protocols and regulatory approvals. The intended inhaled LPS dose was 2 µg. One vial of CCRE was to be reconstituted with 0.5 mL of sterile water, vortexed for 15 minutes, then added to 3.5 mL of sterile saline. The nebuliser chamber would be filled with the resulting 4 mL of 2.5 µg/mL LPS solution if the participant was allocated to inhale LPS. The participants inhaling saline would have the chamber filled with 4 mL of sterile saline.

The participants would perform 36 inspirations with the nebuliser set to a 5 s actuation (delivering ~1.98 µg of LPS to those inhaling LPS). After practice breaths of saline prior to administration of the study drug as allocated, the participants were to be instructed to perform each breath as an exhalation into the room air prior to a slow inspiration on the nebuliser mouthpiece with a short breath hold at maximum inspiration.

2.1.5 Nebuliser decontamination

Calibration procedures and administration of saline to the volunteer were undertaken with different nebuliser sets. The study plan had been to reuse some of these items for future participants, and a protocol for decontamination of the apparatus in a microbiological safety cabinet with disinfecting fluid (Milton Pharmaceutical Company, UK) was available, kindly provided by Dr Laura Barr and Dr Sarah Wiscombe.

2.1.6 Spirometry

Spirometry for volunteer screening prior to inclusion in the study was undertaken in the Sunderland Royal Hospital Lung Function Department by clinical physiologists. Spirometry on the study day itself to assess safety for LPS inhalation and bronchoalveolar lavage was undertaken using a Vitalograph Micro Handheld Spirometer (Vitalograph Ltd., UK). Participant age, sex, ethnicity, and height were entered into the device. The participant wore a nose clip prior to the spirometry procedures. In a standing position, participants were asked to exhale fully, then inhale maximally, then perform a hard and fast expiration to completion with verbal encouragement from the supervising team member. The best spirometry values from three attempts were recorded.

2.1.7 Bronchoalveolar lavage

Bronchoalveolar lavage was performed according to a protocol (**Appendix A, page 182**). The protocol for the research bronchoscopy was designed to correlate closely with the clinical standards and techniques of bronchoscopy and bronchoalveolar lavage. Participants were to be offered sedation with intravenous midazolam, but those participants opting to have sedation had to be accompanied home after the study day (by a friend or relative, for example), and would have been advised not to return to work, cycle, drive, operate moving machinery or sign legal documents for the remainder of the day. The participant who attended before the study was interrupted by the COVID-19 pandemic did not take up the offer of sedation.

The participant was connected to continuous pulse oximetry and 3-lead electrocardiogram monitoring. The participant's clothes were protected by absorbent sheets. Topical anaesthesia to the oropharynx was given by sprays of 10% lidocaine. Topical anaesthesia to the nasal passages was administered by the volunteer sniffing Instillagel (Farco-Pharma

GmbH, Germany). Supplemental oxygen was applied by nasal cannulae, with a plan to titrate the oxygen during the procedure to maintain the participant's oxygen saturations above 90%. Yankauer suction was available to remove excessive oral secretions during the procedure. Several 5 mL syringes filled with 2 mL of 1% lidocaine and 3 mL of air were prepared for topical anaesthesia of the mucosa during the procedure (the air facilitates injection of the lidocaine down the working channel of the bronchoscope). Warmed saline was used for lavage.

A single-use bronchoscope (aScope 4 Broncho Regular; Ambu A/S, Denmark) was used in conjunction with the aView monitor (Ambu A/S, Denmark). The end of the scope was lubricated with Instillagel and attempts were made to advance it through the left or right nostril. As is sometimes the case in clinical bronchoscopy, the nasal passages were too tight to advance the scope comfortably for the participant, so a mouth guard was inserted, and the scope was advanced via the mouth. Topical anaesthesia was applied to the vocal cords before the scope was advanced between them. Further anaesthesia was then applied to the carina and the left and right main bronchi.

The end of the scope was positioned within the medial segment of the right middle lobe. 20 mL of warmed saline was injected down the working channel of the bronchoscope, then gently suctioned by hand and discarded. Three 50 mL aliquots of saline were intended to be instilled sequentially and aspirated by hand, but the volunteer opted to discontinue the procedure due to an uncomfortable cough after the first aliquot, yielding 20 mL of lavage fluid. The bronchoscope was withdrawn, completing the procedure.

The lavage fluid was collected in a sterile container and placed on ice for transport to the laboratory for analysis with the blood samples. The volunteer was observed for an hour post-procedure before being allowed home.

2.1.8 Lavage fluid processing

It was intended that the BAL fluid would be processed for cell counts, expression of cell surface efferocytosis receptors, efferocytosis assays and fluid cytokine measurements. Protocols were devised which may have required optimisation for optimal results. They are presented below.

2.1.8.1 Alveolar macrophage isolation

Lavage fluid was filtered through a 70-micron cell strainer (Corning Inc., USA) to remove any mucus, then centrifuged at 500g for 10 minutes. The supernatant was carefully aspirated and collected in a separate container for freezing to facilitate future assessments of BAL cytokines by ELISA. The cell pellet was resuspended in 5 mL of sterile phosphate buffered saline (PBS) and samples were taken for haemocytometer cell counts and cytocentrifuge smear. Half the sample was set aside for flow cytometry assays. The remainder was resuspended at 1×10^6 cells/mL in culture medium consisting of RPMI 1640 with L-glutamine (Lonza, USA) supplemented with 10% foetal bovine serum (Merck KGaA, Germany), 25 U/ml penicillin and 25 µg/ml streptomycin (Penicillin-Streptomycin; Merck KGaA, Germany), and 10 ng/ml human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF; Biovision, USA). The cell suspension was plated in 24-well plates at 1 mL/well and incubated at 37 °C for 3 hours to adhere alveolar macrophages to the plastic surface. The medium was then replaced, removing non-adherent cells.

2.1.8.2 Efferocytosis assay

Efferocytosis assays with alveolar macrophages could not be performed before suspension of the study. A version of the intended assay using monocyte-derived macrophages and peripheral blood neutrophils is presented in Section 2.2.8 as used for the work presented in Chapter 4.

2.1.8.3 BAL immunophenotyping and assessments of efferocytosis-associated cell surface markers

Two flow cytometry antibody panels were devised to identify the immune cell populations present in the BAL and assess the expression of alveolar macrophage surface receptors associated with the recognition of apoptotic material. The aim was to test the hypotheses that ageing and pro-inflammatory immune cell polarisation by inhaled LPS impair the expression of efferocytosis receptors on alveolar macrophages in the human lung.

The first panel (**Table 2.1**) was to be used to label the cells in an aliquot of BAL fluid with fluorophore-conjugated antibodies to cell surface markers before acquisition by multicolour flow cytometry using the Symphony A5 flow cytometer (Becton Dickinson, UK). Nested gating of cell populations associated with characteristic cell surface markers would then allow the

innate immune cell populations to be identified. Appropriate isotype control antibodies and unstained samples were to be used to help discern specific antibody staining from background staining, and fluorescence-minus-one (FMO) control samples were to be prepared to facilitate the positioning of the gates. A fixable viability dye was selected (Zombie UV Fixable Viability Kit; Biolegend, USA) to distinguish live and dead cells by fluorescence in the 379/28 detection channel upon excitation by the 355 nm laser.

Fluorochrome-conjugated antibody	Clone	Manufacturer, catalogue number	Laser, detection channel
Anti-human CD16 BV711	3G8	Biolegend, 302044	405 nm, 710/50
Anti-human CD14 BV785	M5E2	Biolegend, 301840	405 nm, 780/60
Anti-human CD206 AF488	15-2	Biolegend, 321114	488 nm, 530/30
Anti-human CD24 eFluor710	eBioSN3	Thermo Fisher, 46-0247-42	488 nm, 670/30
Anti-human CD169 PE	7-239	Biolegend, 346004	561 nm, 586/15
Anti-human CD11c PE-Cy5.5	3.9	Thermo Fisher, 35-0116-42	561 nm, 710/50
Anti-human HLA-DR PE-Cy7	L243	Biolegend, 307616	561 nm, 780/60
Anti-human CD45 AF700	HI30	Biolegend, 304024	635 nm, 730/45

Table 2.1. *Bronchoalveolar lavage fluid immunophenotyping flow cytometry panel. AF = Alexa Fluor; BV = Brilliant Violet; CD = cluster of differentiation; Cy = cyanine; HLA-DR = human leukocyte antigen DR isotype; PE = phycoerythrin*

Light scatter characteristics and expression of cell surface markers were to be used to identify myeloid-lineage cell populations in the BAL fluid (**Table 2.2**), roughly guided by the American Thoracic Society's guideline on Improving the Quality and Reproducibility of Flow Cytometry in the Lung (Tighe *et al.*, 2019).

Cell population	Markers
Alveolar macrophages	$CD45^{+}$, $CD11c^{+}$, $CD169^{+}$, $CD206^{+}$, $CD14^{lo}$
Classical monocytes	$CD45^{+}$, $HLA\text{-}DR^{+}$, $CD14^{hi}$, $CD16^{lo}$
Non-classical monocytes	$CD45^{+}$, $HLA\text{-}DR^{+}$, $CD14^{lo}$, $CD16^{hi}$
Intermediate monocytes	$CD45^{+}$, $HLA\text{-}DR^{+}$, $CD14^{+}$, $CD16^{+}$
Neutrophils	SSC^{hi}, $CD24^{+}$, $CD16^{+}$

Table 2.2*. Cell populations identified by cell surface markers with flow cytometry. Adapted from Tighe et al., 2019. SSC = side scatter*

The second panel (**Table 2.3**) was to be used to discern the expression of cell surface markers on alveolar macrophages where altered expression might represent an alteration in capacity for efferocytosis.

Fluorochrome-conjugated antibody	Clone	Manufacturer, catalogue number	Laser, detection channel
Anti-human CD14 BUV395	MϕP9	Becton Dickinson, 563561	355 nm, 379/28
Anti-human CD169 BUV737	7-239	Becton Dickinson, 748925	355 nm, 740/35
Anti-human MERTK BV421	590H11G1E3	Biolegend, 367604	405 nm, 450/50
Anti-human CD36 BV711	CLB-IVC7	Becton Dickinson, 745470	405 nm, 710/50
Anti-human CD206 BV786	19.2	Becton Dickinson, 740999	405 nm, 780/60
Anti-human CX3CR1 PE-D594	2A9-1	Biolegend, 341624	561 nm, 610/20
Anti-human TIM4 PE-Cy7	9F4	Biolegend, 354006	561 nm, 780/60

***Table 2.3**. Alveolar macrophage efferocytosis receptor flow cytometry panel. BV = Brilliant Violet; BUV = Brilliant Ultraviolet; CD = cluster of differentiation; CX3CR1 = C-X3-C motif chemokine receptor 1; Cy = cyanine; MERTK = MER proto-oncogene tyrosine kinase; PE = phycoerythrin; PE-D = phycoerythrin-Dazzle*

Aside from identification of alveolar macrophages as $CD169^{+}$ $CD206^{+}$ $CD14^{lo}$ cells, this second panel could be used to discern differences in macrophage expression of efferocytosis receptors CD36, MERTK and TIM4 and efferocytosis chemokine receptor CX3CR1 (as measured by median fluorescence intensity in corresponding detection channel minus median fluorescence intensity of FMO control) between older and younger volunteers inhaling LPS or saline.

Although antibody manufacturers often quote guide concentrations of antibodies to be used in flow cytometry experiments, it is to be noted that multicolour flow cytometry panels such as these require optimisation to ensure antibody concentrations in the stains allow for clear resolution and distinction of different cell populations. Ideally these optimisations should be undertaken using the tissue or sample that will be studied, although it is appreciated that difficult to obtain samples (such as BAL fluid) may require optimisation using a surrogate sample (Ferrer-Font *et al.*, 2021). In this case the optimisation work was not done prior to recruitment, but future experiments should include allowances in participant recruitment for samples to contribute to assay optimisation.

2.1.9 Peripheral blood collection and processing

An intravenous cannula (Becton Dickinson, UK) was inserted into a peripheral vein to facilitate repeated sampling over the course of the study day. Blood samples at the baseline time point were taken into Vacuette tubes (Grenier Bio-One, UK): one 4 mL sample taken into a tube containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant; one 3.5 mL sample into a tube containing 3.8% sodium citrate as an anticoagulant; and two 5 mL samples taken into serum separation tubes. This 17.5 mL sampling was repeated at the subsequent time points. The repeat blood sampling was undertaken through the cannula after first flushing it

with 2 mL of saline, then aspirating and discarding the first 3 mL of sample. All blood samples were refrigerated at 4°C until transport to the laboratory at Newcastle University for analysis.

Peripheral blood was to be processed to allow myeloid lineage leukocyte kinetics to be measured at baseline and tracked over the 24-hour period after inhalation of LPS or saline to identify to what extent any inflammation in the lung corresponded to systemic inflammation, and whether this differed with age. Plasma and serum would be separated from the cellular fraction of peripheral blood and stored for future cytokine measurements by ELISA. Flow cytometry would be used to assess leukocyte kinetics.

2.1.9.1 Peripheral blood flow cytometry

A flow cytometry assay using whole blood with lysed red cells was used. The use of whole blood avoids cell washing steps, which minimises activation of immune cells and cell losses during preparation (Petriz, Bradford and Ward, 2018).

Trucount flow cytometry test tubes (Becton Dickinson, UK) were used. Trucount tubes contain a known number of fluorescent beads such that cell numbers per unit volume of original sample can be calculated from the acquired flow cytometry data.

100 µL of whole blood from EDTA-containing blood sample tubes corresponding to each time point were placed into Trucount tubes and stained with a panel of fluorophore-conjugated antibodies to leukocyte cell surface markers (**Table 2.4**), and Zombie UV as a viability dye. 50 µL aliquots of whole blood from the baseline EDTA sample were placed in standard flow cytometry test tubes (Corning Inc., USA) for FMO controls. After staining the red cells were lysed by adding FACS Lysing Solution (Becton Dickinson, UK) to the tubes. The samples were then ready for acquisition on the flow cytometer as a time series.

Fluorochrome-conjugated antibody	Clone	Manufacturer, catalogue number	Laser, detection channel
Anti-human CD16 V500	3G8	Becton Dickinson, 561394	405 nm, 525/50
Anti-human CD3 BV605	7-239	Becton Dickinson, 748925	405 nm, 610/20
Anti-human HLA-DR BV785	L243	Biolegend, 307642	405 nm, 780/60
Anti-human CD45 FITC	HI30	Becton Dickinson, 555482	488 nm, 530/30
Anti-human CD14 PE-D594	HCD14	Biolegend, 325634	561 nm, 610/20
Anti-human CD19 APC-Cy7	SJ25C1	Biolegend, 363010	635 nm, 780/60
Anti-human CD20 APC-Cy7	2H7	Biolegend, 302314	635 nm, 780/60

***Table 2.4**. Flow cytometry panel to track monocyte and neutrophil kinetics in peripheral blood. APC = allophycocyanin; BV = Brilliant Violet; CD = cluster of differentiation; Cy = cyanine; FITC = fluorescein isothiocyanate; HLA-DR = human leukocyte antigen DR isotype; PE-D = phycoerythrin-Dazzle*

Neutrophils and mononuclear cells were to be identified by light scatter characteristics and CD45 expression, and monocytes would be identified as the mononuclear cells expressing CD14 and CD16 (to varying degree depending on subset as in **Table 2.2**) and HLA-DR. The remainder of the mononuclear cells would represent lymphocytes, which would be positive for CD3 (T lymphocytes) or CD19 and CD20 (B lymphocytes).

2.1.9.2 Plasma/serum separation and storage

The samples in Vacutainer tubes containing EDTA and sodium citrate were centrifuged at 300 g for 10 minutes, yielding a layer of plasma in each tube that was collected by careful aspiration and frozen. Serum separation tubes were centrifuged at 1500 g for 10 minutes, and the serum layer was aspirated and frozen.

2.2 Drug repurposing to enhance the resolution of inflammation

This work seeking to identify resolution-enhancing drugs is presented in Chapter 4. Portions of that work are published as a journal article: Sendama, W. (2020). 'L1000 connectivity map interrogation identifies candidate drugs for repurposing as SARS-CoV-2 antiviral therapies'. *Computational and Structural Biotechnology Journal*, 18, pp. 3947–3949.

2.2.1 The L1000 Connectivity Map

The L1000 Connectivity Map is a progression on the first-generation Connectivity Map, which sought to catalogue the differential gene expression profiles of four different cell lines exposed to a library of 164 perturbagens including both non-drug tool compounds and drugs approved by the United States Food and Drug Administration (Lamb *et al.*, 2006). Having recognised the utility of the connectivity map, the researchers then aimed to expand the library of perturbagens, the range of perturbagen concentrations, the range of tested cell lines and the range of measured genes. To streamline the process of upscaling, the researchers developed the L1000 assay, a high throughput assay that reduces the volume of data required to be captured by measuring the differential gene expression of 978 "landmark" genes, and computationally inferring from those the remainder of the transcriptome with high fidelity, validated against RNA-seq (Subramanian *et al.*, 2017).

The data from the L1000 project are publicly available online via the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo), with accession numbers GSE70138 and GSE92742.

2.2.2 Data analysis

Data from the L1000 Connectivity Map were downloaded and analysed using Genevestigator v8.0.1 (Nebion AG, Zürich). Genevestigator is a computer software platform that incorporates a suite of data analysis tools and a search engine for public high throughput functional genomics data that has been curated, quality controlled, annotated and normalised by the Nebion AG team to facilitate meta-analysis (Hruz *et al.*, 2008). A normalised and annotated version of the L1000 Connectivity Map dataset is available on the Genevestigator platform, derived from the publicly available data on GEO.

The significance of differentially expressed genes in data was tested in the software using the Linear Models for Microarray data (Limma) algorithm to perform a moderated t-test on the normalised data (Ritchie *et al.*, 2015). Unless noted otherwise, genes were considered significantly differentially expressed with at least an absolute $\log_2$ ratio change > 0.58 (roughly 1.5-fold change) to $p < 0.05$. This is an arbitrary threshold selected for screening of the connectivity map due to precedent for 1.5-fold change being considered differentially expressed in other published microarray experiments, and also because differential expression thresholds at that level are likely to represent a good trade-off between allowing the rejection of background noise and identifying biologically meaningful changes (Dalman *et al.*, 2012). Where the differential expressions of several genes were considered in conjunction, false discovery rate (FDR) was calculated to correct for multiple significance testing, and an FDR < 0.05 was considered significant (Benjamini and Hochberg, 1995).

2.2.3 Literature searches

Informal online searches were undertaken to establish which of the drugs in the screen had been approved for a clinical indication in humans. Similarly, literature searches were undertaken using Google Scholar using the drug and gene names as keywords to find literature suggesting effects of drugs on gene expression.

2.2.4 Leukocyte isolation from healthy volunteer blood

Leukocytes were isolated from blood drawn from healthy adult human volunteers. Ethical approval for blood donations in these experiments was obtained after review of the protocol and supporting documents by the North East – Newcastle & North Tyneside 2 Research Ethics Committee (reference 12/NE/0121). Blood was drawn using Vacutainer Safety-Lok blood collection sets (Beckton-Dickinson, UK) into Vacuette tubes (Grenier Bio-One, UK) containing 3.8% sodium citrate as an anticoagulant.

Blood was immediately transported to the laboratory for leukocyte isolation by dextran sedimentation and density gradient centrifugation. Whole blood was centrifuged at 300*g* for 20 minutes to pellet the erythrocytes and leukocytes, then the platelet-rich plasma layer was removed using a Pasteur pipette. 2.5 mL of 6% dextran per 10 mL of cell pellet was added, then the original container volume was reconstituted with warm 0.9% saline. The solution was mixed by gentle inversion, then the vessel was left open and undisturbed for 30 minutes at room temperature to allow erythrocyte sedimentation. The leukocyte-rich upper layer was then transferred to a new tube, mixed with warm saline and centrifuged at 200*g* for 5 minutes to pellet the leukocytes. 2.5 mL of 70% Percoll PLUS (GE Healthcare Biosciences, Sweden) in PBS was layered gently onto 2.5 mL of 81% Percoll in a new tube, then the leukocyte cell pellet was resuspended in 2.5 mL of 55% Percoll and overlaid on the other two layers to complete the Percoll gradient. Centrifugation at 700g for 20 minutes resulted in leukocytes settling between the Percoll layers: peripheral blood mononuclear cells (PBMC) between the top and middle layers and polymorphonuclear cells (PMN; predominantly neutrophils) between the middle and bottom layers.

PBMC and PMN were collected into separate tubes with Pasteur pipettes and counted in a haemocytometer (with viability assessed by Trypan blue exclusion), then washed with Hank's balanced salt solution (HBSS) before use in assays.

2.2.5 Monocyte isolation from PBMC

Monocytes were isolated from PBMC by negative selection immunomagnetic cell separation with the Pan-Monocyte Isolation Kit (Miltenyi Biotec BV, Germany). PBMC were pelleted and resuspended in magnetic separation buffer (PBS containing 2 mM EDTA and 0.5% bovine serum albumin) then Fc receptors were blocked with the reagent included in the kit. Biotin-

conjugated monoclonal antibodies against antigens expressed on leukocytes other than monocytes (also included in the kit) were added, then the cell suspension was mixed and incubated at 4 °C for 5 minutes. Ferromagnetic beads conjugated to anti-biotin monoclonal antibody (also in kit) were added, then the cell suspension was mixed and incubated at 4 °C for a further 10 minutes. The labelled cell suspension was then passed through an MS MACS Column (Miltenyi Biotec BV, Germany), which retained the ferromagnetically labelled non-monocyte cells and allowed unlabelled monocytes through. The MACS Column was rinsed thrice with magnetic separation buffer to maximise monocyte yield.

2.2.6 Monocyte differentiation to monocyte-derived macrophages

Isolated monocytes were centrifuged at 300*g* for 5 minutes to pellet the cells following isolation. The pellet was resuspended at 1×10^6 cells/mL in a macrophage differentiation/culture medium consisting of RPMI 1640 with L-glutamine (Lonza, USA) supplemented with 10% fetal bovine serum (Merck KGaA, Germany), 25 U/mL penicillin and 25 µg/mL streptomycin (Penicillin-Streptomycin; Merck KGaA, Germany), and 10 ng/mL human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF; Biovision, USA). Cells were seeded in a 96-well plate at 200 µl of cell suspension per well and cultured in an incubator at 37 °C with 5% CO_2 and 21% O_2. The culture medium was replaced every four days and assays were only performed after at least one week of culture to allow differentiation into macrophages.

2.2.7 Preparation of labelled neutrophils for efferocytosis assays

Neutrophils isolated from healthy volunteer blood were centrifuged at 300*g* for 5 minutes to pellet the cells following isolation. The cell pellet was resuspended at 3×10^6 cells per mL in warmed serum-free RPMI 1640 with L-glutamine, then CellTracker Green BODIPY (Life Technologies, UK) dye was added at a concentration of 5 µM to stain the cells. The cell suspension was incubated for staining for 45 minutes at 37°C, then the cells were centrifuged once more to pellet, and again resuspended in serum-free medium. The cells were incubated overnight in a at 37 °C shaking incubator oscillating at 200 RPM to induce apoptosis. Preparation in this manner resulted in cell suspensions containing upward of 75% early and late apoptotic cells ($n = 2$) as assessed with flow cytometry using the APC-Annexin V Apoptosis Detection Kit (Biolegend Ltd, UK).

2.2.8 Efferocytosis assay

Monocyte-derived macrophages were treated with flavopiridol hydrochloride (Tocris Bioscience, UK), CGP-60474 (Tocris Bioscience, UK) or vehicle controls for 16 hours (with the exception of cytochalasin D [Tocris Bioscience, UK] used as a negative control, which was added at a concentration of 10 µg/mL for 30 minutes prior to macrophage co-culture with labelled apoptotic neutrophils). Dimethyl sulfoxide (DMSO) was used as a vehicle control with the final concentration in wells being less than 0.1% v/v. Concentrations of flavopiridol and CGP-60474 were selected to provide ranges around the doses producing significant differential gene expression of efferocytosis-associated genes in the L1000 data. The selected concentrations also encompassed reference values for the half-maximal inhibitory concentrations of the drugs for cyclin-dependent kinases, although it is appreciated that cyclin-dependent kinase inhibition may not be the mechanism by which any effects on inflammation resolution might manifest.

The drug-containing cell culture medium was removed after the treatment period, and labelled apoptotic neutrophils resuspended at 3×10^6 cells per mL in RPMI 1640 with L-glutamine and 10% fetal bovine serum were added to the macrophage wells at 200 µl per well, giving a neutrophil-to-macrophage ratio of 3:1. The culture plates were incubated at 37 °C with 5% CO_2 and 21% O_2 for two hours to allow efferocytosis to take place. The co-culture supernatant was then removed, and the wells were washed three times with cold PBS to remove non-adherent cells. Adherent cells were then gently scraped into PBS with a pipette tip to remove them from the culture plate surface. The retrieved cell suspensions were centrifuged at 300g for 5 minutes to pellet, then resuspended in Stain Buffer (FBS) (BD Biosciences, USA). Mouse anti-human CD14 monoclonal antibody conjugated to allophycocyanin (APC; BD Biosciences, USA) was added to label the macrophages for additional confidence in their identification, and following staining for 20 minutes at 4 °C the samples were acquired on a FACSymphony A5 flow cytometer (BD Biosciences, USA) using FACSDiva software (v9.0; BD Biosciences, USA).

Flow cytometry data were analysed in FCS Express 7 (De Novo Software, USA) and RStudio 2021.09.1 (RStudio PBC, USA). Debris were excluded by negative gating of events with low forward and side scatter values. Macrophages were identified on flow cytometry plots by light scatter (using control macrophages that had not ingested neutrophils to provide confidence

in the macrophage gates), and the macrophages that had ingested neutrophils were identified as those exhibiting fluorescence in the CellTracker Green emission detection channel beyond the limit of the macrophages within the control macrophage gate (**Figure 2.1**, **Figure 2.2**). Efferocytic index was calculated as the proportion of macrophages displaying CellTracker Green fluorescence, with a minimum of 1000 macrophage events sampled.

Efferocytosis experiments were undertaken in duplicate, with n = 3 for all experiments reported. Significance testing of comparisons between drug conditions and control was performed by one-way analysis of variance (ANOVA), with $p < 0.05$ taken as the threshold for significance.

Due to constraints on the availability of volunteers, the macrophages were co-cultured with heterologous neutrophils. The SARS-CoV-2 pandemic also reduced the opportunity for optimisation of the efferocytosis assay, and this is discussed in Chapter 4.

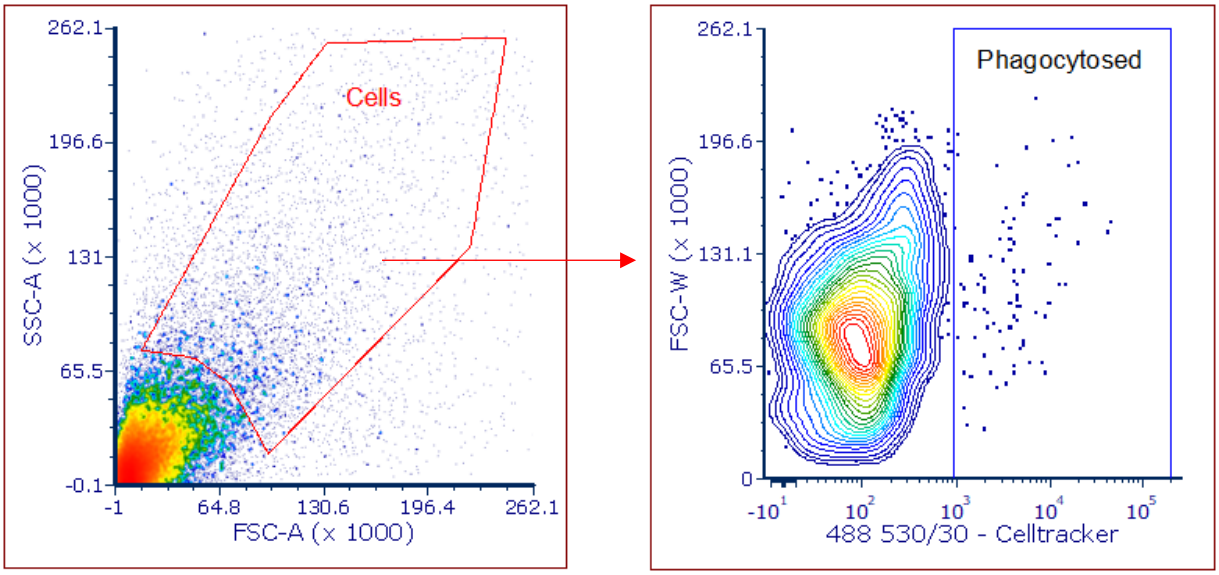

***Figure 2.1**. Debris (events with low light scatter values) excluded by gate, with macrophages included in gate labelled "Cells" (left). Macrophages containing CellTracker Green-stained neutrophils included in "Phagocytosed" gate (right). FSC-A = forward scatter area; FSC-W = forward scatter width; SSC-A = side scatter area*

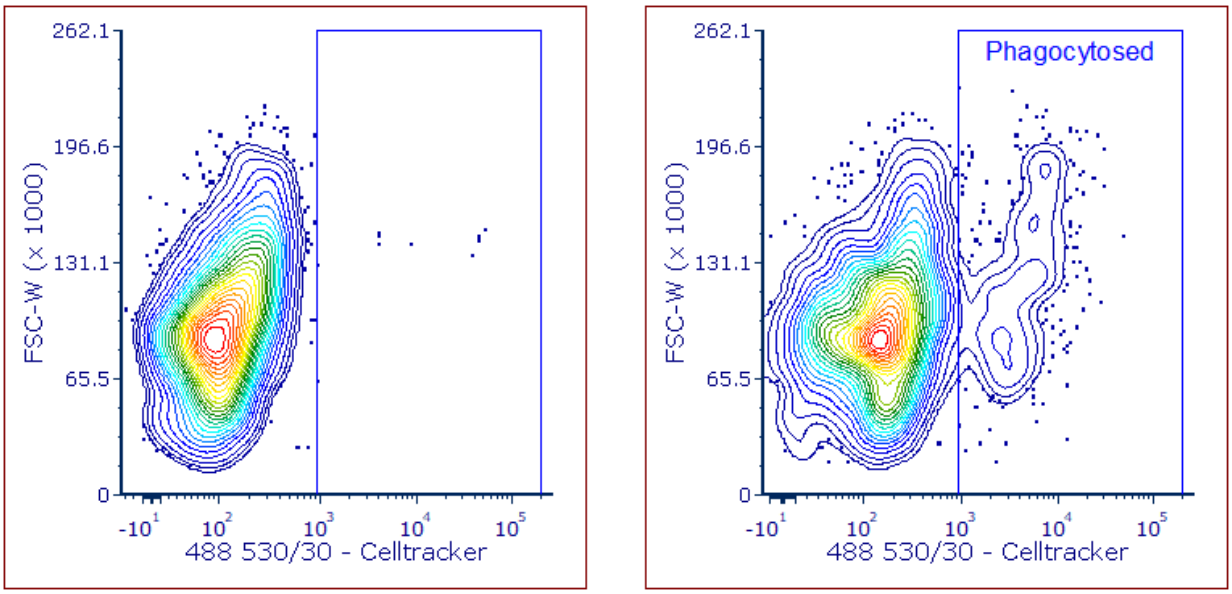

***Figure 2.2**. Setting the gate for macrophages containing phagocytosed stained neutrophils using a control sample of macrophages alone (left). Co-cultured macrophages and stained neutrophils shown on right, with macrophages containing ingested neutrophils represented by events included in gate.*

2.3 Immunosuppression in hypersensitivity pneumonitis

This work seeking to evaluate the effect of immunosuppressive drugs on the progression of chronic hypersensitivity pneumonitis is presented in Chapter 5.

2.3.1 Patient identification

Patients with a diagnosis of chronic hypersensitivity pneumonitis were identified from the Newcastle Interstitial Lung Disease (ILD) service's patient census. The diagnosis for each patient was established by the consensus of the multidisciplinary team that comprises the service. Patients who received their first pulses of cyclophosphamide between May 2015 and December 2020 were included, and electronic medical records were accessed for collection of data, stored in anonymised form. Patients who did not have enough recorded lung function prior to or after initiation of cyclophosphamide to calculate an FVC or TLCO gradient by simple linear regression were excluded from the analysis (with a minimum of two data points required for each regression), as were patients who had either died or undergone lung transplantation before one year of lung function follow-up post cyclophosphamide initiation.

2.3.2 Data collection and analysis

Anonymised data on patient age, the dates of administration of cyclophosphamide treatments, lung function and treatment outcomes were entered from the electronic medical records into a database. FVC and TLCO measurements from up to a year prior to

cyclophosphamide initiation and up to a year after initiation (plus one measurement on either side of that timeframe) were included if available. Data were analysed using RStudio 2021.09.1 (RStudio PBC, USA). Rates of decline in FVC and TLCO before and after initiation of cyclophosphamide were calculated by simple linear regression for each patient (**Figure 2.3**). Significance testing of the differences between rates of lung function decline before and after the initiation of cyclophosphamide was assessed by paired Wilcoxon signed-rank test, with $p < 0.05$ considered significant. Two-sided tests were performed to allow for the possibility of cyclophosphamide increasing as well as reducing the rates of lung function decline.

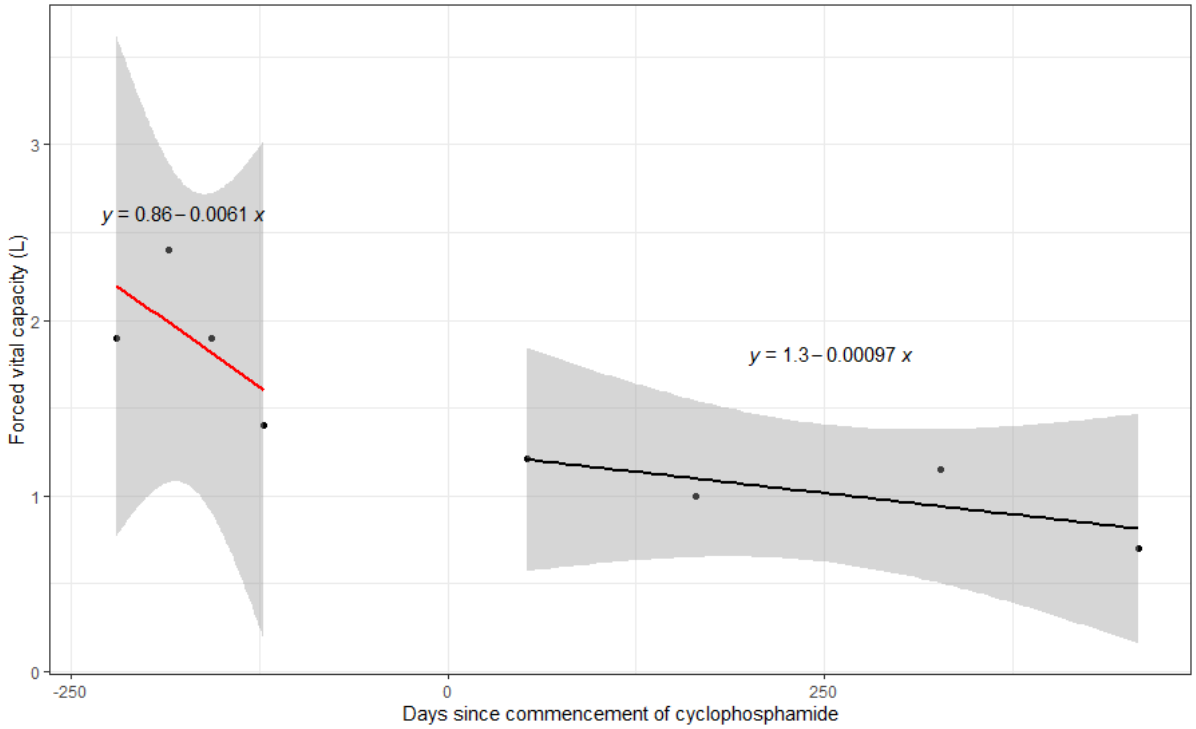

***Figure 2.3**. Illustrative simple linear regression analyses for changes in forced vital capacity prior to (red) and after (black) initiation of cyclophosphamide in one patient. Shaded areas represent standard errors of the regressions. Coefficient of x represents forced vital capacity decline in litres per day.*

2.4 Understanding the role of inflammation in hypersensitivity pneumonitis

This work exploring the transcriptome in hypersensitivity pneumonitis is presented in Chapter 6.

2.4.1 Lung Genomic Research Consortium dataset

The primary source of data for the analysis presented in Chapter 6 is transcriptomic data from the Lung Genomic Research Consortium (LGRC) dataset (National Jewish Health, USA), which is available from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession

number GSE47460. These data are derived from microarray experiments using RNA extracted from homogenised surgical waste tissue of lung biopsy samples from patients with interstitial lung disease, COPD and no chronic lung disease undergoing surgical biopsy, with the diagnosis established by history, radiological examination and surgical pathology. The control lung tissue samples were from patients who underwent surgical biopsy for investigation of a lung nodule and did not have evidence of chronic lung disease as assessed by radiological examination and pathology. The tissue samples and clinical metadata were obtained from the Lung Tissue Research Consortium (LTRC) biospecimen repository (National Heart, Lung and Blood Institute, USA). Tissue samples were processed for the repository by LTRC contributors according to a protocol that was available on the now-defunct LTRC website (which is available via a website archiving tool): briefly, tissue was flash frozen after a maximum ischaemic time of 15 minutes. RNA for the microarray experiments that comprise the LGRC dataset was extracted from the frozen samples.

2.4.2 Microarray data analysis

Microarray data was downloaded and analysed using Genevestigator v8.0.1 (Nebion AG, Zürich). As discussed earlier in this thesis, Genevestigator allows searches of quality controlled versions of public transcriptomic datasets (Hruz *et al.*, 2008). A version of the LGRC data from GEO accession number GSE47460 was accessed. The annotated dataset available through Genevestigator comprises 151 samples acquired on the Agilent Whole Human Genome Array 4x44K G4112F platform and 424 samples acquired on the Agilent SurePrint G3 Human Gene Expression 8x60K Array platform. There can be variation in expression measurements for a given gene across platforms, so data acquired on the latter platform (which also contains samples for a greater number of hypersensitivity pneumonitis patients than the former) were used for analysis. The curated dataset on this platform contains control samples (n = 89); samples from patients with idiopathic pulmonary fibrosis (IPF, n = 122); non-specific interstitial pneumonia (NSIP, n = 14); desquamative interstitial pneumonia (DIP, n = 4); respiratory bronchiolitis-interstitial lung disease (RB-ILD, n = 11); cryptogenic organising pneumonia (COP, n = 2); acute interstitial pneumonia (AIP, n = 1); collagen vascular diseases (CVD, n = 11); hypersensitivity pneumonitis (HP, n = 21); uncharacterised lung fibrosis (UF, n = 6); and chronic obstructive pulmonary disease (COPD, n = 143).

The significance of differentially expressed genes in microarray data was tested in the software using the Linear Models for Microarray data (Limma) algorithm to perform a moderated t-test on the normalised data (Ritchie *et al.*, 2015). Where required, false discovery rate (FDR) was calculated to correct for multiple significance testing (Benjamini and Hochberg, 1995). Genes were considered significantly differentially expressed with at least an absolute $\log_2$-fold change > 0.58 (roughly 1.5-fold change) to $p < 0.05$ or FDR < 0.05. The rationale for this arbitrary threshold is as above in Section 2.2.2. Distance-based clustering of similar genes or conditions was undertaken in the software using the algorithms indicated.

Chapter 3. Ageing and pulmonary inflammation resolution

3.1 Introduction

The work in this chapter seeks to understand whether ageing is associated with a failure of inflammation resolution processes in the human lung, and adequate exploration of this question is contingent upon the presence of reliable experimental models of lung inflammation. Fortunately, as mentioned in the opening section of this thesis, the lung is perhaps one of the more accessible organs for scientific enquiry given its direct communication with the external environment. In addition, the reproducibility of the responses downstream of pattern recognition receptors when ligated by their cognate molecules provides the basis for a robust set of experimental models of inflammation in human volunteers.

The administration of inhaled lipopolysaccharide — the cognate ligand of toll-like receptor 4 (TLR4) — results in a safe, self-resolving pulmonary inflammation. The inflammatory exudate can then be sampled by bronchoalveolar lavage, allowing subsequent interrogation of the inflammatory process using laboratory techniques including flow cytometry. The design of the experiments presented here leans heavily upon these techniques.

The COVID-19 pandemic presented a real-world insight into the perils of disordered pulmonary inflammation — and particularly so in advanced age — and while it did inform some of the work presented later in this thesis, it did disrupt the progress of the experimental work intended for this chapter. As such, this chapter focuses primarily on the background concepts and experimental design, but even with limited results I hope it is evident that valuable insights were gleaned that will inform future inflammation and ageing research.

3.1.1 Modelling human pulmonary inflammation with inhaled lipopolysaccharide

Lipopolysaccharide (LPS) — often referred to as “endotoxin” despite that being more accurate as a term for lipid A, the hydrophobic domain of the molecule (Raetz and Whitfield, 2002) — has been used to model human pulmonary inflammation at least since its implication in respiratory symptoms experienced by textile mill workers (Cavagna, Foa and Vigliani, 1969). Building on previous work suggesting that outbreaks of “mill fever” were associated with the contamination of cotton by Gram-negative organisms, Cavagna and colleagues sought to

reproduce the associated respiratory symptoms in healthy volunteers and volunteers with chronic bronchitis by administering aerosolised endotoxin purified from *Escherichia coli*. The experiment did not include inhalation of a control substance and the sample sizes were small, but the researchers identified transient reductions in lung function in both groups, more so and more readily in the chronic bronchitis group (Cavagna, Foa and Vigliani, 1969).

Experimental evaluations of the pulmonary effects of inhaled endotoxin in humans were limited to observations of clinical parameters perhaps until a study by Sandström and colleagues published in 1992. The researchers performed bronchoalveolar lavage (BAL) on healthy volunteers before and after a challenge with nebulised LPS. The inhalation and lavages were well tolerated by the volunteers, with any symptoms resolving in the hours following inhalation. The results mirrored the results of previous pulmonary endotoxin challenge experiments in animals: LPS inhalation was associated with an increase in BAL cellularity, owing chiefly to a striking rise in the number of neutrophils in the lavage fluid. The retrieval of the immune cells by bronchoalveolar lavage also allowed for an evaluation of cell function, with an observation of reduced phagocytosing capacity of alveolar macrophages after LPS inhalation (Sandström, Bjermer and Rylander, 1992).

An assessment of the safety of LPS inhalation in healthy human volunteers is assisted greatly by dose-response information, and Michel et al. provided useful insights in that regard, having dosed healthy volunteers with up to 50 µg of inhaled LPS. Their volunteers, men and women between the ages of 24 and 50, reported transient malaise, myalgia, shivers and fatigue with the highest dose, and it was noted that the threshold of appreciable immune response to inhaled LPS was 0.5 µg, with detectable changes in sputum and blood neutrophil counts and serum C-reactive protein (CRP) at that dose and higher (Michel *et al.*, 1997).

3.1.2 Administering inhaled lipopolysaccharide

The two primary delivery methods of an inhaled drug are by inhaler devices or nebuliser systems. Typically, however, the latter provides for a high degree of control over the content of the drug delivery system owing to the relative accessibility of the drug reservoir in a nebuliser system compared to that in a metered dose inhaler, for example. This makes a nebuliser system a strong choice for a study such as the one described in this chapter. Once

the decision to deliver the drug by nebulisation is made, the factors that impact upon successful dose delivery by different types of nebulisers must be considered.

3.1.2.1 Jet nebulisation

Nebulisers are systems that convert a liquid solution or suspension into an aerosol that may then be inhaled, typically through the application of a shear force to the liquid. Nebuliser systems are broadly classified by how that force is applied.

Jet nebulisers are perhaps the most common nebuliser systems in clinical application, using force from a compressed gas to draw the liquid through a capillary tube in the drug reservoir, shearing the liquid into an aerosol. The aerosol is directed towards baffles that catch larger aerosol droplets and return them to the reservoir while smaller droplets are allowed through for inhalation (Lavorini, Buttini and Usmani, 2019).

Ultrasonic nebulisers and vibrating mesh nebulisers are less common in clinical practice. Ultrasonic nebulisers apply shear force to the liquid by means of a piezoelectric crystal oscillating at ultrasonic frequencies, and the size of the droplets is related to the oscillation wavelength (O'Callaghan and Barry, 1997). The oscillation may heat the liquid, limiting the utility of ultrasonic nebulisers with heat-sensitive drugs (Lavorini, Buttini and Usmani, 2019). Vibrating mesh nebulisers work on a similar principle although the piezoelectric element is used to oscillate a perforated plate containing apertures that give greater control over the size and flow of the aerosol droplets (Lavorini, Buttini and Usmani, 2019).

Owing primarily to their familiarity, a jet nebuliser was chosen for this study, but when deciding upon a jet nebuliser system it is still necessary to consider the factors that affect the delivery of an accurate dose of the nebulised solution. Many clinical jet nebulisers drive gas through the device continuously, leading to wastage of non-inhaled drug and dissemination of the nebulised solution into the environment. Aside from the undesirable effect of nebulising endotoxin into the research environment, a continuously driven jet nebuliser system would also mean an unpredictable dose of drug would be delivered to the study participant. Intermittent jet nebulisers mitigate this by only entraining gas through the nebuliser on inspiration, maximising the dose delivered to the user and limiting waste (O'Callaghan and Barry, 1997).

After deciding on an intermittent jet nebuliser, it is then necessary to consider that the size of inhaled aerosol droplets and inspiratory flow rate impact upon the site of deposition of the drug in the respiratory tract, with larger particles and higher inspiratory flow rates associated with greater deposition in the oropharynx (Laube, Swift and Adams, 1984; Zeman, Wu and Bennett, 2010). The manufacturers of the Akita Jet nebuliser system (Vectura GmbH, Germany) promote its ability to target inhaled drugs to the peripheral airways using proprietary technology that allows customisation of the nebulisation to the user's lung function (Fischer *et al.*, 2009). A particle deposition study undertaken using the system found that although aerosol was successfully and reproducibly targeted to the lower airways, the largest influence on this was the inspiratory flow rate rather than such technology (Brand *et al.*, 2000), and the device provides the user with feedback to ensure a suitably slow inspiration. Perhaps as a consequence of this successful targeting, the device was demonstrated to facilitate measurable pulmonary inflammatory responses with inhaled LPS doses of as low as 1 μg (Möller *et al.*, 2012). The Akita Jet nebuliser system was therefore selected for this study, with the intention to administer 2 μg of LPS to those participants inhaling LPS.

3.1.3 Bronchoalveolar lavage in the study of human pulmonary inflammation

The use of BAL enables assessments of regional pulmonary inflammation and is therefore an ideal technique for work such as that presented in this part of the thesis. Bronchoalveolar lavage most frequently involves the instillation of saline into segments of the lung followed by retrieval of the fluid by suction, at which point the retrieved saline should contain a roughly representative sample of the cellular and chemical contents of those segments. This is usually done under bronchoscopic guidance, and prior to the invention of the flexible fibreoptic bronchoscope examinations of the human respiratory tract were undertaken by rigid bronchoscopy. Rigid bronchoscopy was limited for only allowing views of larger airways and the requirement for subjects to be under general anaesthesia. The introduction in the 1960s of the "flexible fibrebronchoscope" (as it was known then) likely helped to increase the feasibility of BAL as a diagnostic technique, and the fact that flexible bronchoscopy was more easily performed using local anaesthetic with reduced discomfort may also have facilitated the use of BAL as a research technique (Ikeda, Yanai and Ishikawa, 1968).

Cantrell and colleagues were among the first researchers to employ fibreoptic bronchoscopy and BAL in such a capacity. In a 1973 study, the authors describe the retrieval of pulmonary alveolar macrophages from healthy volunteers (some of whom were cigarette smokers) by BAL with the aim of understanding the effect of cigarette smoking on aryl hydrocarbon hydroxylase enzymes within lung immune cells (Cantrell *et al.*, 1973).

3.1.3.1 Procedure safety

Bronchoscopy is generally considered safe, with complication rates in a multicentre series of 20,986 clinical rigid and flexible bronchoscopies as low as 1.08% with a mortality of 0.02% (Facciolongo *et al.*, 2016). It stands to reason that research bronchoscopies are even safer than clinical bronchoscopies considering that the former are often performed on healthy volunteers, and very often involve additional procedures no more involved than lavage. Procedures involving biopsies are deemed riskier numerically in the above cited series, with complications in 0.97% and 3.43% of cases where bronchial biopsy and transbronchial biopsy were performed respectively, compared to 0.70% of cases where only BAL was performed (Facciolongo *et al.*, 2016).

Collins and colleagues draw on their experience of research bronchoscopies to provide a useful list of adverse events associated with research BAL that can be quoted to inform participants when obtaining consent to undertake the procedure: sore throat/hoarseness (~1 in 4 cases), fever (~1 in 100), infection (<1 in 1000), epistaxis (<1 in 1000), endobronchial bleed (<1 in 5000). The authors also state that in their experience, sedation with the benzodiazepine drug midazolam does not appear to improve the tolerability of the procedure (Collins *et al.*, 2014).

3.1.3.2 BAL volume and cell yields

The number of cells required for use in downstream assays should always be considered when planning studies employing BAL, and a decision must therefore be made about the volume of saline to be used for lavage. Saline volumes of 150-200 ml are typically instilled in sequential aliquots of around 50 ml, but it is certain that a proportion of the total volume instilled will not be recovered, likely due to the structure and compliance of the lung. Retrieval of greater than 50% of the volume instilled is considered a good yield in research bronchoscopy (Collins

et al., 2014), and age correlates negatively with the proportion of fluid recovered, possibly due to a loss of elasticity of the lung with age (Meyer *et al.*, 1998; Heron *et al.*, 2012).

When considering the total volume for instillation, it should also be noted that the first retrieved aliquot (the so-called "bronchial sample") is considered to contain a greater proportion of secretions and epithelial cells from the more proximal airways, and may often be discarded in favour of the subsequent aliquots ("alveolar samples") which more closely reflect the contents of the distal airways and alveoli (Rennard *et al.*, 1990; Heron *et al.*, 2012).

In healthy non-smoking adults, the cellular component of the alveolar sample is roughly composed of 85% alveolar macrophages, 12% lymphocytes and 1% neutrophils, with total cellularity on the order of 100,000 cells per ml of retrieved fluid (Heron *et al.*, 2012). The cellular composition of the BAL fluid changes dramatically after inducing inflammation with inhaled LPS, with one study suggesting 26% macrophages, 23% neutrophils and 20% lymphocytes in BAL fluid 9 hours after the inhalation of 60 µg of LPS by healthy male non-smokers (Brittan *et al.*, 2014). The cellularity of the fluid may also increase, with the same investigators reporting around 300,000 cells per ml in the BAL fluid of the volunteers challenged with 60 µg of inhaled LPS compared to 100,000 cells per ml after challenge with saline control (Brittan *et al.*, 2012).

3.2 Study design

The primary aim of the study was to test the hypothesis that *ageing is associated with a failure of inflammation resolution processes in the immune cells of the human lung*. Comparing younger and older volunteers, the study aimed to discern differences between pro-resolution characteristics and functions of immune cells isolated from the BAL fluid of healthy volunteers exposed to either inhaled LPS or inhaled saline as a control. In addition, the study aimed to model any systemic responses to experimentally induced pulmonary inflammation, again with the aim of identifying differences between responses in younger subjects and older subjects.

In the absence of suitable preliminary data, the study was designed as a pilot study, with arbitrary sample sizes of up to nine volunteers in the younger cohort and nine in the older cohort, with five in each cohort allocated to inhale LPS and the remainder saline. Age

thresholds were also arbitrary, with the younger volunteers aged between 16 and 30, and the older volunteers aged over 65.

Following screening and recruitment, volunteers were to attend the main study day, with clinical procedures undertaken in a bay of a hospital intensive care unit reserved for clinical research. Clinical parameters were to be measured prior to and in the hours following inhalation of a bolus of LPS or saline, and serial blood samples were to be taken to facilitate monitoring of blood leukocyte and serum cytokine kinetics that would allow modelling of the progression of any systemic inflammation.

At eight hours following the inhalation of LPS or saline, volunteers were to undergo a protocolised bronchoscopy and BAL. After a short period of observation post-procedure volunteers were to be allowed home with a plan to attend the following morning for follow-up and one further blood sample.

BAL fluid was to be processed in the laboratory to separate the supernatant from the cells. The supernatants were to be stored to facilitate measurement of BAL fluid cytokine concentrations, and the cells were to be prepared for functional and flow cytometric assays pertaining to the resolution of inflammation. Blood samples were to be prepared for flow cytometry and serum cytokine measurements to track the progression of any systemic inflammatory response associated with LPS inhalation.

Due to the COVID-19 pandemic, only one of the screened volunteers attended the main study day, and laboratory analysis of fresh samples was interrupted due to laboratory closure.

3.2.1 Ethical and regulatory approvals

The chief investigator of the study was Professor John Simpson of Newcastle University. The regulatory sponsor of the study was the Newcastle Joint Research Office (a partnership between the Newcastle upon Tyne Hospitals NHS Foundation Trust and Newcastle University) and ethical approval for the study was granted by the Yorkshire & The Humber – Leeds West Research Ethics Committee (REC) and the NHS Health Research Authority (HRA) with REC reference 18/YH/0088 (*The effect of ageing on acute inflammatory responses in humans*). The Research and Innovation Department at City Hospitals Sunderland NHS Foundation Trust (later South Tyneside and Sunderland NHS Foundation Trust following a merger between City Hospitals Sunderland NHS Foundation Trust and South Tyneside NHS Foundation Trust in April

2019) acted as a partner in research delivery. Clinical procedures were undertaken at Sunderland Royal Hospital.

3.2.2 Study data management

Study data derived from samples were stored in a database in pseudonymised form, with each participant given a unique study identification number only linked to their name and contact details on a list held in a locked drawer at Newcastle University. During the clinical procedures clinical information was documented and stored according to usual clinical data management practices at Sunderland Royal Hospital. Clinical staff therefore had access to participants' demographic information and clinical information from the screening visits that allowed the provision of safe care.

3.3 Results

3.3.1 Participant characteristics

Ten volunteers attended for screening between September 2019 and February 2020. The screened volunteers had a mean age of 23 (standard deviation = 2.5 years). Seven of these volunteers were eligible for inclusion in the study (**Table 3.1**). One participant was excluded due to a diagnosis of asthma and two participants were excluded due to white blood cell counts outside the laboratory reference range.

Characteristic	Screened (*n* = 10)	Eligible (*n* = 7)	Reference range
Mean age at screening date (SD), years	23 (2.5)	22 (2.1)	
Sex, % male	30%	29%	
Mean (SD) % of predicted FEV1	106% (13.8%)	110% (14.4%)	
Mean (SD) % of predicted FVC	106% (10.8%)	107% (12.0%)	
Mean white cell count, 10^9/L	6.57 (2.19)	6.72 (1.37)	4.0 – 11.0
Mean neutrophil count, 10^9/L	3.97 (1.92)	4.05 (1.30)	1.8 – 7.5

Table 3.1*. Characteristics of volunteered screened for inclusion in the study. FEV1 = forced expiratory volume in first second; FVC = forced vital capacity; SD = standard deviation*

3.3.2 A feasible model for the study of pulmonary inflammation with inhaled drug delivery

Although only one participant completed all clinical procedures prior to study suspension, the study as designed uses techniques that have been used in prior studies safely (Brittan *et al.*, 2012; Jardine *et al.*, 2019) and provides an experimental system for assessing pulmonary and systemic inflammation with or without an inhaled drug. The volunteer described here did not inhale LPS, but the cited studies provide evidence of the safety of inhaling larger doses of LPS than planned for this study.

The model described in this study could also easily be adapted for use as part of an early phase trial of a candidate anti-inflammatory or pro-resolution drug, with administration of a candidate drug prior to inhalation of LPS or saline, followed by peripheral blood and BAL sampling. Work to identify candidate pro-resolution drugs that could be trialled in such a system is presented later in this thesis.

3.3.3 Barriers to the participation of older volunteers in experimental medicine studies

Identifying potential participants as described resulted in numerous expressions of interest over the study period, and subsequently ten younger volunteers attended for screening between September 2019 and March 2020 (of whom seven were eligible for inclusion in the study). No older volunteers attended for screening.

To determine if the study design included unforeseen barriers to participation for older people, I attended a meeting of the VOICE Research Support Group at Newcastle University in March 2020. The Research Support Group is composed of patients, carers and other members of the public with an interest in research. The group frequently provides feedback on and strategic guidance for research projects to ensure members of the lay public contribute to research in a manner that maximises the benefits of research to society at large.

The meeting took the format of a presentation on this research project and experimental medicine studies in general to ten members of the Research Support Group all above retirement age, followed by an unstructured group discussion about the themes in the presentation.

Broadly, the group expressed that they appreciated the importance of experimental medicine studies investigating lung inflammation, especially given the developing news of the COVID-

19 pandemic at the time. The prospect of undergoing clinical procedures such as bronchoalveolar lavage was not a deterrent, nor was the inhalation of lipopolysaccharide, but the prospect of a long study day (with a requirement to remain nil by mouth for a large portion of it) was. In addition, the group pointed out that if we were seeking to recruit participants from the Newcastle upon Tyne area the travel to Sunderland for the study day would extend the day further.

Some of the expressions of interest we received from older volunteers were in response to the advertisements in influenza vaccination clinics at general practitioners' surgeries. The research support group mentioned that if we wanted to broaden our reach, we might also wish to advertise in community centres including libraries and leisure centres, where we might have hoped to have our advertisements seen by older people attending for group activities or exercise.

3.4 Discussion

It is unfortunate that the study was interrupted by the COVID-19 pandemic. Prior to the enforced closure of the university laboratory, there were concerns about the extent to which it was possible to guarantee the safety of volunteers who attended the Sunderland ICCU for the study days. Even though the ICCU has a dedicated cubicle with associated monitoring equipment for research, the volunteers would still have been attending a clinical area likely to be involved in the care of patients with COVID-19, increasing the risk of transmission. It had also been recognised that older people were at heightened risk from COVID-19 infection, with a greater risk of developing severe disease in this population (Torres Acosta and Singer, 2020).

An additional safety concern was the risk to the research team from exposure to the then-novel coronavirus during the clinical and laboratory procedures. Asymptomatic infection and transmission had been described at that point (Chan *et al.*, 2020; Pan *et al.*, 2020), and it was likely that bronchoscopy and lavage would result in aerosolisation of respiratory secretions, which is recognised to increase the risk of transmission of respiratory pathogens to healthcare workers (Tran *et al.*, 2012). Where the bronchoscopies were not taking place for essential clinical reasons, it was decided that the risks outweighed the benefits.

Shortly after those issues were considered, the secondary legislation enacting the coronavirus lockdown came into force in England, imposing restrictions on people leaving their homes without a reasonable excuse, the list of which did not include participation in research where the research was not part of medical care (*The Health Protection (Coronavirus, Restrictions) (England) Regulations*, 2020). This resulted in complete cessation of the study, and clinical researchers on the study team were redeployed to assist the pandemic response shortly afterwards (Medical Research Foundation, 2020).

The discontinuation notwithstanding, the volunteer who completed their participation progressed smoothly through screening, recruitment and the clinical procedures, performing the inhalation without difficulty and kindly providing biological samples (although these could not subsequently be processed). A concern — which likely arose due to oversight in the study design — was the slow recruitment of older volunteers. The insights from the unstructured group discussion on how to improve participation of older volunteers were welcome, but the focus group was organised in response to poor recruitment rather than prior to or as part of study design. Although this reactive approach did yield information that will be useful in the design of future studies involving older volunteers, in retrospect a better approach would have been to include lay representatives of older people in the research design process with continued collaboration throughout the research process. This approach has the potential to enhance relationships between researchers, the public and research, maximising the chances of public benefit (Rolfe *et al.*, 2018).

Chapter 4. Drug repurposing to enhance the resolution of inflammation

4.1 Introduction

If enhancing the resolution of inflammation has benefits for health, it becomes necessary to seek interventions with that effect to translate to clinical practice. However, the journey of new drug treatments to clinical application is fraught with difficulty and increasingly so, with fewer drugs completing successful clinical trials despite rising research and development budgets (Scannell *et al.*, 2012).

Drug repurposing — the identification of new indications for drugs that have already entered investigational or clinical stages of use — may increase the efficiency of the drug development pipeline, given that some attributes of established drugs (such as safety/toxicity profiles) will already be known. Furthermore, with increasingly comprehensive databases of drug effects and greater computational power come the possibility for much of the hypothesis generation for drug repurposing to be done *in silico*, allowing improved targeting of subsequent *in vitro* or *in vivo* experimentation (Pushpakom *et al.*, 2019).

The computational prediction of a drug's effects on a cell is made feasible through the tools of systems biology. Systems biology is a discipline that in part aims to facilitate models of biological behaviour by understanding the components of the biological processes, and in practice this often means the collection of large quantities of data regarding a cell's gene or protein expression to give insight into how a cell functions under defined conditions (Butcher, Berg and Kunkel, 2004).

Connectivity maps are examples of how these data may be exploited to aid drug repurposing. Connectivity maps are catalogues of the gene expression profiles of cells exposed to different perturbagens (drugs or small molecules, for example), enabling screening for perturbagens that result in particular patterns of gene expression (Qu and Rajpal, 2012). This approach is better suited to hypothesis generation than hypothesis confirmation, however, for two main reasons: firstly, it is possible that the connectivity map has been at least partially computationally derived rather than directly measured such as in the case of the L1000 Connectivity Map (Subramanian *et al.*, 2017); and secondly, gene expression does not always correlate to functional protein expression.

Nonetheless, computational techniques may be harnessed to focus searches for drugs that may enhance the resolution of inflammation, and as above, using connectivity maps for this purpose requires prior knowledge of gene targets pertinent to biological processes in resolution. Given that efferocytosis plays a crucial role in the resolution of inflammation, the genes known to be involved in regulating the process could be reasonable starting points to identify resolution-enhancing drugs from connectivity maps.

The work in this chapter starts with work evaluating the feasibility of using small panels of genes to search a connectivity map, which is an unorthodox strategy seeing as connectivity maps are designed to use broad gene signatures as inputs in searches. Following that, I attempt to identify drugs that could plausibly modulate efferocytosis by screening the connectivity map using panels of genes known to regulate the process. I then present work that aims to test the hypothesis that the drugs identified in the screen enhance efferocytosis *in vitro*.

4.1.1 Connectivity map screening with small gene panels

The work presented in this chapter relies upon a subversion of the more commonly documented application of connectivity maps in drug discovery. It is often the case that connectivity maps are searched using gene expression signatures, where the differential expression of a large panel of genes in a cell state is documented with respect to a control cell state, and those data are then matched to a group of perturbagens in the connectivity map that either induce a similar gene expression signature (for agonists) or induce a signature similar to the inverse (for antagonists) (Qu and Rajpal, 2012; Han *et al.*, 2018). What is less clear, however, is whether there is still value in performing the screen if perturbation signatures in a connectivity map are disaggregated into panels comprising fewer (or even single) genes. This would allow searches for perturbagens that impact disproportionately upon genes close to the top of the gene regulation hierarchy for a particular biological process — transcription factors, perhaps — which would mean that the drugs found in the screen could have utility as modulators of the "master switches" of the biological processes in question.

It could be posited that considering smaller panels of genes might blind a researcher to off-target effects of the identified drugs, reducing the specificity of the search, but I argue that

this can be mitigated by filtering the identified drugs for compounds that are already in clinical use as treatments, meaning that there will be at least some prior knowledge of undesirable effects *in vitro* and *in vivo*.

The COVID-19 pandemic brought to prominence a disease in which a single gene might have an outsized impact upon the disease process. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which infects a target cell by engaging its spike (S) protein with the host cell receptor ACE2 and subsequently fusing its membrane with that of the cell. In order for the virion to enter the target cell, the S protein must be primed by the cellular serine protease TMPRSS2, the inhibition of which has been demonstrated to limit SARS-CoV-2 infection of a human lung cancer cell line *in vitro* (Hoffmann *et al.*, 2020). Rather usefully for a researcher seeking to reduce the gene expression of this protease, TMPRSS2 has no known physiological function in mammals (Kim *et al.*, 2006).

Given this, I hypothesised that some of the perturbagens in a publicly available connectivity map would cause a reduction in expression of *TMPRSS2* mRNA in the tested cells, and furthermore, some of the identified perturbagens might be drugs with prior approvals for use in humans. If this held true in a manner that could be validated, it would provide a proof of concept for the screening of connectivity maps using limited panels of genes rather than broad gene signatures, which may allow application in other biological processes where process-critical genes might be known, such as inflammation resolution, explored thereafter.

4.2 Results

4.2.1 L1000 Connectivity Map contains approved drugs that are modelled to downregulate *TMPRSS2* mRNA expression

Of the compounds in the curated L1000 dataset that were tested in 24-hour perturbation assays, 40 drugs and tool compounds were identified that significantly downregulated *TMPRSS2* expression in A549 cells (a human alveolar epithelial cell line) in at least one of the tested drug concentrations. Of the drugs identified, 9 are drugs with prior approvals for use in humans for alternative indications at the time of writing: alpelisib/BYL719, crizotinib, fedratinib/TG-101348, neratinib, nilotinib, nintedanib, ruxolitinib, selumetinib, and vemurafenib (**Table 4.1**). None of these compounds significantly affected *ACE2* expression.

Perturbagen	Drug class	Concentration (μM)	log_2 ratio change in *TMPRSS2* expression (perturbagen/DMSO)	*p* value
Alpelisib/BYL719	PI3K inhibitor	0.04	-0.94	0.012 *
		0.12	-0.97	0.010 *
		0.37	-0.69	0.068
		1.11	-0.75	0.046 *
		3.33	-0.50	0.186
		10	-0.52	0.162
Crizotinib	ALK inhibitor	0.12 †	-0.32	0.485
		0.37	-0.57	0.127
		1.11	-0.44	0.243
		3.33	-0.76	0.043 *
		10 †	-0.09	0.844
Fedratinib/TG-101348	JAK2 kinase inhibitor	0.04	-0.60	0.112
		0.12	-0.68	0.069
		0.37	-0.76	0.042 *
		1.11	-0.65	0.083
		3.33	-0.38	0.307
		10	-0.41	0.278
Neratinib	Tyrosine kinase inhibitor	0.04	-0.29	0.441
		0.12	-0.36	0.336
		0.37	-0.60	0.109
		1.11	-0.96	0.011 *
		3.33	-0.22	0.564
		10	-0.59	0.116
Nilotinib	Bcr-Abl tyrosine kinase inhibitor	0.04	-0.81	0.031 *
		0.12	-0.65	0.083
		0.37	-0.60	0.112
		1.11	-0.44	0.243
		3.33	-0.56	0.138
		10	-0.12	0.754
Nintedanib	Tyrosine kinase inhibitor	0.04	-0.85	0.024 *
		0.12	-0.36	0.334
		0.37	-0.42	0.263
		1.11	-0.23	0.541
		3.33	-0.38	0.316
		10	0.21	0.568
Ruxolitinib	JAK1/2 inhibitor	0.04	-0.55	0.141
		0.12	-0.84	0.025 *
		0.37	-0.52	0.168
		1.11	-0.51	0.177
		3.33	-0.61	0.105
		10	-0.82	0.028 *
Selumetinib	MEK inhibitor	0.04	-0.31	0.402
		0.12	-0.51	0.176
		0.37	-0.82	0.030 *
		1.11	-0.55	0.146
		3.33	-0.61	0.104
		10	-0.92	0.014 *
Vemurafenib	B-raf inhibitor	0.04	-0.29	0.444
		0.12	-0.35	0.355
		0.37	-0.90	0.014 *
		1.11	-0.72	0.054
		3.33	-0.66	0.077
		10	-0.43	0.249

***Table 4.1**. Change in TMPRSS2 expression in A549 human alveolar epithelial cells exposed to perturbagens or DMSO control for 24 hours. Asterisk (*) denotes p < 0.05. Dagger (†) denotes data only available from n = 2 experiments; n = 3 for all other experiments. DMSO = dimethyl sulfoxide; PI3K = phosphatidylinositol 3-kinase; ALK = anaplastic lymphoma kinase; JAK = Janus kinase; MEK = mitogen-activated protein kinase kinase.*

4.2.2 Literature searches suggest selumetinib as a potential inhibitor of SARS-CoV-2 cell entry and TMPRSS2 expression

Selumetinib is a mitogen-activated protein kinase (MAPK/ERK) kinase (MAPKK/MEK) inhibitor that has FDA approval for the treatment of neurofibromatosis type 1, a multisystem disorder that is most often characterised by a propensity for neurofibroma formation. Although selumetinib has not been trialled in COVID-19 patients, *in vitro* data suggest it may have some antiviral activity. Selumetinib (as well as some other MAPK pathway modulators) was found to inhibit the infection of a human hepatoma cell line with Middle East respiratory syndrome coronavirus (MERS-CoV) although the modulation of TMPRSS2 was not directly implicated (Kindrachuk *et al.*, 2015). MERS-CoV is a coronavirus related to SARS-CoV-2 that also requires TMPRSS2 for S protein priming before cell entry, and similarly to SARS-CoV-2, inhibition of TMPRSS2 with a serine protease inhibitor limits MERS-CoV infection of simian kidney epithelial cells *in vitro* (Shirato, Kawase and Matsuyama, 2013).

Separately, Zhou and colleagues identified that MEK inhibitors including selumetinib and trametinib attenuate the expression of TMPRSS2 in human lung and colon cell lines (Zhou *et al.*, 2020). These findings provided circumstantial evidence of a successful search for a candidate TMPRSS2 modulator using a limited gene panel rather than a gene expression signature as the search query. I proceeded to apply the approach to a gene relevant to inflammation resolution.

4.2.3 Several cyclin dependent kinase inhibitors upregulate *EDIL3* gene expression

Because of literature suggesting its role in inflammation resolution and because its gene product is a secreted protein that would be convenient to measure in future experiments, I selected the gene *EDIL3,* and I also examined a panel of its associated genes.

EDIL3 codes for the multifunctional protein developmental endothelial locus-1 (Del-1), which is secreted by cells under the influence of endogenously produced pro-resolving mediators such as resolvin D1 as efferocytosis occurs. Del-1 can enhance the efferocytic capacity of macrophages in a manner mediated by the action of the transcription factor liver X receptor (alpha and beta isoforms, LXRα/LXRβ) (Kourtzelis *et al.*, 2019). Del-1 can also interfere with extravasation of activated neutrophils by blocking their adhesion to endothelial cell surfaces (Choi *et al.*, 2008). Both these actions contribute to the resolution of inflammation.

I searched the L1000 dataset for drugs that were able to upregulate the expression of *EDIL3* in the tested cell lines using the arbitrary threshold of a 2 log_2-fold increase in expression to $p < 0.05$. This yielded a list of 40 drugs and tool compounds in various concentrations (**Figure 4.1**).

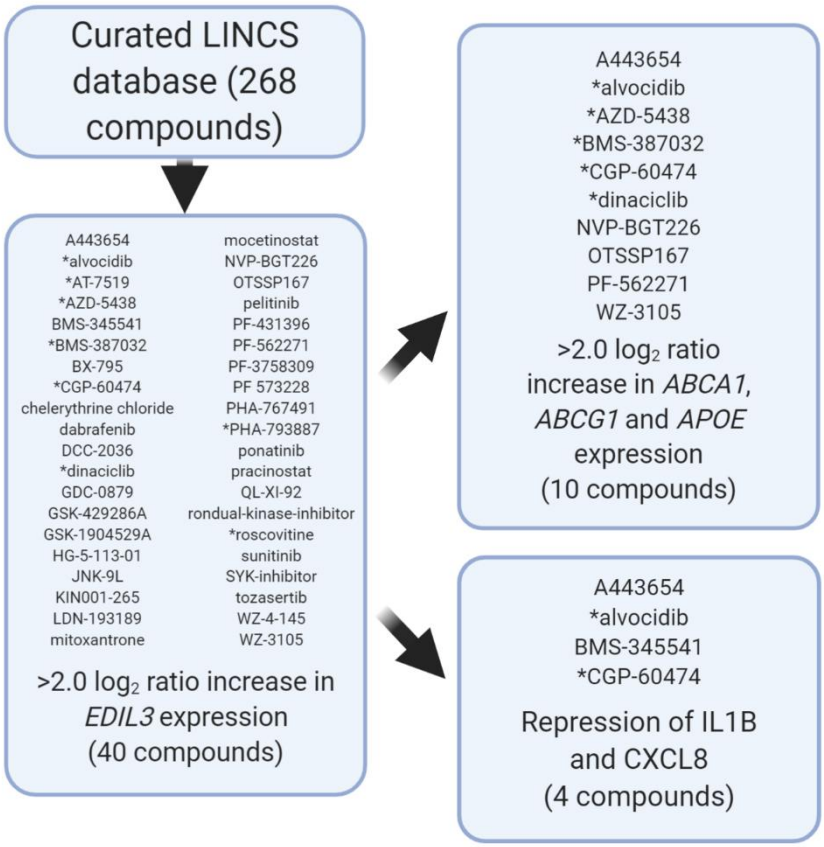

***Figure 4.1**. Screen for candidate pro-resolution compounds from LINCS database. Asterisks denote cyclin dependent kinase inhibitors*

I then aimed to identify the experimental conditions that could have been associated with the secretion and activity of Del-1 by screening for the upregulation of genes relating to products downstream of Del-1. As mentioned above, the efferocytosis-enhancing action of Del-1 is mediated at least in part by activation of LXRα/LXRβ (Kourtzelis *et al.*, 2019). Both LXR isoforms induce the transcription of cholesterol efflux genes such as those encoding the ATP-binding cassette transporters ABCA1 and ABCG1, and the fat-binding protein apolipoprotein E (APOE) (Alonso-Gonzalez and Hidalgo, 2014; Tall and Yvan-Charvet, 2015). As such, I surmised that experiments in which both *EDIL3* and those associated lipid metabolism genes were upregulated were likely to be those in which active Del-1 was being secreted by cells.

In one or more of the tested concentrations in one or more of the treated cell lines, 21 of the identified putative Del-1 inducers were able to increase *ABCA1* expression at least 2 $\log_2$-fold ($p < 0.05$) compared to control, and 12 of those compounds increased expression of both *ABCA1* and *ABCG1* beyond the same threshold. 10 compounds met the arbitrary threshold of at least 2 $\log_2$-fold increases of *ABCA1*, *ABCG1* and *APOE* in the experiments included in the connectivity map (FDR <0.05), suggesting that these were most likely to be associated with Del-1-mediated activation of LXR (although this does not rule out some less potent activity by other drugs in different concentrations).

Eight of the identified compounds (alvocidib, AT-7519, AZD-5438, BMS-387032, CGP-60474, dinaciclib, PHA-793887 and roscovitine) are cyclin dependent kinase (CDK) inhibitors, a drug class containing compounds that have been demonstrated to enhance the resolution of inflammation in experimental contexts (Rossi *et al.*, 2006).

4.2.4 *EDIL3* inducers repress genes encoding pro-inflammatory cytokines

Aside from regulating lipid efflux, LXR activation also suppresses the transcription of pro-inflammatory cytokines including those under the transcriptional control of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Joseph *et al.*, 2003; Ogawa *et al.*, 2005). I screened the list of 40 putative Del-1 inducers for evidence of suppression of a panel of NF-κB-related pro-inflammatory genes (*IL1B*, *IL6*, *CXCL8* and *TNF*). Four compounds (A443654, alvocidib, BMS-345541 and CGP-60474) were able to repress *IL1B* expression significantly in at least one L1000 experiment, and these compounds were also associated with significant repression of *CXCL8* (**Figure 4.2**).

Thus, I identify a short list of candidate compounds with potential to both limit the propagation of and enhance the resolution of inflammation: the Akt inhibitor A443654, the IκB kinase (IKK) inhibitor BMS-345541, and the CDK inhibitors alvocidib and CGP-60474. Of these, alvocidib (also known as flavopiridol) has prior regulatory approval for use in humans.

Figure 4.2*. Gene expression profiles of HME1 human mammary epithelial cells perturbed with candidate compounds for 24hrs vs DMSO control. Values to the right of heatmap highlight differential EDIL3 expression.*

4.2.5 No effect of flavopiridol hydrochloride on efferocytosis over the observed period

Two CDK inhibitors were selected for further evaluation in an *in vitro* efferocytosis assay: alvocidib/flavopiridol and CGP-60474. Flavopiridol was selected because of its prior approvals for use in humans, which would streamline any future experimental medicine research using the drug in the event of positive results. CGP-60474 was selected due to apparently potent anti-inflammatory effects and minimal cytotoxicity in the above cited animal study (Han *et al.*, 2018). The series of experiments aimed to test the hypothesis that the drugs enhance efferocytosis undertaken by human immune cells. The original intention was to undertake efferocytosis assays using human alveolar macrophages donated by volunteers for the immune challenge study detailed earlier in this thesis, but in light of the challenges posed by the COVID-19 pandemic on the recruitment of volunteers for bronchoalveolar lavage, the experiments described in this section used human monocyte-derived macrophages.

Concentrations of flavopiridol and CGP-60474 were selected to provide ranges around the doses producing effects on efferocytosis-associated genes in the L1000 data. The selected concentrations also encompassed reference values for the half-maximal inhibitory concentrations of the drugs for cyclin-dependent kinases, although it is appreciated that cyclin-dependent kinase inhibition may not be the mechanism by which any effects on inflammation resolution might manifest.

Exposure of monocyte-derived macrophages to flavopiridol hydrochloride in the three concentrations used resulted in no change in efferocytic index compared to vehicle control (p = 0.88 by one-way ANOVA) (**Figure 4.3**).

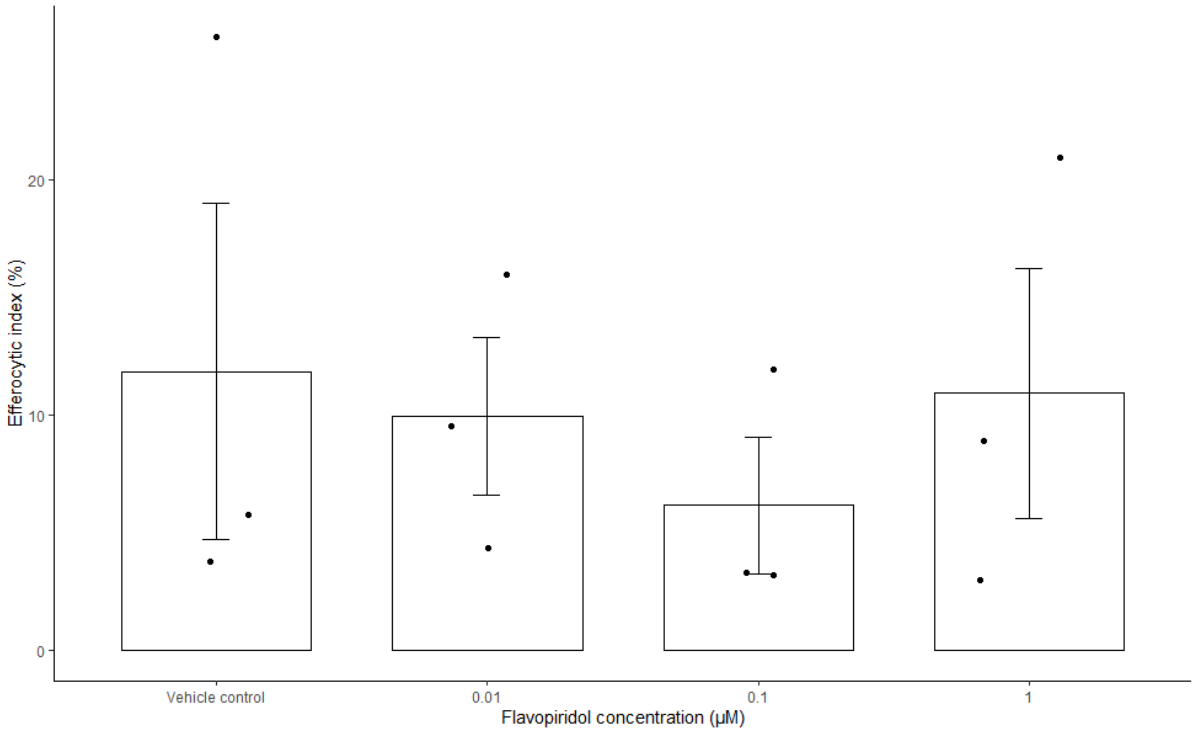

***Figure 4.3**. Effect of flavopiridol hydrochloride and vehicle control on efferocytic index (n = 3, experiments performed in duplicate), with efferocytic index defined as the proportion of monocyte-derived macrophages assumed to have ingested labelled apoptotic neutrophils. Error bars represent standard error. M = molar concentration*

4.2.6 No effect of CGP-60474 on efferocytosis over the observed period

Exposure of monocyte-derived macrophages to CGP-60474 also resulted in no change in efferocytic index compared to vehicle control (p = 0.86 by one-way ANOVA) (**Figure 4.4**).

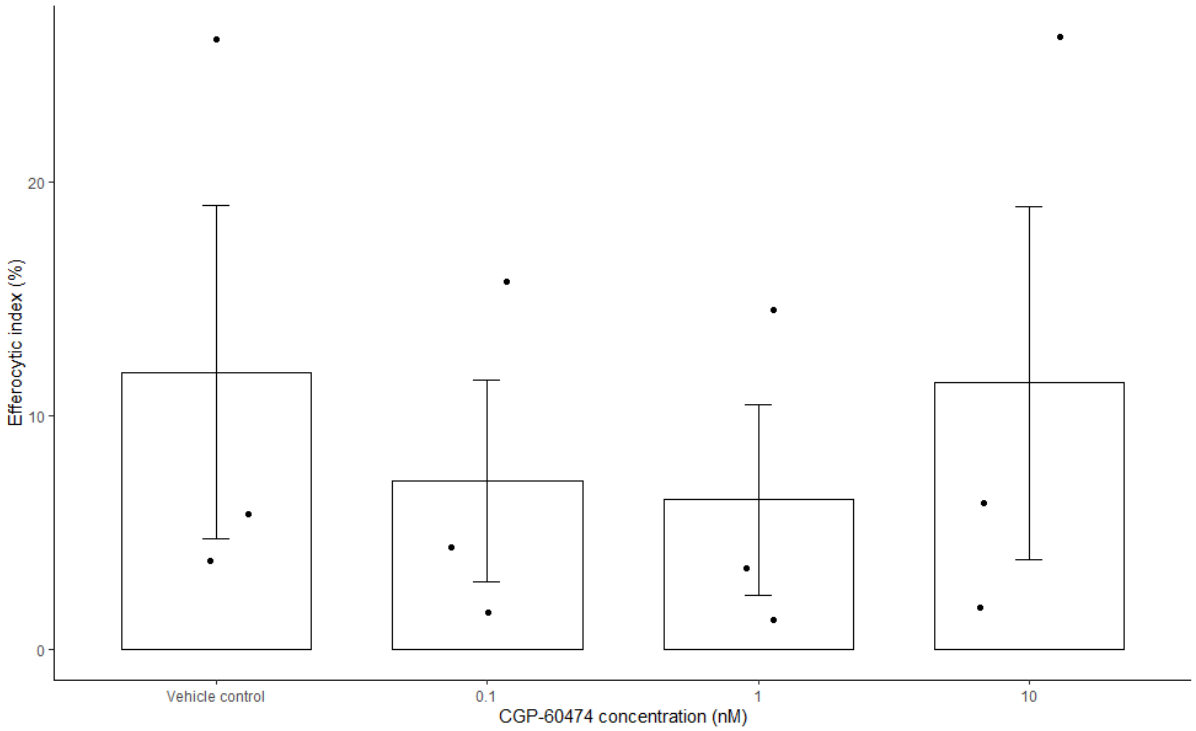

***Figure 4.4**. Effect of CGP-60474 and vehicle control on efferocytic index (n = 3, experiments performed in duplicate), with efferocytic index defined as the proportion of monocyte-derived macrophages assumed to have ingested labelled apoptotic neutrophils. Error bars represent standard error. M = molar concentration*

4.2.7 No effect of negative control on efferocytosis over the observed period

There was also no difference in efferocytic index between macrophages treated with vehicle control and cytochalasin D (p = 0.35), which — among other potential explanations explored in the discussion below — may suggest that efferocytosis was observed over too short a period for differences to become apparent.

4.3 Discussion

The findings showing no effect of the selected drugs on efferocytosis should prompt challenges of the prior assumptions of the assay. It is assumed that exposure of the macrophages to apoptotic cells results in their phagocytosis by efferocytic mechanisms. However, without clearer evidence that the apoptotic cargo is being internalised and, if so, the internalisation being the result of mechanisms known to be related to recognition of apoptotic material, then it is possible that this is an incorrect assumption. One alternative explanation for the appearance of the flow cytometry plots is that some of the labelled neutrophils remain adherent to the culture plate plastic despite the washes and are then captured as CellTracker-positive events on flow cytometry regardless of internalisation. It was initially intended that events representing macrophages with efferocytosed neutrophils

would be positive for both CellTracker and APC fluorescence (with APC conjugated to an anti-CD14 antibody bound to macrophage surface CD14), which would make it more credible that a CD14-positive macrophage contains a CellTracker-positive neutrophil. Upon acquisition, however, I found that the monocyte-derived macrophages expressed too little CD14 compared for APC fluorescence to be used to identify macrophages in addition to using light scatter alone (**Figure 4.5**). This may be consistent with the findings of Schulz and colleagues, who describe a reduction in CD14 expression on human monocyte-derived macrophages following exposure to GM-CSF (Schulz *et al.*, 2019). In retrospect, perhaps a better surface marker to identify the macrophages beyond just light scatter values would have been CD206, expressed highly on monocyte-derived macrophages (Davies and Gordon, 2005) and less so on neutrophils (Yu *et al.*, 2016). That said, an even better method to prove internalisation of CellTracker-labelled cargo would be direct visualisation with fluorescence microscopy.

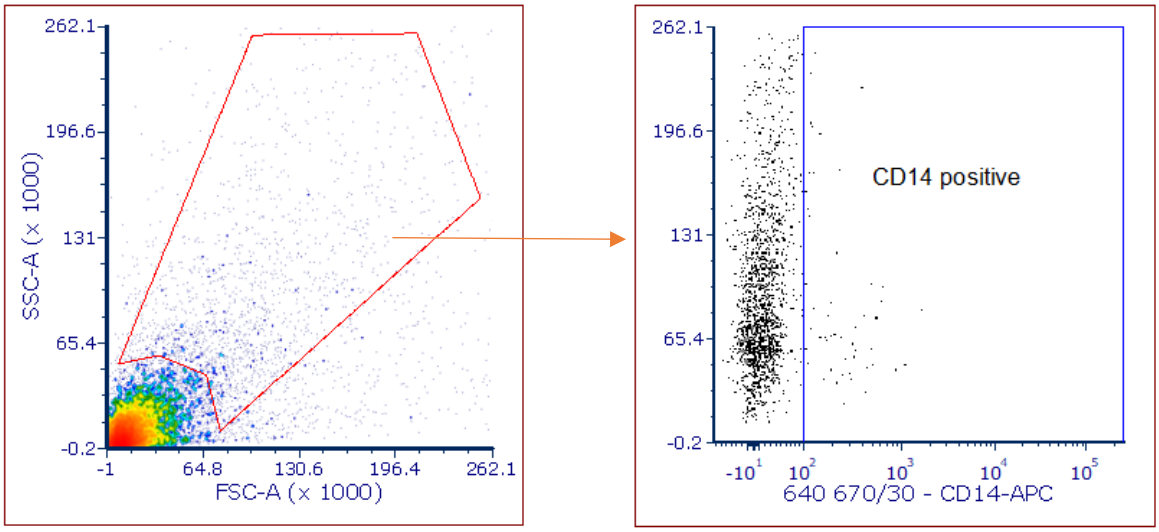

***Figure 4.5**. Representative plot showing low expression of CD14 on cultured monocyte-derived macrophages, with CD14 positive gate set by fluorescence-minus-one control.*

The purpose of the washing step in the assay as performed was to remove non-adherent cells from the culture plates, with the assumption that those cells removed by washing three times with PBS were non-internalised and/or dead cells, as in other published efferocytosis assays (Hulsebus *et al.*, 2016; Kasikara *et al.*, 2021). This is of course an assumption that could lead to a falsely depressed efferocytic index if a sizeable proportion of macrophages included in the total number of cells counted were in fact dead cells that had become apoptotic or necrotic during the assay prior to co-incubation with apoptotic neutrophils. During

optimisation, a live/dead stain could be used on a sample of the macrophages to ensure the majority of cells counted after the wash steps are live.

The absence of effect of cytochalasin D compared to vehicle control calls into question the ability of the assay described to detect differences in efferocytosis at all. Cytochalasin D blocks phagocytosis through a reduction of the extent of actin polymerisation (Goddette and Frieden, 1986). As such, a reduction in efferocytic index would be expected in cells treated with the compound. The large standard deviations of observed efferocytic indices might suggest that the experiment as performed lacked sufficient precision to detect differences between conditions. Having used $n = 3$ leaves the efferocytic index susceptible to skew by a single biological or technical outlier, and this is perhaps suggested by the mean efferocytic index of vehicle-treated macrophages being 11.9% with a standard deviation of 12.4%. Assuming these are reasonable pilot data, though, a future experiment guided by these would require $n = 17$ to provide 80% power to detect a doubling of the efferocytic index (arbitrary effect size) from that of vehicle control with $p < 0.05$.

An alternative explanation for cytochalasin D having no effect would be that efferocytosis was not allowed to take place for long enough for conditions to diverge. Optimisation of the assay would involve using a range of co-culture durations.

Just as the use of an effective negative control in the assay would provide some confidence that detectable differences in CellTracker expression in macrophages was due to internalisation of CellTracker-positive neutrophils, the use of an effective positive control would provide additional confidence that the assay was able to detect an increase in efferocytosis if drugs were able to produce such an effect. The absence of a positive control is an omission. Selection of a positive control would be optimal if it was known that such an agent would reproducibly enhance efferocytosis to an extent greater than the candidate drugs. The drug lovastatin has been demonstrated to increase the uptake of apoptotic neutrophils by human monocyte-derived macrophages, and so would be a potential positive control during optimisation of the assay (Morimoto *et al.*, 2006).

If what is observed does represent no effect of the drugs on efferocytosis, it may then be wise to test other hypotheses generated by the connectivity map screen. It may not necessarily follow that the drugs enhance efferocytosis from the patterns of gene regulation suggested

by the screen, but RNA-sequencing experiments or quantitative polymerase chain reaction experiments may be able to validate the patterns of transcript expression caused by the drugs compared to control (bearing in mind that gene expression in the L1000 connectivity map is for the most part computationally inferred, and also that the results used in the screen were from experiments using epithelial rather than myeloid cells). If measured patterns of gene expression match the inferred patterns from L1000, then a further step would be to discern if patterns of gene expression (perhaps of upregulated *EDIL3* by a drug) correspond to altered expression of the appropriate proteins, for instance using enzyme-linked immunosorbent assays.

Another consideration for the design of the assay is whether the process of differentiation of monocytes to macrophages by GM-CSF changes the phenotype of the cells in a manner that influences efferocytosis. The original experiments were planned using primary alveolar macrophages before redesign due to the COVID-19 pandemic, and given the apparently distinct ontology of alveolar macrophages from monocyte-derived macrophages, primary cells in such experiments would function better as models of behaviour of the tissue macrophages than peripheral blood cells.

It is also worth questioning the assumption that the selected doses are those doses which would detectably influence efferocytosis. Although the dose ranges used include reference values for the half-maximal inhibitory concentrations of the drugs for cyclin-dependent kinases, it is not clear whether cyclin-dependent kinase inhibition is the mechanism by which such drugs have any influence on inflammation resolution. Given more time for optimisation of the assay, the protocol would have included dose-finding and exposure duration studies using these monocyte-derived cells.

If an effect is proven after optimised experiments, then the future work would include considering existing knowledge of the drug characteristics to inform trials in *in vivo* systems. Alvocidib/flavopiridol has been granted orphan drug status by the United States Food and Drug Administration for the treatment of patients with acute myeloid leukaemia, and its anticancer action is at least in part related to the induction of apoptosis in malignant cells through depletion of Mcl-1 (Gojo, Zhang and Fenton, 2002). However, the CDK inhibitor has not been evaluated for effect in patients with inflammatory diseases. Preclinical studies demonstrate efficacy of alvocidib in limiting experimental hepatitis and arthritis in mice,

suggesting it may be an effective counter to inflammation in human models (Sekine *et al.*, 2008; Schmerwitz *et al.*, 2011). Bolus injections of alvocidib followed by intravenous infusions were shown to result in plasma concentrations of the drug in the micromolar range in human phase 1 studies (Byrd *et al.*, 2007) and similar concentrations appeared sufficient in the L1000 experiments to modulate expression of genes of interest to inflammation resolution in some cell lines. A phase 1 study of an oral alvocidib prodrug was underway at the time of writing (ClinicalTrials.gov identifier NCT03604783), which would increase the options for administration routes in experimental medicine studies investigating an immunomodulatory effect. Until such studies, it remains to be seen whether measurable effects on inflammation in experimental or disease contexts would be observed in humans with non-toxic alvocidib doses.

CGP-60474 has been identified as a CDK inhibitor that reduces mortality in endotoxaemic mice by researchers screening the L1000 database using a different strategy to the one described above (Han *et al.*, 2018). The researchers generated a composite differential gene expression signature of sepsis using public microarray data from studies of patients with sepsis, then screened the LINCS database for compounds that had an antonymous effect on the tested cell lines. The candidate drugs identified were tested *in vitro* for anti-inflammatory and cytotoxic effects, and CGP-60474 was identified from among them as having the most potent anti-inflammatory effect with minimal cytotoxicity. Treatment of mice with CGP-60474 after intraperitoneal administration of LPS was associated with increased survival compared to treatment with vehicle control (Han *et al.*, 2018). Unlike alvocidib, CGP-60474 has not undergone testing in humans, so its safety profile remains unknown.

Chapter 5. Immunosuppression in hypersensitivity pneumonitis

5.1 Introduction

The pharmacological management of chronic hypersensitivity pneumonitis is aimed at disrupting the inflammation that is thought to drive the disease. The intense lymphocytic infiltrate that characterises the condition is thought to develop in response to the secretion of interferon gamma and its associated chemokines (Agostini, Calabrese, *et al.*, 2005). The pro-inflammatory IL-17 cytokines secreted by neutrophils and lymphocytes may also be important in lung inflammation in HP (Joshi *et al.*, 2009), and the proportions of neutrophils in HP lung tissue sections appear to correlate with the extent of fibrosis in chronic HP (Pardo *et al.*, 2000).

Although it may then follow from first principles that anti-inflammatory and immunomodulatory drugs might be effective in treating the disease, the efficacy of such drugs in limiting the decline in lung function associated with chronic HP has not been proven in randomised controlled trials. Adegunsoye and colleagues described a subgroup of 20 CHP patients treated with prednisolone in a retrospective study of lung function outcomes in patients from the University of Chicago interstitial lung disease (ILD) registry. Patients treated with prednisone monotherapy had a decline of 10% in forced vital capacity (FVC) over 36 months compared to a decline of 1.3% over the same duration in patients who were not treated with immunosuppression at all. However, untreated patients had significantly higher FVC at the beginning of the study period compared to those who were on immunosuppression, making it difficult to draw direct comparisons between the two subgroups (Adegunsoye *et al.*, 2017).

In data from a single-centre study of the effects of corticosteroid treatment and antigen avoidance in hypersensitivity pneumonitis, De Sadeleer and colleagues described a cohort of 109 HP patients with lung fibrosis, of whom 82 received corticosteroid treatment. Corticosteroid treatment had no effect on the monthly rate of decline of lung function, although the absence of randomisation must be considered when interpreting the results of the cohort study. There was also a trend towards worse survival in the steroid-treated fibrotic HP patients, although this trend was no longer seen when data were adjusted for patient age, gender and baseline FVC (De Sadeleer *et al.*, 2018).

Chronic HP patients may be treated with immunomodulatory agents such as cyclophosphamide alongside corticosteroids, as in other progressive inflammatory lung conditions such as the interstitial pneumonia associated with scleroderma (Hoyles *et al.*, 2006). Selected patients with chronic hypersensitivity pneumonitis treated through the Newcastle Interstitial Lung Disease service are treated in this way, but as above it is not certain whether the therapy alters the rate of decline in lung function. In order to determine the effect of cyclophosphamide on the progression of chronic HP, I undertook a retrospective case series evaluation of the trajectories of lung function decline before and after administration of the immunomodulatory drug.

5.1.1 Measuring the progression of interstitial lung diseases

Chronic interstitial lung diseases often result in reduction of effective total lung capacity (TLC) due to the alteration of the lung parenchyma, with reduced lung compliance occurring as a result of increasing interstitial tissue as the conditions progress (Miguel-Reyes *et al.*, 2015). TLC is less straightforward to measure in practice than vital capacity (VC; the volume of air that can be expelled from the lungs by exhaling to completion after a maximal inspiration), which is often measured with a forceful exhalation during spirometry, yielding the forced vital capacity (FVC). FVC can function as a surrogate of TLC, and partly because FVC trends have been shown to predict mortality in idiopathic pulmonary fibrosis (IPF), FVC has been used as a primary endpoint in clinical trials of treatment for the interstitial lung disease (Wells, 2013).

There is less evidence for using FVC to monitor treatment outcomes in hypersensitivity pneumonitis, although retrospective analysis has demonstrated an increased risk of mortality in chronic HP if a fall in FVC greater than 10% is noted over 6 to 12 months of follow-up (Gimenez *et al.*, 2018). Perhaps due to its utility in IPF studies, FVC has been used as an endpoint for the few retrospective studies seeking to evaluate the effect of immunomodulatory treatment in chronic HP although it is yet to be validated for this specific application.

Another physiological measurement that could be used to monitor progression is the diffusing capacity for carbon monoxide (known as TLCO or DLCO). TLCO is a measure of the ability of the lungs to transfer gas from air to the blood, and the test takes advantage of the high affinity of blood haemoglobin for carbon monoxide (CO). The test is typically performed by the

inhalation of a known volume of a gas containing known proportions of oxygen, nitrogen, CO and an inert tracer gas (Graham *et al.*, 2017). Following a timed breath hold the exhaled gas is collected, and the difference between the relative proportions of gases in the delivered gas mixture and the collected gas mixture provide a measure of how much CO has diffused across the alveolar-capillary barrier per unit of time per unit of partial pressure of CO in the alveoli — the affinity of haemoglobin for CO is so great that the partial pressure of CO in the blood is a negligible part of the pressure gradient (Hughes and Borland, 2015).

The resulting measure is given in units of mmol/min/kPa and is influenced by the effective alveolar surface area available for gas diffusion and the thickness of the alveolar-capillary barrier. The alveolar-capillary barrier is composed of the alveolar epithelium, the capillary endothelium and the intervening interstitium, and given that interstitial lung diseases may result in inflammatory infiltration and/or fibrosis of the interstitium, the TLCO declines with disease progression (Miguel-Reyes *et al.*, 2015).

A decline in TLCO of greater than 10% in the first year after diagnosis of chronic HP is associated with reduced survival (Macaluso *et al.*, 2022). Morisset and colleagues were able to demonstrate an improvement in TLCO after a year of immunosuppressive treatment with mycophenolate mofetil or azathioprine in a retrospective study of chronic HP patients, but it is uncertain if that correlated with increased survival (Morisset *et al.*, 2017).

A downside of using TLCO to measure lung function progression in chronic lung disease is that the breath-hold technique can be difficult for patients to perform in late-stage disease, possibly resulting in increased variability or simply absent results as disease progresses (Nathan and Meyer, 2014).

5.1.2 Cyclophosphamide therapy in hypersensitivity pneumonitis

As mentioned in the introductory chapter, cyclophosphamide is an immunosuppressive agent that preferentially causes the death of cells with low levels of aldehyde dehydrogenase like lymphocytes, which might explain the drug's immunosuppressive effect at least in part (Kastan *et al.*, 1990). The drug is administered alongside the corticosteroid methylprednisolone for the treatment of rapidly progressive ILD on the strength of evidence of efficacy in ILD associated with the connective tissue disease scleroderma (Mathai and

Danoff, 2016). There have been no prospective randomised controlled trials to evaluate the efficacy of cyclophosphamide in treating chronic HP.

Patients treated with cyclophosphamide through the Newcastle Interstitial Lung Disease service receive cyclophosphamide alongside methylprednisolone as combination intravenous therapy in six pulses, with the first three pulses of the drugs at 3-week intervals and the subsequent three pulses at 4-week intervals. Patients are then commenced on maintenance immunosuppression, usually with mycophenolate mofetil, a purine synthesis inhibitor that impairs lymphocyte proliferation (Allison and Eugui, 2000). Patients may be considered for further pulses of cyclophosphamide and methylprednisolone after the initial course depending on tolerability and therapeutic response.

As a potent cytotoxic drug, cyclophosphamide is not without its side effects. Aside from the potential for infection that can arise through immunosuppression, the drug is also associated with a haemorrhagic inflammation of the bladder that is thought to be the result of damage to the urothelium by acrolein, a toxic cyclophosphamide metabolite (Fraiser, Kanekal and Kehrer, 1991). Patients treated through the Newcastle ILD service therefore receive intravenous mesna with each cyclophosphamide dose, which conjugates and inactivates acrolein to reduce the risk of haemorrhagic cystitis.

Usual doses of cyclophosphamide and methylprednisolone for combination therapy at each pulse are 15 mg/kg and 10 mg/kg respectively. To mitigate against side effects, patients over the age of 70 and those with renal impairment receive 10 mg/kg doses of cyclophosphamide alongside the 10 mg/kg methylprednisolone. For the sake of brevity, the remainder of this chapter refers to the combination therapy as “cyclophosphamide”.

5.2 Results

5.2.1 Patient characteristics

The patient census included 25 patients who had a diagnosis of chronic hypersensitivity pneumonitis and were commenced on cyclophosphamide between May 2015 and December 2020. These patients were 36% male and had a mean age of 62 years (standard deviation [SD] = 8.9 years) at the time of cyclophosphamide initiation.

Characteristic	FVC analysis (*n* = 7)	TLCO analysis (*n* = 5)	Excluded (*n* = 18)
Mean age at cyclophosphamide treatment start (SD), years	62 (11)	60 (10)	62 (8.5)
Sex, % male	29%	40%	39%
Mean (SD) % of predicted FVC at treatment start	69% (17%)	72% (19%)	*Unavailable*
Mean (SD) % of predicted TLCO at treatment start	-	56% (8.5%)	*Unavailable*
Reduced cyclophosphamide dose during treatment	3	2	3
Comorbidities			
Coronary artery disease	2	2	-
Osteoarthritis	2	1	-
Psoriasis	2	2	-
Hypertension	1	-	2
Gastro-oesophageal reflux	1	-	1
Frontal fibrosing alopecia	1	-	-
MGUS	1	1	-
Hypothyroidism	-	-	1
Asthma	-	-	1
Type 2 diabetes	-	-	1
Adverse events during treatment			
Respiratory tract infection	2	2	1
Neutropaenia	1	1	-

***Table 5.1**. Characteristics of patients included in analysis; second group is subset of first. FVC = forced vital capacity; MGUS = monoclonal gammopathy of uncertain significance; SD = standard deviation; TLCO = diffusing capacity for carbon monoxide*

Once the patients with insufficient FVC data and those who had died or had lung transplants before 1 year of follow-up were excluded, there were 7 patients suitable for analysis (**Table 5.1**). These patients were 29% (2/7) male and had a mean age of 62 years (SD = 11 years) at the time of cyclophosphamide initiation. FVC was a mean of 69% (SD = 17%) of the predicted reference value at the last recording prior to cyclophosphamide initiation, with the last recordings taken a mean of 71 days (SD = 36 days) prior to the cyclophosphamide initiation. Three patients required reduced doses of the cyclophosphamide component of combination therapy at some point during therapy: one for the development of a low neutrophil count during treatment and two for being aged above 70.

The group for TLCO analysis excluded two further patients for having insufficient recordings to calculate lung function trajectories. These remaining 5 patients were 40% (2/5) male with a mean age of 60 (SD = 10 years). Their TLCO was a mean of 56% (SD 8.5%) of the predicted

reference value at the last recording prior to cyclophosphamide initiation, with the last recordings taken a mean of 59 days (SD = 33 days) prior to cyclophosphamide start. Two of these patients had reduced cyclophosphamide doses during treatment: one was the patient who developed neutropaenia, and the other was older than 70. Patient comorbidities and adverse events during treatment are presented in **Table 5.1**.

5.2.2 No effect of cyclophosphamide on FVC decline

The mean rate of FVC decline before cyclophosphamide initiation (*n* = 7) was 820 mL/year (SD = 940 mL/year). The rate of decline was 57 mL/year (SD = 260 mL/year) after cyclophosphamide initiation, but this was not a statistically significant difference as assessed by paired Wilcoxon signed-rank test (*p* = 0.08).

5.2.3 No effect of cyclophosphamide on TLCO decline

The mean rate of TLCO decline before cyclophosphamide initiation (*n* = 5) was 2.6 mmol/min/kPa per year (SD = 3.8 mmol/min/kPa/year). The rate of TLCO change after cyclophosphamide initiation was an *increase* of 0.11 mmol/min/kPa/year (SD = 0.81 mmol/min/kPa/year) after cyclophosphamide initiation. Again, this was not a statistically significant difference (*p* = 0.19).

5.3 Discussion

These results appear to indicate an absence of effect of cyclophosphamide therapy on the rate of lung function decline in chronic HP, but the results must be taken with strong notes of caution. Retrospective studies such as this (particularly those relying upon medical case notes) are susceptible to compromise by missing data. Here, an already small sample of patients was reduced further by the absence of sufficient data to calculate lung function trajectories. At least part of the absence of data could be explained by the fact that the Newcastle ILD service is a regional service receiving tertiary referrals and some of the information pertaining to the care of patients for the same conditions will be stored by other centres. The spectre of COVID-19 also reared its head; some of the patients commenced on treatment towards the end of 2019 were then followed up for long periods without attending for lung function testing due to the shift away from face-to-face appointments for the sake of infection control during the height of the pandemic. Missing data also hampered the assessment of TLCO decline, but here the likelihood is that the difficulty of the breath-holding

technique required for TLCO measurement in patients with progressive lung disease meant the procedure could not be completed, as some patients had spirometry measurements on occasions that TLCO measurements were missing.

The large standard deviations of the rates of FVC decline — especially those prior to treatment — suggest imprecision in the measurements or strong influence by outlying values. Only two of the patients had at least a full year of lung function test results available in the period prior to cyclophosphamide initiation, and most had two lung function measurements in the six months prior to treatment initiation as their only available pre-treatment lung function tests. Calculated rates of change of FVC by linear regression prior to the initiation of treatment ranged from a decline of 2200 mL/year (likely the result of test variability causing outlier measurements) to an *increase* of 560 mL/year. The normal rate of FVC decline in healthy older adults is on the order of 65 mL/year (Thomas *et al.*, 2019), and even in a cohort of patients with various fibrosing interstitial lung diseases, clinical trial data suggested a rate of decline on the order of 190 mL/year in patients treated with placebo (Flaherty *et al.*, 2019). It is likely that more frequent measurements of lung function over longer durations are required to provide precise enough estimates of the pre-treatment rate of lung function decline, particularly where spirometry is known to demonstrate intrinsic variability when monitoring the progress of interstitial lung diseases (Santermans *et al.*, 2019). Future work could model the most efficient lung function testing strategy (or lung function test sampling strategy, if dealing with retrospective data) to predict future lung function decline in chronic HP patients.

Five patients who died prior to one year of follow-up were excluded from the analysis. The direct causes of death were not available, likely because some of these deaths occurred in institutions with no automatic data sharing arrangements with the Newcastle ILD service. The impact of the exclusion of these patients from this analysis is difficult to discern, but in general there is the risk that exclusion of such patients imparts a bias towards favourable outcomes upon the results.

Finally, there is always the possibility that the small numbers of patients included in the analyses hampered the ability to detect differences between pre- and post-treatment rates of lung function decline. Assuming similar rates of FVC decline in chronic HP to the placebo-treated patients in the trial of nintedanib for fibrosing interstitial lung diseases presented by Flaherty and colleagues (mean rate of decline 187.8 mL/year, SD 269 mL/year) (Flaherty *et*

al., 2019), the MKpower package in RStudio can be used to estimate the sample size required to provide 80% power to detect a 100 mL/year (arbitrary effect size) difference in pre- and post-treatment rates of FVC decline to p = 0.05 by Monte Carlo simulations of Wilcoxon signed-rank tests (Shieh, Jan and Randles, 2007; Kohl, 2020):

```
# Load the MKpower package
library("MKpower")

# Assign simulated values to pre- and post-treatment groups for simulation,
# assuming normal distribution
rx <- function(n) rnorm(n, mean = 190, sd = 269)
ry <- function(n) rnorm(n, mean = 90, sd = 269)

# Run Monte Carlo simulation with 1000 iterations, using 10 as minimum
# sample size, 20 as maximum sample size, using paired Wilcoxon
# signed-rank test
sim.ssize.wilcox.test(rx = rx, ry = ry,
      n.min = 10, n.max = 20, step.size = 1, iter = 1000,
      sig.level = 0.05, power = 0.8, type = "paired",
      alternative = "two.sided")

     Wilcoxon signed rank test

              n = 10, 11, 12, 13, 14, 15, 16, 17, 18
             rx = rnorm(n, mean = 190, sd = 269)
             ry = rnorm(n, mean = 90, sd = 269)
      sig.level = 0.05
      emp.power = 0.504, 0.514, 0.553, 0.618, 0.676, 0.680, 0.742, 0.744,
0.815
    alternative = two.sided

NOTE: n is number in *each* group
```

These simulation results indicate that a sample size of 18 patients (n = 18 in pre-treatment group and n = 18 in post-treatment group for a paired test) would be required.

Chapter 6. Understanding the role of inflammation in hypersensitivity pneumonitis

6.1 Introduction

Hypersensitivity pneumonitis is a syndrome characterised by an excessive pulmonary immune response to inhaled aerosols containing small diameter (<5 μm) organic particles. The antigens that can cause HP in susceptible individuals are various and include bacterial, fungal, protozoal and insect components. The resultant manifestations of HP have often been named for the occupational or environmental settings in which the exposures are likely to have occurred (*e.g.* "farmer's lung" for hypersensitivity to *Saccharospora rectivirgula* from mouldy hay; "pigeon fancier's lung" for avian droppings/feathers) (Selman, Pardo and King, 2012).

The pathophysiology of hypersensitivity pneumonitis is unclear, and options for pharmacological management are therefore limited (Vasakova *et al.*, 2017). The disease frequently takes the form of inflammation of the lung parenchyma with lymphocytic infiltration and granuloma formation, and this may overlap with a fibrotic interstitial pneumonia similar to idiopathic pulmonary fibrosis in a syndrome known as chronic hypersensitivity pneumonitis (CHP) (Vasakova *et al.*, 2019). In some cases the fibrosis of CHP is progressive such that the only effective therapy is lung transplantation (Selman, Pardo and King, 2012). It has been suggested that in HP dysfunctional T regulatory cells allow the proliferation and pro-inflammatory activity of effector T lymphocytes specific to culprit antigens, and this may act in concert with inflammation caused by deposited antibody-antigen immune complexes to result in the clinical syndrome (Barrera *et al.*, 2008; Vasakova *et al.*, 2019).

It is not known whether persistent or repeated inflammation in acute and subacute HP directly contributes to the progression of the lung fibrosis that characterises chronic HP. There is some evidence indicating that in HP inflammatory and pro-fibrotic signals are amplified by bone marrow-derived haematopoietic cells known as fibrocytes, and the influence of such signalling on local fibroblasts and T lymphocytes in the lungs of patients with HP may propagate the disease (García de Alba *et al.*, 2015).

If dysregulated and persistent inflammation results in the clinical syndromes of HP, it might be possible to consider it within the paradigm of dysregulated inflammation *resolution* in order to discern molecular strategies to interrupt the disease process. In this chapter, I present exploratory analyses of public transcriptomic data derived from the explanted lung tissue of patients with HP to elucidate the molecular mechanisms driving the persistent inflammation in CHP.

6.1.1 Public transcriptomics resources in the understanding of inflammatory interstitial lung diseases

Our understanding of the pathogenesis of interstitial lung diseases like HP may be limited by the relative inaccessibility of human tissue specimens and the possibility that animal models of disease do not correspond sufficiently to the human diseases for experimental findings to be applied to patients.

If samples from human patients with rarer diseases such as HP might otherwise be inaccessible for research, a powerful solution may lie in one of the strengths of the approach of systems biology. Systems biology entails the development of models of biological behaviour by deconstructing biological processes into their components and sampling them (Kohl and Noble, 2009), and this might require large amounts of data to be collected to catalogue (for instance) the expression of protein-coding genes in a sample of lung tissue affected by an inflammatory disease. If those data can be stored locally by researchers for use in one study, the impact of those data may be increased by storing them on internet-connected servers for reanalysis by other researchers.

Established in 2000 by the National Centre for Biotechnology Information (NCBI), the Gene Expression Omnibus (GEO) represents one attempt to provide a freely accessible, centralised repository for gene expression and other genomic data to facilitate the dissemination of such data for use by researchers around the world for their own analyses (Clough and Barrett, 2016). Of the myriad datasets available on the GEO website (http://www.ncbi.nlm.nih.gov/geo/), some datasets uploaded by members of the National Institutes of Health (NIH)-funded Lung Tissue Research Consortium (LTRC) serve as a means to obtain data regarding gene expression in hypersensitivity pneumonitis and other interstitial lung diseases. These data from microarray experiments performed by the Lung

Genomic Research Consortium subgroup of the LTRC allow for exploratory analyses of which RNA transcripts are differentially expressed in HP lung tissue compared to other interstitial lung diseases and healthy control lung tissue, which might prove useful in determining whether disordered inflammation resolution processes play a role in the propagation of the disease.

6.1.1.1 Microarrays for transcriptome profiling

Measuring RNA transcripts in tissue can provide hints as to the roles of the corresponding genes in that tissue in relation to each other, or in relation to a different disease state in the same tissue. Although it might not necessarily follow that greater expression of an RNA transcript corresponds to greater expression of the encoded protein in that biological environment, measurement of the transcriptome in this fashion does provide a fair model of the protein expression profile. Work by Lundberg and colleagues suggests that the majority of a cell's measured RNA transcripts are expressed as protein, and changes in transcript abundance are reflected in changes in protein abundance (Lundberg *et al.*, 2010). That said, more caution is perhaps required when attempting to infer *function* from transcript abundance, as genes that are expressed as proteins in the proteome are still subject to differing rates of translation in different circumstances, and may also be subject to additional regulation of function including post-translational modification (Kumar *et al.*, 2016; Uhlén *et al.*, 2016).

Complementary DNA (cDNA) microarrays were developed in the mid-1990s as a method of measuring the abundance of a large number of RNA transcripts in a biological sample in parallel. The technique involves the preparation of cloned cDNAs complementary to RNA of genes and gene fragments from an organism, then the attachment of those cDNAs (with cDNA of each different sequence termed a "probe") to a glass slide in arrays, one probe in each array position. Fluorescently labelled mRNA from the biological sample is then hybridised onto the cDNA attached to the glass slide, resulting in varying fluorescence intensities in each array position corresponding to the abundance of those gene transcripts in the biological sample (Schena *et al.*, 1995).

Microarrays were superseded by RNA sequencing (RNA-seq), which involves recording the sequences of fragmented RNA from the biological sample followed by computational

reconstruction of the original RNA transcripts by aligning those sequences onto a reference genome, with gene abundance inferred from the number of copies of a transcript available to map to the reference genome (Lowe *et al.*, 2017). RNA-seq is more sensitive at detecting low-abundance transcripts and single nucleotide variations, allows for sequencing of an entire transcriptome and requires smaller samples of RNA, but microarrays benefit from lower cost and are less labour-intensive to use (Lowe *et al.*, 2017). As such, despite the advent of RNA-seq much of the transcriptomic data uploaded to GEO remain those from microarray experiments, including the data used for the analyses in this chapter. As described in the methods chapter, the data in these analyses is a processed dataset derived from the raw data uploaded to GEO, normalised for inclusion in the Genevestigator repository to facilitate meta-analysis of diverse datasets.

6.2 Results

6.2.1 Differential gene expression indicates interferon gamma signalling and lymphocyte recruitment in hypersensitivity pneumonitis

Comparison of differentially expressed genes between hypersensitivity pneumonitis and control lung tissue homogenate identified 593 upregulated genes and 699 downregulated genes at FDR < 0.05 and absolute $\log_2$-fold change > 0.58 (**Appendix B**). 15 of the top 20 upregulated genes were immunoglobulin-related genes, suggesting the presence of B-lineage lymphocytes in the lung tissue in HP.

Manhattan distance clustering of the upregulated genes across all conditions in the dataset demonstrated significant overlap between the genes upregulated in the sampled interstitial lung diseases including the immunoglobulin-related genes, but also made apparent a cluster of 19 probes (18 genes and one long intergenic non-protein coding RNA, LINC00892) that were differentially upregulated in HP relative to the other conditions (**Figure 6.1**; **Table 6.1**). Among these were genes coding for lymphocyte chemoattractant cytokines CXCL11 (1.37 $\log_2$-fold change from control, $p < 0.001$), CXCL10 (1.75 $\log_2$-fold change, $p < 0.001$) and CXCL9 (2.13 $\log_2$-fold change, $p < 0.001$). By comparison, these genes were either downregulated or minimally changed in IPF compared to controls (CXCL11 -0.70 $\log_2$-fold change in IPF versus control, $p < 0.001$; CXCL10 -0.42 $\log_2$-fold change, $p = 0.032$; CXCL9 -0.57 $\log_2$-fold change, $p = 0.005$).

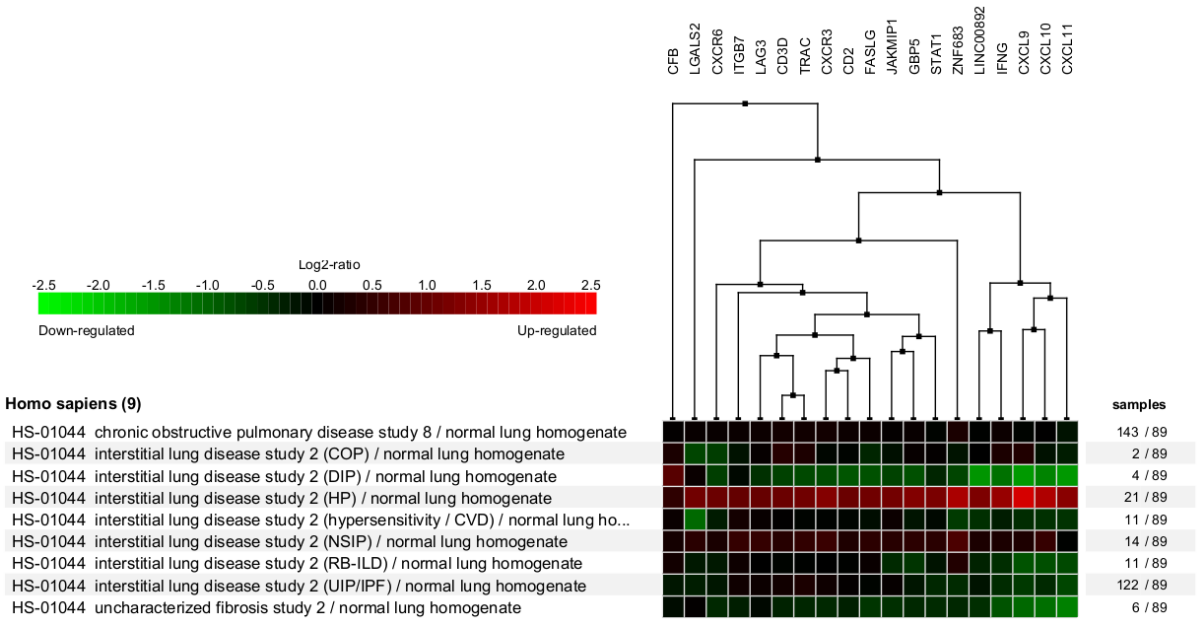

***Figure 6.1**. Cluster (by Manhattan distance clustering) of differentially expressed gene transcripts in hypersensitivity pneumonitis versus controls. COP = cryptogenic organising pneumonia; DIP = desquamative interstitial pneumonia; HP = hypersensitivity pneumonitis; CVD = collagen vascular diseases; NSIP = non-specific interstitial pneumonia; RB-ILD = respiratory bronchiolitis-interstitial lung disease; UIP/IPF = usual interstitial pneumonia/idiopathic pulmonary fibrosis*

CXCL9, CXCL10 and CXCL11 are considered interferon gamma (IFN-γ) induced cytokines (Metzemaekers *et al.*, 2018), and indeed IFNG (the gene coding for IFN-γ) was represented in this cluster (1.52 $\log_2$-fold change in HP versus control, $p < 0.001$). Downstream IFN-γ activity is mediated through activation of the STAT1 transcription factor, and the gene encoding STAT1 was also in the cluster (1.23 $\log_2$-fold change, $p < 0.001$), suggesting the presence of active IFN-γ.

The gene coding for the receptor for CXCR3 (the receptor for CXCL9, CXCL10 and CXCL11), was upregulated and represented in this cluster (1.32 $\log_2$-fold change, $p < 0.001$). CXCR3 is expressed on effector T lymphocytes and natural killer cells, as is the chemokine receptor CXCR6 (Agostini, Cabrelle, *et al.*, 2005; Berahovich *et al.*, 2006), the gene for which was also included in this cluster (2.18 $\log_2$-fold change, $p < 0.001$). The cluster also contains the genes coding for the constant region of the T cell receptor alpha chain (TRAC; 1.13 $\log_2$-fold change, $p < 0.001$) and the T cell co-receptor CD3D (1.06 $\log_2$-fold change, $p < 0.001$). This pattern of transcript upregulation peculiar to HP among other interstitial lung diseases suggests the exaggerated presence of T lymphocytes in lung tissue in the disease.

Gene	Product	$\log_2$ ratio (vs control)	*p* value

CXCL11	C-X-C motif chemokine 11	1.37	< 0.001
CXCL10	C-X-C motif chemokine 10	1.75	< 0.001
CXCL9	C-X-C motif chemokine 9	2.13	< 0.001
IFNG	Interferon gamma	1.52	< 0.001
LINC00892	(long intergenic non-protein coding RNA)	1.25	< 0.001
ZNF683	Homolog of Blimp-1 in T cells	1.71	< 0.001
ITGB7	Integrin beta-7	1.01	< 0.001
LAG3	Lymphocyte activation gene 3 protein	1.02	< 0.001
CD3D	T cell surface glycoprotein CD3 delta chain	1.06	< 0.001
TRAC	T cell receptor alpha chain constant	1.13	< 0.001
CXCR3	C-X-C chemokine receptor type 3	1.32	< 0.001
CD2	T cell surface antigen CD2	1.06	< 0.001
FASLG	Fas ligand/Tumour necrosis factor ligand superfamily member 6	1.21	< 0.001
JAKMIP1	Janus kinase and microtubule-interacting protein 1	1.15	< 0.001
GBP5	Guanylate-binding protein 5	1.31	< 0.001
STAT1	Signal transducer and activator of transcription 1-alpha/beta	1.23	< 0.001
CXCR6	C-X-C chemokine receptor type 6	1.08	< 0.001
LGALS2	Galectin 2	1.13	< 0.001
CFB	Complement factor B	1.32	< 0.001

***Table 6.1**. Cluster (by Manhattan distance clustering) of differentially upregulated genes in hypersensitivity pneumonitis relative to controls. CD = cluster of differentiation*

6.2.2 Gene expression patterns indicate evidence of cytotoxic lymphocyte recruitment in hypersensitivity pneumonitis

The cluster of HP-specific overexpressed genes indicates high expression of the gene coding for the cytotoxic molecule Fas ligand (FASLG; 1.12 $\log_2$-fold change from control, $p < 0.001$). Fas is expressed by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, which store the molecule in cytoplasmic granules prior to degranulation to effect cell-mediated cytotoxicity

(Kojima *et al.*, 2002). To discern whether other effectors of cell-mediated cytotoxicity are transcribed in the lung tissue in HP, we screened for differential expression of Homo sapiens genes catalogued under the "cytolysis" Gene Ontology term (accession number GO:0019835) (Ashburner *et al.*, 2000; The Gene Ontology Consortium, 2019). Among these, genes coding for cytotoxic molecules typically expressed in CTL and NK cells were overexpressed in HP with respect to control including granzyme H (GZMH; 0.66 $\log_2$-fold change, p = 0.003) and granzyme A (GZMA; 0.79 $\log_2$-fold change, $p < 0.001$). Granzyme B trended towards overexpression (GZMB; 0.54 $\log_2$-fold change, p = 0.006). As granzymes are exclusively expressed by CTL and NK cells (Trapani, 2001), I concluded that the lymphocytic infiltrate in hypersensitivity pneumonitis is enriched with CTL and/or NK cells.

6.2.3 Upregulated genes suggest activated tissue residency programming of cells in hypersensitivity pneumonitis

One of the genes in the HP-specific cluster by way of overexpression is ZNF683 (1.71 $\log_2$-fold change from control, $p < 0.001$). ZNF683 codes for the zinc finger protein Homolog of Blimp-1 in T cells (Hobit), a transcription factor that is implicated in activation of the phenotype that enables T lymphocytes to become resident in an epithelial barrier tissue (Mackay *et al.*, 2016). These cells are referred to as tissue-resident memory T cells (T_{RM}). Hobit has also been demonstrated to regulate the expression of granzymes in $CD8^+$ T cells (Kragten *et al.*, 2018). Hobit facilitates residency of T lymphocytes by repressing genes required for egress of lymphocytes from tissue such as S1PR1, CCR7 and TCF7 (Mackay *et al.*, 2016). In this dataset TCF7 and CCR7 expression in HP whole lung tissue were unchanged and minimally changed respectively compared to control (TCF7 -0.05 $\log_2$-fold change, p = 0.657; CCR7 0.52 $\log_2$-fold change, p = 0.009). However, S1PR1 was significantly downregulated (-1.23 $\log_2$-fold change, $p < 0.001$) although this was not unique to HP among the other interstitial lung diseases sampled, and also comes with the caveats of attempting to infer downregulated gene transcription in individual cell populations from transcripts in a whole tissue homogenate.

T_{RM} cells are also prepared for residency through the upregulation of immune checkpoint molecules in order to maintain self-tolerance at sites of residence (Djenidi *et al.*, 2015). One of the genes that appears in the cluster of HP-specific differentially expressed genes is LAG3 (1.02 $\log_2$-fold change, $p < 0.001$). LAG3 (lymphocyte activation gene 3) encodes an immune checkpoint molecule expressed on the surface of tissue T lymphocytes that acts as a negative

regulator of effector T cell function similar to other immune checkpoint molecules such as cytotoxic T lymphocyte antigen 4 (CTLA-4; encoded by the gene CTLA4) and programmed cell death protein 1 (PD-1). LAG3 and PD-1 in particular have been shown to function synergistically in negatively regulating the activity of tumour-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes (Woo *et al.*, 2012). Manual searching of the dataset reveals that the gene encoding PD-1 is significantly overexpressed in HP lung tissue versus control (PDCD1; 0.73 $\log_2$-fold change, $p < 0.001$) as is the gene encoding another checkpoint molecule, B- and T-lymphocyte attenuator (BTLA; 0.93 $\log_2$-fold change, $p < 0.001$). CTLA4 is minimally changed in HP with respect to control (0.54 $\log_2$-fold change, $p = 0.035$) as is TIM3, a gene encoding another similar molecule (0.47 $\log_2$-fold change, $p < 0.001$).

Given the upregulation of suppressive molecules and the transcription factor associated with residency, I concluded that the lymphocytes present in lung tissue in HP are likely to comprise an appreciable proportion of tissue-resident T and/or NK cells.

6.2.4 Meta-analysis of public datasets indicates other diseases with gene expression similarities to hypersensitivity pneumonitis

I was interested to see what other diseases might share a similar gene expression profile in whole tissue samples, which would suggest a similar role of tissue-resident cytotoxic cells in their pathogenesis. The Genevestigator platform allows its compendium of annotated public gene expression data to be used as an input for simultaneous gene and condition clustering by similarity using the BiMax algorithm (Prelić *et al.*, 2006). Using the cluster of 19 HP-specific upregulated probes as an input, I selected all the samples in the Genevestigator compendium acquired on the Affymetrix Human Genome U133 Plus 2.0 Array platform (69363 samples at time of writing) and ran the biclustering algorithm with a discretisation threshold of 0.8 (*i.e.*, anything more than a 0.8 $\log_2$-fold change from control condition is treated as upregulated) using all samples annotated as perturbations (disease states or tissues/cells challenged with an exogenous agent) or controls. The largest bicluster by area encompassed 14 of the 19 HP-specific upregulated probes and included cutaneous sarcoidosis skin biopsy tissue (compared to normal and non-lesional skin tissue; from GEO accession number GSE32887), cutaneous T cell lymphoma (compared to normal skin tissue; from GEO accession number GSE59307), inclusion body myositis (compared to normal biceps femoris tissue; from GEO accession

number GSE39454) and diphenylcyclopropenone (DPCP)-exposed skin (compared to skin exposed to topical placebo; from GEO accession number GSE52360) (**Figure 6.2**).

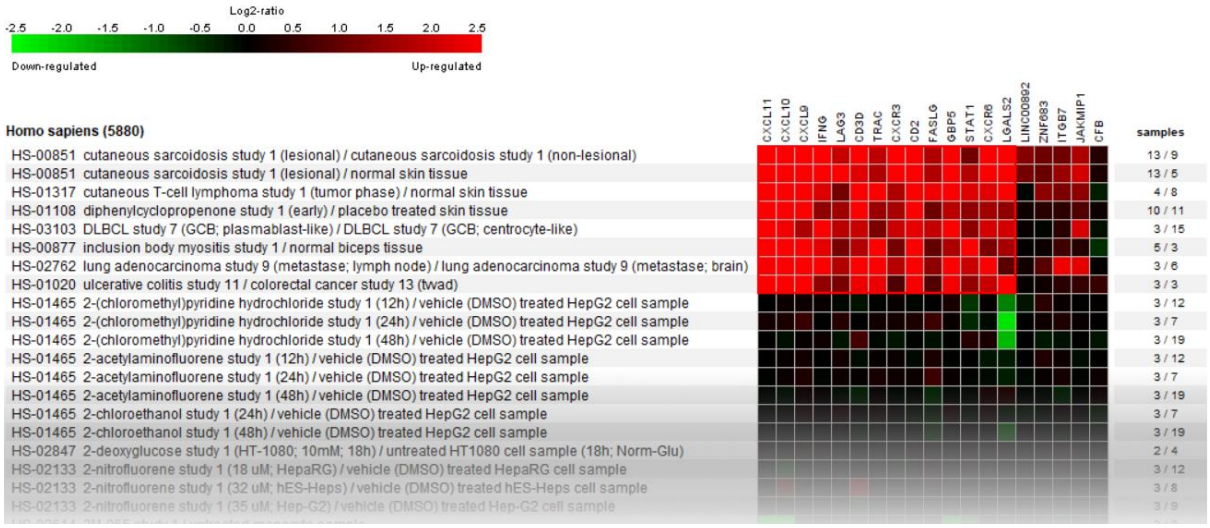

***Figure 6.2**. Biclustering of upregulated hypersensitivity pneumonitis genes and experimental comparisons from Genevestigator compendium encompassing all curated experiments acquired on Affymetrix Human Genome U133 Plus 2.0 Array platform. Maximal cluster highlighted in red*

DPCP is a compound known to induce a delayed-type hypersensitivity reaction in the skin after repeated topical application, leading to an influx of activated T lymphocytes and an associated increase in IFN-γ signalling (Gulati *et al.*, 2014). Cutaneous T cell lymphomas are a heterogenous group of non-Hodgkin lymphomas that arise due to the malignant accumulation of cells with a T_{RM} phenotype in the epidermis (Adachi *et al.*, 2015; Querfeld *et al.*, 2018). Inclusion body myositis is an inflammatory disorder of skeletal muscle that is characterised by muscle infiltration by cytotoxic T lymphocytes with a terminally differentiated effector memory phenotype (Greenberg *et al.*, 2019). Cutaneous sarcoidosis is characterised by the development of granulomatous lesions in the skin, where granuloma formation may be driven by the influence of T cell-secreted IFN-γ on resident macrophages (Damsky *et al.*, 2018).

6.2.5 Upregulation of immune checkpoint genes in conditions associated with resident T lymphocytes provides insight into side effects of immune checkpoint inhibition therapies

Cutaneous sarcoidosis and inclusion body myositis are autoimmune conditions that might share the pathophysiology of a loss of tolerance of a population of T_{RM} cells that have expanded after sensitisation to a self-antigen. Indeed, it has been reported that anti-PD-1 cancer therapy has been associated with the development of sarcoidosis or sarcoid-like

reactions (Birnbaum *et al.*, 2017; Paolini *et al.*, 2018; Tetzlaff *et al.*, 2018), and myositis (including granulomatous myositis, a mimic of inclusion body myositis) (John *et al.*, 2017; Uchio *et al.*, 2018).

Working under the hypothesis that stereotyped side effect syndromes of anti-PD-1 therapy are the result of T_{RM} cells in epithelial barrier tissues losing tolerance when their immune checkpoint molecules are antagonised, I considered some other recurrently reported side effects of PD-1 blockade. Vitiligo is a cutaneous side effect of anti-PD-1 therapy that is frequently reported (Hua *et al.*, 2016). Recent investigation has demonstrated the increased presence of autoreactive $CXCR3^+$ $CD8^+$ T_{RM} in the perilesional skin in vitiligo with the capacity to secrete IFN-γ (Boniface *et al.*, 2018), and evidence of CXCR3 signalling via the IFN-γ-induced ligands CXCL9 and CXCL10 (Rashighi *et al.*, 2014; Wang *et al.*, 2016).

In the gastrointestinal system, anti-PD-1 therapy can induce a colitis similar to ulcerative colitis (Yamauchi *et al.*, 2018). Again, dysregulated tissue CTL are implicated, with pathological examination of the colon in such patients revealing significant infiltration of the colon by $CD8^+$ cells (Yoshino *et al.*, 2019). T cells with a T_{RM} phenotype have been implicated in the inflammation in colon biopsy samples from patients with inflammatory bowel disease and experimental colitis in mouse models (Zundler *et al.*, 2019).

Anti-PD-1 therapy may also result in pneumonitis (Nishino, Giobbie-Hurder, *et al.*, 2016). The pathology of the pneumonitides that can be induced has not been characterised fully, but can include one that appears radiologically similar to hypersensitivity pneumonitis (Nishino, Ramaiya, *et al.*, 2016).

In light of the above observations, I posit that vitiligo, cutaneous sarcoidosis and inclusion body myositis are linked to hypersensitivity pneumonitis by a common pathogenesis that implicates dysregulated cytotoxic T_{RM} cells, where the pathological tissue-resident cells in the former three conditions have clonally expanded in response to an autoantigen and in the latter in response to an exogenous organic antigen. Furthermore, I hypothesise that several side effects of anti-PD-1 therapy also arise due to disinhibited cytotoxic T_{RM} cells.

6.3 Discussion

Hypersensitivity pneumonitis has remained a disease with an elusive pathogenesis, leading to few more effective treatments than antigen avoidance. It is often the case, however, that an antigen cannot be identified despite rigorous attempts, and failure to identify a causative antigen is associated with poorer outcomes in the disease (Pérez *et al.*, 2013).

A lack of randomised controlled trials leaves open the question of whether immunosuppression can modify the course of the disease. It is possible that new hypotheses regarding the pathogenesis of the disease might lead to trials of more precise immune modulation than corticosteroids, and viewing HP as a disease of dysregulation of tissue-resident populations of antigen-specific lymphoid cells may give weight to more nuanced treatment strategies. It appears possible, for example, to interfere with the function of cytotoxic T_{RM} populations by blocking IL-15 signalling (Richmond *et al.*, 2018). Adachi *et al.* reported diminished contact hypersensitivity reactions to a hapten in IL-15-deficient mice (Adachi *et al.*, 2015). Richmond *et al.* reported the impairment of IFN-γ signalling by melanocyte-specific T_{RM} cells in a mouse vitiligo model using short-term IL-15 receptor blockade, with subsequent loss of those T_{RM} cells from the skin after longer term blockade, resulting in resolution of vitiligo (Richmond *et al.*, 2018). It is tempting to speculate that some modulation of the IL-15 signalling pathway might be useful to alleviate symptoms in patients with hypersensitivity pneumonitis where they are unable to avoid their culprit antigen, and if the activity of T_{RM} cells also contributes to the progression of the chronic form of the disease then it may also be wise to measure the effect of IL-15 receptor blockade on lung function decline.

That said, complete leukodepletion of tissue-resident cytotoxic cells may not be desirable given their role in the swift control of recall pathogens. Depletion of resident lymphocytes in hypersensitivity pneumonitis may also make the development of lung fibrosis more likely; there are data from mouse models of hypersensitivity pneumonitis that suggest that NK cell depletion promotes fibrosis after repeated exposures to the sensitising antigen (Denis, 1992). A leukodepletion model may be useful in understanding how lung fibrosis develops in hypersensitivity pneumonitis as it is still not known whether fibrosis is a direct and inevitable consequence of persistent hypersensitivity pneumonitis.

T_{RM} cell modulation also has significance in the field of oncology, and as the use of drugs targeting T_{RM} cell immune checkpoints becomes more widespread in cancer therapy, so will immune-mediated side effects. Understanding these in the context of inappropriate disinhibition of resident memory T cell populations may offer insights into how to mitigate those side effects. Discerning phenotypic differences between tumour-infiltrating lymphocytes and lymphocytes implicated in an ipilimumab-induced colitis, for example, might mean it is possible to target the immunotherapy at the former population of cells without stimulating the latter.

The model of T_{RM} cells expanding in tissues in response to antigens (endogenous or exogenous), remaining durably resident and responding aberrantly to repeat encounters with antigen is one that appears to provide a sound explanation for several diseases that seem disparate at first glance: psoriasis, mycosis fungoides and multiple sclerosis have all been linked to T_{RM} pathology (Clark, 2015). The action of resident memory T cells has been used to model allergic asthma (Hondowicz *et al.*, 2016; Ichikawa *et al.*, 2019), but there is little evidence suggesting these cells play a role in the propagation of hypersensitivity pneumonitis. Further work to validate these *in silico* findings will be useful to enable expansion of the repertoire of treatments for the disease.

Chapter 7. Discussion, future work and conclusions

7.1 Pulmonary inflammation resolution in ageing

The unfortunate interruption of the study presented in Chapter 3 of this thesis means that the question of whether ageing impairs efferocytosis by human alveolar macrophages remains unanswered by the work presented here. To the extent that it is possible to extrapolate from findings from work in other human organ systems, there is evidence from De Maeyer and colleagues that suggests ageing impairs the clearance of neutrophils in experimental sterile skin inflammation induced by the topical application of the vesicant cantharidin (De Maeyer *et al.*, 2020). Although those researchers were able to observe greater numbers of apoptotic neutrophils in skin blisters induced in older people, that only provided circumstantial evidence of a failure of efferocytosis. Efferocytosis assays were therefore undertaken *ex vivo* using peripheral blood monocytes from older and younger volunteers. Although the experiments presented in this thesis similarly used peripheral blood monocytes (differentiated into monocyte-derived macrophages before use in assays), the initial intention prior to study disruption was to use primary alveolar macrophages isolated from the lungs of healthy younger and older volunteers to assess age-related deficits in efferocytosis. This has not previously been done, and experiments to investigate age-related functional changes in primary alveolar macrophages (rather than peripheral blood monocytes or derivatives thereof as models) are needed given the evidence suggesting the majority of human alveolar macrophages are of distinct haematopoietic origin to circulating blood monocytes (Hashimoto *et al.*, 2013; Evren *et al.*, 2021, 2022).

Although the immune stimulus in the planned experiments was due to be lipopolysaccharide, the protocol described in Chapter 3 allows for modification to assess the effect of other immune stimuli on alveolar macrophage function, or indeed the effect of systemic or inhaled drugs on the immune behaviour, stimulated or unstimulated. With that in mind it is possible to imagine a future study in which a candidate pro-resolution drug had been identified for repurposing by the computational screening described elsewhere in the thesis, then validated with *ex vivo* efferocytosis assays using monocyte-derived macrophages, then administered to volunteers in an adapted version of the immune challenge protocol in order to discern a

dampened response to inhaled LPS or enhanced efferocytosis by retrieved alveolar macrophages after treatment with the drug.

As became apparent during attempted recruitment, a significant flaw in the design of the protocol arose from a failure to consider the specific needs of older volunteers, resulting in a low number of expressions of interest in the study by older volunteers and poor conversion from expressions of interest to attendance for screening. The barriers to recruitment of older people to traditional clinical trials are reasonably well-documented, with factors including an increased impact of the required time burden and restrictive inclusion criteria recognised as contributors to poor participation in research among this group (Mody *et al.*, 2008; Carroll and Zajicek, 2011).

There is perhaps less writing on the specific considerations for older people taking part in experimental medicine studies such as the immune challenge study described in this thesis. With populations ageing as they are, understanding the pathophysiology of disease states specific to older people becomes ever more important, and experimental medicine studies will play an important role in filling gaps in such knowledge. Although it was not the case as the study in Chapter 3 began, an additional consideration for the participation of older volunteers was the COVID-19 pandemic and what turned out to be heightened risk from infection for older people. Richardson and colleagues recognise this but also recognise the importance of continued inclusion of older people in research through the pandemic, suggesting that inclusion may be facilitated by incorporating research assessments in routine care visits, or close to usual places of care or residence (Richardson *et al.*, 2020). Although some insights into participation barriers were gleaned in the unstructured focus group discussions presented in Chapter 3, future work could include a formal qualitative assessment of the barriers faced by older people seeking to participate in experimental medicine studies in particular, and factors that might influence their retention or attrition. My preconceptions were that the physical health risks of immune challenge would be a primary deterrent, but that was nowhere near as much a concern for the people in the focus group as social barriers like travel distance and time burden. Exploration of these issues through qualitative research would facilitate ethical and equitable experimental medicine research in future.

Studies of age-related inflammation involving healthy older volunteers may also raise the question of to what extent the observations of the populations being studied could be

generalised to older members of the population at large. If inflammaging is linked to frailty, then it becomes possible that volunteers who meet the inclusion criteria and successfully negotiate the exclusion criteria are more likely to be people in whom age-related inflammation is, in a sense, less active. In that context, the older the volunteer, the more likely they are to be a model of successful ageing rather than a model of the human in advanced age. This should not necessarily preclude the inclusion of such individuals in experimental studies; in my view it suggests that perhaps studies should be designed with the consideration that people in the extremes of advanced age might represent a group of people with a separate inflammatory phenotype again. Arai and colleagues, in a longitudinal study including supercentenarians and their offspring, suggest that although supercentenarians display elevated biomarkers of chronic inflammation in their advanced age, their offspring are likely to have lower inflammatory markers in the earlier stages of their lives. This might imply that either low cumulative chronic inflammation over the lifespan or active compensatory anti-inflammatory (or perhaps pro-resolution) processes are contributors to successful ageing (Arai *et al.*, 2015). For future work, protocols such as those described in this thesis could be adapted to identify surrogates of pro-resolution processes in biological samples (peripheral blood or bronchoalveolar lavage fluid, for example) in the extremes of age to define the phenotype of successful ageing.

7.2 Drug repurposing to enhance the resolution of inflammation

The work presented in Chapter 4 utilised a computational drug screen to identify candidate pro-resolution drugs, then attempted to assess their effect on efferocytosis in an *ex vivo* assay. Although no effect of the drugs was demonstrated, the methods described demonstrate the power of using publicly available systems biology datasets for hypothesis generation. If nothing else, the screen of the L1000 Connectivity Map independently corroborated the association between cyclin-dependent kinase inhibitor drugs and expression of the anti-inflammatory molecule Del-1, which has been shown to contribute to reduced inflammatory cell recruitment in an animal model of LPS-induced lung inflammation (Choi *et al.*, 2008). Cyclin-dependent kinase inhibition has also been found to result in reduced downregulation of Del-1 on endothelial cells in an inflamed tissue environment, and this mechanism was implicated in the effect of CDK inhibition on reducing inflammation in a murine model of inflammatory arthritis (Hellvard *et al.*, 2016). Further work based on the

negative results in Chapter 4 might seek to define the effects of the selected CDK inhibitors on expression of Del-1 in *ex vivo* systems involving human myeloid cells. ELISA could be used to measure relative concentrations of Del-1 in culture supernatants after exposure to a CDK inhibitor or control. If an effect is confirmed, work beyond that might attempt to reproduce the findings of Choi *et al.* in a human model of LPS-induced lung inflammation to test the hypothesis that Del-1 is similarly involved in the modulation of pulmonary inflammation in humans. As above, the protocol for the human challenge experiments described in this thesis is adaptable, meaning that there would be leeway for the CDK inhibitor to be administered systemically (as flavopiridol is when used for treatment of haematological malignancy) or as an inhaled drug.

Given the exploration elsewhere in the thesis of chronic inflammatory and fibrotic interstitial lung diseases, further reasons to consider Del-1 as a drug target in future work are the observations that Del-1 deficiency results in increased pulmonary fibrosis in a mouse model of lung injury by bleomycin, and intravenous supplementation with Del-1 attenuates fibrosis, possibly through suppression of TGF-β activation (Kang *et al.*, 2014; Kim *et al.*, 2020). Given that it is not known whether the fibrosis that develops in chronic hypersensitivity pneumonitis is a direct consequence of the inflammation that characterises the acute and subacute forms of the condition, future work might then start to investigate the role of Del-1 in the progression of hypersensitivity pneumonitis into fibrosis, perhaps attempting to identify the relative levels of expression of the molecule in tissue or BAL samples from patients with hypersensitivity pneumonitis, and correlating this with the subsequent development, progression and extent of fibrosis in the disease.

Such work may then present the possibility of exploring the utility of CDK inhibition as a treatment in conditions characterised by chronic inflammation. Hypersensitivity pneumonitis is a suitable candidate condition based on the work in this thesis and given the above literature that might suggest a connection between CDK inhibition and the prevention of pulmonary fibrosis. In addition, especially in view of the association between chronic inflammation and frailty of ageing, it might be possible to conceive of an early phase clinical trial attempting to discern the effect of repurposed flavopiridol on markers of chronic inflammation in older individuals. It is far from certain that generating some anti-inflammatory effect would recapitulate a successful ageing phenotype, but with the

association between inflammaging and functional decline the potential link may be worth exploring, and computational drug screening may help to identify drugs that are already in clinical use that could be repurposed to this end.

It must be conceded that the efferocytosis assay described in Chapter 4 needs to be optimised to provide confidence in its results, with a priority being the direct visual demonstration of the ingestion of efferocytic cargo and its modulation by positive and negative controls, which could be undertaken by fluorescence microscopy, for example. Once the assay is optimised, it would then be useful to perform work to link deficient efferocytosis to disease outcomes, and link drugs that rescue deficient efferocytosis *in vitro* with improved disease outcomes. Deficient efferocytosis is postulated to contribute to disordered inflammation in the chronic lung condition COPD, and although treatment with the drug lovastatin has been demonstrate to rescue efferocytosis in alveolar macrophages in *ex vivo* experiments (Morimoto *et al.*, 2006), it is not certain whether this means that disease outcomes would be improved by statin treatment, or whether that can be attributed to an improvement in efferocytosis. A double-blind, randomised, placebo-controlled trial at a single Austrian healthcare centre demonstrated an increased time to first COPD exacerbation in patients treated with simvastatin (Schenk *et al.*, 2021), but it would require additional work to link that outcome with improved efferocytosis. This is the niche that could be filled by an optimised efferocytosis assay.

When considering disordered inflammation resolution in COPD, it is notable that macrolide antibiotics are used in clinical practice to reduce the frequency of exacerbations of the disease. The mechanism of action of macrolide antibiotics in this context has long been uncertain, but recent evidence from murine models has suggested the macrolide antibiotic erythromycin suppresses inflammation in LPS-induced lung injury in a Del-1-dependent manner (Maekawa *et al.*, 2020). This provides a further reason to consider Del-1 as a target for drugs repurposed on the strength of computational screens like the ones described in this thesis.

7.3 Immunosuppression in hypersensitivity pneumonitis

Although the randomised controlled trial remains at the top of the hierarchy as a method of evaluating the effect of a treatment (Bothwell *et al.*, 2016), there is still value in retrospective

analyses such as those presented in Chapter 5, even if only for the sake of evaluating past practice to inform the need for further study. As it happened the case series required more data than were available to provide a robust assessment of the effect of cyclophosphamide therapy in hypersensitivity pneumonitis, and at least part of the deficit was the result of missing entries in the electronic medical record. This outlines one of the primary limitations of retrospective studies using medical notes, and aside from a loss of power it is necessary to discern whether any missing data are missing in a random or non-random fashion and whether the absences might be related to other observations (Papageorgiou *et al.*, 2018). If the data are missing completely at random then the likelihood of the absence of the data imparting bias upon the final inference are minimised. Lung function data could be missing as a consequence of a patient being unable to complete a test due to severe symptoms, however, so not considering those patients in the analysis might have biased the results in favour of outcomes observed in less unwell participants.

As well as lung function data that might have been missing due to participants becoming more unwell as the disease progressed, there was also a conspicuous absence of data that might have helped to calculate the lung function trajectory prior to treatment with greater precision. As mentioned in the discussion section of that chapter, an explanation for this could lie in the Newcastle ILD Service's position receiving tertiary referrals, meaning that although lung function in the time prior to referral is likely to have been documented, there is a possibility that it will be documented at a site that is not readily accessible to Newcastle researchers without specific transfer of information.

The question of whether cyclophosphamide is effective in the treatment of chronic hypersensitivity pneumonitis is one that is yet to be answered definitively, which makes it somewhat disappointing that the study presented in Chapter 5 could not consider enough patients to be able to detect a significant difference in rates of lung function decline before and after cyclophosphamide therapy. It is important to note that rates of FVC or TLCO decline may not be the ideal outcome measures to consider in future studies of the efficacy of treatment as there is insufficient evidence that an intervention that alters those physiological parameters would reliably result in an effect on a clinically meaningful endpoint such as mortality (Raghu *et al.*, 2012). This is not to say that measuring the physiological parameters is of no utility, however, as it is intuitive that an alteration in the rate of lung function decline

might suggest an effect of the mechanics of the progression of the disease, which might include inflammation. Small retrospective studies have considered the rate of decline in FVC and TLCO in chronic HP treated with cyclophosphamide and found modest improvements in the rate of change, but not quite to the extent as those seen in ILD associated with connective tissue diseases treated with the same drug (Barnes and Johannson, 2021). Prospective studies are sorely required, and failing that, good retrospective analyses would add to the wisps of evidence that currently exist.

7.4 Understanding the role of inflammation in hypersensitivity pneumonitis

It is uncontroversial to note that the lack of proven treatments in chronic hypersensitivity pneumonitis is likely to be related to an incomplete understanding of the disease. With a prevalence on the order of 1 per 100,000 population (Fernández Pérez *et al.*, 2018) hypersensitivity pneumonitis is a rare disease, meaning that opportunities for pathological studies are limited. The paucity of tissue available for research is aggravated by the fact that biopsy is recommended to establish the diagnosis only in cases of diagnostic uncertainty (Raghu *et al.*, 2020).

Systems biology datasets derived from the biological samples that are available are therefore likely to play a large role in developing our understanding of rare diseases like hypersensitivity pneumonitis, and the work presented in Chapter 6 draws on publicly available transcriptomic data derived from the lung tissue of patients with interstitial lung diseases undergoing lung transplantation. It must be noted that there are limits to the extent that such data can be used to test hypotheses regarding disease processes as opposed to generating them, not least because of the gap between the presence of mRNA in a biological sample and the protein that is eventually translated or biologically active.

Within those constraints, the work presented in Chapter 6 seeks to demonstrate that it is possible to gain some insights into the pathogenesis of a disease with the aid of bioinformatic tools, literature searching and inductive reasoning. By comparison to the other interstitial lung diseases included in the dataset it became apparent that a gene signature compatible with the presence and activity of lymphocytes was present in the hypersensitivity pneumonitis tissue, and moreover, the likely identity of those lymphocytes could be hypothesised, with the likely culprits being tissue-resident memory T cells.

It is then necessary to test this hypothesis by direct observation of HP lung tissue, and further work will certainly involve pathological studies using immunofluorescence microscopy to identify the increased presence of T_{RM} cells in areas of inflammation in HP lungs compared to other interstitial lung diseases and healthy lungs. As above, the availability of tissue would be the limiting factor in designing such a study. Biopsies for interstitial lung disease — and especially surgical lung biopsies yielding relatively large tissue samples — are probably less prominent in diagnostic workups where diagnoses can be made by multidisciplinary teams with the aid of good background clinical information and high resolution computed tomography (Spagnolo *et al.*, 2021), meaning that any such pathological study might come to rely on archived tissue from historical biopsies. To support such studies, the Royal College of Pathologists has produced guidance for research use of specimens from hospital laboratory archives with appropriate ethical approvals after their diagnostic use is complete (The Royal College of Pathologists, 2015).

If indeed T_{RM} cells are proven to be the drivers of disease in hypersensitivity pneumonitis, the next question that could be asked is whether there are any specific treatments that might target these cells to modify the disease. Work from animal models suggests that the persistence of these cells in the lung might be dependent upon IL-15 signalling (Mackay *et al.*, 2015), and if one were to extrapolate from the experiments suggesting that IL-15 blockade can reverse murine models of vitiligo (Richmond *et al.*, 2018) and coeliac disease (Yokoyama *et al.*, 2009) — conditions that appear to be mediated by CD8-expressing T cells resident in epithelial barrier tissues — then reasonable future experiments might aim to discern whether IL-15 blockade could reverse the murine model of hypersensitivity pneumonitis provoked by the repeated inhalation of the Gram-positive bacterium *Saccharopolyspora rectivirgula* (Andrews *et al.*, 2016).

7.5 Conclusions

The work in this thesis describes a viable platform for the assessment of pulmonary inflammation that could be adapted to facilitate experimental medicine studies characterising inflammation in advanced age or early phase trials of immunomodulatory drugs that might alter the course and resolution of inflammation. This thesis also describes a method to

identify candidate immunomodulatory drugs using connectivity maps and puts forward Del-1 as a target for drugs that enhance the resolution of inflammation.

In considering the role of inflammation in chronic lung disease, this thesis also presents an exploration of the mechanisms of inflammation in chronic hypersensitivity pneumonitis, using a systems biology approach to implicate tissue-resident memory T lymphocytes in the pathogenesis of the disease. This opens up the possibility of IL-15 signalling as a potential target for treatment of the disease where few treatments are currently proven to be effective.

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Appendix A. Study protocol and materials for bronchoalveolar lavage study

This appendix contains the most recent version of the study protocol for the bronchoalveolar lavage study detailed in Chapter 3 (submitted for consideration to the NHS Health Research Authority as *The effect of ageing on acute inflammatory responses in man*). Supporting materials are also included in this section: advertisements for volunteers, consent forms, Health Research Authority approval letter and clinical procedure protocols.

Study Protocol

The effect of ageing on acute inflammatory responses in humans.

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Funder and funder reference number	Newcastle NIHR Biomedical Research Centre, BH173352
Sponsor	Newcastle upon Tyne Hospitals NHS Foundation Trust
Protocol	v1.3 (13th November 2018)

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Introduction

Deaths from pneumonia and influenza increase throughout adult life, and are the fourth highest cause of death in people aged >80 in the UK (1). Rodent data suggest that ageing impairs cellular immune responses to infection. Parallel human data are distinctly lacking however, and fundamental differences between immune responses in rodents and humans are well described (2). Furthermore, while early observations suggest age-related impairment of *circulating* neutrophil, monocyte and lymphocyte function, no inferences can be made about immune function in inflamed human *tissues*.

The lung represents an ideal end-organ for studying innate immune responses in humans. The lung is readily accessible via bronchoscopy and bronchoalveolar lavage (BAL), with the advantage that single cells are retrieved from the alveolar space (with no need for biopsy and enzymatic digestion of tissue). Clinically, the lung is highly relevant given that admission rates associated with community-acquired pneumonia (CAP) are increasing steadily, with mortality rates significantly higher in patients >65 years old than in young patients (3). The profile of intensive care unit (ICU) admissions is increasingly dominated by an ageing population, who develop high rates of pulmonary complications, including ventilator-associated pneumonia (VAP).

The significant morbidity, mortality and secondary bacterial infection associated with influenza in older people has driven the development of the influenza vaccination programme. However, immune responses to intramuscular vaccination are blunted and less predictable with increasing age. Significant interest is evolving around inhalational vaccination as a more efficient means of providing protection, but progress is limited by lack of understanding of alveolar immune responses in humans.

With this background in mind, this study seeks to make the most comprehensive dissection of peripheral and alveolar cellular immune function to date, comparing young and old volunteers. The study has 2 components, both comparing younger individuals (aged 16-30) and older individuals (aged >65). In the first component, participants will inhale a low dose of a non-infective chemical (called lipopolysaccharide or LPS) isolated from the cell wall of Gram-negative bacteria, or a control (saline) under controlled conditions in a hospital intensive care unit, and the ensuing immune response in the blood and the lung will be followed over the course of the day.

In the second component, we shall make a preliminary but detailed assessment of the cellular immune response to influenza vaccination in young and old volunteers. It should be emphasised that volunteers will take part in either the inhaled (LPS/saline) part of the study OR the influenza element. No participant will contribute to both elements.

The Human Inhaled LPS Model

Lipopolysaccharide (LPS) is a conserved virulence factor in the cell wall of Gram negative bacteria. LPS is believed to contribute to the pathogenesis of Gram negative sepsis. Low doses of purified LPS, inhaled under controlled conditions, have been used in experimental medicine for over 30 years, in dozens of studies, with an excellent safety record. Inhaled LPS creates a predictable, self-limiting pulmonary inflammation, with associated systemic symptoms. Volunteers typically develop a low-grade pyrexia at around 8 hours and may have mild headache and fatigue. These resolve a few hours later, and almost invariably have disappeared by the next day. The lung inflammation may be accompanied by mild breathlessness.

The inhalation is associated with recruitment of inflammatory cells to the lung, peaking at around 4-8 hours and back to normal by 24 hours. A low grade blood neutrophilia is observed, peaking at 6-8 hours and resolving by 24 hours. While the neutrophil is the predominant cell entering the lung, we and others have demonstrated that a range of leukocyte cell types enter the lung, which can be comprehensively characterised using flow cytometry and single cell functional genomics.

We have performed previous clinical studies using inhaled LPS in human volunteers (4, 5, and Jardine L et al, manuscript in preparation), and we are currently carrying out a study of intravenous LPS in human volunteers. Our studies have had an excellent safety profile, in keeping with the existing literature.

Influenza vaccination

Influenza vaccination will be given as per normal clinical practice.

Objectives

Primary Objective

The primary objective is to determine whether the pattern of immune response differ consistently between younger and older individuals after a challenge with a microbiological stimulus.

Secondary Objectives

Which specific types of immune cells appear in the blood (and the case of LPS, lungs) of volunteers after challenge with a microbiological stimulus?

Are there previously unidentified immune cells in the lung and/or blood after challenge with a microbiological stimulus?

What are the predominant cell types mobilised, activated and expanded in number after immune challenge?

Study Design

Participant Enrolment and Selection

An advert will be placed on email lists and notice boards in Newcastle University, City Hospitals Sunderland NHS Foundation Trust and Newcastle upon Tyne Hospitals NHS Foundation Trust. General medical practices interested in aiding recruitment will be asked to carry advertisements for volunteers. Potential participants will be asked to make contact with the research team only if they consider themselves to be healthy. Interested individuals will be invited to contact the research team who will send out information on the study (participant information sheets and screening consent form). Participants sent information on the LPS inhalation study will be invited to contact the research team to arrange a screening visit (see below) or to decline participation. If no reply is received after 2 weeks, the research team will telephone the volunteer as a reminder. Participants suitable and willing for the LPS and flu vaccination studies will come for a study visit, and return for further blood sampling as indicated below.

(i) INHALED STUDY

Screening Visit

Healthy volunteers aged 16-30, or >65, will be recruited from within Newcastle University, City Hospitals Sunderland NHS Foundation Trust,Newcastle upon Tyne Hospitals NHS Foundation Trust, or via participating general medical practices. The

screening visit will take place in the Pre-Assessment Clinic at Sunderland Royal Hospitals, and will take the form of:

- a short history
- vital signs measurement (temperature, pulse rate, blood pressure)
- measurement of oxygen saturation breathing room air
- cardiorespiratory examination
- blood sample for full blood count
- blood sample for urea & electrolytes assay, liver function tests and C-reactive protein.

The screen is designed to indicate whether participants have any exclusion criteria.

Inclusion criteria

- The individual considers him/herself to be healthy.
- Age >65 (for the older individuals).
- Age 16-30 (for the younger individuals).

Exclusion criteria

We seek to recruit healthy individuals who are free of symptoms of respiratory infection at the time of recruitment and who are not on any medication with an obvious effect on the immune system. Ideally we would prefer individuals to have no regular medications, but experience tells us that some medications are so common in the general population, and have so little obvious effect on immune function, that they will be permissible if necessary. With this in mind, the exclusion criteria are:

- Current smoker
- Ex-smoker (unless smoking stopped >5 years ago, and smoking history was no more than 5 pack-years)
- Current diagnosis of asthma, chronic obstructive pulmonary disease, bronchiectasis, pulmonary fibrosis, cardiac failure, ischaemic heart disease, diabetes mellitus, rheumatoid arthritis, or any other chronic medical condition considered to compromise safety (at bronchoscopy) or scientific quality of the study, as judged by the chief investigator.
- Currently taking medication known to have immunosuppressive or stimulatory effect e.g. oral or systemic glucocorticoids, methotrexate, suphasalazine (where possible volunteers will be on no regular medication, but medicines such as the oral contraceptive pill will be permissible).
- Known HIV infection
- Needle phobia
- Pregnancy or breast feeding
- Abnormal physical signs detected at cardiorespiratory examination
- Clinical evidence of current respiratory tract infection
- Temperature >37.3°C
- Oxygen saturation <95% breathing room air

- Haemoglobin outside the laboratory reference range.
- Platelet count less than 100 x 10^9/l or greater than 650 x 10^9/l.
- Total white cell count outside the laboratory reference range.
- Any deviation of greater than 20% from normal limits in the differential white cell count.
- Serum sodium, potassium, creatinine outside the laboratory reference range.
- Blood urea greater than 10mg/dl.
- Bilirubin greater than 30micromol/l.
- Alanine transferase greater than twice the upper limit of the laboratory reference range.
- Currently participating in a clinical trial that the chief investigator feels would interfere with the analysis carried out as a result of this study.

If a volunteer is excluded on a parameter that may be transient, ie temperature, inter-current respiratory infection, or a blood test parameter(s), he/she may be re-screened once at a later date.

If found eligible for the study, the volunteer will be given time to consider if they wish to continue to study entry and invited to re-attend on a set day. Participants will be asked to attend the integrated critical care unit (ICCU, City Hospitals Sunderland NHS Foundation Trust) for LPS/saline administration.

Sample size

Up to 18 healthy participants will be recruited to the inhaled study. Five young volunteers (aged 16-30) will inhale LPS, 4 will inhale the equivalent volume of saline. Five older volunteers (aged >65) will inhale LPS, 4 will inhale the equivalent volume of saline.

Consent

All eligible volunteers will be given written and verbal information regarding study participation. All will be asked to give informed, written consent. Consent will be taken by a member of the research team who holds GCP.

Ineligible and non-recruited participants

For volunteers found to be ineligible at screening, or eligible but not subsequently entered into the study, the reason for ineligibility or non-recruitment will be entered on the study database. Only pseudo-anonymised data will be entered on to the database and this will include gender, age, “ineligible” or “non-recruitment” and the associated reason. If found ineligible, permission will be sought from participants to contact their GP with the results of screening tests and any further action required.

Study visit

Volunteers satisfying study criteria, wishing to proceed with the study, and providing written informed consent will attend the integrated critical care unit (ICCU), City Hospitals Sunderland on an agreed day. Volunteers will spend a full day (approximately 10 hours) in the unit. Volunteers will be allowed to have breakfast as normal, and to eat and drink up until 6 hours before the scheduled bronchoscopy. After this they should only have sips of water until 3 hours after the bronchoscopy. A brief history (regarding symptoms of any acute illness) will be sought. If a respiratory infection or other acute illness is suspected on clinical grounds, the participant will be excluded, but may return for one further round of screening at a later date, if they wish. In female participants of reproductive age, a urinary pregnancy test will be performed and will be reviewed by a medical practitioner who will offer appropriate advice. A positive test will result in exclusion from the study. Vital signs (pulse rate, blood pressure, temperature, respiratory rate and oxygen saturation) will be monitored at baseline and every at least every 30 minutes for the first 4 hours after inhalation, and then at least hourly until the bronchoscopy. A venous cannula will be inserted into the arm.

Spirometry will be performed at baseline. Whether the volunteer receives LPS or saline will depend on their number in the study – among the 9 younger volunteers the order will be LPS-saline-LPS-saline-LPS-saline-LPS-saline-LPS for the nine participants. The same schedule will be used for older volunteers.

Volunteers will receive either 2 micrograms LPS (from *Escherichia coli* O:113, List Biological Laboratories, California, USA) or sterile 0.9% saline (placebo) using an inhalation-synchronised dosimeter nebuliser. Spirometry will be repeated if volunteers report breathlessness and/or chest tightness after LPS inhalation and immediately before proceeding to bronchoscopy. If symptoms of chest tightness/breathlessness or signs of wheeze arise, or if forced expiratory volume has dropped from baseline, the research team may administer a bronchodilator (e.g. nebulized salbutamol 2.5mg) If FEV_1 has fallen by >15% at the 8-hour time-point, then bronchoscopy will not be performed. If symptoms of headache/fever are troublesome after inhalation the volunteer may be offered appropriate treatment (e.g. paracetamol 1g orally). In event of unexpected hypotension, treatment will be given according to doctors in the research team and/or on the ICCU (e.g. a bag of intravenous saline).

Blood will be drawn just before inhalation of LPS (t=0) and at t=1 hour, 2 hours, 4 hours and 8 hours. At 8 hours bronchoscopy and BAL will be performed using a standardised protocol and procedure employed throughout our work thus far. Local anaesthesia will be applied topically to the throat. Sedation (e.g. with intravenous midazolam) will be optional. Electrocardiogram (ECG) trace and SaO_2 will be monitored continuously. A flexible fibreoptic bronchoscope will be passed per-orum or per-nasum. 20 ml of saline will be instilled into a segment of the right middle lobe, gently aspirated and discarded (as the 'bronchiolar' sample). 120 ml of saline will then be instilled in aliquots and gently aspirated. The volunteer will have vital signs monitored at least half hourly for a further 3 hours after which he/she can eat and drink.

If volunteers have normal observations and cardiorespiratory examination 2 hours following bronchoscopy they will be allowed home and supplied with a number to

contact should they feel unwell. They will then return briefly the following morning for the final blood test (t=24 hours). The study investigators will telephone the volunteer later that day to ensure they remain well.
Burden of time

Volunteers will be required to attend for a screening visit, a ten-hour study day for induction of inflammation, observation and sampling, followed by one further visit for follow-up and a blood sample sampling. In recognition of the burden on the volunteer in terms of time, volunteers will be offered £250 on study completion.

Discharge criteria

The attending clinician will ensure that the participant's symptoms have settled and that their observations are trending to normal (all altered parameters, e.g., elevated heart rate and temperature, demonstrating consistent reduction toward baseline values) prior to sanctioning the end of observation and subsequent discharge. Monitoring will be discontinued, venous cannulae will be removed, and haemostasis will be ensured. It will be confirmed that the participant is happy to be discharged home and has the contact details of the research team in case of any concern.

Participants having sedation as part of bronchoscopy must agree to have a friend or relative pick them up and take them home, and must agree not to drive for the remainder of the day.

The following day the volunteer will return to the Pre-Assessment Clinic for a single blood sample to be taken.

Downstream preparation and analysis of samples generated by the study will be performed in the research labs in Newcastle University (principally the Simpson lab). Processing of screening blood tests and routine clinical tests following administration of LPS will take place in the clinical laboratories at City Hospitals Sunderland.

(ii) INFLUENZA VACCINATION STUDY

The principles of the influenza vaccination study are the same, ie samples are taken before and after an immune stimulus, and the effects in younger and older volunteers are compared. Identification and enrolment of volunteers is as described on page 6. There is no screening study in the influenza vaccination element.

Inclusion criteria

- The individual considers him/herself to be healthy.
- The individual is scheduled to have influenza vaccination as part of his/her routine clinical care.
- Age >65 (for the older individuals).
- Age 16-30 (for the younger individuals).

Exclusion criteria

- Already had an influenza vaccination for the forthcoming/current flu season
- Current smoker
- Ex-smoker (unless smoking stopped >5 years ago, and smoking history was no more than 5 pack-years)
- Current diagnosis of asthma, chronic obstructive pulmonary disease, bronchiectasis, pulmonary fibrosis, cardiac failure, ischaemic heart disease, diabetes mellitus, rheumatoid arthritis, or any other chronic medical condition considered to compromise safety (at bronchoscopy) or scientific quality of the study, as judged by the chief investigator.
- Currently taking medication known to have immunosuppressive or stimulatory effect e.g. oral or systemic glucocorticoids, methotrexate, suphasalazine (where possible volunteers will be on no regular medication, but medicines such as the oral contraceptive pill will be permissible).
- Known HIV infection
- Needle phobia
- Pregnancy or breast feeding
- Clinical evidence of current respiratory tract infection
- Temperature >37.3°C
- Oxygen saturation <95% breathing room air
- Currently participating in a clinical trial that the chief investigator feels would interfere with the analysis carried out as a result of this study
- Known allergy to eggs
- Previous allergy or adverse reaction to influenza vaccination.

Sample size

Up to 8 volunteers will be studied, comprising up to 4 younger and up to 4 older volunteers.

Consent

All eligible volunteers will be given written and verbal information regarding study participation. All will be asked to give informed, written consent. Consent will be taken by a member of the research team who holds GCP.

Ineligible and non-recruited participants

For volunteers found to be ineligible the reason for ineligibility or non-recruitment will be entered on the study database. Only pseudo-anonymised data will be entered on to the database and this will include gender, age, “ineligible” or “non-recruitment” and the associated reason.

Study visits

The volunteer will come to the Pre-Assessment Clinic at Sunderland Royal Hospitals. If a respiratory infection or other acute illness is suspected on clinical grounds, the participant will be excluded, but may return once at a later date, if they wish. If no exclusion criteria are identified a blood sample will be taken, then the volunteer will be given the influenza vaccination. The volunteer's GP will be informed of the vaccination.

The volunteer will return to the Pre-Assessment Clinic 1, 2 and 7 days after the vaccination, and at each of these visits a single blood sample will be taken.

Correspondence after study completion

After an individual has completed the necessary elements of his/her participation, he/she will be sent a thank you letter. All participants will be advised that they may request a summary of the study results, which will be sent once all data have been collected and analysed.

End of study

The clinical element of the study will be completed after the final blood sample is taken from the final volunteer. Scientific analysis of samples in accordance with the experimental plan may be performed after this.

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22 October 2018

Dear Prof Simpson

HRA and Health and Care

Study title:	**The effect of ageing on acute inflammatory responses in man.**
IRAS project ID:	**241028**
REC reference:	**18/YH/0088**
Sponsor	**Newcastle Joint Research Office**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales? You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

Following the arranging of capacity and capability, participating NHS organisations should **formally confirm** their capacity and capability to undertake the study. How this will be confirmed is detailed in the "*summary of assessment*" section towards the end of this letter.

You should provide, if you have not already done so, detailed instructions to each organisation as to how you will notify them that research activities may commence at site following their confirmation of capacity and capability (e.g. provision by you of a 'green light' email, formal notification following a site initiation visit, activities may commence immediately following confirmation by participating organisation, etc.).

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your nonNHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Mr Aaron Jackson
Tel: 01912825789
Email: aaron.jackson@nuth.nhs.uk

Who should I contact for further information?

Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **241028**. Please quote this on all correspondence.

Yours sincerely

Sharon Northey
Senior Assessor

Chief Investigator:
Professor John Simpson
Professor of Respiratory Medicine
Newcastle University

Tel: 0191 208 7770
Email: j.simpson@ncl.ac.uk

The effect of ageing on acute inflammatory responses in humans

PARTICIPANT INFORMATION

HELPING YOU DECIDE WHETHER TO ENTER THIS STUDY

INTRODUCTION

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study, outlines what will happen to you if you take part, and informs you about the conduct of the study.

- Part 2 gives you more detailed information about what will happen to you if you take part.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

Infection of the lung tissue (pneumonia) is one of the most common causes of death in older people across the world. This remains the case despite the fact that we have antibiotics that readily kill the germs that most commonly cause pneumonia, and a vaccine against the commonest cause of pneumonia. This suggests that something is different (and less effective) in the lung's immune response as we get older. If we understood exactly why the lung's immune defences work less efficiently in later life, we could begin to design logical new treatments and/or develop vaccines that are more effective.

The difficulty in studying the immune response in older adults with pneumonia is that it is very difficult to predict who is going to get pneumonia, and when. Therefore, following the immune response from before the pneumonia starts to when it resolves is extremely difficult.

Inhalation of a (non-infectious) bacterial product that we know stimulates lung immunity for a period of a few hours, in a way similar to pneumonia, under carefully controlled medical conditions, provides a unique opportunity to study how the immune response starts, develops, and resolves. New technologies allow us to extract and study individual immune cells from the lung. Knowing which immune cells are "first on the scene" and how they behave when they arrive in the lung should give us important information. By comparing immune responses in younger and older individuals (and by giving bacterial product to some individuals and saline “placebo” to others within each age group), we shall build up a profile of the effect of fundamental differences in the lung’s immune response between younger and older people. This information will provide important first steps in assessing the feasibility of designing new treatments and vaccines.

Why have you been chosen?

Because you appear to be healthy, and you are either aged 16-30 or >65 (the 2 age ranges we wish to compare).

If you are pregnant or breast feeding you should not take part in the study – this is simply because pregnancy affects your immunology and would skew the results of our tests. We will ask that you undertake a pregnancy test to see if you are eligible for inclusion in the study.

What will happen to you if you take part?

You will attend for a screening visit at the Pre-Assessment Clinic at Sunderland Royal Hospital. If the screening visit is satisfactory and you want to proceed to the study you will have one day in hospital (on a day suitable to you), and a brief check up the next morning. The procedures we would like you to have during your day in hospital are

- blood samples

- inhalation of a product called lipopolysaccharide, aiming to cause mild, transient inflammation in the lung
- a ‘camera’ investigation of the lung (called bronchoscopy and bronchoalveolar lavage)

Greater detail about each of these is found in Part 2.

Do you have to take part?

No. It is up to you to decide whether or not to take part.

You are free to withdraw from the study at any time and without giving a reason. A decision not to take part, or withdraw at any time, will not affect the health care you receive at any stage, now or in the future.

How long can you take to decide?

You should take as much time as you wish.

Do you have to complete all of the tests described?

No. While we obviously prefer to obtain all of the samples described, you are under no obligation to have any of the tests. You can complete all of the tests, or you can decline as many of the tests as you wish.

Involvement of the GP

With your permission, should any abnormal clinical results emerge during your tests, we would inform your GP.

Will any genetic tests be done?

Yes. We shall test how gene products (RNA) are made in cells after exposure to the inhaled LPS or placebo, in younger and older individuals. However, we shall not perform diagnostic tests on DNA from individuals.

What are the potential benefits of taking part?

There is no direct benefit to you.

Is there any reimbursement for taking part?

Yes. We provide a fee of £250 if you complete all the study days (travel expenses are incorporated in this fee). If you attend the screening visit or study day and are not eligible to continue, we shall reimburse any travel costs if you provide receipts. If you are eligible for all of the study but fail to complete the study days, we shall reimburse any travel costs if you provide receipts.

Can you access the results of the research?

We shall be happy to send a summary of the overall results of the study after its completion, should you so wish. If you would like us to do this, please contact Prof Simpson at the address shown below.

What will happen to the results of the research study?

We intend for the results to be published in medical/scientific journals and presented at medical/scientific meetings. All information in the public domain will be anonymous (i.e. you cannot be identified from these publications/presentations). We intend that the results of the study will inform the design of future studies seeking to prevent pneumonia (by developing better vaccines) or treating pneumonia (by understanding how to boost immunity in the older lung).

What if there is a problem?

If you have a concern about your treatment by members of staff during the study, you should ask to speak with the researchers who will do their best to answer your concerns (see contact details below for Prof Simpson). If you would prefer to raise your concerns with someone not involved in your care, you can contact the Patient Advice and Liaison Service (PALS). This service is confidential and can be contacted on Freephone 0800 032 0202. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your hospital.

In the unlikely event that something goes wrong and you are harmed during the study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the NHS/Newcastle University but you may have to pay for your legal costs. The normal NHS complaints mechanisms will still be available to you.

How will my samples be used?

The blood samples (in packaging containing no identifying information) will be transported to the laboratories at Newcastle University for analysis by researchers working in Prof Simpson's research group. We will purify blood cell types of interest from the samples and undertake tests of their functions, including tests to identify which gene products are made as described above.

The liquid from the lavage of your lungs will also be transported to the laboratories at Newcastle University in a similar way. Again, the packaging will be secure and contain no identifying information. We will purify cell types of interest from the samples and perform similar tests to the cells taken from blood.

Will any material be stored?

Yes, but only with your permission. We propose to store the liquid portion from blood samples (plasma/serum) and from a 'wash' (lavage) of your lungs. In addition, we shall retain slides containing cells from your blood and lungs. These will be kept in an anonymised

form (ie. you cannot be identified from the samples). We propose to keep your samples for up to 5 years after completion of the study.

At 5 years we propose to destroy the samples. However, as medicine advances and new information becomes available, we occasionally find good reasons to perform additional tests on stored samples in the future. Should this situation arise we may use your samples again, but this would be on the strict conditions that you give permission for this, that the samples would be anonymous (ie. you could not be identified from them) and that we obtain fresh and separate permission from an ethics committee.

If you decide to withdraw from the study, or if you cannot complete the study for another reason, we propose to use any data collected up to that point, unless you inform us in writing that you wish all samples and data collected from you to be destroyed.

Will your taking part in this study be kept confidential?

Yes. Your information and samples will be given a study identity number, and all the information would remain strictly confidential. Your number will only be connected to your personal details in a file kept in a locked drawer in Newcastle University, in a room separate to where your samples are stored. Your name will not appear in any report or publication that arises as the result of this study.

Who is organising and funding the research?

The research team is made up of clinicians and staff from Newcastle University, Sunderland Royal Hospital and Newcastle upon Tyne Hospitals NHS Foundation Trust. The research is funded by the Newcastle National Institute for Health Research (NIHR) Biomedical Research Centre (BRC) in Ageing and Long-Term Conditions.

Who has reviewed the study?

The Newcastle NIHR BRC ran a grant competition open to research groups in the region. Applications were assessed by experts outside Newcastle, and also by a scientific panel in Newcastle. Applications considered to be of the highest scientific quality and importance were funded. The study has also been independently reviewed and approved by the Yorkshire and the Humber – Leeds West Research Ethics Committee and the regional Research and Development Office.

Is there an independent doctor you can approach for further information?

If you would like to discuss any aspect of this research with an experienced researcher who is not linked in any way to this study, please feel free to contact

Dr Malcolm Brodlie
Consultant Paediatric Respiratory Physician
Great North Children's Hospital
Royal Victoria Infirmary
Newcastle upon Tyne

NE1 4LP
Tel:0191 233 6161

This completes Part 1 of the Participant Information

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making your decision.

THANK YOU FOR TAKING TIME TO READ THIS

The effect of ageing on acute inflammatory responses in humans

PARTICIPANT INFORMATION SHEET

PART 2

What will happen to you if you take part?

Screening visit

On a day suitable to you, you would come to the Pre-assessment Clinic at Sunderland Royal Hospital. The purpose of this visit is to check that you do not have any features which might exclude you from our study, and to answer any questions you may have. In addition, you should not be taking part in any clinical trials of medicines known to affect the immune system at the time. Women in the 16-30 age group who are pregnant or breast feeding cannot take part in the study.

A doctor from the research team would take a short medical history and examine your heart and lungs.

You would also have:

- Simple measurement of heart rate, blood pressure, temperature and oxygen level in the blood (the latter involves placing a probe on your finger and does not involve needles)
- a blood sample taken to check your 'full blood count' (which tells us about your white blood cells) and blood biochemistry
- spirometry (a test of lung function which involves taking a deep breath and breathing out as hard and fast as you can into a mouthpiece)

If you are thought to have a respiratory illness (such as a chest infection) or another current medical problem on the screening day, you will not be able to take part. However, if you wish, you will be allowed to return for one new screening visit when the problem has resolved.

The information obtained will be kept confidential by the research team. We shall inform you if we find any unexpected abnormalities and, with your permission inform your general practitioner. If we are satisfied that there are no reasons to prevent you taking part, and if you still wish to participate in the study, you will be asked to return for the actual study.

Study visit summary

On a day suitable for you, you will come to the Integrated Critical Care Unit (ICCU), Sunderland Royal Hospital. The study is performed on an intensive care unit because we perform similar and separate studies using LPS injected into the blood stream (which will not happen to you), which we have run in the ICCU. The unit therefore has good experience of LPS studies, and of monitoring volunteers. The collaboration has been excellent, and it seemed sensible to incorporate this expertise into the inhaled LPS studies. In addition, while LPS inhalation has an excellent safety record, performing these studies in an intensive care unit adds an even greater level of safety.

The study day lasts approximately 10 hours (around 8am-6pm). You should have breakfast as usual. You can eat and drink as you wish until about 10am, as we prefer the stomach to be empty at the time of the bronchoscopy, and this best achieved by having nothing to eat for 6 hours beforehand, though you can have sips of water. You can eat again around 3 hours after the bronchoscopy, ie around 7pm.

The bullet points give a brief summary of the day.

- Consent for your participation will be requested in writing.
- Brief medical history and examination of heart and lungs
- Female participants in the 16-30 group will provide a urine sample to allow a pregnancy test to be done
- Spirometry (simple breathing test)
- Practice inhalation of saline (salty water, 0.9% sodium chloride)

- Blood tests (blood will be taken from a 'cannula' placed in your vein, such that we can take blood more than once from a single use of a needle)
- Controlled "nebulised" inhalation of lipopolysaccharide (LPS) or saline (salty water) through a mouthpiece
- Observation throughout the day
- Bronchoscopy and a "wash" (or lavage) of one of the 23 "segments" of your lung

- Observation after bronchoscopy
- Home about 2 hours after bronchoscopy

If you are thought to have a respiratory illness (such as a chest infection) or some other current medical problem on the study visit day, you will not be able to take part, though you could come back for a new screening visit when the problem has resolved.

More detailed explanation and potential symptoms associated with each procedure are described in the next section.

Blood sampling

Blood samples taken on the screening visit and the day after the study day will be single blood samples taken using a needle. The amount of blood on the screening day will be approximately one standard tablespoonful. On the day after the study the amount of blood taken will be approximately 2 standard tablespoonfuls.

On the study day we take multiple blood samples, through a cannula. A cannula is a plastic tube inserted in a vein using a needle, but while the needle is immediately removed, the plastic tube stays in place. In this way, multiple samples can be taken over time, with the use of a single needle. We propose to take 5 samples through the cannula over the course of the day (ie at 1, 2, 4 and 8 hours after the initial sample). Each sample taken will be approximately 2 standard tablespoonfuls.

Therefore the maximum amount of blood that a volunteer is asked to provide over the course of this study is approximately 13 tablespoonfuls over 3 visits (ie 1 tablespoonful at screening, 5 x 2 tablespoonfuls on the study day, 2 tablespoonfuls the next day). To place this in context, a standard full blood donation to the Blood Transfusion Service is approximately two and a half times more than the amount you would provide during the study.

Inhalation of LPS or saline

A doctor in the research team will ask you a few medical questions and briefly examine your heart and lungs. You will also perform a breathing test (spirometry) which involves taking a maximal deep breath in, then breathing out as fast and hard as you can, into a tube. You will

then proceed to a practice inhalation through a mouthpiece (practicing the correct depth of inhalation), during which a small clip will be placed on your nose. You will not be informed whether you are to breathe the LPS or saline (this is called 'blinding' and is routinely used in clinical science).

You will then be asked to breathe in the LPS or saline through the nebuliser machine you have just practiced on. The actual test usually simply involves 5 slow, controlled breaths through a mouthpiece connected to a nebuliser. LPS is a product made by certain bacteria and is just one small component of the bacterium – i.e., if you are allocated to receive LPS, you would not be inhaling actual bacteria that may cause an infection but a small, non-infectious component of the bacterium's "cell wall". The dose of LPS we use is 60 micrograms. This dose has proved to be informative and very safe in our previous studies. We would be pleased to supply you with further literature from such studies if you wish. Your pulse, blood pressure, temperature and oxygen levels will be recorded regularly after the inhalation.

The LPS is used to cause a mild inflammation in the lung lasting a few hours only. Up to a third of volunteers may feel tired or have a warm/flushed/'flu-like' feeling a few hours afterwards, but not all of these volunteers actually have fever. Some volunteers get headache a few hours after the LPS. Rarely a transient sensation of tightness in the chest can develop but it is unusual for this to be associated with any change in lung function tests.

If you do have uncomfortable symptoms of fever, or a high temperature, or headache, you may be offered paracetamol. If you have chest tightness or breathlessness, a member of the team will listen to your chest and perform further spirometry, and if there is evidence of wheeziness we may offer you another nebuliser containing medicine (e.g. salbutamol) to open up the bronchial tubes.

Nebulised saline is unlikely to cause any symptoms.

After the nebulised LPS/saline you will rest for approximately 8 hours. During this period you will have blood samples (as described above) and intermittent recording of your pulse, blood pressure, temperature and oxygen levels.

Bronchoscopy and Bronchoalveolar Lavage (BAL)

Bronchoscopy is a routine medical diagnostic procedure, in which a thin, soft, flexible plastic tube is passed into the lung. The tube carries a light source, fibre-optic bundles, and a channel through which we can introduce fluid and/or suck fluid from the lung. This test allows us to visualise your lungs directly (images are projected to a television screen in the room, which you are welcome to watch), and to 'wash' a very small part of one of your lungs, allowing us to obtain cells for study in the lab. The procedure is carried out in your room in the integrated critical care unit.

Preparation for the procedure takes about 20 minutes. You will be given a low dose of oxygen to breathe through your nose, an oxygen detection probe will be placed on your finger and your heart trace will be recorded throughout (as is standard practice with all diagnostic bronchoscopies in the NHS). Some local anaesthetic spray will be sprayed into your mouth (to numb the back of the throat so that it is not sore during the procedure). You will be offered sedation, in the form of a medicine such as midazolam (given through the cannula in your arm) which has the combined functions of making you sleepy and relaxed. If the bronchoscope is passed through your mouth (as opposed to your nose) you will be asked to wear a small mouth guard (to prevent you biting the bronchoscope). When you are comfortable, the bronchoscopy and lavage will commence.

The procedure itself usually takes 10-20 minutes. The tube is passed through your mouth (or nose if you prefer) and to your vocal cords ('voice box'). We spray some local anaesthetic on the vocal cords (in order to numb them and limit coughing). About a minute later we pass the telescope through the vocal cords into the windpipe and lungs. This transiently induces coughing. Once this has settled, we perform the **bronchoalveolar lavage.** This involves introducing some sterile saline through the telescope into a single 'segment' of the lung (the 2 lungs typically have 23 segments, and so saline enters only a small proportion of the lung). We then gently suck the fluid back through the telescope. You may have the sensation of a salty taste when the lavage is being performed. The salty water we retrieve contains cells from deep inside your lungs that we can analyse. We then remove the telescope.

Bronchoscopy and BAL are considered extremely safe procedures, and even more so in healthy volunteers. The continuous oxygen monitoring and cardiac trace are part of the safety checks. In the extremely unlikely event that your oxygen levels cause concern, we remove the scope and administer extra oxygen until oxygen levels are satisfactory again. Although bronchoscopy is very safe, some elements may be transiently unpleasant.
a) the local anaesthetic spray tastes bitter and makes your eyes water for a few minutes. It then leaves the sensation that the mouth is 'swollen' (which it is not) and a sensation that it is difficult to swallow – you may have experienced this sensation if you have had dental work with local anaesthetic. The spray wares off after about 2-3 hours. If saliva pools in your mouth because of the spray, we suck it away with a suction catheter (again, you may have experience of this from having dental work).
b) in a very small minority of patients the natural 'gag reflex' is particularly strong and makes it hard for us to reach the voice box. It is impossible for us to predict whether you will be in this small minority until we do the test. If you are, and you find this particularly unpleasant, we simply abandon the procedure.
c) almost all people have a bout of coughing and sometimes a sensation of choking, immediately after the scope has entered the lung for the first time. In nearly all cases this is accompanied by normal oxygen levels throughout, and lasts less than 10 seconds. We talk to you throughout the procedure and during this period you may hear us confirming that your oxygen levels are satisfactory and counting slowly to 10 – in our previous studies this has proved an effective means of reassurance that this period is safe and transient.

d) All volunteers cough at c) above – the majority have only minor cough after that. A small minority have more frequent cough – again this is unpredictable.

Most patients who have bronchoscopy have a mild sore throat for a few hours after the anaesthetic spray has worn off. Some volunteers have mild feverish symptoms for a few hours after bronchoscopy, particularly those who inhale LPS earlier (this is because the BAL, like the LPS, can cause mild feverish symptoms of itself). Some people have a cough that disappears within 24 hours – occasionally tiny specks of blood can be seen in the sputum, but this is entirely normal after bronchoscopy. The majority of patients relax for the rest of the day and resume full normal activities the following day.

After bronchoscopy and bronchoalveolar lavage you will rest and be observed. Your pulse, blood pressure, temperature and oxygen levels will be monitored. Three hours after administration of the local anaesthetic spray you can eat (the delay is to allow the local anaesthetic spray to wear off – when it is active food or liquid can go down the wrong way). You will be seen by a doctor from the medical team and allowed home unless any unexpected findings arise.

If you have **not** been given sedative medicine, you may go home unaccompanied, where we recommend you rest for the remainder of the day. If you have accepted sedation (midazolam) you must be taken home by a friend or relative, as the sedation means that you must rest for the remainder of the day and NOT return to work, drive, cycle, operate moving machinery (e.g. DIY tools), or sign any legal documents. This is because the sedation has subtle effects on your coordination, judgement and memory for a few hours afterwards.

You will leave with a telephone number to contact in the unlikely event that you feel unwell.

Contact Details

Once you have read and considered the information here, we would be most grateful if you would contact us to let us know if you wish to take part or not, or if you want further information before making a decision. The best way to contact us is by phone or email, but the full contact details are

Professor John Simpson
Professor of Respiratory Medicine
Institute of Cellular Medicine
4th Floor, William Leech Building
Medical School
Newcastle University
Framlington Place
NE2 4HH

Tel: 0191 208 7770
Email: linda.ward@newcastle.ac.uk

THANK YOU FOR TAKING THE TIME TO READ THIS INFORMATION AND FOR CONSIDERING TAKING PART

Chief Investigator:
Professor John Simpson
Institute of Cellular Medicine
3rd Floor, William Leech Building
Medical School, Newcastle University
NE2 4HH, Tel: 0191 208 7770
Email: j.simpson@newcastle.ac.uk

The effect of ageing on acute inflammatory responses in humans (screen)

PARTICIPANT NUMBER | | | | | |

TO BE COMPLETED BY THE PARTICIPANT (please initial each box):

1. I confirm that I have read and understood the Participant Information Sheet dated 17/09/19 (version 1.4) for the above study. I have had the opportunity to consider the information, ask questions and have had those questions answered fully. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. I understand that information gathered for the purpose of the study will be kept in a secure confidential file. I agree that this file may be looked at by researchers involved in this study or by regulatory authorities overseeing the research. I understand that my personal data will be processed and stored in compliance with the Data Protection Act 2018. [ ]

4. I agree to a clinical examination of my heart and lungs. [ ]

5. I agree to have blood tests to assess my suitability for further participation in this research. [ ]

6. I agree that if any abnormalities are found that make me ineligible for the study, my GP can be informed. [ ]

_____	_____	_____
Participant's Name	Signature	Date
_____	_____	_____
Researcher's Name	Signature	Date

One copy to be kept by participant, one copy for the research file, one copy for the medical records.

Version 1.3, 17th September 2019

IRAS ref: 241028

Chief Investigator:
Professor John Simpson
Institute of Cellular Medicine
3rd Floor, William Leech Building
Medical School, Newcastle University
NE2 4HH, Tel: 0191 208 7770
Email: j.simpson@newcastle.ac.uk

The effect of ageing on acute inflammatory responses in humans (LPS/saline)

PARTICIPANT NUMBER | | | | | |

TO BE COMPLETED BY THE PARTICIPANT (please initial each box):

1. I confirm that I have read and understood the Participant Information Sheet dated 17/09/19 (version 1.4) for the above study. I have had the opportunity to consider the information, ask questions and have had those questions answered fully. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. I understand that information gathered for the purpose of the study will be kept in a secure confidential file. I agree that this file may be looked at by researchers involved in this study or by regulatory authorities overseeing the research. I understand that my personal data will be processed and stored in compliance with the Data Protection Act 2018. [ ]

4. (For female participants aged 16-30) I agree to provide a urine sample for a pregnancy test [ ]

5. I agree to having blood samples taken. [ ]

6. I agree to have an inhalation of lipopolysaccharide or saline. [ ]

7. I agree to have a bronchoscopy and bronchoalveolar lavage. [ ]

8. I agree that if I choose to have a sedation for the bronchoscopy test, I shall arrange to be taken home by a friend/relative, and will not drive. [ ]

9. I agree that if any abnormalities are found that make me ineligible for the study, my GP can be informed. [ ]

10. I agree that my samples may be used in future studies, on condition that I cannot be identified from my samples and that suitable ethical permissions are in place. [ ]

_____	_____	_____
Participant's Name	Signature	Date
_____	_____	_____
Researcher's Name	Signature	Date

One copy to be kept by participant, one copy for the research file, one copy for the medical records.

Version 1.3, 17th September 2019

IRAS ref: 241028

Chief Investigator:
Professor John Simpson
Professor of Respiratory Medicine
Newcastle University

Tel: 0191 208 7770
Email: j.simpson@ncl.ac.uk

Are you aged 65 or older?

Opportunities to be involved in medical research

Our research group at Newcastle University is interested in the immune system and how the body defends itself from infections like flu and pneumonia, especially since these defences are thought to become less efficient as we get older. In order to understand this better (ultimately with a view to designing new treatments to keep the immune system in better shape as we age) **we are seeking volunteers aged 65 or above** to help with our research studies.

If you are **not taking steroid tablets or aspirin** and you **do not smoke**, you might be exactly the sort of person who could help with our research. If you are interested to hear more about what this would involve, please feel free to **get in touch** on the contact details below:

Dr Wezi Sendama
07930 760 335
wezi.sendama@newcastle.ac.uk

Many thanks. We look forward to hearing from you.

Chief Investigator:
Professor John Simpson
Professor of Respiratory Medicine
Newcastle University

Tel: 0191 208 7770
Email: j.simpson@ncl.ac.uk

Are you aged 18 to 30?

Opportunities to be involved in medical research

Our research group at Newcastle University is interested in the immune system and how the body defends itself from infections like flu and pneumonia, especially since these defences are thought to become less efficient as we get older. In order to understand this better (ultimately with a view to designing new treatments to keep the immune system in better shape as we age) **we are seeking young volunteers aged 18 to 30** to help with our research studies.

If you consider yourself **healthy** and you **do not smoke**, you might be exactly the sort of person who could help with our research. If you are interested to hear more about what this would involve, please feel free to **get in touch** on the contact details below:

Dr Wezi Sendama
07930 760 335
wezi.sendama@newcastle.ac.uk

Many thanks. We look forward to hearing from you.

Date of screening

Name **MRN**

Sex **Participant no.**

DOB

PMH (inc. clinical trial participation)

Asthma ☐	COPD ☐	Bronchiectasis ☐
Pulm fibrosis ☐	Cardiac failure ☐	IHD ☐
Diabetes ☐	Rheum arth ☐	HIV ☐
Pregnancy ☐	Breastfeeding ☐	

DH

Steroids ☐	Methotrexate ☐	Sulfasalazine ☐
Aspirin ☐		

Smoking status Never ☐ Current ☐ Ex ☐ _____

Examination (inc. obs)

Spirometry

The effect of ageing on acute inflammatory responses in humans

Study day protocol

Introduction

This serves as a guide protocol for obtaining samples (time course blood samples and single BAL sample) from healthy human volunteers in the above study undergoing inhalation of LPS or saline. It assumes eligibility for the main study day after a screening assessment as described in the main protocol.

***

1. Participant arrives for 08:00.

2. Participant confirms no major changes to health status, medication or smoking status since screening visit, including confirming no recent respiratory illness.

3. Participant signs consent form.

4. If female and of reproductive age, ask the participant to complete a urine pregnancy test. A positive test excludes the participant from the rest of the study.

5. Take observations at baseline and document. Observations are blood pressure, heart rate, respiratory rate, oxygen saturations and temperature, as well as open question asking of any constitutional symptoms.

6. Take spirometry at baseline.

7. Insert intravenous cannula, preferably large bore in antecubital fossa to facilitate repeated sampling. Connect 3-way tap to cannula. Take baseline bloods. (Except where noted, bloods are 1x EDTA 4ml, 1x citrate 3.5ml, 2x 5ml SST.) Subsequent blood sampling from cannula should be after flushing with 2ml saline and discarding the first 3ml aspirated. All bloods to be refrigerated until transport to Newcastle.

8. Fill nebuliser chamber with saline for practice inhalations. Device will provide feedback, but in general ensure exhalation prior to slow inhalation with breath hold at maximum inspiration.

9. Empty nebuliser chamber and fill with saline/LPS solution. Participant inhales as practiced. Clock started; t=0 hrs, aim for 08:30.

10. Observations (t=0.5).

11. Observations and bloods (t=1).

12. Observations (t=1.5).

13. Observations and bloods (t=2), real time circa 10:30. **Participant to be nil by mouth apart from sips of water from this point.**

14. Observations (t=2.5, t=3, t=3.5).

15. Observations and bloods (t=4).

16. Observations (t=5, t=6, t=7).

17. Observations and bloods (t=8). Real time circa 16:30. Bloods will also include 4x 9ml EDTA tubes.

18. Repeat spirometry and document. If FEV1 has fallen by ≥15%, then participant is ineligible for bronchoscopy.

19. Bronchoscopy and BAL with warmed saline as per protocol.

20. Samples transported to Newcastle for analysis; nebuliser transported for overnight decontamination.

21. Obs (t=9, t=9.5, t=10). Participant allowed home with phone number if well; accompanied if received sedation. Participant can eat and drink 3 hours after bronchoscopy (circa 19:30).

22. Return next morning for bloods (t=24).

23. Complete form for participant reimbursement.

Supplemental recording form

Date of study

MRN

Participant no.

Initial spirometry

Pre-bronchoscopy spirometry (t=8)

FEV1 change ≥15%?

***

Symptom recording

Any other symptoms? Record times

The effect of ageing on acute inflammatory responses in humans

Bronchoscopy protocol

Introduction

This serves as a protocol for obtaining bronchoalveolar lavage samples from healthy human volunteers in the above study undergoing inhalation of LPS or saline. It assumes eligibility for bronchoscopy after repeat spirometry as set out in the study day protocol. The participant will have had intravenous access established and will have been nil by mouth except for sips of water for the six hours prior to the procedure.

Potential complications are to be explained to the participants: sore throat/hoarseness (~1 in 4), fever (~1 in 100), infection (<1 in 1000), epistaxis (<1 in 1000), endobronchial bleed (<1 in 5000).[1]

Cardiorespiratory depression is rare with midazolam sedation for research bronchoscopy, but flumazenil must be available during and after the procedure.[1] If participant opts for sedation, the participant must be accompanied home (by friend or relative, for example) at the end of the study day and should be advised not to return to work, cycle, drive, operate moving machinery or sign legal documents for the rest of the day.

10% lidocaine (Xylocaine) spray should be available for oropharyngeal local anaesthesia, and Instillagel should be available for local anaesthesia to the nasal passages. Spray-as-you-go local anaesthesia is to be provided by 2ml aliquots of 1% lidocaine prepared in 5ml syringes (syringes should contain 3ml of air to facilitate injection down the working channel of the scope). There is no additional increase in participant satisfaction with the procedure if 2% lidocaine is used instead of 1%, despite the likely additional dosage.[2]

Lavage should be undertaken with warmed saline. One 20ml syringe (for the bronchial sample to discard) and three 50ml syringes of warmed saline (for the bronchoalveolar samples) should be prepared.

***

1. Participant is connected to continuous pulse oximetry and 3-lead ECG monitoring. Participant's clothes are protected by the use of hospital gown and/or absorbent sheets.

2. Local anaesthesia to oropharynx is given by two to three sprays of 10% lidocaine (Xylocaine) at the back of the mouth.

3. Participant anaesthetises nasal passages by sniffing Instillagel. The participant should be warned that this can cause a stinging sensation.

4. Supplemental oxygen is applied by nasal cannulae and titrated through the procedure to keep saturations >90%.

5. If midazolam is desired, it is administered in doses at the discretion of the physician to achieve light sedation.

6. The end of the scope is lubricated with Instillagel and passed through the left or right nostril. If nasal intubation is difficult or unduly uncomfortable for the participant, then a mouth guard is inserted and the scope is passed through the mouth.

7. The bronchoscope is advanced and the vocal cords are identified. The participant is warned that spraying the vocal cords with lidocaine will cause a short-lived cough. Local anaesthesia is administered to the vocal cords with two to three aliquots of 1% lidocaine pushed down the working channel of the scope.

8. The participant is asked to take a few slow, deep breaths. The cords part further on inhalation, making it easier to advance the scope between them into the trachea. Lidocaine is used for topical anaesthesia to the carina, the left and right main bronchus and the right middle lobe bronchial orifice.

9. The end of the scope is positioned within the medial segment of the right middle lobe.

10. The 20ml syringe of warmed saline is instilled and aspirated (with gentle hand suction if possible). This sample, the bronchial sample, is discarded.

11. The three 50ml aliquots of saline are sequentially instilled and aspirated. The retrieved samples are collected in sterile containers and placed on ice.

12. The bronchoscope is withdrawn and the procedure is complete.

References

1. Collins AM, Rylance J, Wootton DG, et al. Bronchoalveolar Lavage (BAL) for Research; Obtaining Adequate Sample Yield. J Vis Exp. 2014;(85). doi:10.3791/4345

2. Madan K, Biswal SK, Mittal S, et al. 1% Versus 2% Lignocaine for Airway Anesthesia in Flexible Bronchoscopy Without Lignocaine Nebulization (LIFE). J Bronchol Interv Pulmonol. 2018;25(2):103-110. doi:10.1097/LBR.0000000000000458

Appendix B. Differential gene expression: HP vs control lung tissue

Log-ratio	p-value	minFDR	Gene Symbol
2.386624336	2.49068E-13	1.80007E-10	UBD
2.145256996	0.000105608	0.001108837	ITLN1
2.130993843	3.63836E-08	1.79302E-06	CXCL9
2.001555443	8.83961E-08	3.66874E-06	IGKV6-21,IGKV6D-21
1.963585854	5.14099E-07	1.49737E-05	MMP7
1.914785385	3.47027E-08	1.75784E-06	IGKV6D-41
1.883901596	4.67239E-15	6.45561E-12	SLAMF7
1.87444973	6.40973E-07	1.78039E-05	IGKV4-1
1.873261452	2.82005E-07	9.18712E-06	IGKV1D-8
1.864600182	3.75316E-07	1.14495E-05	IGKV3OR2-268,IGKV3-7,IGKV3D-7
1.862315178	1.64537E-07	5.97264E-06	IGLV3-19
1.854318619	5.81015E-07	1.63958E-05	IGKV3D-15,IGKV3-15
1.826432228	3.52434E-07	1.09147E-05	IGKV1-16
1.815361977	4.59848E-08	2.17194E-06	IGKV1D-16
1.815113068	5.79936E-11	1.06695E-08	IGLV5-45
1.797786713	2.48684E-07	8.23315E-06	IGKV2D-28,IGKV2-28
1.7948246	1.34968E-06	3.22158E-05	IGHA2
1.776727676	1.27345E-07	4.92199E-06	IGKV1D-33,IGKV1OR-3,IGKV1OR9-1,IGKV1D-39,IGKV1-17,IGKV1OR1-1,IGKV1D-27,IGKV1-33,IGKV1OR2-1,IGKV1OR-2,IGKV1-39,IGKV1OR9-2,IGKV1-27,IGKV1OR2-118,RP11-295P22.2,AC134879.2,IGKV1OR10-1,IGKV1OR22-5,IGKV1OR2-2
1.773388863	2.33308E-07	7.76613E-06	IGKV1D-43
1.765972137	4.54858E-06	8.62678E-05	PLA2G2A
1.760728836	1.07953E-06	2.72006E-05	IGKV1OR2-3,IGKV1OR2-9,IGKV1OR2-11
1.757297516	9.27642E-07	2.39154E-05	IGHV3-49,IGHV3-65
1.75333786	6.68486E-07	1.83973E-05	CXCL10
1.753194809	3.13117E-06	6.38394E-05	IGHM
1.750444412	6.99901E-07	1.91114E-05	IGLV2-14,AC244157.1,IGLV2-11,MIR650,IGLV2-8,AC244250.3
1.745113373	1.08307E-06	2.72616E-05	COL17A1
1.741480827	1.85111E-06	4.17413E-05	CXCL14
1.738563538	1.21196E-06	2.95781E-05	IGLV2-23,AC244250.1
1.736124992	0.00182051	0.010774214	IGHD
1.732629776	2.15577E-06	4.68828E-05	IGLV8OR8-1
1.729577065	5.13249E-07	1.49668E-05	IGKV1OR2-108
1.720791817	3.12411E-08	1.60576E-06	IGKV2D-24,IGKV2-24
1.720739841	5.59689E-10	6.64726E-08	ADAMDEC1
1.717638969	6.13745E-07	1.72466E-05	AC136616.2,IGHV3OR16-7,IGHV3-15
1.710428238	8.79199E-08	3.66448E-06	MZB1
1.70971489	9.04909E-08	3.72447E-06	ZNF683

Log-ratio	p-value	minFDR	Gene Symbol
1.706726074	1.50376E-06	3.52068E-05	AC141272.1
1.697430611	5.87304E-08	2.62687E-06	TNFRSF17
1.696770668	6.18249E-07	1.73265E-05	IGHV3-35
1.689846039	5.66783E-07	1.60126E-05	IGHV3-48
1.687458992	1.88467E-06	4.24197E-05	IGLV4-69
1.686927795	2.19743E-06	4.75353E-05	IGLV1-47
1.686056137	1.51629E-06	3.54661E-05	IGHV3OR16-10,IGHV3OR16-11, RP11-1166P10.8,AC142381.1, RP11-812E19.7,RP11-989E6.8
1.675474167	2.61753E-06	5.51598E-05	IGLV1-44
1.672257423	1.91529E-06	4.29117E-05	IGHV3-30,IGHV3-38
1.668294907	1.99378E-11	5.36043E-09	TIFAB
1.668128014	5.88334E-10	6.85438E-08	IL4I1
1.667053223	4.47347E-06	8.51072E-05	IGKV3D-11,IGKV3-11
1.660517693	1.6549E-07	5.98203E-06	IGHV3OR16-6
1.65470314	3.09899E-06	6.33416E-05	IGHV3-33,IGHV3-23
1.651481628	2.42175E-12	1.11794E-09	CD38
1.638401031	6.02213E-10	6.91725E-08	C15orf48,MIR147B
1.633870125	7.70827E-05	0.000870284	IGLV8-61
1.633663177	7.55765E-07	2.03353E-05	IGLV4-60
1.631948471	1.02904E-06	2.61438E-05	IGHV3-20
1.623677254	4.11844E-07	1.24244E-05	IGHV3-73
1.618900299	4.25568E-12	1.62687E-09	CTD-2227E11.1
1.611823082	8.80515E-07	2.3065E-05	AC233755.2
1.607635498	3.90162E-07	1.18286E-05	IGKV2-30,IGKV2D-30
1.602032661	2.33358E-06	5.01259E-05	IGHV3-74
1.601364136	0.000387351	0.003152036	SAA2
1.591021538	5.84633E-06	0.000106426	IGHV3-66,IGHV3-53
1.588472366	1.72327E-06	3.93297E-05	CCL18
1.585333824	6.70536E-09	4.9563E-07	IL13RA2
1.584984779	8.46948E-07	2.23532E-05	IGLV3-27
1.581846237	3.11942E-08	1.60576E-06	FAM26F,CALHM6
1.576257706	1.82742E-06	4.13213E-05	BMS1P20,IGLV1-51
1.572759628	4.89555E-07	1.4396E-05	IGHV3OR16-12
1.55412674	3.64232E-08	1.79302E-06	IGLV10-54
1.535243034	1.16847E-07	4.58871E-06	CTSE
1.534632683	2.10019E-06	4.59533E-05	IGHV3-16
1.515657425	2.10392E-07	7.15918E-06	IFNG
1.510660172	3.61149E-05	0.000465855	IGLV3-9
1.506080627	1.47157E-06	3.45523E-05	COMP
1.505567551	8.83096E-07	2.30832E-05	IGHV5-51
1.49579525	1.03556E-14	1.33348E-11	WNT10A
1.493055344	5.63926E-06	0.000102963	CCL19
1.491583824	1.02841E-06	2.61438E-05	IGLV1-36
1.487541199	5.93584E-05	0.000703698	CXCL13
1.48743248	6.81904E-07	1.87034E-05	IGHV3-13
1.483522415	4.07766E-06	7.9115E-05	IGLL3P

Log-ratio	p-value	minFDR	Gene Symbol
1.480471611	3.39091E-06	6.80574E-05	IGHV3-72
1.478095055	9.03038E-07	2.33796E-05	TUBB3,TUBB3P1,RP11-566K11.2, AC092143.1
1.469963074	2.08745E-07	7.13289E-06	IGFL2
1.453894615	3.80422E-07	1.15855E-05	IGKV2-29,IGKV2D-29
1.443861008	1.21438E-05	0.000193054	IGLC6
1.443675995	7.98734E-05	0.000897237	IGHG3
1.440795898	1.17654E-05	0.000187894	IGLL5,IGLC1
1.437615395	1.78716E-05	0.00026532	POU2AF1
1.437085152	6.27868E-07	1.74959E-05	IGHV1-18
1.433283806	3.86371E-08	1.88306E-06	AC103563.2,AC103563.3
1.429314613	6.97637E-09	5.12564E-07	DIO2
1.429097176	0.000343387	0.002862459	IGLV3-10
1.428792	3.52079E-08	1.76878E-06	LILRB4
1.428174973	0.00016968	0.001620571	IGLV3-21
1.419616699	9.86168E-05	0.001054528	IGHG2,IGHG4
1.411931038	1.44629E-05	0.000223248	IGLV3-25
1.411661148	3.23772E-06	6.56479E-05	IGLV6-57
1.397935867	0.001796904	0.010657711	MMP1
1.390636444	2.7199E-07	8.89642E-06	IGKV2D-26
1.386870384	7.53665E-05	0.000855209	IGLV3-16
1.377664566	1.70428E-08	1.00233E-06	MMP11
1.372357368	5.54906E-11	1.05243E-08	DCANP1
1.371949196	1.71339E-05	0.000256705	CXCL11
1.367277145	1.60949E-08	9.67514E-07	LDHAL6FP
1.36249733	3.27907E-07	1.0325E-05	FNDC1
1.356258392	9.46434E-06	0.000157306	IGHV3-47
1.353852272	2.32327E-09	2.08209E-07	MMP13
1.351356506	8.84721E-08	3.66874E-06	IGHV1-3
1.347158432	1.23288E-05	0.000195825	FCRL5
1.345468521	1.163E-09	1.19555E-07	CD27
1.342494011	1.38707E-10	2.13435E-08	MYO7A
1.341730118	1.0479E-05	0.000171033	JCHAIN
1.333284378	0.00794759	0.034679106	MIR205HG,MIR205
1.332457542	0.000691464	0.00499479	LY6D
1.330386162	1.39286E-05	0.000216367	IGLV7-43
1.329710007	2.03419E-05	0.000291897	IGKV1-8,IGKC
1.329461098	5.39853E-07	1.54842E-05	CH17-212P11.5,IGHV1-69-2
1.320496559	1.39862E-11	4.37317E-09	CXCR3
1.319190979	2.42636E-14	2.69833E-11	CFB
1.315246582	2.11406E-05	0.000299841	IGLC3,IGLC2
1.310341835	0.000175598	0.00166197	IGLV9-49
1.308353424	3.34054E-06	6.7336E-05	IGHV5-78
1.308233261	8.3873E-08	3.51979E-06	GBP5
1.303116798	5.46888E-08	2.49136E-06	SCG5
1.288785934	6.40575E-09	4.77814E-07	RAPH1
1.286301613	7.30596E-08	3.14144E-06	RP5-1028K7.2

Log-ratio	p-value	minFDR	Gene Symbol
1.282599449	1.20371E-07	4.68947E-06	JSRP1
1.281852722	2.97642E-07	9.60701E-06	IGHV1-46
1.278351784	4.61358E-10	5.61572E-08	THBS4
1.278324127	0.000420514	0.003364251	IGHG1
1.270845413	1.08531E-08	7.10144E-07	CYP27C1
1.26338768	6.24477E-08	2.76783E-06	TDO2
1.260286331	0.002697677	0.014729159	FDCSP
1.255689621	2.1159E-07	7.18994E-06	CH17-262H11.1,IGHV7-4-1
1.252802849	4.98118E-06	9.28177E-05	IGHV4-61,IGHV4-59
1.252530098	4.81444E-08	2.25651E-06	LINC00892
1.244015694	2.52463E-07	8.33572E-06	FCRL1
1.241973877	2.59773E-09	2.24711E-07	AIM2
1.241422653	3.54244E-11	7.80804E-09	APOBEC3H
1.231715202	1.34766E-13	1.13696E-10	P2RY6
1.230693817	3.30109E-08	1.68259E-06	GJB2
1.226237297	2.5942E-09	2.24711E-07	STAT1
1.225099087	4.20153E-07	1.26439E-05	IGHV6-1
1.218378067	7.21992E-09	5.22682E-07	SMR3A
1.203597069	3.83423E-08	1.87242E-06	ADAMTS14
1.200766563	8.067E-12	2.74121E-09	LYPD1
1.199841499	0.003525181	0.018284307	SAA1
1.192457199	0.000148236	0.001460627	IGHA1
1.19179821	1.79396E-09	1.70782E-07	KCNJ10
1.1823349	1.9897E-06	4.41341E-05	SLC2A5
1.174170494	6.26315E-06	0.000112424	AMPD1
1.163369179	1.36372E-06	3.2393E-05	PNOC
1.15678978	1.91425E-11	5.25596E-09	IGLV5-37
1.154931068	8.95363E-10	9.56592E-08	CCR5
1.154831886	6.24175E-11	1.12287E-08	PCP4
1.154764175	0.002058466	0.011886099	SLN
1.151883125	1.28273E-07	4.93447E-06	JAKMIP1
1.151276588	0.000166728	0.001598527	STRA6
1.133211136	3.66227E-06	7.25515E-05	CDCA7
1.132922173	1.87305E-05	0.000273642	LGALS2
1.127840042	8.53016E-09	5.92895E-07	TRAC
1.126320839	1.3446E-08	8.39206E-07	HAPLN3
1.121700287	1.22764E-08	7.84216E-07	FASLG
1.121440887	5.54399E-07	1.58088E-05	SLAMF8
1.119677544	0.003876984	0.019752479	CR2
1.11592865	0.001105739	0.007254762	IGLV3-12
1.115208626	8.76878E-06	0.000147753	COL3A1
1.109284401	5.19902E-05	0.000630833	COL1A1
1.108561516	5.37505E-05	0.000648451	HS6ST2
1.092074871	7.21312E-10	7.91374E-08	CLNK
1.090680122	1.01446E-08	6.76287E-07	SIT1
1.084327698	2.3202E-08	1.26993E-06	TNRC18
1.076720238	3.0816E-06	6.30388E-05	CXCR6

Log-ratio	p-value	minFDR	Gene Symbol
1.073104858	1.42398E-05	0.000220501	KIAA0125
1.067865372	0.000420635	0.003364251	CILP
1.066847801	1.82811E-08	1.04992E-06	TRAJ3,TRAJ4
1.064699173	2.72721E-08	1.43185E-06	LTB
1.064292908	8.741E-09	6.04117E-07	CD2
1.062119484	3.1613E-09	2.67628E-07	CD3D
1.059700966	2.00707E-06	4.43986E-05	COL10A1
1.058015823	3.71218E-07	1.13386E-05	CPNE7
1.057617188	4.04255E-05	0.000510195	GABBR2
1.053620338	0.001138698	0.007429171	PLA2G7
1.04907608	1.54352E-10	2.31679E-08	TNFRSF18
1.048683167	2.9395E-06	6.06901E-05	CCL11
1.048573494	1.58297E-07	5.82389E-06	CEP55
1.0483284	0.001244239	0.00798153	CRLF1
1.039450645	3.28036E-11	7.50069E-09	PTPN7
1.036634445	1.02361E-05	0.000167741	CYP2C9
1.036559105	2.73605E-12	1.16345E-09	ZBP1
1.029515266	6.83649E-08	2.97004E-06	MYBPC2
1.02841568	7.88508E-09	5.59178E-07	LINC00426
1.026333809	3.15525E-06	6.42233E-05	STEAP1
1.024680138	1.09598E-08	7.15044E-07	LAG3
1.022571564	4.70787E-05	0.000580323	MIA
1.018949509	4.57774E-09	3.63633E-07	GPR84
1.01851368	6.17945E-08	2.74385E-06	KCNN4
1.017323494	0.00061646	0.004567631	CP
1.014738083	7.4307E-10	8.11605E-08	RP11-16E12.2
1.010606766	1.13311E-06	2.82193E-05	LILRB5
1.009610176	1.5132E-11	4.51487E-09	ITGB7
0.99626112	1.63183E-07	5.94033E-06	IGHV3-64
0.994552612	2.89848E-08	1.50242E-06	CD3G
0.994464397	0.005365049	0.025487631	KRT6A
0.989866257	3.05946E-10	3.93962E-08	PLD1
0.989245415	0.004418158	0.021917002	TIMD4
0.988260269	2.06695E-09	1.90113E-07	SLAMF1
0.988246918	4.51423E-08	2.13627E-06	TRBC2
0.986256599	1.90943E-05	0.000277907	SERPINI2
0.985913277	0.001676087	0.010077941	XAGE2
0.984182358	2.16815E-08	1.20014E-06	ASCL2
0.983774185	0.000659232	0.004801662	AADAC
0.981618881	1.1135E-06	2.78879E-05	IRF4
0.979853153	2.5139E-05	0.000346898	CCL7
0.978682518	0.000756472	0.005366148	CTD-2114J12.1,AC104966.1
0.977856636	3.91629E-06	7.65915E-05	ASPM
0.975253105	8.55769E-11	1.43406E-08	TNFSF13B
0.974134445	3.53538E-07	1.09351E-05	DCLK1
0.973732948	6.28805E-08	2.77696E-06	UBASH3A
0.970277786	1.59367E-08	9.60361E-07	TMEM238

Log-ratio	p-value	minFDR	Gene Symbol
0.970092773	1.21718E-07	4.71943E-06	HLA-DOB,XXbac-BPG246D15.9, AL669918.1
0.968438148	6.57385E-07	1.8153E-05	CTC-231O11.1
0.968111992	8.75751E-06	0.000147753	AHSA2,AHSA2P
0.967978477	7.95839E-08	3.36287E-06	HOXC4
0.966270447	0.000138927	0.001384521	THY1
0.965445518	0.00045279	0.003578156	HAMP
0.958375931	1.15778E-08	7.43197E-07	JAK3
0.957873344	8.52209E-10	9.1448E-08	SALL4
0.956338882	4.24359E-10	5.19118E-08	FKBP11
0.950383186	0.000306042	0.002607111	HLA-DQA2
0.947761536	2.10732E-09	1.931E-07	LAX1
0.947245598	8.94639E-05	0.000976718	CTHRC1
0.945146561	7.77836E-09	5.53213E-07	NLRP7
0.942230225	1.10549E-05	0.000178528	CD40LG
0.941695213	4.31223E-06	8.24568E-05	HJURP
0.941669464	6.3627E-10	7.24045E-08	TRAF3IP3
0.940348625	2.99042E-06	6.15775E-05	RP11-81H14.2
0.939535141	7.47688E-08	3.20367E-06	TRBC1,TRBV25-1
0.937610626	5.61267E-07	1.59064E-05	GPR18
0.936670303	0.000826705	0.005757517	LGSN
0.931550026	1.99888E-07	6.91007E-06	KIAA0101,PCLAF
0.930994034	0.000967375	0.006514667	CHI3L1
0.928900719	4.4704E-06	8.51072E-05	SPRR1A
0.928661346	1.98131E-06	4.3988E-05	NCF1B,NCF1
0.928588867	1.01823E-06	2.59501E-05	BTLA
0.928076744	0.001718681	0.010296097	COL11A2
0.925887108	3.83132E-06	7.54119E-05	CD70
0.923797607	1.14701E-06	2.84612E-05	CD6
0.923540115	1.2849E-05	0.000202164	UNC5B-AS1
0.923359871	1.24196E-09	1.26082E-07	ARNTL2
0.921834946	0.000117005	0.001206845	EBF3
0.91908741	3.83482E-05	0.000488659	MS4A14
0.916594505	6.63238E-08	2.89764E-06	CD96
0.913188934	3.35351E-06	6.75283E-05	NCF1C
0.913176537	5.19776E-07	1.50673E-05	CENPF
0.911413193	1.4816E-10	2.23758E-08	C9orf139
0.909650326	5.29939E-09	4.05171E-07	GRIN3A
0.909537315	1.87052E-07	6.55645E-06	MIAT
0.909381866	1.36401E-12	7.25476E-10	IL26
0.908965111	0.000158797	0.001537442	CKMT1A,CKMT1B
0.907840729	2.28936E-08	1.25868E-06	NDC80
0.906508446	0.000207491	0.001894915	FER1L4
0.902198792	1.41398E-07	5.30588E-06	PSAT1
0.9018507	2.26573E-05	0.000317305	RAB42P1,RAB42
0.901709557	0.00485532	0.023565332	ACBD4
0.901115417	3.0936E-07	9.89386E-06	LCK

Log-ratio	p-value	minFDR	Gene Symbol
0.899766922	5.66721E-05	0.000677684	CD24
0.899224281	0.000653429	0.004773603	RP11-296O14.3
0.898513794	0.000318309	0.00268914	KCNK2
0.898463249	2.81902E-06	5.8748E-05	CD5
0.898292542	4.88645E-06	9.16106E-05	HMMR
0.89706707	2.85668E-05	0.000385502	IGLV7-46
0.895763397	0.000291094	0.002505064	ELOVL2
0.895104408	0.008542587	0.036615792	KRT17P3
0.893759727	3.37464E-06	6.78421E-05	EPHA10
0.889847755	0.01133593	0.045608431	SFRP2
0.888775826	5.71214E-09	4.31337E-07	TRAV13-1
0.888767242	1.11605E-09	1.157E-07	ACAP1
0.88795948	0.003869633	0.019727544	MMP9
0.886396408	9.04382E-08	3.72447E-06	FYB,FYB1
0.885753632	1.88074E-09	1.76299E-07	MEI1
0.885277748	0.01132588	0.045575492	KLK7
0.880224228	3.91823E-08	1.89454E-06	IGKV1D-42
0.876204967	1.10429E-08	7.18551E-07	MMP3
0.875700951	1.02247E-08	6.79775E-07	EMB
0.875128746	2.14159E-09	1.94781E-07	DPF3
0.875052452	1.95574E-06	4.36698E-05	CCL5
0.87387085	1.32134E-06	3.1694E-05	CD79A
0.873293877	0.011984786	0.047616073	KRT17P6,KRT17P1,KRT17P2
0.87127018	0.000189063	0.0017608	SFRP4
0.870821953	2.06652E-07	7.09109E-06	CFHR1
0.870610237	0.000149336	0.001468515	KIAA1324
0.869454384	2.62153E-05	0.000359118	CD8B
0.868416786	0.004620501	0.022698765	S100A2
0.867395401	0.000211555	0.001924848	GPR87
0.858785629	1.35532E-06	3.2256E-05	RAB26
0.855266094	3.27322E-09	2.74255E-07	C15orf53
0.854640961	6.20419E-06	0.000111612	PAMR1
0.854551315	2.46786E-07	8.1814E-06	EMBP1
0.85340023	8.86194E-07	2.31147E-05	FPR3
0.851187706	2.97045E-09	2.53223E-07	RP5-1031D4.3
0.843337059	1.11826E-08	7.24894E-07	CTD-2371O3.3
0.842469215	0.009013676	0.038163949	HIF3A
0.840552807	1.53118E-05	0.000234724	CLLU1OS
0.839253426	0.000117922	0.00121426	SPIB
0.83898735	4.21324E-07	1.26635E-05	RMI2
0.838544846	5.50931E-06	0.000101043	CPNE5
0.834740162	0.000150795	0.001478108	ABCA12
0.829903603	5.47842E-08	2.49136E-06	MS4A6A
0.829597473	2.11892E-06	4.62871E-05	IGHV3OR16-13
0.827589035	2.32813E-07	7.76167E-06	SEPT1,RP11-297C4.6
0.827422142	1.82772E-10	2.59982E-08	RARRES3,PLAAT4
0.825895309	3.51673E-09	2.91406E-07	BLNK

Log-ratio	p-value	minFDR	Gene Symbol
0.825734138	5.74411E-05	0.00068487	ASPN
0.82516098	0.000175007	0.00165823	CLIC6
0.824202538	7.02951E-10	7.78208E-08	CTD-2165H16.4
0.823150635	2.63454E-06	5.54227E-05	SERPINF1
0.822629929	2.3446E-10	3.16922E-08	TRIM59
0.820655823	0.003604458	0.018600857	CD19
0.820266724	8.53919E-07	2.24645E-05	CDCA2
0.819229126	0.007606563	0.033561375	HTRA4
0.819056511	1.44221E-07	5.39526E-06	NYX
0.814310074	7.01786E-07	1.91202E-05	CDKN3
0.814256668	2.73363E-06	5.7261E-05	RP5-890E16.2
0.813895226	6.049E-06	0.000109557	CD8BP,CD8B2
0.813366413	1.2982E-07	4.97835E-06	BAAT
0.813339233	1.68975E-08	9.96177E-07	CCR2
0.811617851	3.00322E-07	9.6553E-06	RP11-65D24.2
0.810650826	0.011074438	0.0448811	PROP1
0.809502602	3.49728E-05	0.000454164	KLHDC7B
0.808246613	3.27387E-07	1.0325E-05	ZNF831
0.808002472	5.30412E-08	2.44389E-06	FAM225A
0.807201385	0.000166739	0.001598527	SULT1C2
0.805006027	1.11365E-06	2.78879E-05	NUSAP1
0.803733826	9.12916E-05	0.000991803	PCSK2
0.801962852	6.65909E-09	4.94109E-07	EAF2
0.801527977	4.81398E-10	5.80191E-08	CFI
0.799229622	1.08038E-05	0.000175166	CLEC10A
0.798501968	1.05239E-07	4.22789E-06	RP11-465B22.8
0.797966003	4.02915E-08	1.93668E-06	PTPRCAP
0.796420097	2.40879E-05	0.000333712	CCNB2
0.795746803	2.22892E-09	2.01228E-07	SUSD3
0.79524231	9.71824E-06	0.000160544	DLGAP5
0.795000076	3.68613E-10	4.60127E-08	OSBPL3
0.794746399	0.0004646	0.003652604	IGF1
0.794543266	1.78226E-07	6.37497E-06	IL12RB2
0.793810844	3.54671E-07	1.09563E-05	TLR10
0.792879105	3.57823E-06	7.11749E-05	ETV7
0.791519165	2.06911E-08	1.15577E-06	CTC-527H23.3
0.79031086	1.03187E-06	2.61885E-05	FMO1
0.789875031	5.96521E-06	0.000108348	GZMA
0.78813839	2.01814E-08	1.13247E-06	EPSTI1
0.788077354	1.06021E-05	0.000172757	THEMIS
0.787360191	8.3902E-09	5.84828E-07	PDK1
0.787158012	6.12256E-05	0.000721055	COL15A1
0.785360336	2.21249E-09	2.00485E-07	RASSF4
0.78468895	3.02076E-05	0.000404299	UBE2C
0.782997131	1.04652E-08	6.90141E-07	PTTG1
0.781179428	7.05277E-05	0.000810109	S100B
0.780491829	3.24665E-06	6.57554E-05	SNAP25

Log-ratio	p-value	minFDR	Gene Symbol
0.779937744	2.3761E-10	3.1767E-08	ADAP2
0.776935101	0.00086151	0.00594911	SAA4,SAA2-SAA4
0.775306702	3.19898E-06	6.50051E-05	STYK1
0.774094582	3.42744E-05	0.000447948	SOWAHD
0.771485329	2.87185E-12	1.19089E-09	FAM69A,DIPK1A
0.770563126	0.000209482	0.001910246	MEOX1
0.769734383	1.40176E-07	5.28435E-06	TXNDC5,BLOC1S5-TXNDC5
0.769223213	0.002284268	0.012942772	C6orf222
0.767802238	5.43963E-05	0.000654629	TOP2A
0.76764679	7.05276E-09	5.16625E-07	SDSL
0.763701439	6.8829E-09	5.0722E-07	PSTPIP1
0.763298988	0.000171627	0.001633228	TNF
0.763262749	0.007957459	0.034703601	PFDN6
0.762832642	6.61874E-07	1.82564E-05	LPAR5
0.762607574	3.89958E-08	1.893E-06	ASPHD2
0.760788918	0.002213262	0.012619361	RUNDC3B
0.760203362	0.000380522	0.003107425	IGKV5-2
0.758338928	0.000110932	0.001155664	WFDC2
0.756892204	2.0952E-05	0.00029803	F2RL2
0.756813049	1.75694E-05	0.000262201	TYMS
0.756653786	4.10381E-07	1.23955E-05	NLRC3
0.755537987	0.001683278	0.010111238	CTA-299D3.8
0.755362511	0.003183331	0.016887114	CPXM1
0.754924774	2.88122E-07	9.33669E-06	SLC17A9
0.754620552	1.16507E-06	2.87513E-05	FGL2
0.753162384	6.56499E-08	2.87333E-06	APOBEC3G
0.752932549	1.20822E-07	4.69955E-06	TMEM59L,AC003112.1
0.750796318	3.42011E-07	1.06866E-05	FBN2
0.750597954	4.91076E-05	0.000602082	RP11-23P13.6
0.749209404	6.13985E-07	1.72466E-05	SAMD9L
0.748900414	0.000676083	0.004902501	CHRDL2
0.746935368	6.93162E-05	0.000799572	HS6ST3
0.746206284	6.92672E-07	1.89563E-05	ASIC4
0.745661736	4.35069E-07	1.29968E-05	RP11-37C7.3
0.743895531	3.24656E-07	1.02889E-05	MIR155,MIR155HG
0.743697643	1.39437E-05	0.000216464	IGHV1-58
0.743113518	3.21512E-07	1.02157E-05	TMEM176B
0.742601395	2.92433E-10	3.80568E-08	GNAO1
0.738735199	2.58871E-12	1.15155E-09	SYNGR3
0.737716675	2.5571E-08	1.36301E-06	IL2RG
0.73575592	0.000169227	0.001617941	P2RX5
0.735039711	0.000130771	0.001319898	TNFSF14
0.73387146	0.004229048	0.021176399	TNFAIP6
0.733804703	2.1207E-08	1.17921E-06	CBLN3
0.732575417	0.000143563	0.001423755	BATF
0.73251915	6.67334E-07	1.83863E-05	E2F2
0.732488632	1.63322E-07	5.94033E-06	MESP1

Log-ratio	p-value	minFDR	Gene Symbol
0.732268333	0.010906465	0.044373835	KCTD19
0.731332779	2.0991E-06	4.59533E-05	COL7A1
0.731028557	1.59688E-05	0.000241914	SPAG4
0.729763508	2.88365E-08	1.50109E-06	COL22A1
0.728610992	4.38145E-11	9.15289E-09	TGFBI
0.72809124	0.001321383	0.008338021	HES2
0.727939606	0.000502793	0.003886678	TMEM229A
0.727890015	0.000273214	0.002383053	PDCD1
0.727771759	0.007641732	0.033674824	NPB
0.726546288	0.00115075	0.007489827	SLC1A3
0.726103783	0.003213019	0.017002334	CXCR2P1
0.723018646	0.000133	0.00133688	RARRES1
0.722450256	8.72141E-09	6.04117E-07	RP11-298E9.7
0.721542358	0.000341977	0.002853176	CCL21
0.720428467	3.494E-12	1.38691E-09	KCNA2
0.718882561	3.46814E-05	0.000451338	RASGRP1
0.71691227	0.000568865	0.004289012	CDH2
0.715876579	0.000347663	0.002893175	CRABP2
0.715626717	4.94648E-13	3.11609E-10	CCDC167
0.715227127	1.54914E-06	3.61309E-05	CFHR3
0.714441299	1.16984E-06	2.8823E-05	FAM225B
0.711780548	0.000265474	0.00232882	C21orf58
0.70979023	3.45501E-07	1.07682E-05	RP13-895J2.3
0.708963394	2.57766E-06	5.46018E-05	ASB2
0.708331108	4.42332E-07	1.31496E-05	UHRF1
0.70791626	0.000238444	0.002126786	BIRC5
0.707900047	7.60471E-08	3.24706E-06	NR1H3
0.707242012	1.00217E-05	0.000165223	CYP27B1
0.706474304	0.000206839	0.001890372	RP11-554I8.2
0.706345558	0.000283787	0.002452536	BCAT1
0.704281807	0.00069752	0.005022229	NFAM1
0.703160286	0.000200461	0.001842407	AARD
0.699279785	1.1338E-06	2.82193E-05	CIITA
0.699178696	8.27317E-05	0.00092286	SCML4
0.698698044	0.000128366	0.001302611	IGDCC4
0.695343971	1.31358E-05	0.000205882	CDT1
0.694470406	8.72673E-08	3.64348E-06	RP11-345J4.6,AC133555.4
0.694141388	1.8501E-07	6.52227E-06	CTD-2020K17.1
0.693681717	2.52382E-05	0.00034807	CDC45
0.693627357	6.78762E-05	0.000785925	GALNT1
0.689213753	0.006792681	0.030821537	CCDC80
0.688883781	6.11242E-06	0.000110366	TLR7
0.688573837	7.11261E-05	0.000815068	LPPR4,PLPPR4
0.68830204	0.000504485	0.003898523	GUSBP10
0.68729496	4.99442E-10	5.98988E-08	GBP2
0.686965942	5.43691E-11	1.0474E-08	SEMA4D
0.686151505	4.39244E-05	0.000547455	TPX2

Log-ratio	p-value	minFDR	Gene Symbol
0.684282303	0.000181148	0.001700025	SPC25
0.684223175	3.30569E-09	2.76031E-07	ITM2C
0.684144974	2.31238E-05	0.000321995	BLACAT1
0.683281898	6.5047E-07	1.80027E-05	MAP4K1
0.683258057	1.53284E-05	0.00023483	BCL11B
0.681909561	9.76954E-08	3.98369E-06	GINS2
0.680450439	7.91434E-07	2.10699E-05	E2F7
0.679727554	4.55893E-05	0.000564754	IL21R
0.679650307	1.82692E-10	2.59982E-08	APOBEC3F
0.67934227	4.44527E-05	0.000553106	KCNK12
0.677554131	0.000118097	0.00121555	LGI2
0.676063538	9.68722E-05	0.001039051	TRPM2
0.67578125	9.53368E-06	0.00015824	AQP5
0.674505234	4.13982E-05	0.000521012	ABCC3
0.67346096	5.59471E-06	0.000102302	FAM46C,TENT5C
0.672734261	0.000138465	0.001380475	C4B,C4B_2,C4A
0.672556877	0.006870472	0.031076532	SUSD4
0.672279358	0.009966005	0.041361878	LUZP2
0.671316147	1.64731E-05	0.000248324	KRT81
0.671186447	0.007794327	0.034187164	NMU
0.67089653	0.000935319	0.006338476	SNX10
0.670597076	9.71538E-05	0.001041158	D4S234E,NSG1
0.667599201	0.000714316	0.005123	PCSK1
0.667536736	0.00818606	0.03543203	ZNF843
0.667127609	8.59432E-06	0.000145414	RP11-247C2.2
0.665786743	3.80508E-06	7.50767E-05	HLA-DOA
0.665662289	2.07661E-05	0.000295729	IGHV1-45
0.664982796	8.52216E-06	0.000144493	RAD54L
0.663576126	0.002659013	0.014562876	GZMH
0.661917686	4.39129E-05	0.000547455	TIGIT
0.66133213	3.20486E-08	1.64382E-06	AMPD3
0.65955162	0.000140798	0.001399839	CD84
0.658483505	3.15401E-05	0.000418018	ABCC6P1
0.658341408	6.49608E-06	0.000115756	WNT5A
0.657834053	1.34145E-05	0.000209845	SIRPG
0.657587051	8.30184E-05	0.000924827	PDE6G,AC139530.1
0.656686783	0.001454426	0.008999441	THBS2
0.655964851	6.35633E-06	0.000113763	SNORA11D,MAGED4,SNORA11E, MAGED4B
0.655852318	0.001051963	0.006966539	TRAT1
0.655423164	3.61676E-05	0.000465855	ANLN
0.653879166	0.006893009	0.031143927	RBAKDN
0.653562546	0.00144763	0.008961975	IGSF9
0.653362274	8.36839E-09	5.84828E-07	IFITM1
0.65301609	4.23797E-05	0.000530908	MELK
0.652770996	6.05556E-05	0.00071469	PTCHD1
0.65201664	2.19511E-06	4.7527E-05	FRMD5

Log-ratio	p-value	minFDR	Gene Symbol
0.650125504	0.000312513	0.002649323	RGMA
0.64872551	1.48824E-06	3.49102E-05	ZWINT
0.647768974	1.47667E-09	1.47462E-07	APOBEC3C
0.647603989	4.38653E-06	8.35969E-05	TRBV5-1
0.646953583	4.53679E-06	8.6111E-05	C16orf59,TEDC2,MIR6768
0.64564991	0.000100941	0.001075617	PLEKHN1
0.644321442	9.20165E-06	0.000153461	TROAP
0.642393112	5.26999E-07	1.52065E-05	UBE2T
0.642027855	0.00068388	0.004948771	FAM131C
0.641486168	3.61484E-05	0.000465855	IGLV3-22
0.640380859	1.3674E-06	3.2449E-05	ADA
0.639903069	4.58361E-07	1.3593E-05	CD226
0.639532089	0.001099767	0.007222352	GBP1
0.639308929	2.0688E-08	1.15577E-06	AC022182.3
0.638381958	2.4933E-07	8.2434E-06	ARHGAP22
0.6379776	1.18836E-05	0.000189533	BMP7
0.637498856	9.96487E-11	1.6473E-08	SSR4
0.637372971	1.95238E-05	0.000283017	CENPE
0.636827469	0.000238438	0.002126786	IGFBP5
0.636136055	0.00381671	0.019490635	RP11-872J21.3
0.635745049	7.95455E-05	0.000894125	NUF2
0.635692596	5.39407E-05	0.000650105	REEP2
0.635485649	3.37087E-05	0.000441904	CTSZ
0.634196281	0.00050051	0.003875149	CPNE4
0.633650303	1.62461E-08	9.71827E-07	TRBV7-4
0.633509636	0.005248689	0.025044297	RP11-32B5.8
0.633365631	0.003678895	0.018889369	FAM43B
0.632731438	4.45872E-05	0.000554304	CFH
0.632725716	0.00025359	0.002239833	IDO2
0.632461548	9.45373E-06	0.000157236	TSPEAR
0.630661011	5.38955E-05	0.00064988	C9orf173,STPG3
0.630616188	1.74884E-05	0.000261215	KIF20A
0.630384445	3.3412E-06	6.7336E-05	CKS2
0.630023956	1.51894E-09	1.49849E-07	SEL1L3
0.62924099	0.002573645	0.014216936	C1QTNF4
0.62895298	0.000110956	0.001155664	TIMP1
0.628576279	6.76657E-06	0.000119704	TRBV20-1
0.628481865	6.76501E-05	0.000784049	MYCL
0.627799988	9.32825E-06	0.000155467	CD200R1
0.62744236	2.08351E-06	4.56766E-05	CENPK
0.627214432	1.37299E-07	5.19994E-06	RAB33A
0.626544952	0.007125665	0.031994224	SYNGR4
0.625984192	0.000448624	0.003550965	SLC37A2
0.625784874	0.000522164	0.004004784	TNC
0.625227928	1.8523E-05	0.00027202	MND1
0.625013351	0.000745306	0.005303853	IGSF6
0.624619484	1.00427E-08	6.73165E-07	NFE2L3

Log-ratio	p-value	minFDR	Gene Symbol
0.623123169	2.86748E-06	5.95045E-05	PTGFRN
0.622635841	3.05424E-06	6.25838E-05	STEAP3
0.62234211	9.68423E-10	1.02127E-07	CCDC109B,MCUB
0.622186661	0.000103934	0.001096527	AGMAT
0.621733665	2.80709E-08	1.47063E-06	TNFRSF25
0.621098518	0.007463658	0.033141464	TUBBP5
0.620894432	5.45322E-09	4.14343E-07	LDLRAD4
0.618250847	0.000813803	0.005683843	OR10H2
0.618213654	0.005502817	0.026030919	VCAM1
0.618062019	4.03678E-06	7.85711E-05	ESCO2
0.617782593	0.002610053	0.014359691	APOE
0.617589951	5.61723E-07	1.59064E-05	ANKRD65
0.617482185	3.92278E-06	7.66572E-05	NFKBIE
0.617159843	1.49362E-08	9.18161E-07	PIF1
0.61665535	2.69818E-05	0.00036756	TRIM36
0.616389751	5.07683E-06	9.43843E-05	MYH15
0.61570549	2.76148E-05	0.000374722	TRBV18
0.615053177	1.01134E-08	6.7605E-07	RGS10
0.614093781	0.000175068	0.00165823	IL2RB
0.613794327	1.75459E-06	3.997E-05	C3
0.612402916	6.04969E-06	0.000109557	CD3E
0.61214447	4.90805E-09	3.79405E-07	C4orf48
0.6112957	4.82813E-08	2.25656E-06	C1S
0.610376358	6.41717E-06	0.0001146	HLA-DMB
0.609544754	1.24887E-05	0.000197697	TAP1
0.609302521	3.31302E-06	6.68782E-05	GBP1P1
0.609242439	4.46481E-11	9.15289E-09	TRIM46
0.608808517	0.000641658	0.004704467	STEAP2
0.608781815	7.15082E-09	5.22245E-07	RNF207
0.608174324	0.000132448	0.001333531	TF
0.607980728	5.63047E-07	1.59254E-05	PPP2R2B
0.607627869	3.88234E-06	7.60942E-05	GINS1
0.607625961	0.011237938	0.045333453	FSD2
0.607599258	0.004489087	0.022191929	YJEFN3
0.605946541	1.92689E-07	6.70602E-06	EZH2
0.604930878	0.000873396	0.00600998	AK4P3
0.604664803	2.57138E-07	8.46721E-06	TSPAN33
0.604261398	0.006864964	0.031063105	SIX4
0.604239464	1.22273E-06	2.97666E-05	SNHG3,SNORA73B
0.603193283	0.003315868	0.017407043	CCNB2P1
0.602072716	9.62997E-06	0.000159301	CTSB
0.601902008	3.10098E-07	9.9045E-06	SP140
0.601787567	0.001695422	0.010171703	VWCE
0.600880623	8.79763E-05	0.000966081	IL27
0.600623131	0.003595675	0.0185712	GPR171
-0.600707054	2.65057E-05	0.00036188	ABCG2
-0.600710392	0.000870206	0.005995621	DAO

Log-ratio	p-value	minFDR	Gene Symbol
-0.601137161	9.84616E-08	3.99496E-06	WFS1
-0.602849007	0.001053118	0.006968609	ACSM6
-0.603081703	4.38199E-07	1.30425E-05	GPX6
-0.60363245	0.004854211	0.023565332	IL1R2
-0.604418755	4.70833E-05	0.000580323	HULC
-0.605270386	7.82127E-08	3.32214E-06	INMT-FAM188B,INMT-MINDY4
-0.60632515	7.70503E-09	5.49595E-07	EPB41L4A-AS2
-0.606433868	8.42102E-06	0.000143274	USP53
-0.607068062	9.63229E-05	0.001035884	G0S2
-0.607275009	1.68378E-11	4.7351E-09	BAIAP2
-0.607943535	1.94553E-08	1.10183E-06	RP11-680F8.3
-0.608034134	5.18783E-06	9.60828E-05	EXOC3L1
-0.608322144	3.87855E-09	3.12147E-07	SHROOM1
-0.608413696	5.80006E-11	1.06695E-08	SPRED2
-0.608533859	4.25641E-05	0.000532944	WFDC1
-0.608921051	0.00566244	0.026611076	ANXA8L1,ANXA8
-0.610920906	0.012434206	0.048980082	ALDH1L1
-0.611591339	2.85974E-07	9.27937E-06	CAMK2N1
-0.612086296	1.14144E-06	2.83518E-05	CACNA1H
-0.6132164	3.50482E-08	1.76438E-06	PRICKLE2
-0.613257408	3.25213E-07	1.029E-05	MTURN
-0.613443375	2.19444E-06	4.7527E-05	LINC00922
-0.613898277	0.001404017	0.008738405	PTPN5
-0.614450455	0.004452202	0.022054582	AC002480.5
-0.614834785	0.001628977	0.009847923	MT1A
-0.615695	7.75087E-11	1.31896E-08	NDEL1
-0.615825653	1.83906E-06	4.15462E-05	HSPA2
-0.616333961	4.38725E-06	8.35969E-05	ARL4D
-0.616346359	8.09541E-06	0.000139089	B3GALNT1
-0.616484642	0.003632842	0.018715752	GLDN
-0.616500854	6.5798E-05	0.000765484	TMEM35,TMEM35A
-0.617508888	0.001256709	0.008028524	PTPRR
-0.618587494	1.34035E-08	8.39206E-07	FGFR4
-0.618668556	0.000388223	0.003157666	C5AR1
-0.619589806	0.002356177	0.013236793	KLK11
-0.619859695	9.41219E-08	3.86374E-06	TP53INP2
-0.620450974	0.000199626	0.001836113	C2orf72
-0.621082306	8.36318E-07	2.21204E-05	AC144450.2
-0.621216774	1.55863E-06	3.62831E-05	KCNQ1-AS1
-0.621310234	0.000152652	0.001487959	WDR11-AS1
-0.621434212	2.07808E-08	1.15814E-06	NKD1
-0.623332024	2.37412E-08	1.29366E-06	FERMT2
-0.623512268	0.000181064	0.001699894	SRRM4
-0.623832703	0.002653439	0.014549315	ARG2
-0.624229908	0.000945393	0.006384209	CYP2G2P,CYP2G1P
-0.624839783	5.24976E-09	4.02635E-07	PGM1
-0.625235558	9.43769E-05	0.001018987	RP11-195B21.3

Log-ratio	p-value	minFDR	Gene Symbol
-0.625886917	0.000104851	0.001104774	RP3-403A15.5,SOGA3,AL096711.2, KIAA0408
-0.628661156	8.63627E-05	0.000951538	FGF12
-0.628936768	1.95264E-05	0.000283017	KCNJ12
-0.629457474	2.11045E-05	0.000299676	TNNT2
-0.630079269	8.42005E-05	0.000935111	CREB5
-0.630224228	9.6776E-09	6.58215E-07	TSC22D1
-0.63031292	0.003634871	0.018720324	PLA2G1B
-0.630548477	6.41105E-07	1.78039E-05	SEMA3B
-0.630918503	1.12327E-07	4.46606E-06	JAM3
-0.631531715	1.39889E-08	8.6866E-07	KLC3
-0.632485867	1.33142E-07	5.08184E-06	PRX
-0.633081436	3.73949E-05	0.000478255	SH3GL2
-0.633888245	9.68516E-09	6.58215E-07	RALA
-0.634588242	4.83549E-05	0.000594199	FAM95B1,AL669942.1
-0.636610985	2.21731E-06	4.79229E-05	LONRF1
-0.638085365	9.7949E-06	0.000161593	MSX1
-0.638394356	0.000995183	0.006661602	EMP1
-0.639947891	0.000154266	0.001501302	JPH2
-0.640658379	9.929E-08	4.0219E-06	CCNB3
-0.64108181	3.60904E-07	1.10928E-05	XKR6
-0.641531944	0.000157253	0.001523709	FAM124B
-0.64162159	4.06313E-09	3.24865E-07	AOC4P
-0.641926765	0.000376843	0.003083557	NOS2
-0.64231205	0.000156702	0.001519564	AC073464.11
-0.642608643	6.63044E-06	0.000117551	BMPR2
-0.642883301	1.96282E-05	0.000284156	THSD1
-0.643428802	0.000812843	0.005679504	AKAP12
-0.643661499	4.36154E-08	2.07606E-06	NDRG2
-0.643921852	4.63685E-09	3.65038E-07	HPCAL1
-0.645545006	1.26334E-06	3.06029E-05	GHR
-0.645781517	3.56225E-05	0.000460644	NOSTRIN
-0.646098137	4.06626E-05	0.00051281	EFR3B
-0.646304131	3.2131E-06	6.52379E-05	SHANK3
-0.646450996	0.002144439	0.012281332	NCOA7
-0.646567345	1.52947E-12	7.79583E-10	PROSER2
-0.646697998	4.44392E-05	0.000553106	ARHGEF4
-0.646790504	3.15882E-07	1.00761E-05	SH3BP5
-0.647638321	1.64096E-10	2.41854E-08	MAFK
-0.648012161	1.77759E-08	1.02444E-06	AVPI1
-0.648861885	6.58444E-06	0.000116905	TCAP
-0.649773598	8.51657E-07	2.24336E-05	CLDN11
-0.651010513	0.000191684	0.001780346	GNAZ
-0.651323318	1.6809E-08	9.96177E-07	SHROOM2
-0.652913094	1.2874E-07	4.94467E-06	RARRES2
-0.653347969	5.55545E-08	2.50774E-06	HEG1
-0.653863907	8.47343E-05	0.000938759	B3GNT5

Log-ratio	p-value	minFDR	Gene Symbol
-0.654148102	4.64019E-09	3.65038E-07	CEBPB
-0.654809952	4.12852E-06	7.99117E-05	COL13A1
-0.655777931	7.01995E-08	3.03445E-06	AGPAT9,GPAT3
-0.656157494	5.74763E-09	4.32681E-07	PRELP
-0.657077789	0.004376947	0.021778805	ZC3H12A
-0.657698631	1.41548E-05	0.000219463	MAP2
-0.658275604	2.59446E-06	5.48153E-05	RSPO1
-0.658498764	6.43919E-07	1.78618E-05	RADIL
-0.658685684	1.73264E-08	1.01167E-06	AMOTL2
-0.658977509	6.30451E-08	2.77921E-06	RNF152
-0.659365177	4.98854E-06	9.28841E-05	SPACA4
-0.65938282	4.91194E-05	0.000602082	EIF5
-0.659517288	4.74118E-07	1.39925E-05	SVEP1
-0.660254478	1.88697E-06	4.24326E-05	MIDN
-0.660408974	2.28542E-07	7.68065E-06	RP11-61N20.3,AC135977.1, SLC25A33
-0.66061306	0.000151935	0.001485707	FMO2
-0.661128044	2.37509E-06	5.0839E-05	NEDD9
-0.661429405	1.39793E-06	3.3077E-05	RP11-244F12.3
-0.662178993	1.86851E-07	6.55645E-06	DNAJC6
-0.662722588	5.04679E-07	1.47521E-05	RXFP1
-0.66318512	4.92776E-08	2.29206E-06	SRGAP1
-0.663918495	0.000423132	0.003380914	FABP4
-0.665207863	1.39881E-08	8.6866E-07	FBLN5
-0.66593647	0.002062695	0.011896724	KLRF1
-0.665979385	0.000112353	0.001166241	HAPLN2
-0.666175842	0.007591028	0.033519906	TMCC2
-0.666343689	1.88745E-05	0.000275363	RND3
-0.666631699	4.28224E-06	8.21075E-05	SAMD5
-0.667020798	5.59188E-07	1.58898E-05	RP11-736K20.5
-0.668287277	2.41847E-10	3.21578E-08	PHLDB1
-0.670399666	1.60208E-06	3.69431E-05	STC1
-0.671524048	4.70203E-05	0.000580131	DAPK2
-0.671537399	2.39376E-07	7.94652E-06	MFAP4
-0.672157288	7.14868E-06	0.000125466	COL21A1
-0.673212051	2.01392E-06	4.44699E-05	LAMA3
-0.673887253	1.19266E-06	2.9238E-05	CAB39L
-0.674720764	5.88866E-07	1.65982E-05	CTD-2600O9.1
-0.675899506	1.80347E-07	6.43204E-06	PITPNM2
-0.676049232	1.5435E-05	0.000235726	CACNA2D3
-0.6762743	6.00701E-07	1.69123E-05	TAS1R1
-0.677074432	9.73353E-07	2.48841E-05	CRYAB
-0.67783165	0.000189156	0.001760994	DYRK3
-0.679244041	3.4917E-08	1.7614E-06	FAM134B,RETREG1
-0.679522514	7.79271E-05	0.000878601	TSPAN18
-0.679616928	4.62986E-07	1.3697E-05	JDP2
-0.680300713	1.38749E-06	3.28619E-05	PALD1

Log-ratio	p-value	minFDR	Gene Symbol
-0.680678368	5.21623E-06	9.63965E-05	RP11-736K20.4
-0.681036949	6.142E-06	0.000110656	TRNP1
-0.681637764	3.82347E-08	1.87242E-06	CASZ1
-0.68169117	1.89199E-06	4.25064E-05	MCL1
-0.682575226	1.32383E-07	5.06077E-06	ARHGEF10
-0.683311462	1.83108E-12	8.95986E-10	SPRY2
-0.683953285	0.000673037	0.004883307	PCOLCE2
-0.684994698	4.84337E-08	2.2571E-06	SIK2
-0.685713768	6.41662E-06	0.0001146	ARHGEF26
-0.686461449	2.59864E-08	1.38053E-06	PAQR5
-0.687055588	2.18011E-06	4.73682E-05	GPR146
-0.688457489	8.6932E-06	0.000146782	CDO1
-0.689139366	8.06657E-08	3.4027E-06	MAL
-0.689679146	1.89269E-07	6.61522E-06	RP11-172H24.4
-0.690049648	0.001756628	0.01046194	KLK1
-0.690444946	3.29117E-07	1.03498E-05	PARD6B
-0.691340446	8.55752E-06	0.000144892	KALRN
-0.691459656	1.97174E-08	1.1141E-06	FLYWCH1
-0.692390442	4.83299E-08	2.25656E-06	TEAD4
-0.69252491	2.87008E-06	5.95079E-05	PDGFB
-0.69316864	8.95476E-07	2.32577E-05	CCNL1
-0.693358421	3.27664E-05	0.000431466	SLC51B
-0.694117546	3.74733E-08	1.84101E-06	ID3
-0.695257187	0.007750197	0.034011896	MEG8_2
-0.695355415	7.62523E-05	0.000862102	RP11-102K13.5
-0.695905685	1.82445E-09	1.73011E-07	IFNLR1
-0.697357178	3.63484E-12	1.41159E-09	GATA6
-0.697363853	7.56359E-07	2.03353E-05	CLEC14A
-0.697412491	3.51588E-07	1.09023E-05	ADGRB3
-0.697450638	2.3384E-08	1.27704E-06	PEAR1
-0.697976112	1.99975E-06	4.42769E-05	ST6GALNAC5
-0.699202538	5.03969E-06	9.3765E-05	CACNA2D2
-0.700237274	7.49465E-08	3.20567E-06	ARAP3
-0.700294495	0.000150587	0.001478025	ANKRD37
-0.700823784	5.40688E-08	2.48189E-06	RPS27
-0.701087952	1.09913E-05	0.000177736	CHRM2
-0.701986313	3.78371E-09	3.06531E-07	PAPSS2
-0.702750206	6.24207E-07	1.74336E-05	FAXDC2
-0.704720974	5.30774E-07	1.52955E-05	SLC27A6
-0.705426216	9.89204E-09	6.6856E-07	CBSL,CBS
-0.705600739	2.7082E-07	8.86999E-06	AC011742.3,AC011742.2
-0.706474304	0.000393812	0.003192515	CALCRL
-0.707414627	2.68394E-06	5.6365E-05	ACKR3
-0.70745945	1.73821E-05	0.000259786	ADRA2C
-0.710478306	7.65205E-07	2.05055E-05	SYCP2L
-0.711350441	2.43021E-09	2.13876E-07	CLEC1A
-0.712133408	2.10513E-05	0.000299269	DOK7

Log-ratio	p-value	minFDR	Gene Symbol
-0.712295532	6.86662E-10	7.67117E-08	FADS3
-0.712547779	1.6897E-08	9.96177E-07	HUS1B
-0.712745667	1.06486E-05	0.000173108	MFAP3L
-0.714371681	0.001770171	0.010529782	CTSV
-0.714909554	7.0421E-08	3.03866E-06	STON1
-0.715403557	7.88846E-08	3.34487E-06	LGALSL
-0.716597557	4.0266E-06	7.84354E-05	C3orf36
-0.71767807	3.87535E-09	3.12147E-07	C1orf198
-0.717973709	5.08346E-08	2.36E-06	ZSWIM4
-0.718883514	3.65486E-09	2.98066E-07	WNT7B
-0.719640732	0.00506728	0.024392373	PF4
-0.719913483	1.18355E-09	1.21158E-07	HOMER1
-0.720520973	8.46431E-06	0.000143811	KCNAB1
-0.720636368	0.000115794	0.001195871	PTCRA
-0.720793724	1.41346E-08	8.75488E-07	DOCK6
-0.721146584	6.34899E-06	0.000113715	ADGRL2
-0.721610069	4.99954E-11	1.00261E-08	SMAD7
-0.721652985	5.99706E-10	6.91725E-08	FRY
-0.722260475	1.25227E-05	0.000197921	HCAR3
-0.722846031	7.52716E-07	2.02819E-05	SPIN4
-0.724080086	1.3415E-08	8.39206E-07	WNT7A
-0.725710869	0.000280973	0.002435096	GS1-600G8.5
-0.725935936	0.000189245	0.00176115	ODF3L1
-0.728801727	1.08556E-08	7.10144E-07	PFKFB3
-0.729050636	1.80633E-07	6.43285E-06	DOK6
-0.729364395	3.70687E-05	0.000474829	OR2A42,OR2A1
-0.730659485	0.000496695	0.003855376	MS4A15,AP004243.1
-0.730689049	0.000168229	0.001610288	S1PR5
-0.733120918	1.04454E-06	2.64551E-05	CETP
-0.73453331	4.67412E-06	8.81704E-05	OR2A1-AS1,OR2A20P,OR2A9P
-0.734662056	0.000120998	0.001241233	KCNK17
-0.734787941	2.7281E-05	0.000371221	STAC
-0.735600471	3.50726E-07	1.08958E-05	RP4-575N6.4
-0.736317635	2.06455E-05	0.000294424	CYP4F25P,CYP4F60P
-0.73657608	0.000968073	0.006517577	LINC00487
-0.736820221	7.40863E-05	0.000843888	NLGN4X
-0.737193108	2.02198E-07	6.96756E-06	KIAA1211L
-0.737268448	4.26814E-05	0.000533866	TMEM26
-0.737816811	2.18275E-08	1.20549E-06	GALNT18
-0.738089561	1.91909E-09	1.79208E-07	SHE
-0.739134789	1.37126E-05	0.000213553	PCP4L1
-0.739391327	1.13241E-06	2.82193E-05	RAMP2
-0.739521027	4.06737E-08	1.94584E-06	C1orf115
-0.742811203	2.04017E-05	0.000291936	KDM6B
-0.744679451	4.66169E-08	2.19332E-06	AC092066.1
-0.745872498	1.13133E-08	7.30323E-07	SASH1
-0.746635437	6.20843E-05	0.000728864	ULBP1

Log-ratio	p-value	minFDR	Gene Symbol
-0.74724865	2.11283E-05	0.000299841	DKK2
-0.747548103	7.72179E-08	3.28559E-06	DCUN1D3
-0.748096466	8.01276E-09	5.64957E-07	NEBL
-0.749002457	4.70323E-10	5.69649E-08	MAOA
-0.749773026	1.14833E-09	1.18544E-07	BFSP1
-0.749955177	6.23393E-12	2.3109E-09	OTUD1
-0.750193596	3.91248E-06	7.65782E-05	PIK3R3,AL358075.4
-0.752352715	6.26026E-08	2.76968E-06	PDZD2
-0.752435207	0.012032783	0.047767981	IL13
-0.752552986	7.89406E-06	0.000136306	EXD1
-0.75278759	0.000692145	0.004996759	LTK
-0.754384041	0.002028511	0.011741082	CHI3L2
-0.756337166	1.20562E-05	0.00019191	CHST1
-0.757102013	3.69756E-05	0.000473885	SEMA3E
-0.757117271	4.7695E-08	2.23974E-06	MME
-0.757242203	6.98674E-05	0.000804034	GBA3
-0.757514954	5.60002E-07	1.58944E-05	CLEC3B
-0.759500504	7.77107E-07	2.0711E-05	ART4
-0.759807587	8.88155E-05	0.000971372	CTNND2
-0.760094643	4.98305E-07	1.46135E-05	PDE1C
-0.762564659	5.72608E-10	6.7353E-08	P3H2
-0.763374329	2.32046E-07	7.76167E-06	MCAM,AP002956.1
-0.763561249	3.21845E-10	4.0819E-08	FGD5
-0.765806198	0.000406508	0.003275898	MT1X
-0.76584053	0.000303018	0.002588557	RD3
-0.765971184	6.6646E-09	4.94109E-07	CCBE1
-0.766726494	0.000568172	0.004286668	GP9
-0.767572403	2.00315E-05	0.000288799	TNFRSF10D
-0.768619537	1.90649E-08	1.08223E-06	SH2D3C
-0.770476341	0.000350229	0.002907605	GPD1
-0.772013664	3.20905E-07	1.02108E-05	MAP3K8
-0.772805214	0.012473636	0.049064306	PSPHP1
-0.77440834	0.000332584	0.002789514	U2,RNU2-2P,RNU2-1,RNU2-59P
-0.776087284	2.25027E-07	7.5938E-06	C11orf91
-0.776955605	3.21397E-08	1.64504E-06	TGFBR3
-0.778249741	1.80122E-07	6.43204E-06	CSPG4
-0.779187202	5.18637E-11	1.0233E-08	HSPB8
-0.779208183	1.05803E-05	0.000172571	MGAM
-0.780641556	0.00013684	0.001368179	IL7R
-0.780672073	4.36093E-07	1.30115E-05	SPOCK2
-0.78112793	1.91741E-10	2.66541E-08	HYAL1
-0.783037186	0.000101448	0.001077043	RAMP3
-0.784265518	5.4916E-08	2.49272E-06	FRAS1
-0.784754753	1.50803E-05	0.000231465	LINC00163
-0.787547112	2.91636E-06	6.038E-05	FAM69B,DIPK1B
-0.789024353	5.74174E-06	0.000104756	ANKRD20A17P
-0.789629936	8.28792E-09	5.81009E-07	SLIT2

Log-ratio	p-value	minFDR	Gene Symbol
-0.790273666	1.9088E-07	6.65251E-06	KCNK3
-0.791892052	3.9814E-05	0.000503927	TIPARP
-0.791999817	2.81334E-07	9.17751E-06	P2RY1
-0.792497635	6.99431E-10	7.7783E-08	AOC3
-0.794914246	1.54493E-08	9.42597E-07	LMCD1
-0.795088768	5.05953E-05	0.000617081	KIAA1683,IQCN
-0.796602249	5.21278E-08	2.41088E-06	EMP2
-0.796808243	2.04198E-06	4.50081E-05	TBX3
-0.797290802	2.29079E-07	7.68812E-06	TNNC1
-0.797506332	1.63242E-07	5.94033E-06	EPB41L5
-0.798365593	4.81087E-06	9.02628E-05	CYP1A2
-0.798556328	6.93741E-05	0.000799862	RP11-141J13.5
-0.798586845	5.86126E-06	0.000106618	BEX5
-0.798908234	2.23761E-07	7.56153E-06	SLC5A1
-0.7992239	3.91286E-08	1.89454E-06	ANXA3
-0.799280167	1.1589E-08	7.43197E-07	PTPN21,AL162171.1
-0.800534248	0.011740437	0.046896579	ASPG
-0.800652504	2.40995E-09	2.12859E-07	TCF21
-0.800944328	5.57817E-07	1.58877E-05	RP11-434D9.1
-0.801603317	3.07217E-11	7.15844E-09	ESAM
-0.802089691	9.45792E-07	2.4332E-05	HSPA12B
-0.802702904	5.16177E-05	0.000628297	HRCT1
-0.803408623	5.66101E-05	0.000677272	TSPAN15
-0.803567886	0.00010206	0.001081889	SLC26A9
-0.803593636	0.000157107	0.001522889	SLC1A7
-0.804865837	1.84187E-06	4.15713E-05	STC2
-0.805190086	4.52669E-11	9.15289E-09	DENND3
-0.808262825	3.5146E-12	1.38691E-09	TUFT1
-0.808668137	2.60388E-06	5.49195E-05	CHRM3
-0.810056686	1.14015E-05	0.000183399	LINC-PINT
-0.810070038	0.001479549	0.009114749	MMP19
-0.810124397	3.47948E-06	6.94931E-05	CMTM2
-0.812345505	5.65479E-11	1.06423E-08	ETS2
-0.814442635	9.41616E-07	2.42501E-05	CA2
-0.815702438	7.42981E-06	0.000129841	VEGFA
-0.816913605	2.79692E-05	0.000378481	ANKRD20A1,ANKRD20A3,ANKRD20A4, ANKRD20A2
-0.817326546	3.02922E-08	1.5644E-06	CITED4
-0.818371773	3.49813E-09	2.91106E-07	PCDH1
-0.81864357	3.20941E-07	1.02108E-05	VGLL3
-0.818827629	5.39634E-11	1.0474E-08	CASP12
-0.819400787	0.000246309	0.002188163	IER3
-0.820438385	1.96149E-09	1.8178E-07	AFAP1L1
-0.823986053	6.30097E-09	4.71436E-07	FLT4
-0.826405525	1.66233E-09	1.59493E-07	PALM2-AKAP2,AKAP2
-0.826591492	1.75962E-08	1.02016E-06	ITPRIP
-0.8269701	7.27709E-12	2.54345E-09	SPTBN1

Log-ratio	p-value	minFDR	Gene Symbol
-0.830456734	3.88464E-06	7.60942E-05	MUC15
-0.830471992	0.000125879	0.001280559	FAM150B,ALKAL2
-0.830478668	1.82791E-07	6.4745E-06	LIMS2
-0.830719948	2.99255E-06	6.15775E-05	IL1A
-0.833148003	4.5225E-11	9.15289E-09	CD93
-0.834306717	4.89739E-06	9.16663E-05	RNF122
-0.83571434	4.14194E-14	4.22236E-11	CITED2
-0.837762833	1.32604E-06	3.17445E-05	COL6A6
-0.838006973	1.0099E-05	0.000165939	LINC00313,CH507-42P11.5
-0.838014603	0.001963988	0.011432531	CLEC4M
-0.839101791	2.34319E-06	5.02881E-05	BDNF
-0.839788437	4.01971E-08	1.93595E-06	C1QTNF5,MFRP
-0.841981888	1.50605E-07	5.59134E-06	EFCC1
-0.842842102	0.000358272	0.002955322	MT1HL1
-0.843366623	7.18221E-09	5.22682E-07	FZD4
-0.845758438	8.23642E-10	8.8772E-08	CDH5
-0.845840454	1.84737E-09	1.73838E-07	EFNB2
-0.84663105	3.87775E-08	1.88614E-06	LPL
-0.847635269	4.7771E-09	3.73408E-07	SULT2B1
-0.848090172	0.000731859	0.005229459	DNASE1L3
-0.850755692	1.99945E-05	0.000288434	SEMA5B
-0.850822449	2.53484E-06	5.40135E-05	EDIL3
-0.850989342	2.24946E-05	0.00031575	ECEL1P2
-0.851471901	1.53144E-08	9.38956E-07	AC113949.1
-0.852519989	4.90204E-06	9.16663E-05	ELN
-0.85286808	0.002292454	0.012983146	RTKN2
-0.853240013	5.26587E-11	1.03068E-08	MIR1182,FAM89A
-0.854058266	1.95063E-07	6.77899E-06	NTNG1
-0.856658936	2.04666E-05	0.000292657	FMO5
-0.857048988	1.31076E-08	8.26524E-07	TMIE
-0.858186722	1.61169E-05	0.000243857	RGS9
-0.858249664	1.17066E-10	1.85983E-08	NPR1
-0.85857296	9.77906E-10	1.02684E-07	FAM46B,TENT5B
-0.858672142	3.88518E-07	1.17934E-05	CYS1
-0.858674049	1.08765E-07	4.35524E-06	MYOZ1
-0.860077858	0.000190156	0.001768287	RP11-510N19.5
-0.861180305	1.7591E-09	1.68118E-07	BCL6B
-0.863506317	1.26674E-14	1.47581E-11	LIN7A
-0.865559578	7.93368E-09	5.60998E-07	VEGFC
-0.867817879	3.84422E-11	8.36238E-09	CDC42EP2
-0.868477345	1.56697E-05	0.000238565	SLC10A4
-0.868631363	1.48836E-07	5.53565E-06	LRP4
-0.870366096	2.57713E-06	5.46018E-05	RBP2
-0.87068367	3.51861E-05	0.000456148	NPTX1
-0.871653557	1.03738E-07	4.18819E-06	MYRF
-0.873598099	3.62077E-07	1.11149E-05	LINC00162
-0.873840332	1.77811E-07	6.36945E-06	FAM222A

Log-ratio	p-value	minFDR	Gene Symbol
-0.874319077	8.39251E-06	0.000143088	RP11-755J8.1,ANKRD20A21P
-0.875386238	0.000844008	0.005849716	SOSTDC1
-0.880187035	3.48324E-08	1.76076E-06	TMEM178A
-0.881030083	0.000369183	0.003035987	CD69
-0.881375313	0.000517227	0.003975648	CLEC1B
-0.882282257	3.5517E-09	2.92579E-07	DACH1
-0.883812904	0.000462915	0.003641701	ARG1
-0.884801865	0.000252007	0.002229066	RP11-343H19.2
-0.885107994	1.44684E-11	4.39739E-09	LINC00472
-0.885343552	5.41722E-08	2.48198E-06	SDR16C5
-0.886311531	2.36973E-10	3.1767E-08	RCAN1
-0.887148857	8.83758E-06	0.000148809	TNFRSF12A
-0.888448715	2.60349E-06	5.49195E-05	DFNB31,WHRN
-0.888615608	1.27767E-05	0.000201155	ANKRD20A8P,ANKRD20A18P
-0.891215324	8.07722E-07	2.14568E-05	PDE6A
-0.891530037	1.23222E-08	7.85089E-07	TMEM204
-0.891634941	4.31746E-06	8.24568E-05	RP11-1094H24.4
-0.892411232	1.1584E-06	2.86567E-05	LRRN3
-0.892580032	0.000400503	0.003239243	MT1CP
-0.893330574	7.90378E-08	3.34557E-06	C8orf4,TCIM
-0.894790649	0.000310381	0.002634904	AWAT2
-0.89493084	3.24078E-05	0.000427895	FAM3D
-0.89541626	1.07745E-05	0.000174807	SYNDIG1L
-0.895755768	0.000145135	0.001434699	IL1B
-0.896060944	1.45383E-05	0.00022427	IL18R1
-0.897006989	1.61426E-06	3.71538E-05	GJA5
-0.898042679	2.07977E-07	7.12222E-06	CYP3A7-CYP3A51P
-0.899620056	7.25752E-07	1.96636E-05	RSPO2
-0.902118683	1.15626E-07	4.56277E-06	INMT
-0.906838417	0.0010144	0.006764328	GPA33
-0.906971931	9.66494E-05	0.001037116	RP11-249C24.11,MT1B
-0.908381462	1.56016E-06	3.62841E-05	SLC2A14
-0.908475876	1.1169E-06	2.79407E-05	KCTD16
-0.908959389	3.06443E-06	6.274E-05	F11
-0.90908432	1.53145E-06	3.57522E-05	C2orf71,PCARE
-0.909262657	1.92514E-06	4.30929E-05	RP11-544L8__B.4
-0.909645081	1.34341E-05	0.000209884	NFIL3
-0.909662247	2.62811E-08	1.38576E-06	ZNF503-AS1
-0.912914276	7.63044E-09	5.46503E-07	COLGALT2
-0.913361549	6.84666E-08	2.97004E-06	ACVRL1
-0.915500641	5.17019E-07	1.50409E-05	SHISA3
-0.917103767	1.38049E-09	1.38992E-07	NOTCH4
-0.917563438	3.93964E-05	0.000499157	FPR2
-0.918902397	6.8039E-11	1.18903E-08	KCNK7
-0.919917107	5.42224E-10	6.47125E-08	MFSD2A
-0.92113018	0.002942586	0.01583921	S100P
-0.922307014	9.35192E-06	0.000155649	ANGPTL7

Log-ratio	p-value	minFDR	Gene Symbol
-0.922347069	1.86002E-05	0.000272989	MOGAT1
-0.928026199	9.01581E-08	3.72447E-06	SEMA5A
-0.92825222	1.24825E-11	3.96619E-09	GFOD1
-0.929355621	3.23543E-07	1.02669E-05	PCDH12
-0.929684639	1.58698E-15	3.49895E-12	PXDC1
-0.933133125	1.85311E-11	5.15206E-09	STARD8
-0.933472633	1.91849E-05	0.00027906	SNX22
-0.934127808	4.31139E-07	1.28952E-05	BMP6
-0.934833527	0.000136466	0.001366669	FGFBP2
-0.936607361	7.76856E-07	2.0711E-05	ADARB2
-0.937086105	1.72472E-08	1.0095E-06	LDLR
-0.938097	0.000546372	0.004159158	GPR42,FFAR3
-0.93935585	4.53309E-09	3.61259E-07	GPM6B
-0.939656258	4.83375E-05	0.000594199	MT1E
-0.940178871	1.3236E-08	8.32472E-07	SLC1A1
-0.940355301	2.46788E-08	1.32121E-06	FSTL3
-0.942260742	1.7247E-08	1.0095E-06	TSC22D3
-0.942400932	0.00024551	0.002183444	MCEMP1
-0.943267822	5.62172E-08	2.52369E-06	KANK4
-0.946286201	8.81495E-05	0.000967114	MT1L
-0.947748184	1.60323E-13	1.30749E-10	ADRB2
-0.948087692	6.44012E-12	2.31712E-09	DLC1
-0.948097229	2.23545E-11	5.81835E-09	PKNOX2
-0.949241638	1.76541E-06	4.01791E-05	CXCR1
-0.949613571	0.000856555	0.005919905	HAPLN1
-0.95148468	4.4855E-11	9.15289E-09	RAPGEF5
-0.953739166	2.49922E-08	1.33506E-06	FOXQ1
-0.956378937	6.8473E-06	0.000120957	CELA2A
-0.956510544	1.65942E-11	4.72086E-09	HYAL2
-0.96216011	1.88975E-10	2.64198E-08	GRIA1
-0.962234497	0.000445885	0.00353157	HSD17B2
-0.962241173	1.06039E-10	1.74118E-08	TMEM2,CEMIP2
-0.964857101	4.18085E-06	8.07331E-05	LRRC32
-0.967316628	1.57992E-09	1.54001E-07	SH3GL3
-0.96743679	3.03085E-08	1.5644E-06	LEFTY2
-0.968788147	1.07525E-10	1.7538E-08	ADAMTS8
-0.96945858	1.19437E-10	1.88397E-08	MPP3
-0.973171234	1.54036E-09	1.51351E-07	NOVA2
-0.975409508	7.94825E-05	0.000894078	AJAP1
-0.976067543	1.2164E-07	4.71943E-06	ADGRE3
-0.9760952	5.20096E-06	9.62532E-05	NFKBIZ
-0.976642609	5.43096E-08	2.48362E-06	MAP1LC3C
-0.978310585	6.35504E-05	0.000742159	THBS1
-0.980587006	2.27272E-08	1.25235E-06	EPHA2
-0.982430458	5.6748E-08	2.54285E-06	AC124789.1
-0.982446671	6.27168E-07	1.74959E-05	HEY1
-0.982753754	1.45051E-07	5.41803E-06	RN7SL69P

Log-ratio	p-value	minFDR	Gene Symbol
-0.982805252	3.5845E-07	1.10376E-05	EDNRB
-0.982981682	1.03661E-05	0.00016953	PDK4
-0.983804703	3.56923E-08	1.77489E-06	SERTAD1
-0.984174728	1.06418E-05	0.000173108	HPGD
-0.986246109	7.16391E-10	7.89514E-08	ID1
-0.987442017	2.26547E-07	7.63458E-06	ADM
-0.991894722	1.37744E-07	5.20872E-06	RP11-241F15.7,AC119751.1, RP11-1281K21.1,AC118282.1
-0.992675781	4.01728E-10	4.96398E-08	HEYL
-0.9955163	0.00240217	0.013457609	FGA
-0.996646881	1.11199E-06	2.78879E-05	SOCS2
-0.999558449	2.60826E-10	3.43084E-08	INSC
-1.00062561	3.22001E-10	4.0819E-08	KL
-1.001483917	1.20637E-09	1.22979E-07	RNF182
-1.004473686	1.62486E-07	5.94033E-06	SEMA6A
-1.005206108	0.000100755	0.001074107	ENHO
-1.011896133	2.28061E-10	3.09986E-08	CASKIN2
-1.012084961	2.60141E-08	1.38053E-06	NFKBIA
-1.012613297	1.59716E-07	5.86112E-06	CX3CL1
-1.013413429	1.58972E-08	9.6035E-07	LINC01552
-1.013979912	9.01064E-06	0.000150996	DPP6
-1.014363289	2.05905E-07	7.07537E-06	SLC2A3
-1.018417358	1.589E-11	4.57371E-09	TAL1
-1.018460274	7.763E-11	1.31896E-08	ATOH8
-1.019195557	3.86229E-11	8.36238E-09	DLL4
-1.019856453	1.55942E-07	5.75457E-06	PTPRQ
-1.020494461	3.64429E-06	7.22539E-05	CELA2B
-1.023412704	0.000152184	0.001485807	ANGPTL4
-1.02652359	1.57952E-09	1.54001E-07	GATA2
-1.02660656	4.21253E-06	8.10887E-05	CTD-3247F14.2
-1.029102325	3.21225E-09	2.70072E-07	BMP2
-1.029670715	1.00079E-09	1.04638E-07	PRKCE
-1.031592369	3.48068E-10	4.38961E-08	TEK
-1.036281586	3.40611E-07	1.06565E-05	SLC16A12
-1.036296844	1.15192E-07	4.55295E-06	CRTAC1
-1.039381981	1.21036E-11	3.89639E-09	GPX3
-1.040403366	5.58365E-08	2.5112E-06	ATP8B5P
-1.04070282	2.45662E-08	1.31806E-06	PIGA
-1.042171478	1.99925E-07	6.91007E-06	TRIB1
-1.046709061	1.51133E-07	5.60246E-06	NPR3
-1.051348686	0.009421366	0.039530636	SOST
-1.057494164	4.6945E-05	0.000579494	ESM1
-1.058496475	2.01851E-12	9.68329E-10	EPAS1
-1.061562538	2.09367E-07	7.14419E-06	BHLHE40
-1.067422867	1.92629E-13	1.52028E-10	KLF6
-1.067793846	1.36763E-05	0.000213124	CD300LG
-1.068806648	5.37359E-07	1.54489E-05	FOSL2

Log-ratio	p-value	minFDR	Gene Symbol
-1.07314682	1.75939E-08	1.02016E-06	SLC19A3
-1.073386192	2.50153E-13	1.80007E-10	AC046143.3
-1.073722839	1.62583E-09	1.57848E-07	ITGA10
-1.074011803	4.64921E-05	0.000574773	C10orf10,DEPP1
-1.0741539	3.06359E-05	0.000408467	ITPKC
-1.074803352	6.36343E-08	2.80014E-06	NIM1K
-1.079090118	0.000403172	0.00325545	RGS1
-1.087613106	1.48553E-09	1.47743E-07	LIFR
-1.088093758	8.81806E-07	2.30741E-05	MGAT5B
-1.090362549	7.66031E-06	0.000133014	MT2A
-1.093467712	1.33805E-07	5.09919E-06	ANKRD20A11P
-1.093848228	2.95375E-07	9.54642E-06	bP-2189O9.2,CR381670.1
-1.095333099	6.51205E-13	3.88594E-10	RASIP1
-1.095337868	3.5398E-08	1.77106E-06	SLCO2A1
-1.100941658	4.94958E-06	9.22991E-05	DUSP2
-1.101031303	2.40281E-09	2.12859E-07	NCKAP5
-1.101493835	5.23116E-05	0.000633278	PROK2
-1.102286339	9.2773E-13	5.04397E-10	IP6K3
-1.105710983	9.13036E-10	9.67028E-08	OLFML2A
-1.107240677	1.82969E-12	8.95986E-10	PLLP
-1.10911274	1.30542E-10	2.0343E-08	PTPRB
-1.109453201	2.81665E-11	6.88831E-09	ROBO4
-1.113288879	3.87909E-10	4.81756E-08	CDH19
-1.125237465	9.98365E-09	6.71044E-07	WNT3A
-1.130819321	2.84362E-11	6.88831E-09	ZNF331
-1.136296272	1.09035E-10	1.76666E-08	SGK1
-1.136375427	2.12877E-10	2.92598E-08	LINC00961,SPAAR
-1.137413979	5.24909E-09	4.02635E-07	BTG2
-1.139140129	0.003305179	0.017367806	CXCL1
-1.141334534	1.6032E-10	2.37721E-08	CLDN5
-1.143054962	6.06513E-13	3.70974E-10	F3
-1.146542549	7.16931E-11	1.244E-08	GPM6A
-1.147045135	1.84486E-08	1.05213E-06	SOX7
-1.14804554	3.9206E-11	8.41417E-09	THBD
-1.150087357	9.62621E-11	1.60214E-08	CCDC85A,RP11-482H16.1
-1.152509689	5.06997E-07	1.48021E-05	RGS2
-1.158525467	1.65529E-07	5.98203E-06	AGER
-1.165658951	6.62598E-10	7.47056E-08	LAMC3
-1.166249275	8.22906E-08	3.45932E-06	CXCR2
-1.166412354	1.11996E-08	7.24894E-07	RIPPLY3
-1.168138504	8.72837E-05	0.000960199	OSM
-1.173175812	7.569E-09	5.44657E-07	IHH
-1.178406715	3.60748E-08	1.78749E-06	SLC25A25
-1.180171967	1.04607E-07	4.21635E-06	SSTR1
-1.181246758	3.20848E-09	2.70072E-07	BEX1
-1.181484222	7.7529E-07	2.07077E-05	EGR2
-1.184472084	6.80299E-12	2.4122E-09	FOXF1

Log-ratio	p-value	minFDR	Gene Symbol
-1.190254211	6.61154E-11	1.16373E-08	IER2
-1.194820404	1.20126E-10	1.88397E-08	GDF10
-1.195119381	3.39734E-11	7.62562E-09	C1QTNF1-AS1
-1.196555138	2.26575E-11	5.83515E-09	DUSP6
-1.201609612	0.00014422	0.001428539	PTX3
-1.206638336	1.45585E-11	4.39739E-09	KCNMB4
-1.214282036	8.93141E-08	3.69739E-06	RGS16
-1.216849327	3.67876E-07	1.12647E-05	CPB2
-1.218144417	1.49659E-12	7.79057E-10	PHACTR3
-1.218767166	2.64463E-07	8.68504E-06	NXF3
-1.223843575	7.55611E-11	1.30189E-08	CDH13
-1.227349281	4.92519E-12	1.85384E-09	SDPR,CAVIN2
-1.22796154	1.39807E-07	5.27859E-06	AGRP
-1.228985786	0.005896409	0.027478389	SELE
-1.229832649	6.4062E-12	2.31712E-09	FAM189A1
-1.23188591	2.67879E-15	4.68138E-12	S1PR1
-1.237390518	2.56744E-12	1.15155E-09	TIMP3
-1.239431381	4.65599E-08	2.19332E-06	XXbac-BPG300A18.13
-1.244757652	0.000784211	0.005522888	CT45A10
-1.248085022	3.07776E-07	9.85607E-06	SLC19A2
-1.250460625	3.42616E-06	6.86522E-05	RETN
-1.25178051	2.82219E-06	5.87641E-05	SYNPO2L
-1.253267288	9.54637E-06	0.00015824	MYC
-1.255192757	3.44032E-11	7.65189E-09	C10orf67
-1.257173538	7.66428E-07	2.05158E-05	SMAD6
-1.262506485	8.89454E-16	2.41793E-12	RGCC
-1.267017365	2.19141E-10	2.99525E-08	SGCG
-1.269378662	6.22448E-07	1.74044E-05	SLC5A9
-1.274327278	8.13962E-13	4.526E-10	TMEM88
-1.274440765	1.55571E-06	3.62494E-05	APOH
-1.274500847	4.08614E-10	5.02369E-08	KLF9
-1.277770042	3.03862E-10	3.93348E-08	SOX17
-1.281978607	1.04503E-09	1.08799E-07	GCOM1
-1.282022476	0.001519463	0.009296118	CT45A3,CT45A5,CT45A7, CT45A9,CT45A2,CT45A8, CT45A1,CT45A6
-1.285447121	7.50279E-10	8.15837E-08	HECW2
-1.288219452	1.12446E-07	4.46606E-06	F2RL3
-1.290509224	4.52033E-13	3.07206E-10	GPR4
-1.293181419	2.75811E-12	1.16345E-09	BAALC
-1.29431057	9.04101E-10	9.61727E-08	MYZAP
-1.295077324	2.79593E-05	0.000378481	GRM8
-1.298112869	4.60587E-09	3.64683E-07	SERTM1
-1.300457954	3.68105E-15	5.62879E-12	KLF2P1,KLF2P2,KLF2P4
-1.304333687	8.25825E-12	2.76776E-09	JUND
-1.306818008	2.13957E-17	8.72445E-14	IRS2
-1.314865112	6.81683E-07	1.87034E-05	S100A12

Log-ratio	p-value	minFDR	Gene Symbol
-1.318433762	1.41208E-11	4.37317E-09	PPP1R15A
-1.31925869	1.9669E-06	4.37475E-05	MT1M
-1.32516098	4.82445E-13	3.11609E-10	SPRY4
-1.331969261	8.06324E-05	0.000903689	C2CD4B
-1.347049713	5.7489E-06	0.000104808	TMSB15A
-1.349451065	3.37516E-07	1.06003E-05	SEMA3G
-1.349931717	1.51816E-09	1.49849E-07	C11orf96
-1.351055145	1.69072E-06	3.86951E-05	RASD1
-1.353055954	6.46567E-07	1.7915E-05	PTGS2
-1.356583595	1.15253E-10	1.843E-08	FIBIN
-1.359541416	5.42349E-06	9.9918E-05	BRINP1
-1.360461235	1.64007E-09	1.57976E-07	FENDRR
-1.362214088	2.15509E-08	1.19561E-06	NAMPTP1
-1.381303787	1.43024E-10	2.18702E-08	MATN3
-1.383310318	1.81416E-10	2.59982E-08	ERRFI1
-1.395345688	1.73787E-17	8.72445E-14	KLF2
-1.40222168	0.000100141	0.001068952	BTNL8
-1.403780937	1.45541E-15	3.49895E-12	KLF2P3
-1.404700279	2.98889E-14	3.1794E-11	RHOB
-1.420445442	1.0417E-08	6.8882E-07	INHBA
-1.420607567	2.75122E-11	6.83574E-09	FAM167A
-1.4316082	2.59925E-09	2.24711E-07	GPIHBP1
-1.433699608	3.56054E-08	1.77418E-06	IL20RA
-1.440769672	1.80693E-05	0.000267767	NR4A1
-1.447304726	7.81929E-05	0.000880381	AGTR2
-1.454687119	1.16988E-14	1.43112E-11	STXBP6
-1.475019455	1.12923E-05	0.000181881	SLCO4A1
-1.477529526	2.69821E-12	1.16345E-09	SP6
-1.49052906	2.41812E-08	1.30312E-06	CCK
-1.49789238	5.67814E-14	5.55685E-11	FIGF,VEGFD
-1.498827934	6.07859E-11	1.10162E-08	CYP3A7
-1.501476288	9.20126E-07	2.37717E-05	AZGP1
-1.501912594	4.20581E-05	0.000528347	FAM71A
-1.504670143	0.000152188	0.001485807	AREG
-1.509148598	3.22932E-12	1.31681E-09	NDRG4
-1.513878822	1.71616E-15	3.49895E-12	JUN
-1.527457237	8.85053E-05	0.000969388	CXCL8
-1.529819489	2.32312E-11	5.92056E-09	CYP3A5
-1.541508675	3.50931E-07	1.08958E-05	APLN
-1.543532372	1.85208E-10	2.60419E-08	SNAI1
-1.552172661	6.54947E-11	1.16115E-08	GADD45B
-1.555409431	1.64814E-07	5.97383E-06	ZBTB16
-1.557231903	2.76603E-11	6.83574E-09	AC129492.1,PER1
-1.563394547	5.09831E-11	1.01411E-08	BTNL9
-1.56778717	5.52832E-11	1.05243E-08	EDN1
-1.574362755	1.24311E-16	4.34485E-13	GRASP
-1.587080956	1.80696E-10	2.59982E-08	CA4

Log-ratio	p-value	minFDR	Gene Symbol
-1.591917038	1.53846E-07	5.68618E-06	ITLN2
-1.595482826	1.25667E-05	0.000198487	RND1
-1.621752739	6.3249E-14	5.95173E-11	ADRB1
-1.62448597	0.000159251	0.001539407	NR4A3
-1.627428055	1.41692E-09	1.42075E-07	PNMT
-1.643855095	0.002451519	0.013662609	ADAMTS4
-1.644469261	2.06602E-12	9.72061E-10	CTGF,CCN2
-1.647453308	1.90037E-07	6.6326E-06	EGR3
-1.648776054	0.000147437	0.001454512	CHIAP2
-1.664306641	4.74949E-15	6.45561E-12	HHIP
-1.708102226	3.11861E-11	7.19811E-09	SYN2
-1.726131439	6.71876E-10	7.54043E-08	MAFF
-1.732595444	1.55679E-10	2.32246E-08	ADAMTS1
-1.740005493	2.56033E-09	2.23718E-07	TMEM100
-1.754311562	7.10382E-07	1.93113E-05	APOLD1
-1.797333717	3.608E-09	2.95229E-07	FCN3
-1.815040588	8.13015E-07	2.15273E-05	LINC00551
-1.829701424	1.93796E-09	1.80282E-07	CXCL3
-1.831422806	7.99716E-11	1.34937E-08	JUNB
-1.834760666	1.84697E-10	2.60419E-08	ANKRD1
-1.84044838	3.01607E-11	7.15844E-09	SIK1,CH507-42P11.8,SIK1B
-1.87036705	2.4323E-13	1.80007E-10	CYR61,CCN1
-1.926895142	1.6419E-08	9.79771E-07	CHRM1
-2.00637722	1.72764E-07	6.21594E-06	SERPINE1
-2.029716492	2.9824E-13	2.08478E-10	EGR1
-2.057840347	3.20971E-16	9.81608E-13	FAM107A
-2.092997551	2.57995E-11	6.50732E-09	VIPR1
-2.155185699	9.15693E-09	6.3108E-07	C8B
-2.1742239	1.79888E-17	8.72445E-14	HBEGF
-2.21232605	6.57943E-10	7.45243E-08	ATF3
-2.285676956	0.000600884	0.004476622	FGG
-2.302476883	7.45517E-20	9.11991E-16	KLF4
-2.329281807	1.32881E-13	1.13696E-10	NR4A2
-2.339277267	6.32043E-10	7.22596E-08	SOCS3
-2.39358902	8.68133E-18	7.07991E-14	ARC
-2.411261559	4.9672E-13	3.11609E-10	ZFP36
-2.527475357	4.58104E-20	9.11991E-16	DUSP1
-2.553723335	3.35169E-11	7.59282E-09	CXCL2
-2.615704536	3.00464E-15	4.90078E-12	CSRNP1
-2.71426487	8.82429E-08	3.66874E-06	IL6
-2.765246391	5.94577E-11	1.08559E-08	IL1RL1
-3.012392044	4.39808E-11	9.15289E-09	SLC6A4
-3.10669899	7.14658E-14	6.47586E-11	FOS
-3.166447639	1.83962E-08	1.05159E-06	CSF3
-4.680952072	2.41234E-15	4.54003E-12	FOSB

Publications arising from this thesis

Sendama, W. (2020). 'The effect of ageing on the resolution of inflammation'. *Ageing Research Reviews*, 57, 101000.

Sendama, W. (2020). 'L1000 connectivity map interrogation identifies candidate drugs for repurposing as SARS-CoV-2 antiviral therapies'. *Computational and Structural Biotechnology Journal*, 18, pp. 3947–3949.

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