The role of Siglec-F as a regulator of alveolar macrophage function and adaptation

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Abstract

Alveolar macrophages are found in a truly unique microenvironment of the lung alveoli, where they are in close contact with the respiratory epithelium. This close contact with the epithelium is essential as it is how these highly inflammatory cells (cells that respond quickly to immune response as dysregulation of homeostasis exacerbates pathology) are regulated to prevent excessive inflammation, which otherwise would damage the lung, possibly leading to Asthma or COPD. Meaning that in the absence of pathology, the inflammatory response of alveolar macrophages are limited. Interaction with the lung occurs when macrophages bind to the epithelium through a specific receptor such as CD200R and possibly Siglec-F. Siglec-F is a lectin on the surface of macrophages that binds glycoconjugates containing sialic acid on the lung epithelium and on mucins, which is speculated to negatively regulate alveolar macrophages. Though the specific role of Siglec-F as a regulator of macrophage is not fully understood.

The aim of this project is to explore changes in gene expression and functional outcomes of signalling through Siglec-F. An exogenous system was used to stimulate this receptor using antibody crosslinking, which is then confirmed by analysing recruitment of SHP-1 in western blot. We also aim to measure differences in cytokine production using a multiplex kit which can simultaneously measure several different cytokines. Lastly, we aim to use RNA sequencing to analyse changes in gene expression. This data will then be used to propose functional outcomes of Siglec-F signalling; improving the understanding of the role of the receptor in negative regulation of alveolar macrophages.

The western blot results showed successful recruitment of SHP-1 and thus successful targeting of Siglec-F. We also observed a reduction in proinflammatory cytokines TNF-α, IL-6, CXCL1 and anti-inflammatory cytokine TGF-β1 when crosslinked with Siglec-F in the presence of a known strong stimulator of the innate immune response (LPS). This reduction of cytokines indicates a negative regulatory function of the Siglec-F receptor, though potential functional effector functions of Siglec-F is not understood. Looking at changes in expression we found interesting putative targets of the Siglec-F signalling pathway including immune related genes such as *TREM2*, *Axl*, and *IL-6* as well as other genes associated with RNA synthesis and the control of protein translation. This study will provide information about the effector functions of Siglec-F and creates new avenues for future study.

Statement of Originality

There is evidence to support the role of CD200R, TGF-β and IL-10 as regulators of homeostasis, however there is only partial evidence for Siglec-F. In order to gather more evidence for the role of Siglec-F as a macrophage regulator; the changes in whole genome expression in alveolar macrophages after stimulation with crosslinked Siglec-F will be explored. It is then possible to link changes in genome expression to effector functions. These effector functions could overlap with existing regulatory functions or could be new regulatory functions not currently associated with other regulators.

This research aims to investigate the outcome of signalling through Siglec-F in mouse alveolar macrophage by whole genome expression using a highly specific antibody crosslinking system, with the possibility of creating new avenues for specialised treatment in diseases such as COPD and asthma.

Declaration

I declare that this thesis has been composed solely by myself and that no material contained in this thesis has been used in any other submission for an academic award. Except where otherwise stated by reference or acknowledgment, the work presented is entirely my own.

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Abbreviations List

SF	Siglec-F
Ig	Immunoglobulin
COPD	Chronic Obstructive Pulmonary Disease
CD	Cluster of Differentiation
SHP-1	Src Homology region 2 domain-containing Phosphatase-1
SOCS3	Suppressor of Cytokine Signalling 3
IL	Interleukin
TNF-α	Tumour Necrosis Factor alpha
TGFβ	Transforming Growth Factor beta
IFN-γ	Interferon gamma
GCSF	Granulocyte Colony Stimulating Factor
MARCO	Macrophage Receptor with Collagenous Structure
ITIMs	Immunoreceptor Tyrosine-based Inhibitory Motifs
ITAMs	Immunoreceptor Tyrosine-based Activation Motifs
LTB4	Leukotriene B4
HRP	Horseradish Peroxidase
IBMS	Institute of Biomedical Science
RNA-seq	Ribonucleic acid - Sequencing
DAP	DNAX activation protein
BAL	Bronchoalveolar Lavage
NPL	Naïve Peritoneal Lavage
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione
LPS	Lipopolysaccharide
UNS	Unstimulated
IC	Isotype Control
PRRs	Pathogen Recognition Receptors
DAMPs	Damage-Associated Molecular Patterns
PAMPs	Pathogen Associated Molecular Patterns
RPKM	Reads Per Kilobase of transcript per Million mapped reads
FLDAM	Foetal Liver Derived-Alveolar Macrophage-like cells
PCA	Principal component analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
RCF	Relative Centrifugal Force
UCL	University College London
ACK	Ammonium-Chloride-Potassium

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1. Introduction

1.1 An introduction into resident macrophages

Tissue resident macrophage function is tailored to the tissue/organ they reside in; this allows macrophages to become specialised to the tasks required by the microenvironment. For example, Kupffer cells whose primary function is to remove protein complexes, small particles, dying red blood cells, and cell debris from portal blood flow *(Kawada and Parola, 2015).* Whereas osteoclasts have become so specialised that they are the only cell capable of bone degradation *(Ross, 2011)*. Alveolar macrophages are another type of resident macrophages which are specialised to live outside the body in the lumen of the lung alveoli. These cells have also adapted to be highly inflammatory sentinels *(Mathie et al., 2014)* as well as efficient removers of dust particles, pathogens, pollutants and dead cells that could interfere with the diffusion of gases. The clearance and recycling of the cells being essential to the host survival *(Mosser and Edwards, 2008)*.

Macrophages are characterised by their diversity and plasticity, allowing macrophages in tissues to respond to different environmental triggers such as: microbes, dead or damaged cells, cytokines produced by activated leukocytes and epithelial cells with distinct functional phenotypes *(Hussell and Bell, 2014)*. This ability to adapt and to carry out several different functions led to the now limited and broad classification of the two main types of polarised macrophages: the classically activated M1 macrophage and the alternatively activated M2 macrophage. Exposure to pro-inflammatory molecules such as IFN-γ has a strong association with classically activated macrophage phenotypes (M1) (*Mosser and Edwards, 2008)*. These macrophages are responsible for host protection against intracellular bacteria or viruses *(Martinez, 2008; Wang et al., 2018).* In comparison, alternatively activated macrophages (M2) are split into four sub populations. M2a macrophages are induced by IL-4 and IL-13 *(Mantovani et al., 2004)* and express high levels of the mannose receptor, IL-R and CCL17. Also named wound-healing macrophages they secret TGF-β, insulin-like growth factor and fibronectin allowing M2a macrophages to contribute to tissue repair. M2b macrophages are induced by combined exposure to immune complexes and TLR agonists or by IL-1R agonists *(Mantovani et al., 2004).* A primary feature of M2b macrophages is that they express and secrete large amounts of IL-10 *(Anderson and Mosser, 2002; Yue et al., 2017)* with these macrophages having a regulatory function. M2c macrophages (acquired deactivation macrophages) are induced by IL-10. These macrophages are primarily anti-inflammatory, accomplished by releasing large amounts of IL-10 and TGF-β *(Mantovani et al., 2004; Rőszer, 2015).* Lastly M2d macrophages are induced by co-stimulation with TLR ligands and the A2 adenosine receptor *(Wang et al., 2010).* M2d cells produce high levels of IL-10, TGF-β, vascular

endothelial growth factor, and contribute to the angiogenesis and cancer metastasis in neoplastic tissue *(Ferrante and Leibovich, 2012)*.

Interestingly research has shown that alveolar macrophages fall into neither category with a large proportion of resting alveolar macrophages expressing a duo M1/M2 phenotype *(Mitsi et al., 2018;* characteristics of M1 and M2 macrophages is shown in table 1), with alternate classification being suggested in 2008 (*Mosser and Edwards, 2008)*. This proposed classification (shown in figure 1) is based on three fundamental macrophage functions involved in maintaining homeostasis: host defence, wound healing and immune regulation (*Mosser and Edwards, 2008)*. This concept of nomenclature insists on being shades of activation which results in a spectrum of macrophage characteristics instead of two defined categories.

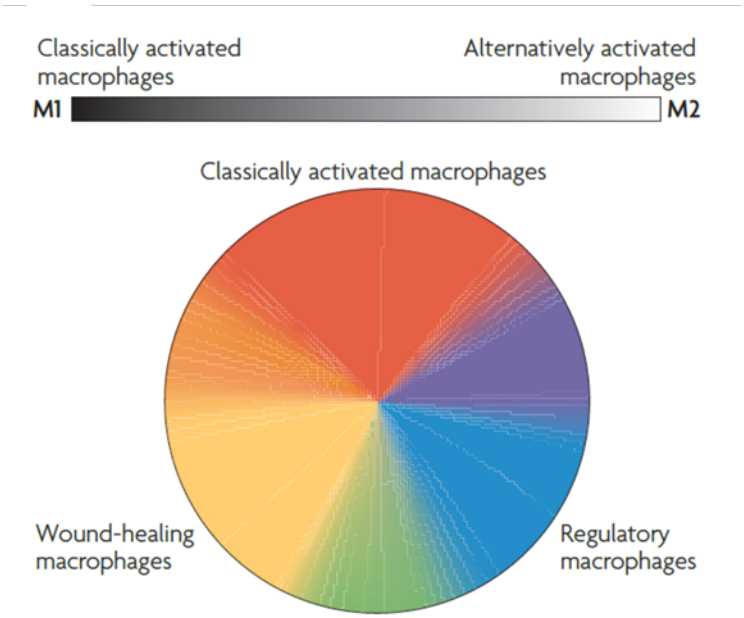


Figure 1: Colour pin wall of macrophage characteristics. A depiction of the questionable nomenclature of the M1 and M2 macrophage designations, followed by a pin wheel graphic. The pin wheel graphic suggests a spectrum of macrophage function, and that classically activated macrophages may also have many characteristics associated with wound-healing macrophages (*edited from Mosser and Edwards, 2008)*.

Table 1: This table contrasts the characteristics of the two types of macrophages *(Gordon, Plüddemann and Martinez Estrada, 2014)*. As mentioned previously this classification of macrophages should be seen as less definitive as macrophages have been observed to show a M1/M2 duo phenotype.

M1 Macrophages	M2 Macrophages
Classically activated by IFN-γ or LPS.	Alternatively activated by exposure to certain cytokines such as IL-4, IL-10, or IL-13.
Produce proinflammatory cytokines, phagocytize microbes, and initiate an immune response.	Produce polyamines to induce proliferation, and proline to induce collagen production.
Responsible for killing of intracellular pathogens.	Associated with tissue repair and building the extracellular matrix.
Responsible for tumour resistance.	Responsible for encapsulation and killing of extracellular parasites.
Require antigen presentation.	Does not require antigen presentation.

Macrophages have several effector functions including the ability to produce relatively large amounts of cytokines and chemokines involved in the immune response, commonly referred to as pro-inflammatory or anti-inflammatory cytokines (*Arango Duque and Descoteaux, 2014*). Cytokines are small proteins that are released from an activated macrophage where they can act on distant cells (endocrine) or in some instances act on themselves (autocrine) or nearby cells (paracrine; *Arango Duque and Descoteaux, 2014*). Examples of cytokines produced by alveolar macrophages include: TNFα, a cytokine involved in systemic inflammation and is present in the acute phase *(Idriss and Naismith, 2000)*; Granulocyte colony stimulating factor (G-CSF) known to induce differentiation of bone marrow stem cells to neutrophils and activate mature neutrophils (*Roberts, 2005*); IL-6, which in synergy with IL-4 and IL-13 enhances phosphorylation of M2 macrophages (*Fernando et al., 2014*); IL-12, a promotor of Th1 responses and inducer of IFNγ production by T and NK cells (*Arango Duque and Descoteaux, 2014*); IL-27, which amplifies cytokine production is response to gram negative bacteria (*Petes et al., 2018*); IL-1β, a cytokine vital in maintaining host response to infection and injury (*Dinarello, 1996*); CXCL1 a chemokine essential in neutrophil recruitment (*Altmann et al., 2012*) and TGF-β, an anti-inflammatory cytokine involved in the resolution phase of tissue injury (*Khalil et al., 1989*). As alveolar macrophages are mostly pro-inflammatory cells it is possible to measure production of these cytokines as an assessment of their activity (*Arango Duque and Descoteaux, 2014).*

Macrophages play an essential role in phagocytosis which is the ingestion of large pathogens (>0.5μm) namely bacteria, dead cells or other material by phagocytes *(Aderem and Underhill, 1999),* another key effector function. This is commonly mediated by pattern recognition receptors (PRRs) interacting with pathogen associated molecular patterns (PAMPs). Once inside the macrophage the pathogen is enclosed in a phagosome which fuses with a lysosome containing: enzymes, Reactive Oxygen Species and Reactive Nitrogen Species which digest the pathogen. Products of this process are either used by the macrophage or removed by exocytosis.

Macrophages are also key mediators of efferocytosis, the process of engulfing and digesting dead, dying or stressed cells (*Grabiec et al., 2018*). This process ensures rapid clearance of dead cells preventing the release of DAMPS, which if not removed would be recognised by PRRs. This process is particularly important in alveolar macrophages to prevent excess neutrophil recruitment into the alveoli (*Mahida and Thickett, 2018*).

1.2 Alveolar macrophages

Residing in the alveolus of the lung, alveolar macrophages are the first line of the cellular immune response of the lung microenvironment (*Mathie et al., 2014)*. It is due to their location that these macrophages are tasked with removing pathogens as well as harmful air pollutants which if not removed, would have a major impact in lung function (*Sibille and Reynolds, 1990; Gonzalez et al., 2018*). Alveolar macrophages can remove these toxic and infectious particles from the lung by secretion of oxygen metabolites, proteases and lysozymes coupled with processes such as phagocytosis *(Rubins, 2003)*. When faced with large amounts of infectious particles these macrophages are able to produce a large variety of inflammatory cytokines and chemokines, which recruit other immune cells, such as neutrophils, to the area *(Arango Duque and Descoteaux, 2014).*

Alveolar macrophages express both Axl and MERTK receptors linked to clearance of apoptotic cells (*Grabiec et al., 2018*). The requirement to efficiently remove apoptotic cells is particularly important to alveolar macrophages due to their highly inflammatory nature, which otherwise would induce secondary necrosis and the release of cellular components (*Grabiec et al., 2018*). Impaired clearance of apoptotic cells would cause accumulation of secondary necrotic cells prolonging inflammation, a possible cause of: Acute Respiratory Distress Syndrome (*Mahida and Thickett, 2018*) cystic fibrosis, COPD and asthma (*Vandivier, Henson and Douglas, 2006*).

The diversity of surface receptors is a well-documented trait of macrophage tissue specificity that is associated with functional outcomes (*Davies et al., 2013*). Alveolar macrophages are able to act as sentinels of the respiratory mucosal surfaces (*Mathie et al., 2014*), maintain immunological homeostasis (*Holt et al., 2008*) and quickly respond to PAMPs such as LPS and peptidoglycan to become highly inflammatory. These characteristics are most likely the result of the high levels of CD68, CD11c, MARCO, TLR 2 and 4 which are expressed as the result of tissue adaptation, and are not present in such levels by macrophages from other sites (*Davies et al., 2013*).

Alveolar macrophages are known to be highly inflammatory cells (*He et al., 2016*) capable of self-renewal (*Tarling, Lin and Hsu, 1987; Zasłona et al., 2014*). A possible reason for alveolar macrophages being described as highly inflammatory is because TLR4-LPS signalling increases the surface expression of IL-1RI (*He et al., 2016*). The upregulation of IL-1RI sensitises alveolar macrophages to IL-1β resulting in alveolar macrophage pyroptosis, exaggerating lung inflammation (*He et al., 2016*).

However as alveolar macrophages reside in such a delicate tissue, their activation needs to be closely regulated by contact with epithelial cells (*Hussell and Bell*). Epithelial cells provide negative signals by expressing CD200, which binds to CD200R present on alveolar macrophages (*Hatherley and Barclay, 2004; Snelgrove et al., 2008*). Macrophages can also receive negative signals when exposed to anti-inflammatory cytokines produced by or tethered onto epithelial cells, such as TGF-β and IL-10 (*Morris et al., 2003*; figure 2). This negative signalling is essential, to avoid unnecessary or dysregulated inflammation. Excess inflammation will damage the delicate surrounding tissue leading to a loss of function (*Knapp et al., 2003*).

Siglec-F is another alveolar macrophage cell surface receptor thought to be involved in alveolar macrophage negative regulation. However, there is only partial evidence to support this hypothesis (*Feng and Mao, 2012;* figure 2).

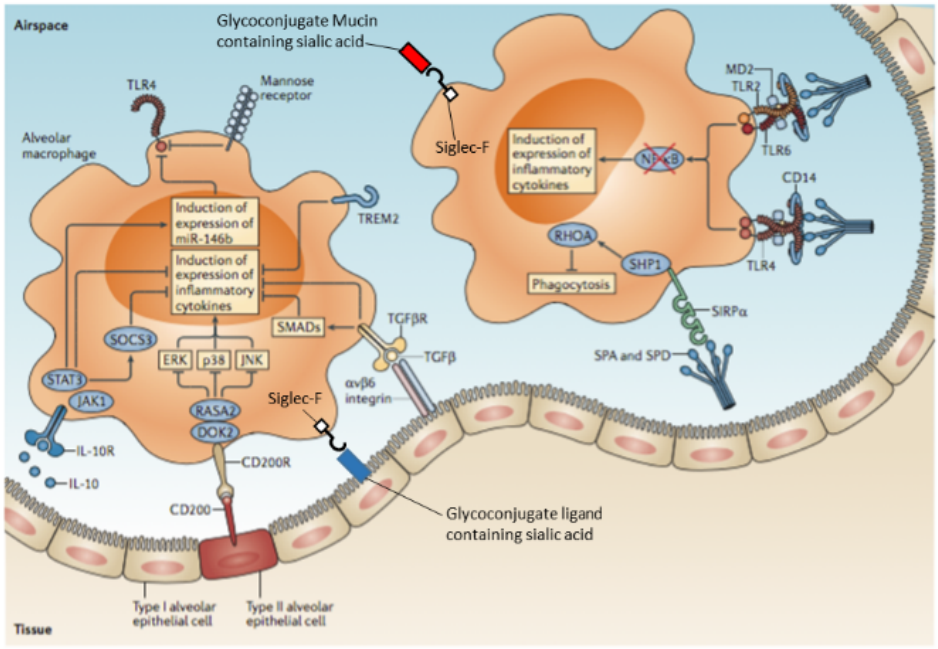


Figure 2: Alveolar macrophages close contact with the respiratory epithelium. This figure shows how Siglec-F binds to the respiratory epithelia (blue) as well as soluble glycoconjugates mucins (red; *edited from Kiwamoto et al., 2013; Hussell and Bell, 2014; Janssen et al., 2016).*

1.3 Alveolar macrophages in COPD

COPD is an umbrella term for emphysema (damage to the alveoli) and bronchitis (long term inflammation of the bronchial tubes) both resulting in breathing difficulties as a result of airflow restriction which is not fully reversible (*Vogelmeier et al., 2017*). The primary trigger of COPD is inhalation of tobacco smoke caused by smoking (*Eapen et al., 2017*). Tobacco smoke is a mixture of solid and liquid particles and includes many well-characterised toxins and carcinogens (*Smith and Fischer, 2001*). Long term exposure to tobacco smoke can result in a 5-10 fold increase in the number of alveolar macrophages, demonstrated in patients diagnosed with COPD (*Tetley, 2002*), with there being a direct correlation between macrophage number and COPD severity (*Di Stefano et al., 1998*). Alveolar macrophages are activated by the tobacco smoke and other irritants found in the smoke resulting in production of pro-inflammatory mediators increasing macrophage number (*Davis et al., 1988; King, Savici and Campbell, 1988; van der Vaart et al., 2004*). These activated alveolar macrophages secrete proteases leading to destruction of the lung parenchyma (*Barnes, Shapiro and Pauwels, 2003).*

As a result of the high rate of inflammation, the antibacterial capabilities of alveolar macrophages are reduced and this explains why patients with COPD are more susceptible to bacterial infections (*Monsó et al., 1995*). These “low-quality macrophages” have impaired phagocytosis which allows for easier bacterial colonisation in the lung. Impaired efferocytosis is also a key factor in COPD (*Hodge et al., 2007*) as it results in continued recruitment of neutrophils further increasing the severity of bronchitis. A chronic effect of cigarette smoke is

the depletion of the GSH stores in the lung (*Muller and Gebel, 1998*), a major antioxidant leading to increased oxidative stress in alveolar macrophages.

GM-CSF is a cytokine required for alveolar macrophage function and pulmonary homeostasis (*Trapnell and Whitsett, 2002*). The importance of this cytokine is demonstrated using GM-CSF knockout-mice where the activity and maturation of alveolar macrophages are impaired resulting in a phenotype similar to emphysema or pulmonary alveolar proteinosis (*Stanley et al., 1994*). The impairment of alveolar macrophage maturation allows for infection with a wide range of opportunistic bacterial and fungal species (*Stanley et al., 1994*). GM-CSF knockout-mice are characterised by an increased accumulation of lung surfactant when compared to alveoli in wildtype (*Dranoff et al., 1994*). This increased surfactant enlarges the alveolar structures resulting in a similar histology to emphysema (*Yoshida and Whitsett, 2006*). It is therefore evident that GM-CSF plays a critical role in the pulmonary homeostasis of alveolar macrophages (*Dranoff et al., 1994*).

1.4 Alveolar macrophages in asthma

Asthma is a syndrome in which the airways become chronically inflamed due to an overreaction to non-harmful allergens but can also result from irritant chemicals, cold and exercise (*Martinez and Vercelli, 2013*). The inflammation leads to thickening of the bronchi due to smooth muscle contraction and increased mucus production resulting in shortness of breath (*Niimi et al., 2003; Dey and Bloom, 2004*).

Alveolar macrophages serve in the initial response after allergen exposure having an enhanced capacity for phagocytosis. In Asthmatics, alveolar macrophages promote the production of higher levels of the proinflammatory Th-2 cytokine and IL-5 by CD4 T-cells *(Tang et al., 1998),* leading to enhanced recruitment of eosinophils and the pathology seen in eosinophilic asthma. Mathie et al (2014) showed, using a house dust mite mouse model of asthma, that depletion of alveolar macrophages with clodronate liposomes, increases IL-13 eosinophilic and Th-2 inflammation and reduces resolution in an IL-27-driven manner *(Mathie et al., 2014)*. The data in this report might be partially confounded by an incomplete depletion of alveolar macrophages and the potential inflammatory response normally associated with clodronate treatment.

Alveolar macrophages from asthmatic patients also demonstrate a higher rate of apoptosis as well as an impaired phagocytic ability *(Fitzpatrick et al., 2008).* Additionally, the epithelium of asthmatic patients produces greater levels of GM-CSF when compared to healthy controls *(Sousa et al., 1993).* Altogether, this data highlights the potential role of an allergic airway disease in altering the turnover of alveolar macrophages and their ability to remove pathogens

and particulates from the lung; possibly resulting in an altered microbial environment *(Fricker and Gibson, 2017).*

Alveolar macrophages in asthmatic patients are also defective in efferocytosis, resulting in elevated levels of dead cells and DAMPs in the upper and lower airways *(Simpson et al., 2012).* This causes a continued state of activation of innate immune cells via PRRs binding to DAMPs, as well as a sustained production of proinflammatory cytokines such as TNF-α, IL-1β, IL-16 and IL-18 *(Ackerman et al., 1994).*

1.5 Siglec-F

Siglecs are immunoglobulin -like cell-surface lectins that bind to glycoconjugates containing sialic acids as well as secreted glycoproteins. There are 14 different Siglec receptors in humans (*Crocker, Paulson and Varki, 2007*), expressed in varying amounts on immune cells, however they do exist on other mammals including mice where they follow a lettering system (*Crocker, Paulson and Varki, 2007*).

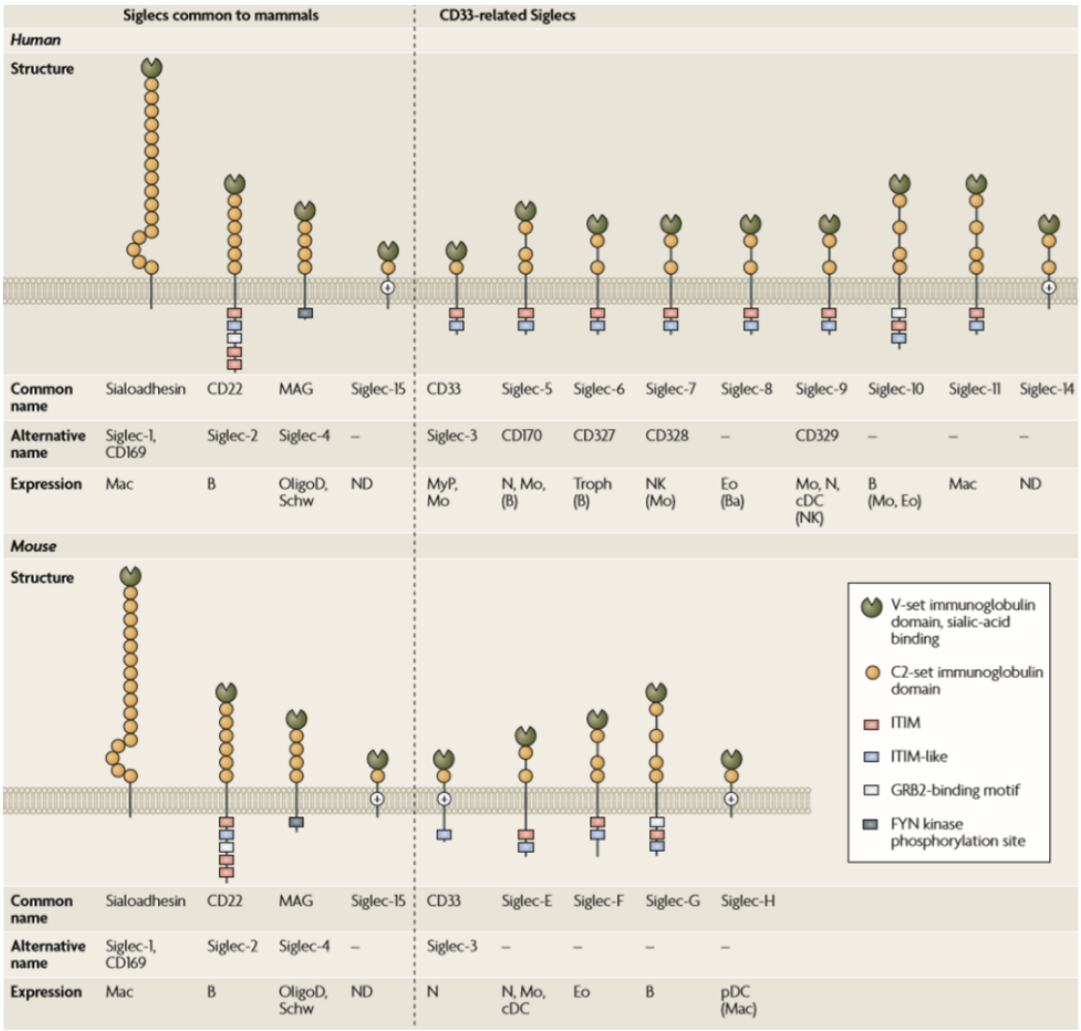
Human Siglec-5 to 11 are CD33-related Siglecs, which can be identified by the ITIM and/or ITIM like motifs in their cytoplasmic domains. Thus, these Siglecs are thought to have a primarily regulatory role. In contrast, Siglec-4, 14, 15 and 16 do not have an ITIM or ITIM like motif, but instead signal through the association of DAP12 and are therefore called activating Siglecs. DAP12 associates with the ITAM present in the cytoplasmic tail of these activatory Siglecs resulting in PI3k recruitment *(Angata et al., 2007)*. These functional characteristics are broadly conserved in both humans and mice (*Crocker, Paulson and Varki, 2007*). Siglec-F and its human paralog Siglec-8 are found on the cell surface of alveolar macrophages in the lung, where they act as regulators of the activity of these cells *(Angata et al., 2006)*. It putatively does this by binding to the epithelial of the lung or soluble ligands in mucins and collectins *(Janssen et al., 2016).*

Although the specific role of Siglec-F as a regulator of alveolar macrophage function is not fully understood *(Feng and Mao, 2012),* it has been shown to act as a negative regulator similar to TGF-β, CD200R and IL-10 *(Morris et al., 2003).* Potential outcomes of Siglec-F signalling could be an increase in transcription of genes already associated with negative regulatory pathways such as Smad7 in TGF-β signalling *(Yan, Liu and Chen, 2009)*.

In an in vivo environment, Siglec-F ligands are glycoconjugates containing sialic acids (*Janssen et al., 2016*). These molecules are long and complex and could be signalling other cell surface proteins simultaneously. Recent developments have shown that Siglecs can be more specifically targeted using crosslinked monoclonal antibodies specific for a Siglec receptor, thus mimicking

natural signalling *(Zhang et al., 2007; Feng and Mao, 2012)*. This system has been previously established using flow cytometry but literature is yet to explore whether successfully targeting Siglec-F using monoclonal antibodies results in intracellular signalling.

Table 2: This table contrasts Siglecs present on human cells with those found on mice cells (*Crocker, Paulson and Varki, 2007*). All known Siglecs can be divided into two groups according to their structure and presence of similar genes, portions of genes, or chromosome segments between different species. The CD33 related Siglecs often share similar extracellular structure and often contain tyrosine-based signalling motifs in their intracellular domains *(Crocker, Paulson and Varki, 2007)*. This is displayed in Siglec-F (humans) and Siglec-8 (mice) where they are described as paralogs (*Crocker, Paulson and Varki, 2007*). The other group of Siglecs consisting of sialoadhesin, CD22, myelin-associated glycoprotein and Siglec-15 often have lower similarity in their structure between mammals (*Crocker, Paulson and Varki, 2007*).



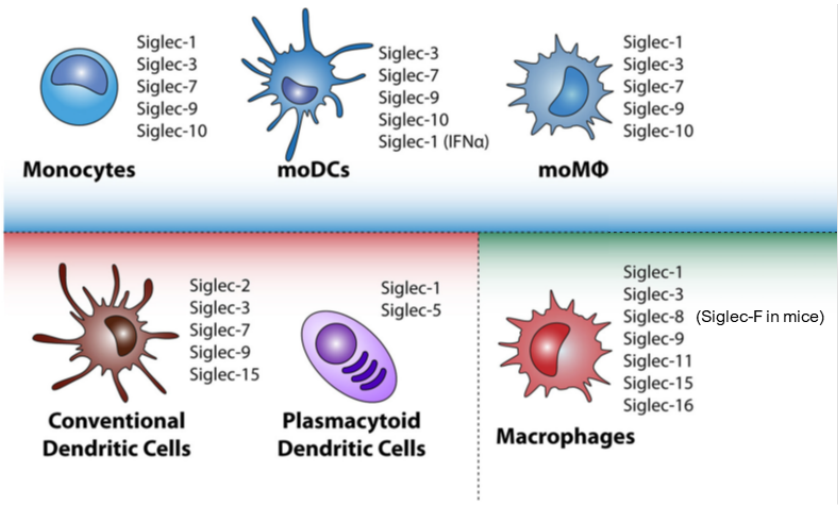


Figure 3: The different Siglecs expressed in five types of human immune cells *(edited from Lübbers et al., 2018).* Shown with a blue background are monocytes and monocyte derived dendritic cells (moDCs) and monocyte derived macrophages (moMQ). Highlighted in red are types of dendritic cells that appear in peripheral blood and lastly in green are macrophages. Differences in microenvironment could lead to alternated Siglec expression.

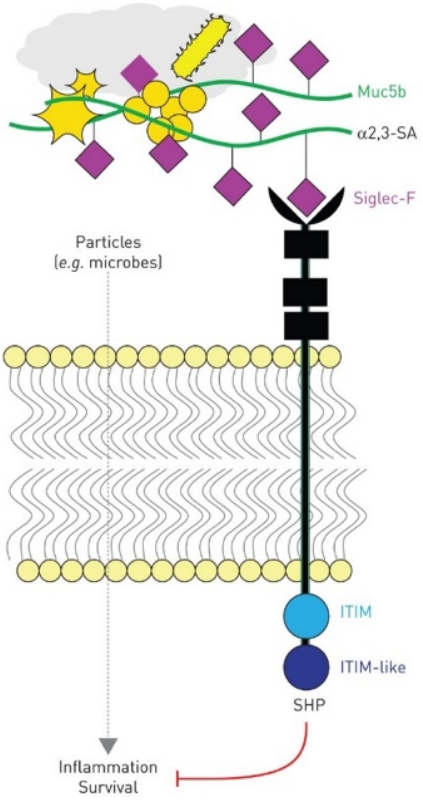


Figure 4: Artists impression of the Siglec-F receptor (Janssen et al., 2016). α2,3-sialic acid on the epithelium protein Muc5b, binds to the N-terminal lectin domain of the Siglec-F receptor. This drives ITIM and ITIM-like domain activation which recruit SHP enzymes that suppress kinase-activated inflammatory signals.

The inhibitory function of Siglec-F is likely caused by the presence of ITIMs in the cytoplasmic tail of Siglec-F shown in figure 4 (*Angata et al., 2006*). ITIMs work by suppressing the activation signals that come from ITAMs, through recruitment of phosphates and tyrosine to the ITAMs *(Khatua, Roy and Mandal, 2013).* The signalling pathway of ITIMS is initiated by the

binding of a sialylated domain to the Siglec-F receptor; leading to phosphorylation of the ITIM in the cytoplasmic tail of the receptor by SRC-family tyrosine kinases (*Tourdot et al., 2013*). SHP1 and SHP2 protein then bind to the Siglec, becoming activated (*Tourdot et al., 2013*). This initiates several functional outcomes shown on figure 5. Recruitment of SHP-1 to Siglec-F would therefore be a way to confirm intracellular signalling.

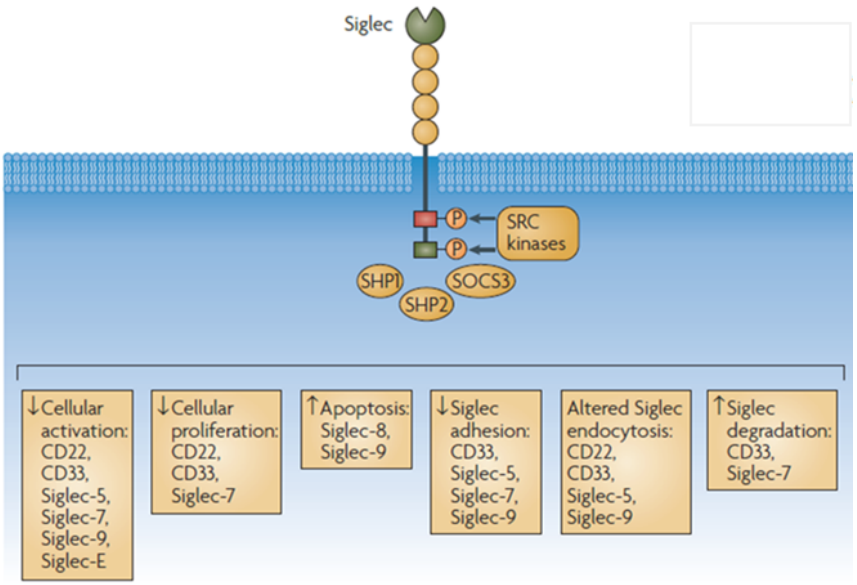


Figure 5: Proposed intracellular signalling pathway for CD33-related Siglecs. *(edited from Crocker, Paulson and Varki, 2007).* This figure shows the proposed intracellular pathway for ITIM, the intracellular signalling portion of CD33-related Siglecs.

Recent evidence (*Janssen et al., 2016*) suggests that there are functional links between alveolar macrophage function and the mucus present in the airways. This is demonstrated by the large number of immunoglobulins and complement found in secreted mucus (*Janssen et al., 2016*), from this it can be hypothesised that mucus is an important carrier of these defensive molecules and that it allows for the immune response to spread faster. There are also links being made between secreted mucins and alveolar macrophages evident by their coordination when resolving inflammation and physical binding of mucins and Siglecs (*Janssen et al., 2016*). Interestingly, alveolar macrophages activated by inflammation decrease in their Siglec-F surface expression *(Janssen et al., 2016),* possibly due to there not being a need for negative regulation. Siglec-F expression increases during the resolution of inflammation alongside apoptosis *(Janssen et al., 2016).*

1.6 The functional role of Siglec-F in alveolar macrophages

The specific role of Siglec-F on alveolar macrophages is not fully understood, though Siglec-F is known to be expressed on the surface of eosinophils where the targeting of Siglec-F with crosslinking antibodies has been proposed to induce eosinophil apoptosis *(O'Reilly and Paulson, 2009; Feng and Mao, 2012).* This is not observed in alveolar macrophages. Instead,

when Siglec-F is crosslinked in alveolar macrophages alongside apoptotic cells the phagocytic activity of resting alveolar macrophages is not alerted *(Feng and Mao, 2012)*. Despite this, the phagocytic activity of Siglec-F crosslinked alveolar macrophages is significantly lower when compared with peritoneal macrophages *(Feng and Mao, 2012).* Suggesting a suppressive role of alveolar macrophages.

Lung macrophages have been proposed to be central in regulating type 2 responses (IL-4-mediated; *Van Dyken and Locksley, 2013*), as IL-4 activation results in regulatory macrophages. Arg1 expressed on alternatively activated macrophages competes with enzyme inducible nitric oxide synthase (iNOS), an enzyme seen on the surface of M1 macrophages *(Lowenstein and Padalko, 2004)*. However, there is new evidence to suggest that Siglec-F may not be involved in regulating type two hyporesponsiveness of alveolar macrophages in the lung (*Svedberg et al., 2019*). When removed from the lung the type 2 hyporesponsiveness of alveolar macrophages is lost (*Svedberg et al., 2019*). Flow cytometry data can be used to measure the hyporesponsiveness of alveolar macrophages to IL-4, as mice injected with IL-4 show elevated expression of markers associated with IL-4: RELMα, Ki67 and EdU on the surface of alveolar macrophages (*Jenkins et al., 2011; Svedberg et al., 2019*). Alveolar macrophages that had been removed from the lung and rapidly expanded with IL-4 did not upregulate RELMα, Ki67 or EdU (*Svedberg et al., 2019*). This lack of response to IL-4 suggests that this response is regulated by the lung environment. Ki67 is a protein present in the nucleus during interphase and mitosis, but is absent when the cell is at rest, it is for this reason that Ki67 is used as proliferation marker (*Scholzen and Gerdes, 2000*).

Mucin5b is the dominant pulmonary airway mucin present on the epithelial cells, the expression of which is increased after treatment with IL-4 in vivo (*Svedberg et al., 2019*), speculating that the two might be linked. However, comparison of gene profiles of wildtype mice to mucin5b knockout-mice showed similar gene profiles and little change in upregulation and downregulation of selected pathways from KEGG analysis (*Svedberg et al., 2019*). This lack of change suggests that mucin5b is not a dominating factor in hyporesponsiveness to IL-4 (*Svedberg et al., 2019*). IL-4 hyporesponsiveness is therefore independent of Siglec-F interactions as mucin5b is the main source of sialylated ligands of this receptor. The regulation of IL-4 responsiveness is also seen to be independent of specific organisms present in the lung microbiome, as expression of RELMα and Ki67 was similar in germ-free and specific pathogen free mice after IL-4 administration (*Svedberg et al., 2019*). In contrast, very little is known about Siglec-F as a regulator of either type 1 (IFNγ-mediated) or general pathogen associated inflammation by alveolar macrophages.

1.7 Hypothesis

The specific effector functions of Siglec-F on the surface of alveolar macrophages is currently not fully understood, although it is suspected to act as a negative regulator of alveolar macrophage function and adaption. It is hypothesised that Siglec-F crosslinking would induce changes in the gene expression programme of these cells. Analysis of gene ontology and cytokine production could give a greater understanding into the mechanism of how Siglec-F acts as a negative regulator of alveolar macrophage function and adaptation.
Targeting and modulating this pathway could uncover new therapeutic targets or biomarkers for diagnosis and treatments in disease such as Asthma and COPD where alveolar macrophages play a key role as sentinel cells with regulatory activity.

1.8 Aims

The overarching aim of this project is to investigate the outcome of signalling through Siglec-F in mouse alveolar macrophage by whole genome expression and cytokine production. To do this, a highly specific antibody crosslinking system previously established using flow cytometry will be used to specifically target Siglec-F.

1.9 Objectives

The following are objectives for this project together with the experiments that will be used to complete them:

- To ascertain proof of signalling through Siglec-F by measuring recruitment of SHP-1 by Western blot.

- To determine the most suitable timepoint for collection of macrophages from the stimulation cultures, by measuring cell death and the ability to produce cytokines. For use in RNA-seq experiments.

- To analyse changes in gene expression associated with the Siglec-F signalling pathway, using RNA-seq, to identify gene targets and alterations in functional or differentiation pathways.

- To characterise a primary cell-line based system of foetal liver-derived alveolar macrophages *(Fejer et al., 2013)* as a substitute to mouse derived alveolar macrophages.

1.10 Significance and project impact

Asthma has been identified as one of the major non-communicable diseases, with an estimated 235 million people suffering from the disease worldwide with 383,000 dying from the disease in 2015 *(World Health Organisation, 2017*). Research into alternative therapeutic techniques has been a priority for Asthma UK, a member-based charity who reported that in 2015 5.4 million people in the UK suffer from the disease, with 70,888 of these asthmatics requiring emergency hospital aid *(Asthma UK, 2018)*. A potential biomarker for the diagnosis of asthma is the significant increase in CCL17 mRNA produced by alternatively activated (M2) macrophages *(Staples et al., 2012)*. M2 macrophages have been shown to regulate Th-2 responses in asthma *(Girodet et al., 2016)*. A possible treatment for asthma could therefore be reprogramming alveolar macrophages towards an alternatively activated lineage, where Siglec-F could then be utilised to keep the macrophage bound to the lung epithelia. RNA-seq experiments could provide information on how to manipulate the negative signalling as an alternative way of inactivating the macrophage.

In COPD there is a marked increase of alveolar macrophages in the lung *(Pesci et al., 1998)* with a positive correlation between number of alveolar macrophages and COPD severity *(Di Stefano et al., 1998*). As alveolar macrophages produce neutrophil chemotactic factors, neutrophils produce proteases which breakdown lung connective tissue, resulting in COPD. A biomarker for COPD could therefore be the large increase in IL-8 and LTB_4. Possible treatments for COPD are anticholinergic drugs such as tiotropium bromide which have been proposed to have anti-inflammatory effects (*Barnes and Stockley, 2005)*. From RNA-seq experiments it could also be possible to pair anticholinergic drugs with Siglec-F stimulants to prevent the release of neutrophil chemotactic factors and production of proteases by neutrophils.

2. Methods and Materials

2.1 Materials

Mice

Inbred, C57BL/6J females of 6-10 weeks of age were purchased from Envigo UK.

Bronchoalveolar lavage

- RPMI media was purchased from Gibco.
- Red-blood cell lysis buffer (ACK) was purchased from Thermofisher.
- Trypan blue 0.4% solution was purchased from Lonza group.
- Versene was purchased from Gibco.

Naïve Peritoneal Lavage

- PBS pH: 7.2 was purchased from Gibco.
- Red-blood cell lysis buffer.

Dynabeads immunoprecipitation

- Dynabeads suspended commercial buffer purchased from Thermofisher.
- Antibody binding and washing buffer Purchased from Thermofisher.
- Washing buffer purchased from Thermofisher.
- Elution buffer purchased from Thermofisher.
- NuPage LDS sample buffer from Thermofisher.
- Lysis buffer purchased from Thermofisher.
- Dynamag magnet purchased from Thermofisher.

Western blot experiments

- Versene was purchased from Gibco.
- LPS (at a concentration of 0.5mg/ml) was purchased from Sigma-Aldrich.
- SDS-PAGE Gel purchased from Thermofisher.
- Page ruler+ purchased from Thermofisher.
- Transfer buffer (0.025M Tris, 0.192M glycine, pH 8.5) was purchased from Thermofisher.
- Running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) was purchased from Thermofisher.
- Blocking buffer (PBS 5% skimmed milk) was purchased from Thermofisher.
- Whatman paper was purchased from Sigma-Aldrich.
- PVDF paper purchased from Thermofisher.
- Methanol was purchased from Sigma-Aldrich.

● Peroxide solution purchased from Thermofisher.

● Luminol enhancer solution purchased from Thermofisher.

Cell culture of FLDAM and AM

● Penicillin/streptomycin was purchased from Thermofisher.

● L-Glutamine was purchased from Thermofisher.

● Complete RPMI (RPMI media 10% FCS 20 mM glutamine, 1000U/ml penicillin) was purchased from Thermofisher.

● G-CSF (0.2mg/mL) was purchased from BioLegend.

● Trypan blue 0.4% solution was purchased from Lonza group.

● Foetal calf serum was purchased from Gibco.

Generation of BMDM

● Mice (inbred, females C57BL/6J).

● Conditioned media (containing 30% M-CSF from transduced L929 cells) was purchased from Thermofisher.

● Ethanol was purchased from Sigma-Aldrich.

● PBS pH: 7.2 was purchased from Gibco.

ELISA

● TNF-α ELISA Kit Purchased from BioLegend (cat: 430907).

● FACS buffer: PBS 10% FCS 0.1% sodium azide was generated at the university.

Materials for LEGENDplex experiments

● LEGENDplex mouse macrophage/microglia panel 8-plex (CXCL1, IL-1β, IL-23, IL-12p70, IL-12p40, IL-6, TNF-α, and TGF-β1) + G-CSF kit purchased from BioLegend.

RNA extraction

● Picopure RNA extraction kit was purchased from Thermofisher. (cat: KIT0204)

RNA-seq

● RNA sequencing will be performed using the illumina NextSeq 500 platform and services will be provided by UCL genomics.

Non-conjugated antibodies

Table 3: The table below contains the details for non-conjugated antibodies used throughout this MRes.

Supplier	*Catalogue Number*	*Specificity*	*Isotype*	*Dilution*	*Application*
BD Biosciences	552125	Mouse Siglec-F	Rat IgG2a	1:100	Crosslinking
BD Biosciences	553926	Isotype Control	Rat IgG2a	1:100	Crosslinking
Biolegend	553926	Rat IgG	Goat IgG	1:100	Crosslinking
Biolegend	101319	Mouse CD16/32	Rat IgG2a	1:100	Flow cytometry
R&D Systems	AF1878	Mouse SHP-1	Goat IgG	1:1000	Western blotting

Conjugated antibodies

Table 4: This table contains the details for fluorochrome conjugated rat anti mouse antibodies used throughout this MRes, all purchased from Biolegend.

Catalogue number	*Specificity*	*Attached Fluorochrome*	*Clone*
123907	CD200r	PE	OX-110
151507	MERTK	APC	2Bioc42
155505	Siglec-F	PE	S17007L
123147	F4/80	BV711	BM8
137005	CD68	FITC	FA-11
101207	CD11b	PE	M1/70
117313	CD11c	Alexa Fluor	N418
652403	Ki-67	PE	16A8
102711	CD38	APC	90

Table 5: This table contains the enzyme-conjugated antibodies used throughout this MRes.

Supplier	*Catalogue number*	*Specificity*	*Isotype*	*Attached Enzyme*
Rockland	18-8844-33	Goat IgG	Rabbit IgG	HRP

2.2 Methods

In this project a Siglec-F antibody crosslinking system, established in previous experiments, has been used to signal through the Siglec-F receptor.

The system is depicted in figure 6 and involves the binding of cell surface Siglec-F by a highly specific anti mouse Siglec-F monoclonal antibody. It is hypothesised that signalling will be induced when two Siglec-F receptors are brought together by the specific binding of the Fc portion of these monoclonal antibodies by a secondary anti rat IgG monoclonal antibody.

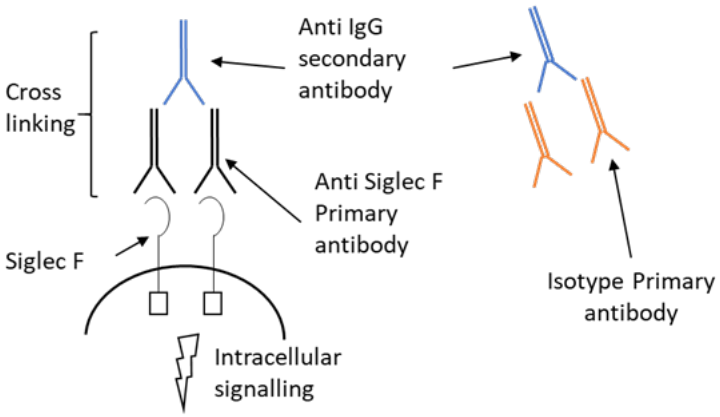


Figure 6: Anti-Siglec-F antibody crosslinking system. This figure shows how crosslinking using anti-Siglec-F antibodies (left) and the isotype control (right) works.

2.2.1 Extraction of alveolar macrophages from mice

Mice are killed by cervical dislocation (at UEL) or lethal pentobarbital injection (100mg/Kg; at UCL). Scissors were then used to remove skin, create a lung pneumothorax and remove the peritracheal membrane to make the trachea accessible. Using a scalpel an incision was made in the trachea allowing for the tip of a venous catheter to be inserted which was used to flush the lungs with RPMI media (+0.1% EDTA); this was done to collect the alveolar macrophages. This process was repeated twice for each mouse.

The pooled contents of the bronchoalveolar lavage (BAL) was spun at 350 RCF for 5 minutes after which the supernatant was discarded. When necessary, the pellet was resuspended in 5mL of ACK (a red blood cell lysis buffer) for 30 minutes in to order to rupture any red blood cells. Five millilitres of RPMI media was then added to the sample to neutralise the ACK. The sample was then spun at 350 RCF for 5 minutes after which the supernatant was removed and the sample resuspended in complete RPMI for further use. Cell numbers were estimated using trypan blue exclusion.

2.2.2 Extraction of peritoneal cavity cells

Mice were killed by cervical dislocation or lethal pentobarbital injection (100mg/Kg). Scissors were then used to cut the outer skin of the peritoneum and gently pull it back to expose the inner skin lining the peritoneal cavity. Cold PBS was then injected into the peritoneal cavity being careful not to puncture surrounding organs. A syringe was then used to collect the maximum volume of fluid which was then deposited into a centrifuge tube kept on ice. For maximum yield insertion of PBS was repeated at least once per mouse.

After all the mice had undergone this process the contents of the peritoneal cavity in PBS media was spun at 350 RCF for 8 minutes after which the supernatant is discarded. When necessary, the pellet was resuspended in 5mL of ACK for 30 minutes in to order to rupture any red blood cells present. Five mL of PBS media was then added to neutralise the ACK. The sample was then spun at 350 RCF for 5 minutes after which supernatant was removed and the sample resuspended in PBS. Number of cells was then estimated using trypan blue exclusion. Peritoneal cavity cells were used to compare the expression of macrophage marker F480 and alveolar macrophage marker Siglec-F with alveolar macrophages using a C6 Accuri flow cytometer.

2.2.3 Differentiation of macrophages from mouse bone marrow using M-CSF

Mice were killed by cervical dislocation or lethal pentobarbital injection (100mg/Kg). The abdomen and hind legs were then sterilised using 70% ethanol. An incision was made in the midline of the abdomen outwards, which exposed the legs. Scissors were then used to remove all muscle from the bone after which the bones are cut at both ends to free them. The femur was separated from the tibia by cutting at the knee joint before flushing the bone marrow with ice-cold PBS from both bones. The cell suspension was then passed through a cell strainer (70µm) with the strainer being washed afterwards with lymphocyte medium to ensure maximum yield. Once all bone marrow cells have been harvested the cell suspension was spun at 350 RCF for 5 minutes, with the pellet then being resuspended in conditioned media (with M-CSF-transduced L929 cells). A haemocytometer was then used to count the cells with a minimum of $7x10^6$ cells being incubated in a culture plate with conditioned media. Culture was then incubated at 37^oc with 5% CO_2. After 7 days the culture was split (1/2) with fresh conditioned medium. Cells were used for experimental analysis when percentage of CD11b+ cells is higher than 90% as measured by FACS analysis (*Zanoni, Ostuni and Granucci, 2009*). Bone marrow derived macrophages were used to compare expression of macrophage marker F480 and alveolar macrophage marker Siglec-F using a C6 Accuri flow cytometer.

2.2.4 Culturing FLDAM

Mice foetal liver cells, acquired from the embryos of C56BL/6 mice 18 days post fertilisation, were added to RPMI with G-CSF (50ng/mL; *Fejer et al., 2013*). The mixture was then added to a cell culture flask and incubated at 37°c and 5% CO_2 for a minimum of 14 days. During the 14 days fresh RPMI and G-CSF were added where appropriate. After the 14 days cells were removed using a cell scraper, the cells were then spun at 350 RCF for 5 minutes and resuspended in RPMI. 10µl of cell suspension was then added to an equal volume of trypan blue, this mixture was placed underneath a cover slide on a haemocytometer. Using a microscope, the number of non-blue cells was counted in the four corner squares, to get cell number per mL the numbers are averaged and multiplied by 20,000.

To continue cell culture G-CSF was added to the culture (50ng/mL) and placed back in a cell culture flask in an incubator. If cell number is >1,000,000 the culture was split between two cell culture flasks.

2.2.5 Cell staining using fluorescently conjugated antibodies

Samples (including: alveolar macrophages, peritoneal cells or derived macrophages) were spun at 350 RCF for 5 minutes, following either BAL, NPL or successful generation of BMDAM, after which supernatant is discarded. The pellet was then resuspended in FACS buffer with CD16/32 antibody in order to block Fc receptors and placed on ice for 10 minutes. Relevant conjugated antibodies were then added to stain specific cell surface receptors. The sample was left to stain in the dark for an hour. Excess antibody was removed by washing with FACS buffer with the pellet being resuspended in FACS buffer. Samples were analysed using a C6 Accuri flow cytometer.

2.2.6 Using dynabeads for crosslinking Siglec-F antibodies and for immunoprecipitation

Removing the cells from culture and continuing culture

Macrophages used for these experiments were grown in sterile conditions (37°c, 5% CO_2) in 5ml complete RPMI and without activation. At least 500,000 were used for each experiment.

A sterile cell scraper was used to remove the cells from the culture plates and cells were then pipetted into a centrifuge tube, which was spun at 350 RCF for 5 minutes. The pellet was resuspended in 3mL RPMI media. Cell number was estimated using trypan blue. This estimate is used to calculate the correct volume needed to successfully continue the culture. The remaining cell sample was then spun again and resuspended into 600µL PBS.

Preparing beads and binding antibody

Dynabeads were resuspended by vortexing after which 50µL was pipetted into each Eppendorf. All Eppendorf tubes were placed on the magnet to separate beads from solution and remove supernatant. Eppendorf tubes were then removed from the magnet. 10µL of the relevant antibody dissolved in 200µl of washing and binding buffer was added to each Eppendorf and incubated at room temperature with vortexing for 10 minutes. Eppendorf tubes were then placed on the magnet and supernatant was discarded after which the Eppendorf tubes were removed from the magnet. The beads were resuspended in 200µL binding and washing and washed by gentle pipetting.

Immunoprecipitation and lysing of Siglec-F and isotype control

Eppendorf tubes were placed on the magnet and supernatant is discarded after which the Eppendorf tubes are removed from the magnet. Approx. 1 million cells were added to each Eppendorf tubes and incubated with rotation for 10 minutes. The Eppendorf tubes are placed on the magnet and supernatants removed, after which the Eppendorf tubes were removed from the magnet and resuspended in washing buffer (this is repeated three times). After the final wash the pellet was resuspended in lysis buffer and incubated on ice for 30 minutes. Eppendorf tubes were then placed on the magnet, where the supernatants were removed and the Eppendorf tubes were taken away from the magnet; resuspended in PBS and left on ice.

Immunoprecipitation and lysing of unstimulated cells

The remaining cells (approx. 1 million cells) were lysed in lysis buffer for 10 minutes on ice, after which the sample was then spun at 13,793 RCF for 10 minutes. The supernatant was carefully pipetted into the Eppendorf containing the beads and the Siglec-F antibody (see preparing beads and binding antibody). The sample was then incubated on ice for 10 minutes. This Eppendorf was then placed on the magnet, where the supernatant was then removed after which the Eppendorf was taken away from the magnet. The pellet was then resuspended in PBS.

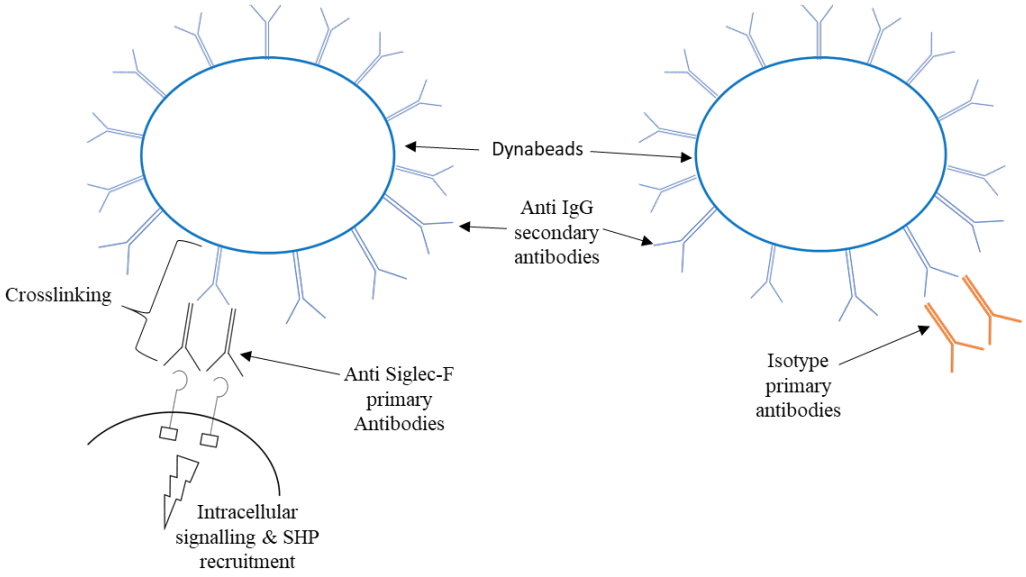


Figure 7: Artist's impression of the anti-Siglec-F antibody Dynabeads crosslinking system. This figure shows how crosslinking using anti-Siglec-F antibodies (left) and the isotype control (right) works, when using anti IgG secondary antibodies bound to magnetic Dynabeads.

Eluting the antigen

All Eppendorf tubes were placed on the magnet where the supernatant was then removed after which the Eppendorf was taken away from the magnet and resuspended in 20µL Elution buffer and 10µL NuPage LDS sample buffer. In order to resuspend, the contents of the Eppendorf tubes were gently pipetted. The samples were then heated for 10 minutes at 70^{o}c.

Removing the DNA from isotype control and Siglec-F Eppendorf tubes

Eppendorf tubes Siglec-F and isotype control were spun at 13,793 RCF for 5 minutes, afterwards the supernatant was carefully removed and pipetted into separate Eppendorf tubes.

2.2.7 Western blot

Samples

Samples from this experiment were Siglec-F crosslinked and immunoprecipitated using Dynabeads.

Preparing gel

To prepare the samples for Western blot 25µL of the sample (approx.: 5x10^{5} cells), 25µL Tris-glycine SDS sample buffer and 10µL 2-ME were pipetted into a labelled Eppendorf tube. The Eppendorf was then placed on a heat block at 100°C for 5 mins. In order to prevent gas build up a small incision was made at the top of the Eppendorf. Running buffer was poured into the tank.

The pre-prepared gel was gently taken out of the packet and the comb removed, running buffer was then used to wash wells. After washing the white tape was removed and gel placed into the tank. The gel was then locked in place and the tank topped up with running buffer so that wells were fully submerged before loading.

25µL of each sample present was placed into individual wells and 10µL of ladder in the relevant well. For the empty columns 10µL of sample buffer was added, the lid was then secured, and the gel electrophoresis ran at 100v for 1 hour.

Transfer

The running buffer was discarded, and tank washed with distilled water. The gel was then removed from the case and placed into a large dish containing transfer buffer.

In order to transfer the contents of the gel to a blot, 2 blotting papers were soaked in the transfer buffer alongside 2 black sponges. A sheet of PVDF similar size to the gel was soaked in methanol. The following configuration is then prepared inside the cassette black side down: the sponge, paper, gel, PVDF. It is at this stage that any air bubbles between the gel and PVFD were removed. The second filter paper was then placed on top of the PVFD and lastly the black sponge. The cassette was then locked.

Running transfer and blotting

An ice pack was placed inside the transfer tank followed by the cassette with black side of the cassette facing the black side of the tank. The tank was then filled with running buffer and ran at 100v for 1 hour. After running PVDF paper was removed and soaked in blocking buffer for 1 hour at room temperature. This step helps reduce non-specific primary antibody binding and reduces background.

The membrane was then incubated with SHP-1 primary antibody (1:1000) overnight at 4°C. After which the membrane was washed 3 times with 0.05% tween 20/ PBS. The membrane was allowed to soak for 10-15 minutes each time. The membrane was then incubated with anti-goat TrueBlot IgG HRP (1:1000) in blocking buffer at room temperature for 1 hour. After which the membrane was washed 3 times with 0.05% tween 20/ PBS. With the membrane being allowed to soak for 10-15 minutes each time. Four millilitres of fresh substrate were then prepared by mixing peroxide solution and luminol enhancer (1:1). The membrane was then incubated with substrate for 5 minutes in the dark. Lastly the membrane was placed in a plastic sleeve for imaging.

2.2.8 Apoptosis assay

Approx. 1 million alveolar macrophages in RPMI media was added to 8 individual wells, with two of these wells containing LPS at a concentration of 10ng/mL, anti-Siglec-F antibody at a concentration of 1:100 being added to two of the wells, isotype control antibody at a concentration of 1:100 being added to two wells and lastly the remaining two wells were left unstimulated. To successfully crosslink anti-IgG antibody was added to the Siglec-F and isotype control wells at a concentration of 1:100. The 96 well plate was then incubated at 37^{o}C and 5% CO_2 for 18 hours.

After incubation the well was removed from the incubator and supernatant was carefully removed and pipetted into labelled Eppendorf tubes and placed into the -20^{o}C freezer for use in the TNF experiment. To detach the macrophages from the bottom of the well plate versene was added to each well, after which the plate was stored in the fridge for 30 minutes to further dislodge cells from the bottom of the well. To dislodge maximum number of cells each well was pipetted up and down before being pipetted into separate labelled Eppendorf tubes. To minimise background CD16/32 was added to each Eppendorf at a concentration of 1:100 and was left on ice for 5 minutes. 50µL annexin V (diluted in FACS buffer 1:100) was then added to each Eppendorf and left to stain on ice for 25 minutes in the dark.

To remove excess annexin V all Eppendorf tubes were spun at 350 RCF for 5 minutes with the pellet then being resuspended in FACS buffer. To further remove background all Eppendorf tubes were washed twice. Eppendorf tubes were run on a C6 flow cytometer.

2.2.9 Cytokine production: TNF ELISA

The well plate

The well plate was constructed by adding 100µL of diluted capture antibody (in assay diluent; 1:200) to 48 wells, with the plate then being incubated overnight in a fridge (2-8^{o}c) to maximise capture antibody binding to the well. After refrigeration the plate was allowed to rise to room temperature after which each of the 48 wells was washed with wash buffer four times to remove excess capture antibody. After washing 200µL assay diluent was added to each well, the plate was then sealed and incubated for an hour at room temperature with shaking. Each well was then washed four times with wash buffer. 150µL of each sample was added to two separate wells alongside a serial dilution with the TNF standard (1:2). The plate was then sealed and incubated at room temperature for an hour with shaking.

Each well was then washed with wash buffer. 100µL diluted detection antibody (in assay diluent; 1:200) was added to all 48 wells and the plate incubated at room temperature for an hour with shaking. Each well is then washed with washing buffer four times. 100µL of diluted Avidin-HRP (in assay diluent; 1:1000) is added to each of the 48 wells and plate incubated at room temperature with shaking for 1 hour. The plate was then washed with washing buffer five times with 5-minute intervals between washing, allowing the wells to soak. 100µL of TMB solution was then added to each well, the plate was then incubated in the dark for 30 minutes or until a blue colour developed. To stop the reaction, 100µL of stop solution was added to each well, turning the wells yellow.

The absorbance of each well was then read at 450nm within 15 minutes of adding the stop solution using a Synergy HTX Multi-Mode Reader. A calibration curve of standards was then used to find TNF-α concentration (pg/mL).

2.2.10 Cytokine production: multiplex cytokine bead array

25µL assay buffer and 25µL sample/standard was added to the relevant wells, with standard wells undergoing a serial dilution of 1:4. For this experiment beads were used instead of IgG antibodies though the beads needed to be diluted by 13x in assay diluent, with an extra 20% being prepared to account for loss. The bead cocktail was vortexed for 10 seconds with 25µL being added to each well. The plate was then sealed and covered with aluminium foil to protect from light and shaken at 140 RCF for 2 hours.

After shaking, the plate was spun 240 RCF for 5 minutes. The supernatant was removed, and the pellet resuspended in wash buffer, the plate was then left to incubate for 1 minute. The plate was spun again at 240 RCF for 5 minutes. The pellets were then resuspended in 25µL of detection antibody, with the plate then being sealed and covered with aluminium foil and shaken at 140 RCF for 1 hour. Without washing 25ul of SA-PE was added to each well and plate sealed and covered with aluminium foil, the plate was then shaken at 140 RCF for 30min. After which the plate was spun at 240 RCF for 5 minutes. The pellet was resuspended in wash buffer in each well and left for one minute, after which the plate was spun at 240 RCF for 5 minutes with pellet being resuspended in 150µL of wash buffer. Samples were read on a flow cytometer.

2.2.11 RNA sequencing

RNA sequencing was carried out as described *(Solanki et al., 2017).* Alveolar macrophages were extracted from female C57BL/6J mice acquired via BAL with an estimated 95% purity. Alveolar macrophages were incubated in culture with RPMI with either IgG antibody crosslinked anti-Siglec-F antibody, IgG antibody crosslinked isotype antibody or without antibody (unstimulated) for 18 hours. RNA was then extracted using Arcturus PicoPure RNA Isolation kit with the quantity and quality determined by Bioanalyser 2100 (Agilent). RNA was also extracted from a population of macrophages immediately after BAL. Extracted RNA was sequenced by UCL Genomics on the Illumina Next Seq 500. Genomic alignment was carried out by the UCL Genomics teams using STAR v2.5b. The RNA sequencing dataset was processed and standardised using the Bioconductor package DESeq2, which was used to generate normalised estimates of transcript abundance. Data analysis was carried out as described *(Sahni et al., 2015; Solanki et al., 2017).* PCA was performed using normalised transcript expression values, using the built-in R function for PCA. Graphical representations of gene ontology and functional classification gene lists were carried our using Panther (www.pantherdb.org).

3. Results

3.1 Comparative analysis of Siglec-F expression between different types of macrophages

Alveolar macrophages are known to express Siglec-F *(Feng and Mao, 2012; Svedberg et al., 2019)* however, the levels of expression of this receptor between alveolar macrophages and other tissue residents or haematopoietic subsets has not been directly compared.

Resting, non-elicited peritoneal macrophages and alveolar macrophages are two subsets of tissue-resident myeloid cells that differ largely in function and morphology (*Guth et al., 2009*). Bone marrow-derived macrophages (BMDM), differentiated in the presence of M-CSF, are commonly used as an in vitro model in macrophage biology and are considered a good approximation to study the phenotype and function of resting macrophages (M0). Flow cytometry was used in order to compare the levels of expression of Siglec-F between these populations, and also to determine if the expression of this receptor is restricted to a particular population. Furthermore, the selection of an additional Siglec-F positive population would allow for use of multiple macrophage populations for further analysis. This is important as the extraction of alveolar macrophages from mouse lungs yields relatively low cell numbers, limiting downstream experimental work.

Figure 8 shows representative data of the level of expression of Siglec-F in the above-mentioned macrophages. These results showed that 72% of macrophages isolated by BAL expressed Siglec-F (reproduced in figure 9) at resting state, whereas less than 3% of BMDMs and peritoneal macrophages expressed the receptor. The comparatively high and restricted level of expression of Siglec-F could be a defining characteristic of alveolar macrophages as they adapt to the lung microenvironment.

3.2 F4/80 + CD11c+ cells express receptors akin of an M1 phenotype

In order to have an approximate assessment of the resting naïve alveolar macrophage differentiation phenotype (M1 or M2), resident macrophages were extracted from residual resting lung tissue. Lung cells were stained for the macrophage specific markers F4/80 and CD11c (highly expressed by alveolar macrophages) together with two characteristic phenotypic markers CD38 (M1) and Arg1 (M2). The results in figure 10 show that the vast majority of alveolar macrophages (92.1%) express the M1 macrophage receptor CD38 and not Arg1, interestingly the second largest population of alveolar macrophages express both M1 and M2 markers (2.51%; shown in figure 11 as a bar chart).

After assessing the expression of cell surface receptors consistent with the functional phenotypes of macrophages; the level of expression of Siglec-F in each subset was assessed. Figure 12 shows that the expression of Siglec-F is high with 80.8% of CD38+ cells expressing the receptor and 81.8% of the CD38+Arg1+ cells also expressing Siglec-F. From this data it was concluded that Siglec-F expression does not segregate between airway macrophage subsets with potentially different differentiation phenotypes.

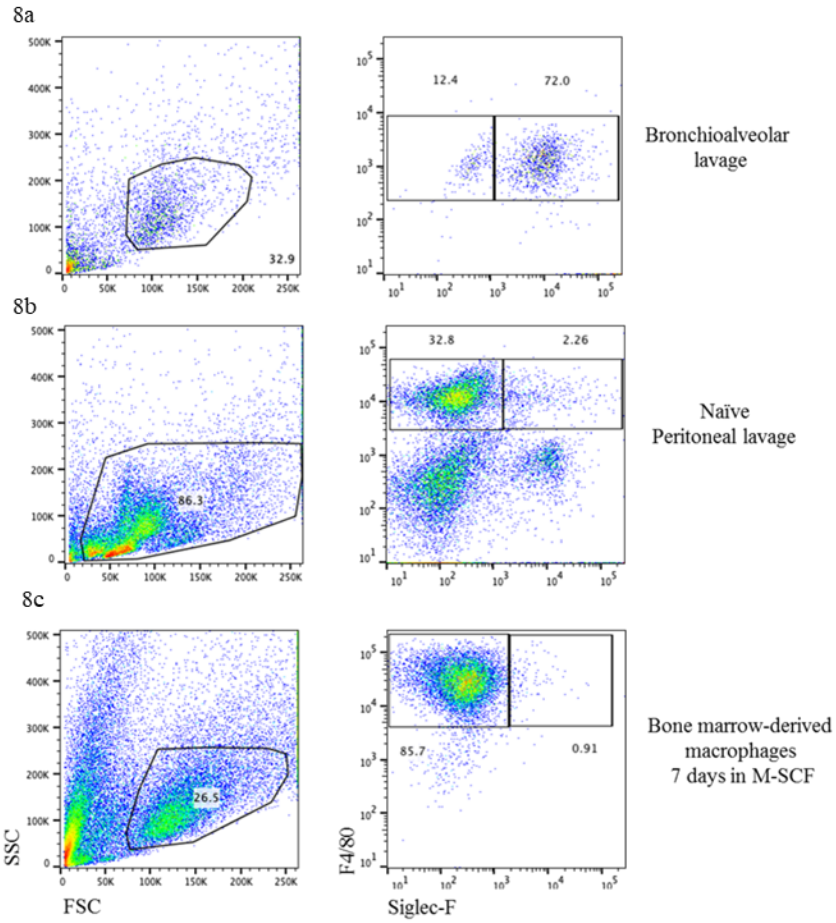


Figure 8: Flow cytometry analysis of three different types of macrophages in mice. Cells shown were isolated via mice BAL (8a), mice NPL (8b) or from bone marrow of mice femurs (8c). The BMDM shown in 8c were 95% F4/80+ after one week in MCSF. Cell populations were then gated from the FSC vs. SSC dot plot to have the size and complexity of living macrophages. Cells were further gated for Siglec-F and F4/80 expression as these are markers expressed by alveolar macrophages. Data representative of three repeats.

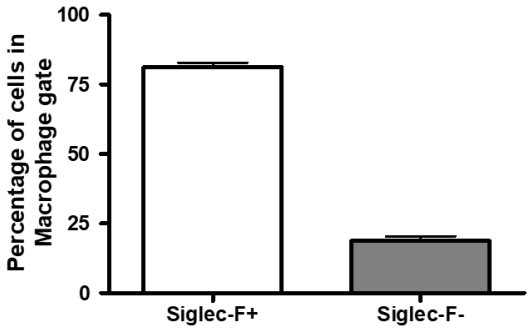


Figure 9: A high proportion of F4/80+ alveolar macrophages express Siglec-F. This bar chart displays the percentage of Siglec-F positive and negative cells obtained via BAL in figure 8a. Bar chart shows the mean +/- SD of three independent experiments.

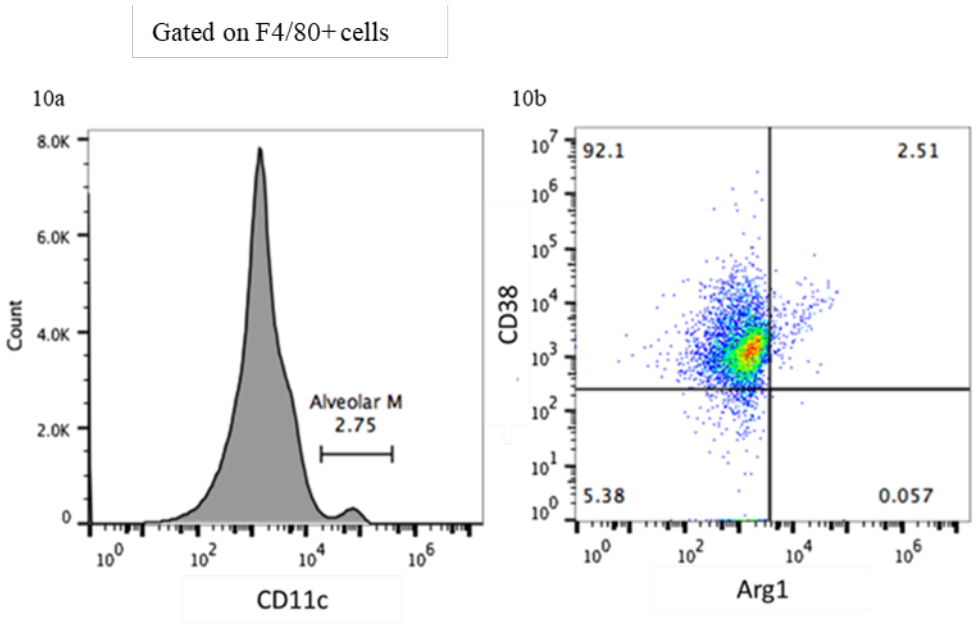


Figure 10: Macrophages in the lung selectivity express CD38 when at rest. In 10a cells were acquired from resting lung tissue, and macrophage population identified on the basis of F4/80 and CD11c expression. 10b shows the percentages of F4/80+ CD11c+ macrophages that are positive or negative for CD38 and Arg1. Data representative of three independent experiments.

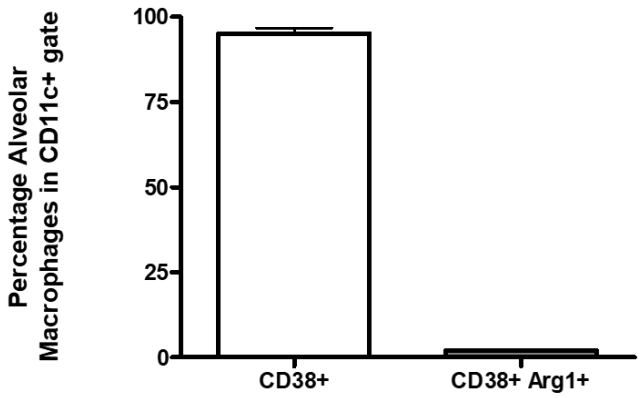


Figure 11: Graphical representation of alveolar macrophages from Q1 and Q2 from figure 10. Bar chart shows the average percentage (n=3) of F4/80+ CD11c+ lung cells, that are either CD38+ or CD38+Arg1+.

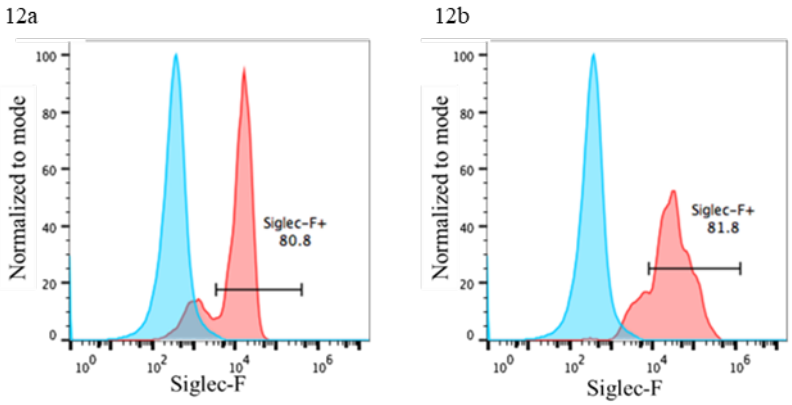


Figure 12: Siglec-F expression by M1 and M2 lung macrophages. Flow cytometry histograms show the percentage of Siglec-F positive CD11c+CD38+Arg1- cells (12a) and CD11c+CD38+Arg1+ cells (12b). Blue overplayed histograms correspond to unstained background controls. Data is representative of three independent experiments.

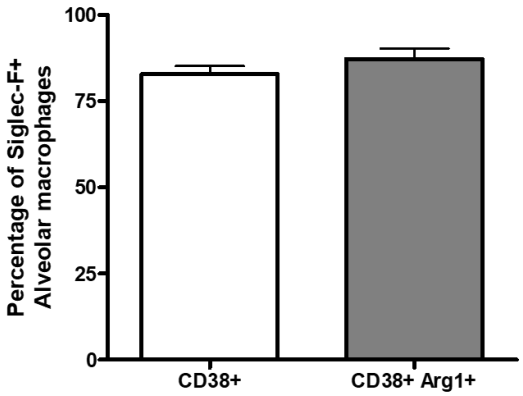


Figure 13: Graphical representation of Siglec-F+ alveolar macrophages from histograms shown in figure 12. Bar chart shows percentage of CD38+ or CD38+Arg1+ that are Siglec-F+. Bar chart shows the mean +/- SD where n=3.

3.3 Assessment of Siglec-F engagement using antibody crosslinking

The aim of this project is to investigate the outcome of signalling through Siglec-F using a highly specific antibody crosslinking system (see figure 6 in methods), to assess changes in gene and protein (cytokine) expression. Flow cytometry was used to establish the extent of Siglec-F receptor targeting by performing a FACS stain using a PE-conjugated anti-Siglec-F antibody on cells. These cells have been previously crosslinked or treated with an isotype control as well as secondary antibodies.

Using flow cytometry, an average percentage shift of 63% in MFI was observed when comparing Siglec-F crosslinked and the isotype control. This is equivalent to 63% of the Siglec-F receptors being targeted by Siglec-F crosslinking. It is important to mention that although arbitrary, this method of exogenous ligand-independent signalling aims to avoid the saturation of all of the Siglec-F receptors on the surface of the cell as this could produce an unphysiological signal transduction strength.

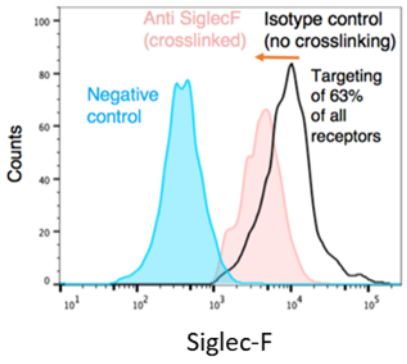


Figure 14: A proportion of Siglec-F receptors can be successfully targeted using antibody crosslinking. Alveolar macrophages were acquired via BAL. F4/80+ macrophages were treated with an unconjugated anti-Siglec-F, or isotype control and an anti IgG secondary antibody prior to FACS staining with a PE-conjugated anti-Siglec-F antibody. The FACS histograms represent the extent of the loss of staining due to crosslinking and was measured by mean fluorescence intensity (MFI). The blue histogram represents the negative unstained background control, the black open histogram represents cells that have been "crosslinked" with an isotype and the red histogram represents cells that were crosslinked with anti-Siglec-F antibodies. The representative % targeting shown on the plot was estimated as the proportional difference in MFI between isotype-treated (representative MFI 12912) and Siglec-F (representative MFI 4691) crosslinked cells. Data representative of two individual experiments.

3.4 Alveolar macrophages undergo minimal levels of apoptosis 18 hours after Siglec-F crosslinking

It has been established that Siglec-F is a negative regulator of eosinophil activation in vivo *(O'Reilly and Paulson, 2009)*. However, when targeted with anti-Siglec-8 antibodies in the presence of secondary antibodies, eosinophils undergo apoptosis due to signalling through a caspase-dependent pathway (*Nutku, 2003*). As Siglec-F has been documented as a functional paralog of Siglec-8 *(Angata et al., 2006*) it is essential to see if this process happens when targeting Siglec-F in mice alveolar macrophages. To test this, alveolar macrophages were crosslinked between 18 and 24 hours. These time points are consistent with the crosslinking times considered for the RNA-seq experiments. After the above-mentioned time points, early apoptosis events were measured by assessing the translocation of phosphatidylserine Annexin V.

Data in figure 15 shows that crosslinking Siglec-F on alveolar macrophages does not induce apoptosis like in eosinophils as the translocation of phosphatidylserine in the Siglec-F and isotype treated cells are comparable. Siglec-F crosslinking also induces similar levels of apoptosis to that seen in unstimulated macrophages both at 18 and 24 hours. Cell death does occur minimally at 18 hours, but is greater at 24 hours. For this reason, 18 hours was used as a suitable time point for RNA sequencing.

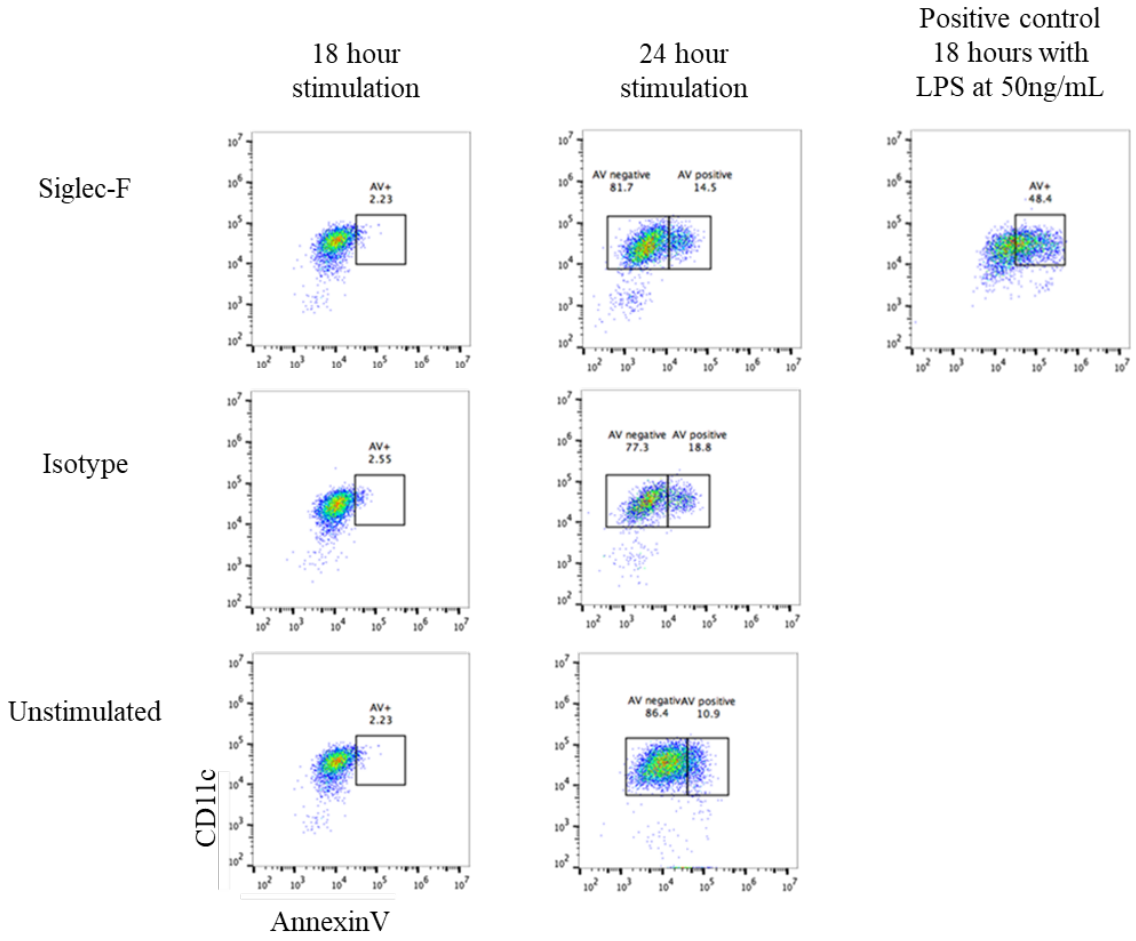


Figure 15: Crosslinking Siglec-F receptors using antibodies does not result in self-apoptosis. Mouse alveolar macrophages isolated by BAL had their Siglec-F receptors crosslinked using antibodies for 18 hours and 24 hours. Cells were gated on an FSC vs. SSC and F4/80 plots (not shown) then displayed on a dot plot for CD11c vs Annexin V. A positive control was created by incubating with LPS at 50ng/mL with a negative control being alveolar macrophages cultured alongside with media alone. Data is representative of two independent experiments.

3.5 Antibody crosslinking causes SHP-1 recruitment

From previous experiments involving flow cytometry it was concluded that Siglec-F crosslinking was successful in targeting of the Siglec-F receptor, though it does not give evidence that antibody crosslinking results in intracellular signal transduction. To test this, resting naïve alveolar macrophages were crosslinked with anti-Siglec-F antibodies, treated with isotype antibodies or left unstimulated for 18 hours. Magnetic beads were used to immunoprecipitate and signal Siglec-F by acting as a secondary antibody. As a negative signalling control, unstimulated cells were lysed and later their Siglec-F receptor immunoprecipitated. The blot was then stained for presence of SHP-1 as this protein should have been recruited to the intracellular domain of Siglec-F during crosslinking (*Tourdot et al., 2013*).

Results showed a band near 70kDa (68kDa for SHP-1) in samples where alveolar macrophages had been crosslinked with anti-Siglec-F antibodies. This suggests the antibody crosslinking is successful in recruiting SHP-1 to the intracellular domain of the Siglec-F receptor in alveolar macrophages. Samples treated with the isotype control had low levels of SHP-1 bands present, with all samples having bands around 55kDa and 25kDa, suspected to be the IgG heavy chain and IgG light chain respectively.

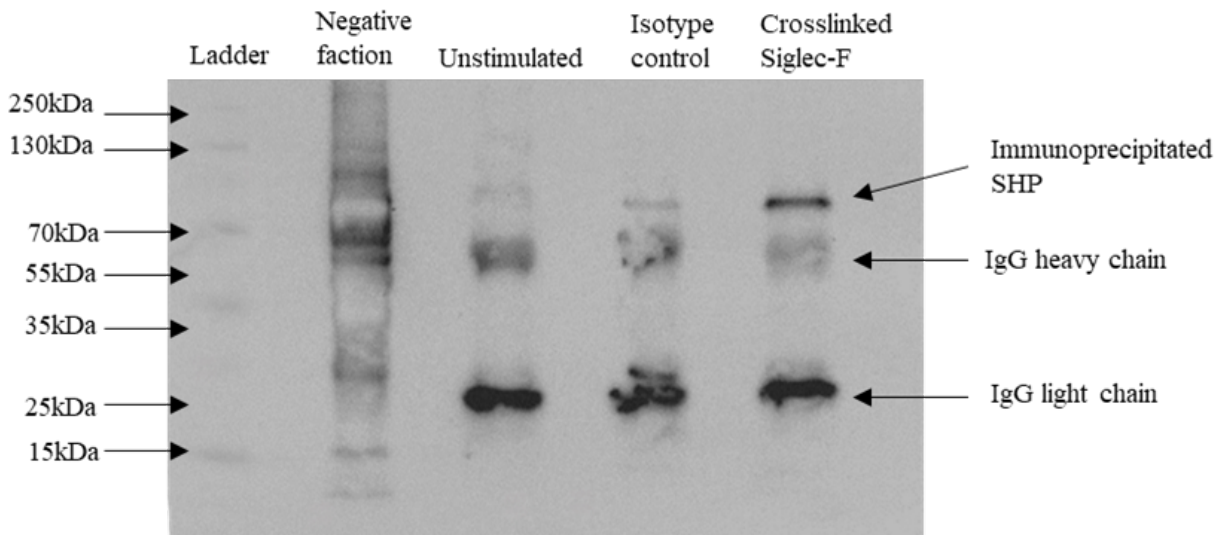


Figure 16: Crosslinking alveolar macrophages using anti-Siglec-F antibodies results in intracellular recruitment of SHP-1 to the receptor. Alveolar macrophages isolated from lungs of mice via BAL were crosslinked with anti-Siglec-F antibodies, crosslinked with an isotype control antibody or left unstimulated. Siglec-F cell lysets were immunoprecipitated and run on an SDS page gel for western blot analysis. Blots were incubated with an anti-mouse SHP-1 primary antibody and an anti-goat TrueBlot IgG antibody conjugated to HRP. The expected molecular weight of SHP-1 is 68kDa. Reactivity of the secondary reagents act as an internal loading control.

3.6 Siglec-F crosslinking alone does not activate alveolar macrophages to release TNF-α

When macrophages are exposed to inflammatory stimuli, in this case LPS, they secrete pro-inflammatory cytokines such as; TNF-α, IL-1β, IL-6, IL-23 and IL-12. TNF-α (a 185-aminoacid glycoprotein) is a cytokine produced during the early acute phase of the inflammatory response *(Idriss and Naismith, 2000)*. It is therefore a good readout of macrophage activation due to TLR agonists.

Figure 17 shows the concentration of the pro-inflammatory cytokine TNF-α after an 18-hour incubation with either crosslinked anti-Siglec-F antibodies, isotype antibodies, LPS (50ng/mL) or unstimulated. Siglec-F crosslinking shows no notable increase in the amount of TNF-α produced by alveolar macrophages after an 18-hour culture. This data suggests that crosslinking Siglec-F does not have a pro-inflammatory effect on alveolar macrophages as far as TNF-α production is concerned.

Siglec-F has been proposed to have an inhibitory function due to the presence of ITIMs in its cytoplasmic domain. Figure 18 shows that signalling through Siglec-F using crosslinking induces a small decrease in TNF-α production when cells are activated with LPS. This reduction in TNF-α is consistent with the hypothesis that Siglec-F has an inhibitory function.

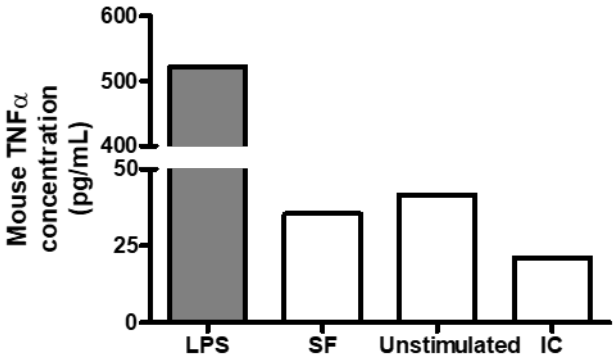


Figure 17: Alveolar macrophages crosslinked with anti-Siglec-F antibodies do not increase in TNF-α production in the absence of stimulation. Alveolar macrophages acquired via BAL were Siglec-F crosslinked for 18 hours, the supernatant was then used to measure TNF-α by ELISA. Data representative of one experiment.

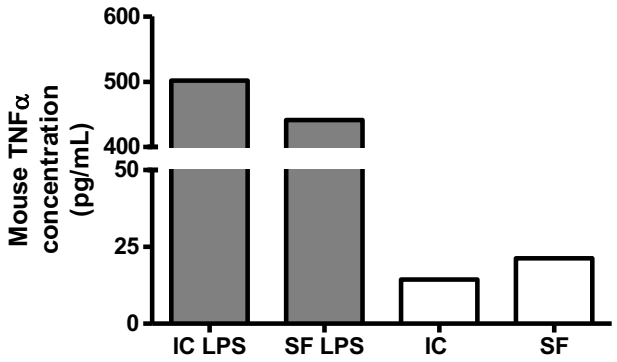


Figure 18: Alveolar macrophages crosslinked with anti-Siglec-F antibodies in the presence of LPS results in a decrease in TNF-α production. Alveolar macrophages acquired via BAL were crosslinked with anti-Siglec-F or isotype control with and without LPS at 50ng/mL for 18 hours, the supernatant was then used to measure TNF-α by ELISA. Data representative of one experiment.

3.7 Siglec-F crosslinking induces changes in a panel of different cytokines.

Following on from the previous preliminary TNF-α experiment, the outcome that receptor crosslinking has on the production of several cytokines was further explored. In order to accomplish this a LEGENDplex kit was used to simultaneously look at the concentrations of 9 different pro-inflammatory cytokines. A total of one million macrophages were then stimulated with and without LPS in the presence of Siglec-F or isotype control crosslinking.

The production of G-CSF showed a moderate increase when crosslinked with Siglec-F when compared to the isotype control. The expression of chemokines and cytokines like CXCL1, IL-6, TGF-β1 and TNF-α decreased when Siglec-F is crosslinked on the surface of alveolar macrophages under LPS stimulation supporting the hypothesis of this receptor as negative regulator. It should be noted that the levels of the cytokine IL-1β increased when macrophages were crosslinked with Siglec-F. However, it is interesting that this appears to be independent of LPS stimulation. IL-12p70, IL-12p40 and IL-23 were also measured but concentrations were undetected by the multiplex bead array assay.

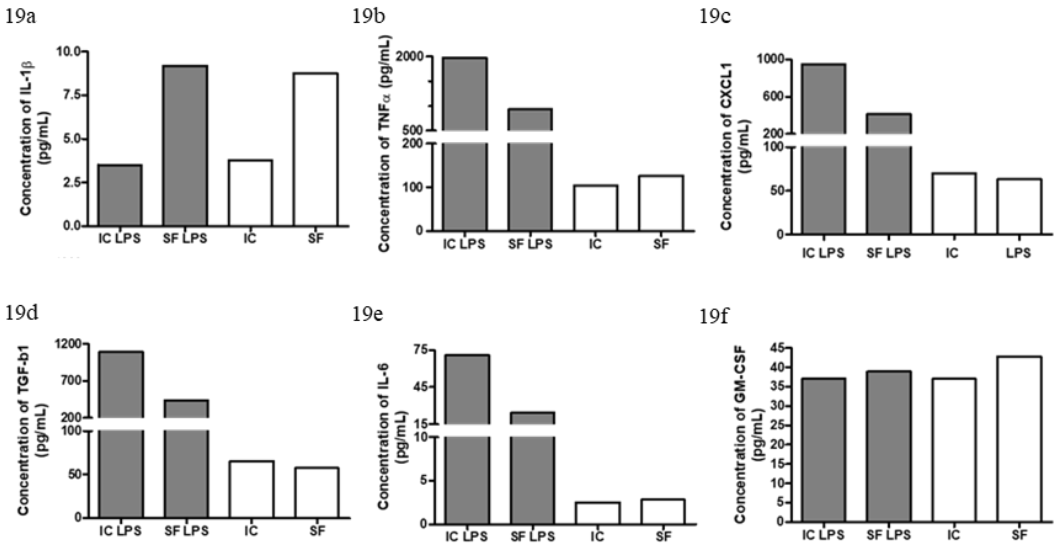


Figure 19: Crosslinking Siglec-F antibodies results in a change in the production of multiple cytokines. Mouse alveolar macrophages acquired via BAL were crosslinked using anti-Siglec-F antibodies or an isotype control in the presence (grey bars) and absence of 50ng/mL of LPS (white bars) for 18 hours. Supernatants were collected and cytokine production measured using a cytometric bead array assay. Data shown is the mean of triplicate wells of one experiment.

Table 6: Table reports the mean cytokine concentrations produced by macrophages incubated with/without LPS and with/without Siglec-F crosslinking.

Cytokine	**Median Cytokine Concentration** (pg/mL)			
	Isotype control + LPS	**Siglec-F + LPS**	**Isotype control**	**Siglec-F**
CXCL1	950.52	410.87	69.77	63.77
IL-6	70.91	24.19	2.54	2.82
GM-CSF	37.02	38.87	37	42.75
TGF-β1	1087.11	436.24	65.48	57.34
TNF-α	1976.02	931.32	105.18	126.91
IL-1β	3.51	9.2	3.76	8.76

3.8 Foetal liver cells can adopt macrophage morphology and potentially be used to replace primary cells

The use of animals in science is paramount but the potential induction of pain and stress has been of great concern to the general public. That is why in 1959 *the principles of humane experimental technique (Russell, Burch and Hume, 1959)* first proposed the three Rs, standing for: Replacement, Reduction and Refinement. It is the concept that as researchers we should always be looking for ways to carry out research with a minimal use of animals.

The average number of alveolar macrophages acquired using BAL is ~150,000 per mouse, requiring up to 15 mice for single experiments. In order to replace the need for acquiring primary alveolar macrophages, a previously-established protocol was investigated that differentiates primary untransformed but self-renewing alveolar macrophage-like cells from the foetal livers of mice (FLDAM; *Fejer et al., 2013).*

Foetal liver cells obtained from 18-day old C57BL/6 mouse embryos were cultured in 50ng/mL of GM-CSF in order to see if the cells would adopt macrophage characteristics after one month in culture. After 30 days, cells started to produce clusters similar to those produced by primary BMDMs and other macrophages, an identifying characteristic of macrophages. Although cells in cultures were morphologically heterogeneous, they were large and showed an elongated morphology, a phenotype that is consistent with macrophage characteristics.

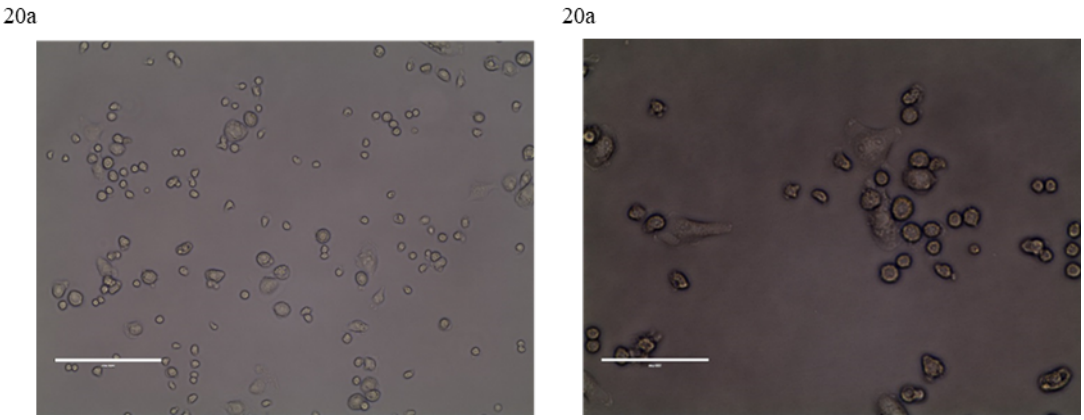


Figure 20: Mice foetal liver cells incubated with G-CSF for 30 days show macrophages morphology. Microscopic pictures of FLDAM incubated with G-CSF at 20x (left) and 40x (right). Pictures shown is a selection of one of three separate cultures.

3.9 Foetal liver-derived alveolar macrophage-like cells express markers naturally present in primary alveolar macrophages

To further support mouse foetal liver cells adopting an alveolar macrophage-like phenotype after incubation with G-CSF, cells were removed from the culture and stained for alveolar macrophage markers CD11c, CD200R and Siglec-F. Cell populations were gated according to the morphology of macrophages using an FSC vs. SSC dot plot. The expression of these markers was then compared to that from alveolar macrophages extracted via BAL. Foetal liver cells showed expression of CD11c, CD200R and Siglec-F. Siglec-F expression in alveolar macrophages is not shown here, though from literature it has been shown that >89% of alveolar macrophages are Siglec-F^{+} *(Svedberg et al., 2019)*. The expression of Siglec-F was tested in alveolar macrophages used in RNA-seq shown in figure 23.

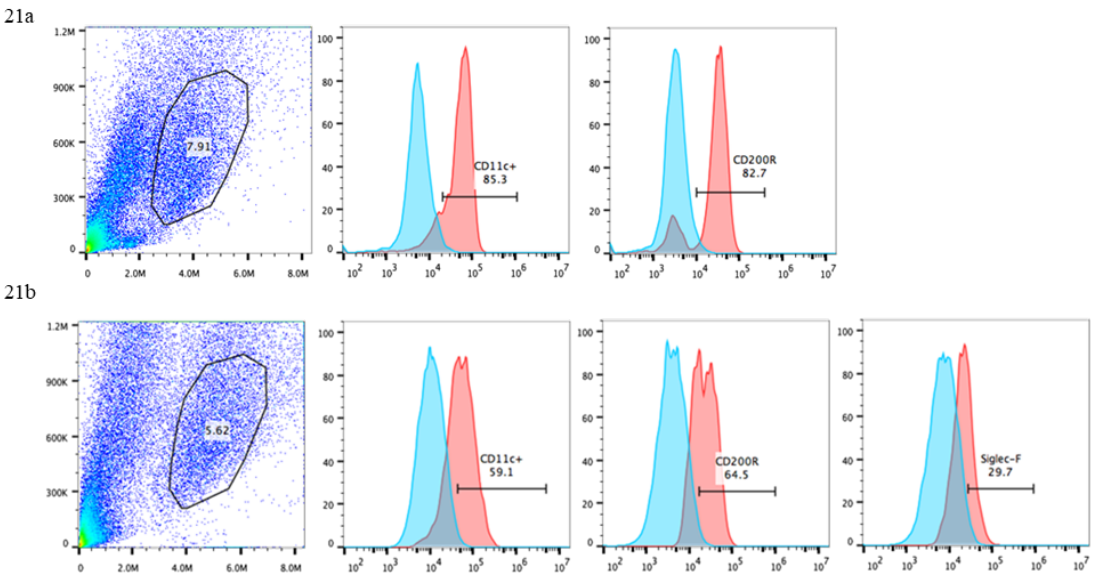


Figure 21: Foetal liver cells incubated with G-CSF show similar phenotype to alveolar macrophages. Comparative flow cytometry histograms are gated on FSC and SSC and show the expression of the common alveolar macrophage markers by FLDAMs after 30 days in the constant presence of 50ng/mL of G-CSF. 21a shows representative histograms overlays of lung alveolar macrophages whilst 21b shows cell-surface marker expression of FLDAMs. Blue histograms represent unstained background controls and the numbers within each plot show the frequency of cells that are positive for the relevant receptor. Data shown is representative of two independent observations.

3.10 Foetal liver-derived alveolar macrophage-like cells proliferate in the presence of G-CSF media

If FLDAM are to be considered a possible substitute for primary alveolar macrophages, there needs to be a constant supply of cells. To assess whether these cells are able to proliferate in the presence of GM-SCF, the expression proliferation marker Ki67 was analysed *(Scholzen and Gerdes, 2000)*. After 10 weeks in culture an average 50.8% of FLDAM showed positive levels of Ki67 compared to the unstained population suggesting that FLDAM do proliferate when cultured with GM-CSF. It is important to mention that cells die in culture in the absence of GM-SCF (data not shown).

Though these cells show promise for being a substitute for primary alveolar macrophages the decision was made to use primary alveolar macrophages for RNA-seq to be more confident in conclusions.

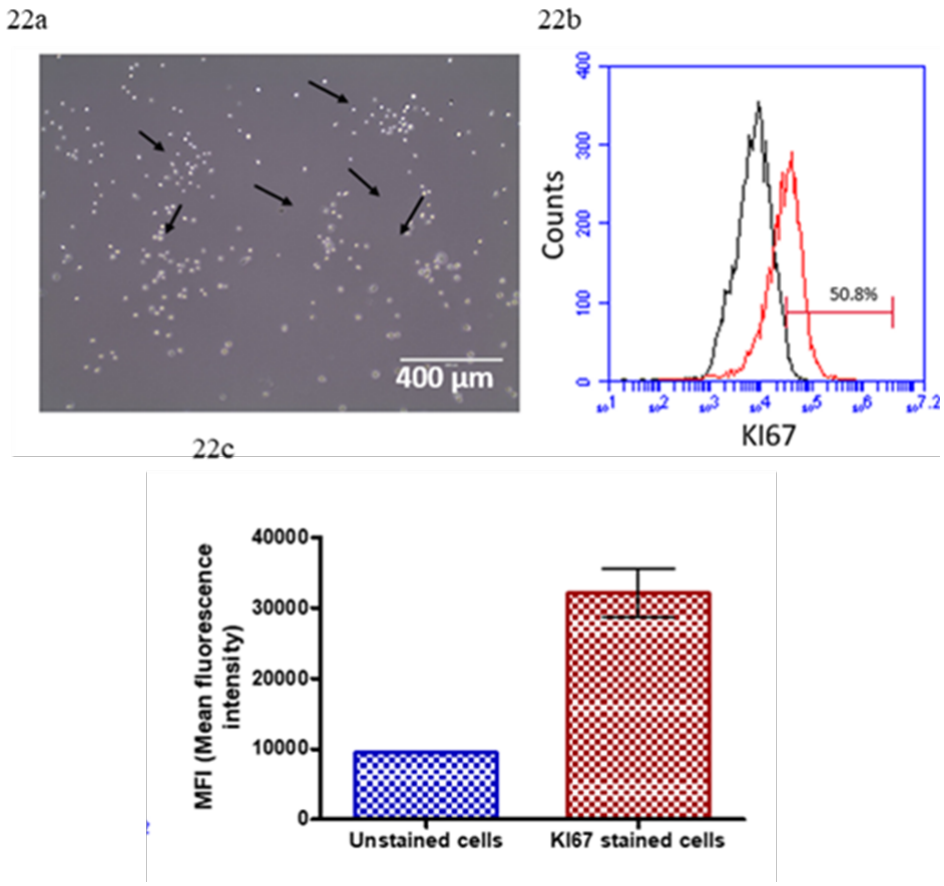


Figure 22: FLDAM expresses the proliferation marker Ki67. 22a shows a light microscopy image of FDMs clustering (arrows; Scale bar 400µm) when grown in the presence of GM-CSF 50ng/ml for 30 days. 22b shows the frequency of Ki67 expressing cells in the culture as a FACS histogram overlay comparing the unstained cells (black) and FLDAMs stained with anti-Ki67 antibody (red). The numbers within each plot show the frequency of cells that are positive for Ki67. 21c is a bar chart showing cumulative Ki67 expression (MFI ± SD) from three independent experiments. Data produced in collaboration with MSc students Emily Rosebery Briggs, Adina Trefas and Rumaysa Ahmad.

3.11 RNA sequencing

3.11.1 The experimental design

To obtain RNA for sequencing, a crosslinking experiment was designed that would both allow for an informational set of samples as well as biological repeats. The sample defined for this analysis are shown in table 7. These included a group of samples in culture that were crosslinked or left unstimulated and a "reference" sample consisting of RNA from alveolar macrophages straight out of the lung.

The composition of the cell suspension obtained by BAL was assessed prior to culturing using FACS to assess the proportion of cells that are alveolar macrophages. Figure 23 shows that the proportion of cells used in the culture and, therefore, taken for sequencing corresponds to cells expressing alveolar macrophage surface markers.

After 18 hours in culture, RNA was extracted and taken for sequencing (see methods). Once the sequence data was obtained, statistical analysis were carried out and included: data normalisation, graphical exploration of raw and normalised data and testing for differential expression for each feature between the conditions. For the purpose of this MRes dissertation a preliminary analysis of this data will include: a visualisation of the variability between replicas in each condition using PCA and gene ontology analysis of normalised differential expressed genes. This will be performed between samples from macrophages that received Siglec-F crosslinking or isotype control treatment.

Table 7: This table shows the conditions and dates of labelled RNA-seq data.

SAMPLE LABEL	CONDITIONS	DATE
JST246	Straight out of the lung	24/6/2019
JST266	Straight out of the lung	26/6/2019
JSU246	Unstimulated	24/6/2019
JSU266	Unstimulated	26/6/2019
JSIC246	Crosslinked with Isotype control	24/6/2019
JSIC266	Crosslinked with Isotype control	26/6/2019
JSSF246	Crosslinked with anti-Siglec-F antibodies	24/6/2019
JSSF266	Crosslinked with anti-Siglec-F antibodies	26/6/2019

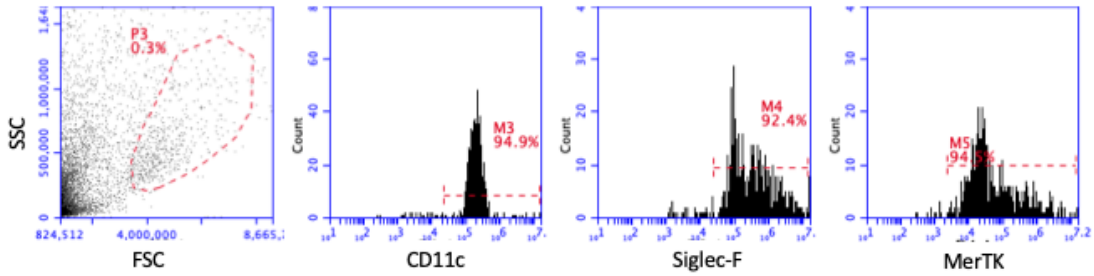


Figure 23: Cells obtained by BAL are alveolar macrophages with significant purity. FACS plot showing the expression of markers characteristic of alveolar macrophages. Numbers in the plots show the frequency of cells positive for each cell surface receptor. Data representative of one experiment

3.11.2 Assessment of variability by principal component analysis

The main source of variability between samples should be due to the differences between biological conditions. One way of analysing this is to look at the first principal components of the PCA, as shown on the figure 24. On this figure, the first principal component (PC1) is expected to separate samples from the different biological conditions, meaning that the biological variability is the main source of variance in the data.

The data shows that alveolar macrophages straight out of the lung cluster together tightly, suggesting that there is very little variance between these samples. These samples also segregate away from cultured samples as expected. The unstimulated and Siglec-F replicas seem to also cluster closely together, this also suggests little variance between data. However, the isotype control treated replicas are separated which might limit the differential gene expression analysis. This batch variability was taken into consideration in the statistical analysis that underpins the gene expression data.

It is expected that when removed from the lung and placed on plastic in culture for 18 hours there will be a variation in gene expression. This is confirmed in figure 25, whereby in all volcano plots for JST vs 18 hour alveolar macrophages cultures, many genes experience a large fold change. This is demonstrated by the low dashed grey line and large number of red dots.

Cultures for crosslinked Siglec-F vs crosslinked isotype control show a more defined number of genes which have been linked to biological processes. Interestingly there is variation between unstimulated vs crosslinked isotype, even though the crosslinked isotype should not bind to alveolar macrophages.

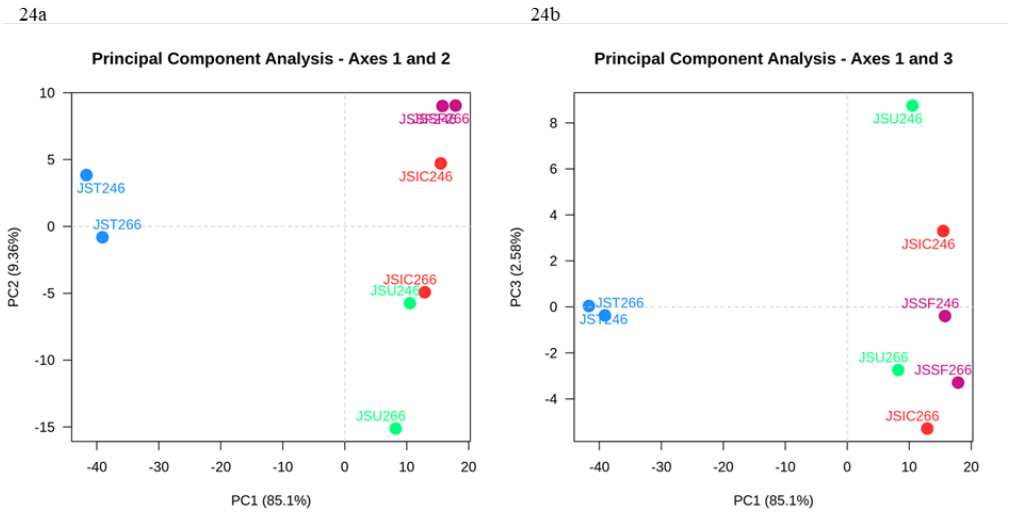


Figure 24: Sample variability expressed as a dot plot of principal components. Data obtained from regularised log transformed data. These scatter plots look at the first principal components of PCA. The first graph compares PC1 (representing the central 85.1% of the data) vs. PC2 (representing 9.36% of the data) and the second figure comparing PC1 vs. PC3 (representing 2.58% of the data). Data is shown as coloured dots: Blue; straight out of the lung, Green; unstimulated, Red; isotype control and Purple; Siglec-F crosslinked. Numbers are representative of dates 246, 24th of June and 266, 26th of June.

Table 8: Number of upregulated, downregulated and total of differentially expressed genes for each comparison.

Test vs Ref	Upregulated genes	Downregulated genes	Total
Isotype control vs Straight out of the lung	2385	2722	5107
Siglec-F vs Straight out of the lung	2806	3066	5872
Unstimulated vs Straight out of the lung	2186	2476	4662
Siglec-F vs Isotype control	154	142	296
Unstimulated vs Isotype control	224	285	509
Unstimulated vs Siglec-F	1240	1190	2430

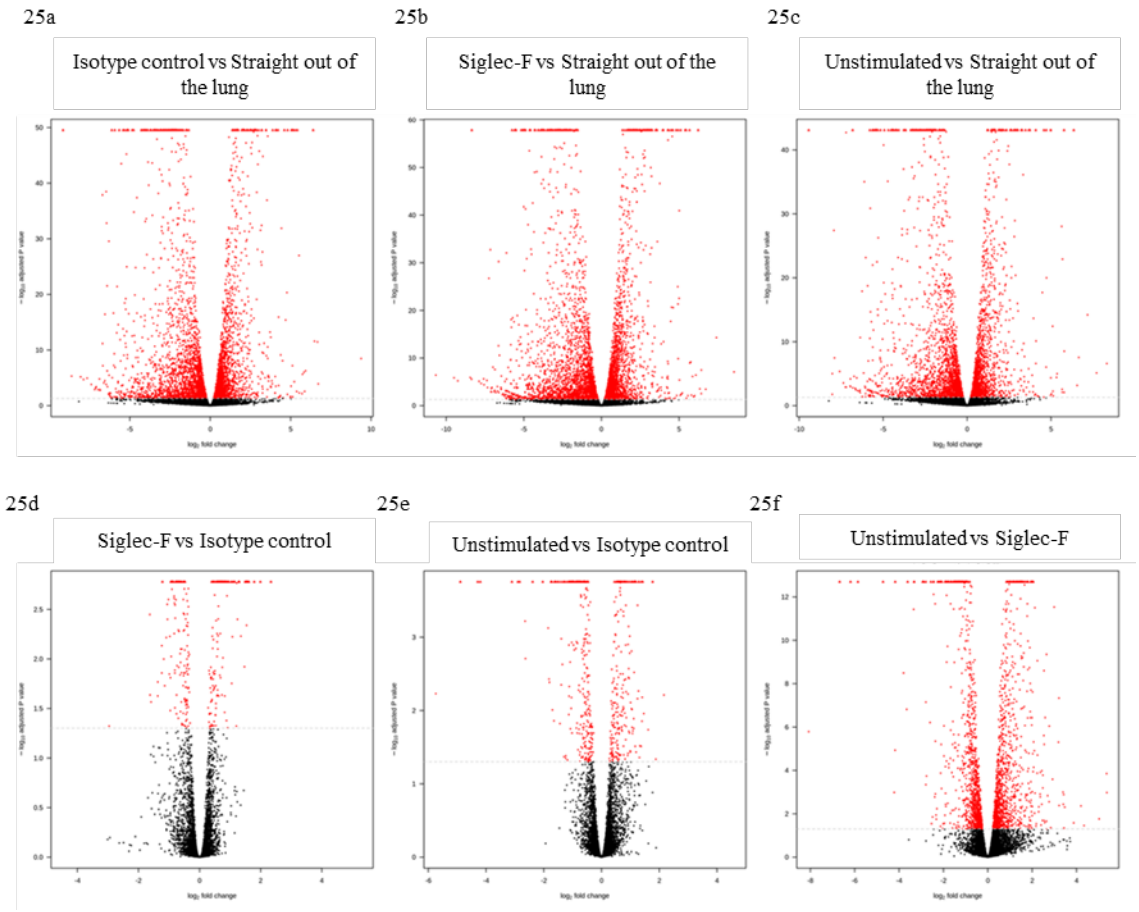


Figure 25: Volcano blots of comparisons with the red representing significant changes in gene expression. The red dots represent genes that have a large fold change when compared with the test (>2) and a high statistical significance (-log10 of p value, y axis). The dashed grey line shows where p = 0.05 with points above the line having p < 0.05 and points below the line having p > 0.05. The black dots represent genes that have an adjusted P value <0.05.

3.11.3 Preliminary gene ontology analysis

A global gene ontology analysis was carried out, with the aim of exploring the different genes that were either differentially upregulated or downregulated by signalling through Siglec-F. To recapitulate, these datasets correspond only to the pairwise comparison between cells crosslinked with an anti-Siglec-F antibody and its corresponding isotype negative controls (JSSF vs JSIC). The complete gene lists can be found in the appendices.

Figure 26 shows the types of proteins encoded by the genes that were significantly downregulated. Among the most frequent were genes that corresponded to enzyme modulators with functional relevance to immune cells, such as Lair1 and importantly the kinase activator G1-S Cyclin D1. Another important group of genes with immunological function relevant to alveolar macrophages include: the chemokine *Cx3cl1*, the TLR signalling modulator *TREM2* and the *IL-6* receptor alpha. It is important to highlight that the data shows that signal transduction though Siglec-F modulates its own expression as well as the expression of Siglec-

E, suggesting a potential regulatory feedback loop and crosstalk between members of the same protein family respectively.

Some pathways can be associated with Siglec-F signalling via the downregulation of gene targets. Some of these pathways include: the inflammation mediated by cytokine and chemokines, integrin signalling and the gastric-cholecystokinin signalling pathway, the latter involved in mucosal biology (see figure 27).

It is evident that there might be a potential link between Siglec-F signalling and efferocytosis via downregulation of the cell surface receptor tyrosine kinase Axl which is part of the TAM family of kinases including TYRO3 and MERTK.

In contrast, figure 28 shows the types of proteins encoded by genes that were significantly upregulated. Among these were important genes that encode nuclear acid binding proteins with translation and transcription regulatory activity. Examples of these genes are the translation initiation factor *Eif4a* and *Eif4b.* Additionally *Casp-4*, an important gene associated with apoptosis, was upregulated upon Siglec-F signalling.

Finally, figure 29 shows that are several pathways linked with Siglec-F signalling via upregulation of gene expression. In this category important pathways such as TGF- β signalling (via the inhibin *Inhba*), TLR signalling and apoptosis were uncovered.

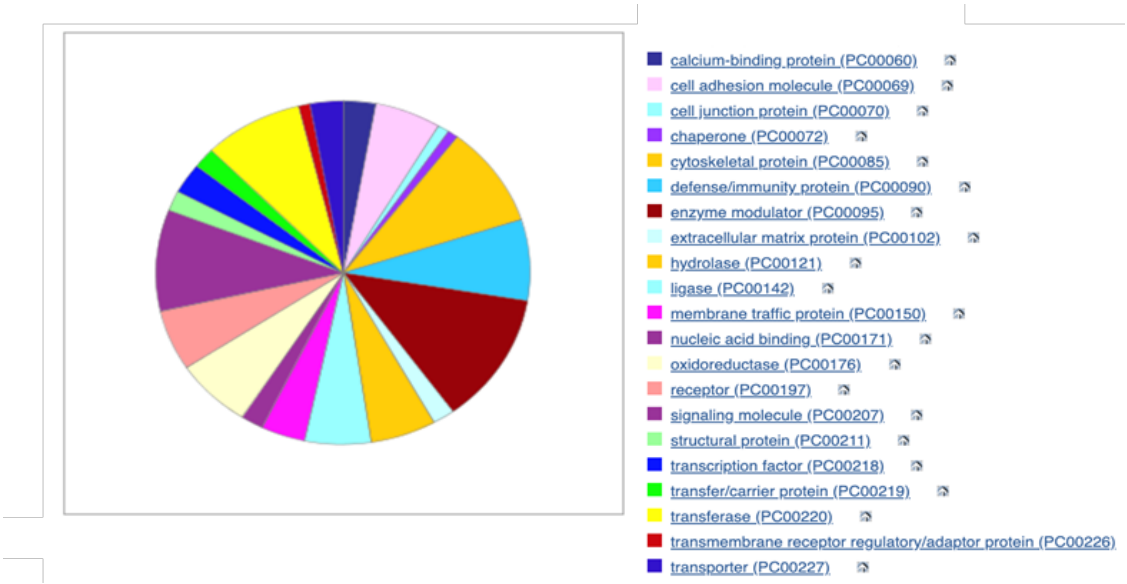


Figure 26: Genes that were downregulated upon Siglec-F crosslinking compared to isotype control. This pie chart shows the different genes that were downregulated upon Siglec-F crosslinking, whereby area of pie chart represents relative amount of downregulation. Genes appear on the pie chart in a clockwise order starting with calcium-binding protein. Gene lists can be found in appendix two. Graph was created using PANTHER.

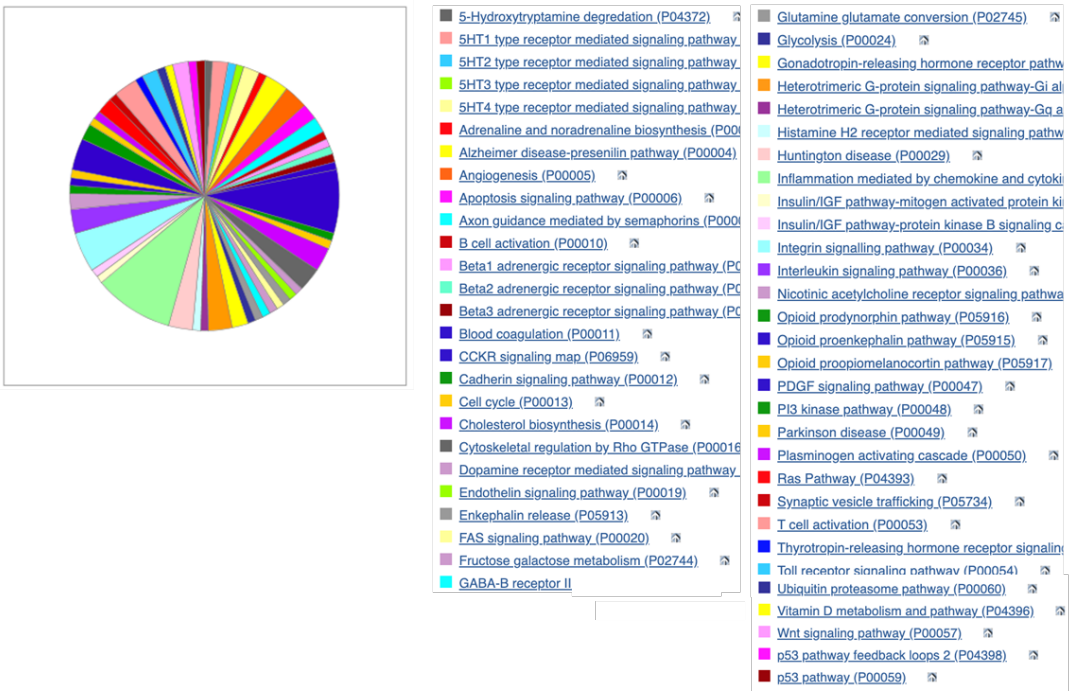


Figure 27: Pathways associated with the genes that were downregulated upon Siglec-F crosslinking compared to isotype control. This pie chart shows the different pathways that were downregulated upon Siglec-F crosslinking, whereby area of pie chart represents relative amount of downregulation. Pathways appear on the pie chart in a clockwise order starting with 5-hydroxytryptamine. Graph was created using PANTHER.

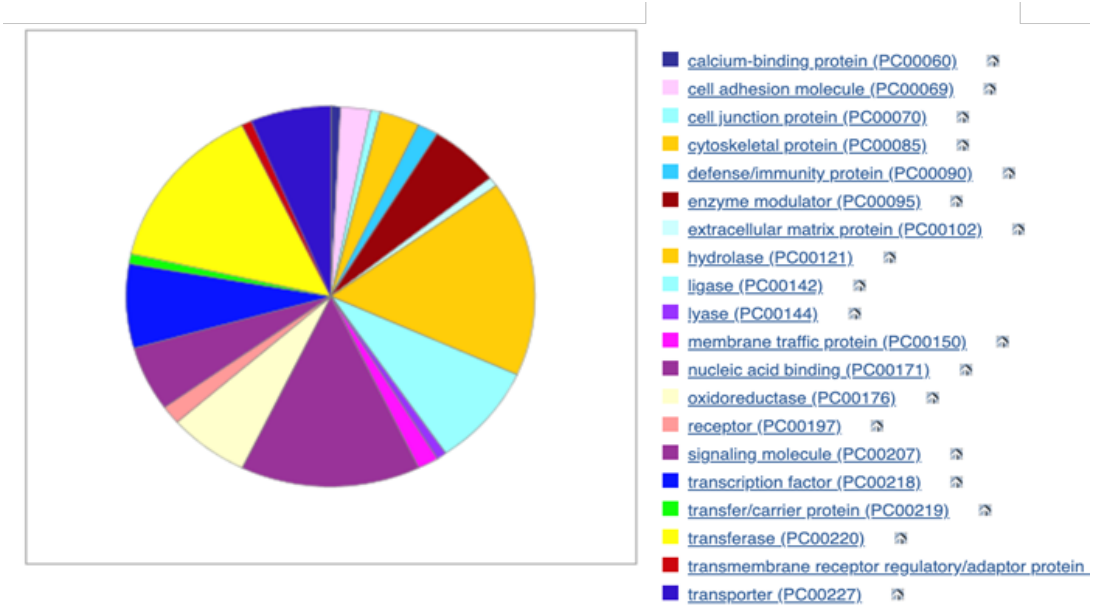


Figure 28: Genes that were upregulated upon Siglec-F crosslinking compared to isotype control. This pie chart shows the different genes that were upregulated upon Siglec-F crosslinking, whereby area of pie chart represents relative amount of upregulated. Genes appear on the pie chart in a clockwise order starting with calcium-binding protein. Gene lists can be found in appendix one. Graph was created using PANTHER.

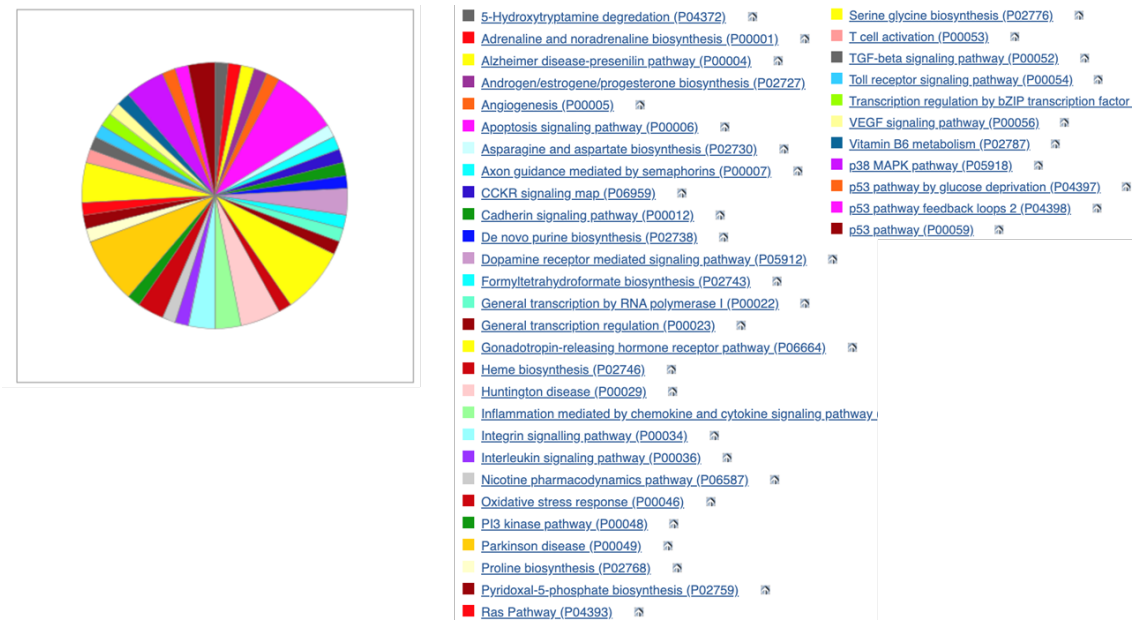


Figure 29: Pathways associated with genes that were upregulated upon Siglec-F crosslinking compared to isotype control. This pie chart shows the different pathways that were upregulated upon Siglec-F crosslinking, whereby area of pie chart represents relative amount of upregulation. Pathways appear on the pie chart in a clockwise order starting with 5-hydroxytryptamine. Graph was created using PANTHER.

4. Discussion

4.1 The rationale for characterising the outcomes of Siglec-F signalling

The overall aim of the project was to design a system that could induce specific signals through Siglec-F and explore the outcomes which, to date, are very poorly understood. Siglec-F has been proven to be expressed in high amounts on alveolar macrophages, eosinophils and some activated T cells *(Zhang et al., 2007)* being attributed as a modulator of apoptosis in eosinophils *(O'Reilly and Paulson, 2009)*. Preliminary analysis of alveolar macrophage function have failed to address the role of this molecule in regulating phagocytosis and to date no data has been produced to support its role in the regulation of cytokine production *(Feng and Mao, 2012)*.

The lung microenvironment is rich in molecules that contain the natural ligands for Siglec-F and studies have shown that there is a regulatory interaction between mucins containing sialic acids and alveolar macrophage activity (*rev in Janssen et al., 2016)*.

The data generated in this study, although preliminary, could provide further evidence to support the role of this molecule as a regulator of alveolar macrophage activity.

4.2 Siglec-F is highly expressed on alveolar macrophages

When comparing macrophages obtained via BAL with macrophages obtained from the peritoneal cavity and in-vitro-differentiated macrophages; the majority of alveolar macrophages expressed the Siglec-F receptor. Existing literature shows that Siglec-F is expressed on upwards of 90% of cells extracted via BAL *(Svedberg et al., 2019)*, whereas in the current data only 70-80% of cells are expressing Siglec-F (shown in figure 8). This lower percentage yield is likely due to a small blood contamination likely caused by cervical dislocation. This contamination results in a myeloid population which have similar FSC vs. SSC properties to macrophages as well as also expressing F4/80. To control and minimise the consequences of having this confounder, mice used for RNA-seq analysis were sacrificed using lethal injection with pentobarbital (100mg/Kg; at UCL). To check for contaminations of samples used for RNA-seq, FACS staining was used for several alveolar macrophage-specific cell surface receptors to identify and gate on these cells. This is demonstrated in figure 23 where markers CD11c, MERTK and Siglec-F were used to identify alveolar macrophages for RNA-seq analysis. These markers can also be used to distinguish between alveolar macrophages, interstitial macrophages and monocytes if there was a contamination *(Svedberg et al., 2019)*.

As shown in figure 8, the high level of expression of Siglec-F seen in alveolar macrophages contrast with the low levels seen in lung interstitial macrophages. This result suggests that the

presence of the receptor is not expressed in all tissue resident macrophages, with Siglec-F also being absent in conventional dendritic cells and monocytes when at a steady state *(Misharin et al., 2013; Sabatel et al., 2017; Gibbings et al., 2017).* This data combined alveolar macrophage ontology and cell fate studies, which support the idea that interstitial macrophages originate from circulating monocytes and may be an intermediate stage in the development and migration to the airways (*Landsman and Jung, 2007*), and in this process, they acquire a robust expression of Siglec-F.

4.3 Are alveolar macrophages M1 or M2

The results show that most of the Siglec-F expressing alveolar macrophages exist as classically activated macrophages (Figures 11 and 12: 92.1%), this supports the highly inflammatory nature of alveolar macrophages. Though it is important to mention that 2.51% of alveolar macrophages are both CD38+ and Arg1+. This observation has been previously reported as alveolar macrophages can exhibit characteristics of both pro-inflammatory and tolerogenic macrophages (*Quillay et al., 2014*). However this data suggests that most macrophages exist as M1 macrophages, this is in contrast to previous research that states the majority of the alveolar macrophage population exist as a duo phenotype (*Mitsi et al., 2018*). Nevertheless, this data provides evidence that not all alveolar macrophages, and possibly not all tissue macrophages, fall into the M1/M2 classification and perhaps have a more diverse plasticity (See Figure 10). It is possible that this duo phenotype is essential in maintaining a balance between immune response against pathogens and immune tolerance (*Mitsi et al., 2018*).

Figure 13 shows that both CD38+ and CD38+Arg1+ cells express Siglec-F (>80%), this is to be expected as in figure 8, 72% of alveolar macrophages obtained via BAL express Siglec-F. The expression of Siglec-F in CD38+ and CD38+Arg1+ cells are comparable and thus Siglec-F cannot be used as a distinguishing feature for M1 or M2 alveolar macrophages.

Alveolar macrophages are heterogeneous with a small proportion of them expressing distinct phenotypic markers which could be due to macrophage plasticity (*Hussell and Bell, 2014*). Expression of these receptors might change under specific situations; like allergen exposure inducing Arg1 or bacterial or viral infections which would induce expression of CD38 *(Gordon, Plüddemann and Martinez Estrada, 2014)*.

4.4 Siglec-F can be successfully targeted

The Siglec-F antibody crosslinking system was shown to successfully target the Siglec-F receptor demonstrated in figure 14. This figure shows a decrease in florescence after antibody crosslinking of the Siglec-F receptor. It is important to state that this decrease is not 100%, this is important as it is unlikely that in vivo mucin5b or other ligands will bind to all the Siglec-F

receptors on an alveolar macrophage. The levels of receptor targeting in these experiments were kept constant by keeping the same concentration of crosslinking reagents across all experiments. However, the physiological level of signal strength provided by crosslinking cannot be easily compared with that of the natural ligands.

Data from the western blot experiment (Figure 16) showed that Siglec-F can be signalled using antibody crosslinking, by assessing that SHP-1 was recruited to the Siglec-F receptor. Interestingly SHP-1 was also recruited to a receptor in the case of the isotype control. This behaviour can be hypothesised to have occurred as the FC region located on all antibodies could have bound to a FC receptor on the alveolar macrophages, leading to SHP-1 recruitment to the ITAM located in the cytoplasmic tail *(Abram and Lowell, 2017).* To further explore this hypothesis this experiment would need to be repeated using CD16/32 or another FC blocker.

It is important to note that the presence of SHP-1 on the western blot is not definitely caused by the protein being bound to the intracellular domain of the Siglec-F receptor. This is due to the methodology as the blot was never stripped and incubated with fluorescently conjugated Siglec-F antibodies. Further work could therefore develop on this method and strip the blot after the incubation and reading of SHP-1 as well as repeating the incubation with primary anti-Siglec-F antibodies. Confirmation of the presence of both the Siglec-F receptor and SHP-1 would strengthen this claim.

4.5 Do alveolar macrophage undergo apoptosis upon Siglec-F crosslinking?

A well-documented effector function of Siglec-F crosslinking in eosinophils is that they undergo apoptosis *(Zhang et al., 2007)*. However, it is unknown whether crosslinking Siglec-F on the surface of alveolar macrophages stimulates apoptosis. To test whether this occurs in alveolar macrophages populations were incubated with: crosslinked anti-Siglec-F antibodies, an isotype control, left unstimulated or incubated with known immune stimulant LPS. The levels of apoptotic cell marker, phosphatidylserine, was then measured at different time points by binding annexin V.

Data showed that antibody crosslinking of the Siglec-F receptor on alveolar macrophages does not induce apoptosis like in eosinophils as shown in figure 15. In this figure, the expression of phosphatidylserine in alveolar macrophages where Siglec-F receptors were crosslinked were comparable to that of the unstimulated population. Siglec-F crosslinking also induces similar levels of apoptosis to that seen in macrophages incubated with the isotype control, meaning that the use of antibodies does not cause apoptosis. Cell death does occur minimally at 18 hours, but cell death is greater at 24 hours, this is likely due to extended absence from the lung

environment. It is for this reason 18 hours was chosen as a suitable time point for RNA sequencing.

4.6 Proinflammatory cytokine production is reduced by Siglec-F crosslinking

From the apoptosis data it was concluded that alveolar macrophages do not apoptose after Siglec-F crosslinking and natural cell death is minimal at 18 hours. It is for this reason that alveolar macrophages were incubated for 18 hours for the cytokine experiments.
Cytokine production was used to measure the immune activity of alveolar macrophages as alongside chemokines their production are some of the initial effector functions of an activated macrophage (*Arango Duque and Descoteaux, 2014*). Initially the production of TNF-α was considered, a cytokine produced in the acute phase of inflammation *(Idriss and Naismith, 2000)* and will thus be at relatively high concentrations after 18 hours of stimulation with and without LPS. The data shows a relatively large concentration of TNF-α when incubated with LPS, as to be expected as LPS is a strong stimulator of macrophage immune response (*Han et al., 2017*). When Siglec-F is crosslinked, the concentrations of TNF-α are similar to that of the unstimulated macrophage population. This suggests that crosslinking Siglec-F does not result in a proinflammatory effector function. Concentrations of TNF-α were comparable when crosslinked with anti-Siglec-F antibodies or with isotype control suggesting that using antibodies has little or no effect on production of this cytokine. When alveolar macrophages are crosslinked with Siglec-F in the presence of LPS there is a decrease in the amount of TNF-α produced when compared to that of just LPS. This means that crosslinking Siglec-F results in negative regulation of the production of proinflammatory cytokine TNF-α. To further support this theory a multiplex cytokine bead array was performed that looked at nine different cytokines; G-CSF, CXCL1, IL-1β, IL-23, IL-12p70, IL-12p40, IL-6, TNF-α, and TGF-β1. In all repeats of this experiment IL-12p70, IL-12p40 and IL-23 concentrations were undetected by the multiplex kit. Lack of IL-12p70 production has been observed in alveolar macrophage activation by LPS (*Isler et al., 1999*), however the same paper observed an increase in the production of cytokine IL-12p40 (*Isler et al., 1999*). IL-12p70 has been shown to be produced by alveolar macrophages when activated by LPS alongside an IL-10 production inhibitor (Isler et al., 1999). In an in vitro environment LPS binds to TLR4, inhibiting IL-10 production though this is alongside TLRs 2 and 9 (*Fernandez et al., 2004*). IL-23 production has been seen to be induced by LPS (*Bosmann et al., 2013*). However, in this instance mice inhaled LPS, inducing lung inflammation in vitro (*Bosmann et al., 2013*).

CXCL1 is a chemokine playing a pivotal role in recruiting and activating neutrophils *(Altmann et al., 2012),* and thus is produced in relatively small amounts by resting macrophages as seen in figure 19. When activated the alveolar macrophages begin to produce an increased amount of

the chemokine *(Altmann et al., 2012),* with reductions in CXCL1 production being used to measure decreases in lung inflammation *(Altmann et al., 2012)*. Increases in CXCL1 are also seen in acute lung injury *(Altmann et al., 2012)*. The crosslinking of Siglec-F reduces the production of CXCL1 (shown in figure 19) indicating inhibition of the CXCL1 production pathway.

TGF-β is generally considered a central mediator of the resolution phase of tissue injury, inhibiting tissue inflammation and enhancing scarring (*Khalil et al., 1989*), with signalling of the TGF-β receptor being required for the homeostasis of alveolar macrophages (*Yu et al., 2017*). These results showed an increase in concentration of the cytokine when alveolar macrophages were activated with LPS. This suggests an important role in the initial stages of development, where the cytokine acts as a potent chemoattractant for monocytes, lymphocytes, neutrophils and fibroblasts (*Breit and Wahl, 2001*). This imbalance between the immune resolution and proinflammatory activities of TGF-β can cause chronic states of inflammation (*Breit and Wahl, 2001*). In figure 19 there is a percentage decrease of 60% in concentration when crosslinked with Siglec-F in the presence of LPS, suggesting an anti-inflammatory role for the receptor during the innate stages of inflammation. Decreases in TGF-β expressing cells after crosslinked Siglec-F has been seen in previous research (*Song et al., 2009*). However, this finding was observed using eosinophils and as discussed previously, there are differences in functional outcomes as a result of Siglec-F signalling in macrophages vs eosinophils (apoptosis). Therefore, more research into Siglec-F crosslinking effecting TGF-β production by alveolar macrophages is needed to strengthen existing literature.

IL-6 has been previously shown to enhance the polarisation of alternatively activated macrophages *(Fernando et al., 2014)* in synergy with cytokines IL-4 and IL-13, with more recent research suggesting IL-6 might also play an essential role in the Th2-mediated allergic response (*Deo et al., 2010*). The role of this cytokine for polarisation of alternatively activated macrophages may explain the cytokines lower concentrations when compared to that of TNF-α, CXCL1 and TGF-β1, as alternatively activated macrophages represent a small percentage of the population (seen in figure 11). Alveolar macrophages have been shown to contribute the largest fraction of IL-6 in the lungs (*Gubernatorova et al., 2018*) with elevated levels of the cytokine being a major contributor to the pathogenesis of asthma (*Gubernatorova et al., 2018*). The reduction in the presence of Siglec-F crosslinked suggests an anti-inflammatory role and shows opportunity for Siglec-F being a possible treatment for asthma.

When looking at results for LEGENDplex there are two graphs that are different from all others, the first being IL-1β, a cytokine that is involved in pain, inflammation and autoimmune conditions. The results for this cytokine are an increased concentration when crosslinked with

Siglec-F independent of LPS. Currently it is unknown why this occurs though due to the relatively low concentrations this could have occurred due to chance. The lack of CXCL1 production could be due to the absence of neutrophils in the culture. Neutrophils are thought to be the sole source of the cytokine though neutrophils have been suggested to indirectly mediate IL-1β release in macrophages (*Peiró et al., 2017*). Levels of G-CSF seem to be consistent across all samples regardless of crosslinking and LPS, this can be explained as G-CSF is a colony stimulating factor that stimulates cells to proliferate which within an alveolar macrophage population is essential as these cells possess the ability of self-renew without a contribution from the bone marrow (*Hashimoto et al., 2013*). The relatively low though similar concentrations highlight this fact as alveolar macrophages need to maintain a consistent population to prevent over colonisation. This is in contrast with published literature that concluded alveolar macrophages produced large amounts of G-CSF following endotoxin exposure *(Tazi et al., 1991),* though this was in the case of exposure to bacterial pneumonia and sarcoidosis.

It was concluded that alveolar macrophages are active after 18 hours and alongside the apoptosis data, 18 hours of incubation was chosen for the RNA-seq experiments.

4.7 FLDAM, a viable alternative to primary alveolar macrophages

Alveolar macrophages are cells crucial to the immune response in the lung (*Davies et al., 2013*). Though due to the low yield acquired from the lungs of mice coupled with the plasticity of macrophages, the study of their distinct effector functions is somewhat hampered. The establishment of an in vivo self-replicating culture of alveolar macrophage-like cells is therefore essential in the further study of alveolar macrophages both for potential cost savings and to be in line with the 3Rs (*Russell, Burch and Hume, 1959)*.

After a 30-day incubation with GM-CSF the mice liver foetal cells display traits associated with macrophages. Flow cytometry analysis (figure 21) showed that these cells are heterogenous and seem to resemble primary alveolar macrophages and therefore could be used as an alternative. Though the percentage of foetal liver cells expressing the Siglec-F receptor (29.7%) is far fewer compared to the high 90% expression of Siglec-F seen by other researchers *(Svedberg et al., 2019)*. Previous work on foetal liver cells cultured with GM-CSF observed the presence of alveolar macrophage receptors MARCO, Chl3l and CD11c *(Fejer et al., 2013)* with the latter also being observed in this data (59.1%). To further support the claim of being alveolar macrophage like cells more functional and activation analysis is needed.

An important trait to consider when finding a suitable alternative for alveolar macrophages is the ability of the FLDAM to self-replicate. When in the presence of the GM-CSF these cells can

be seen to replicate demonstrated by the presence of Ki67, a marker of cell proliferation. Foetal liver cell cultures can repopulate after populations are removed and used in experiments, as long as initial population is $>1x10^6$ cells, and seem to grow exponentially *(Fejer et al., 2013)*. With this evidence for self-renewal, it could be possible to calculate the rate at which FLDAM replicate, thus being able to take populations for use more regularly once a population has reached an estimated number.

4.8 Crosslinking Siglec-F results in downregulation of immune related pathways

RNA-seq is a widely used practice that allows researchers to profile gene expression (*Chu and Corey, 2012; Wang, Gerstein and Snyder, 2009*), with this information then being able to be linked with pathways. The data showed that a total of 296 genes were either up or downregulated as a result of Siglec-F crosslinking with key genes such as *TREM2* and *Axl* being downregulated. Axl is an important mediator of efferocytosis (*Grabiec et al., 2018*), suggesting that downregulation of this receptor would reduce the efferocytotic capacity of alveolar macrophages when Siglec-F is crosslinked, with the alveolar macrophages that are deficient in Axl displaying reduced efferocytotic capacity *(Grabiec et al., 2018).* A reduction in *Axl* mRNA is also seen in patients with moderate-to-severe asthma, a disease where efferocytosis is impaired *(Grabiec et al., 2017).*

The downregulation of TLR signalling modulator TREM2 could suggest a regulatory function as TREM2 expression on macrophages promotes phagocytosis of apoptotic neurons and bacteria (*Humphrey, Xing and Titus, 2015*). TREM2 does this with minimal proinflammatory cytokine production (*Humphrey, Xing and Titus, 2015*). The receptor is also seen to be upregulated during induced pulmonary inflammation (*Turnbull et al., 2006*). Interestingly the downregulation of TREM2 has been seen to increase the production of TNF-α and IL-6 in response to LPS in BMDM and peritoneal macrophages (*Turnbull et al., 2006*). More research is needed to see if this occurs with alveolar macrophages.

TLR8 is an endosomal receptor found inside macrophages (*Guiducci et al., 2013*) and is primarily responsible for recognising single-stranded RNA (*Eng, Hsu and Lin, 2018*). When activated TLR8 has been linked to an increase in the mRNA levels of *TNF-α* and *IL-6* (*Eng, Hsu and Lin, 2018*), this is mirrored whereby both cytokines were reduced when Siglec-F was crosslinked in the presence of LPS (figure 19).

4.9 Concluding remarks

This project uncovered significant data of how crosslinking Siglec-F in alveolar macrophages effects production of cytokines and gene expression, which could create new avenues for future study.

4.10 Future research

The research performed in this dissertation has looked at the effect of signalling alveolar macrophages through the cell surface receptor Siglec-F, the results of which would benefit from further research.

RNA-sequencing of Siglec-F crosslinked alveolar macrophages showed a decrease in *Axl*, a marker associated with efferocytosis. To establish cause and effect, future research should consider performing an efferocytosis assay after alveolar macrophages are crosslinked with Siglec-F for 18 hours.

Research into FLDAM being a possible substitute for primary alveolar macrophages looks promising however as discussed earlier more work needs to be done solidifying this claim. Similar to how production of cytokines was measured in this discussion, a multiplex kit can be used to measure the concentration of cytokines in the presence of LPS and/or crosslinked Siglec-F antibodies. The comparison of this data with that obtained in this dissertation could further support the claim of FLDAM.

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6. Appendix 1

List of differentially up-regulated genes resulting from a pairwise comparison between cells crosslinked with an anti-Siglec-F antibody and its corresponding isotype negative control (JSSF vs JSIC). The list reports normalised counts, fold change, Log2Fold change, p and adjusted p values for samples labelled as 246 and 266 each corresponding to one independent biological replica.

Id	norm.JSIC246	norm.JSIC266	norm.JSSF246	norm.JSSF266	FoldChange	log2FoldChange	pvalue	padj
Ddit3	509	492	1513	1456	2.965	1.568	1.10E-29	1.17E-25
Aars	4715	3959	8527	8213	1.934	0.952	4.50E-21	2.40E-17
Mthfd2	1953	1563	4051	4420	2.422	1.276	1.14E-19	4.04E-16
Asns	1272	955	3202	3483	3.068	1.617	1.45E-17	3.87E-14
Cyb5r1	935	893	1948	1814	2.056	1.04	8.16E-17	1.74E-13
Gbp8	161	221	476	644	2.934	1.553	9.62E-16	1.71E-12
Hspa9	7188	6581	12390	13243	1.867	0.901	1.64E-15	2.49E-12
Trib3	425	335	1497	1464	3.969	1.989	4.81E-15	6.41E-12
Slc7a5	966	712	1830	1877	2.222	1.152	2.36E-14	2.80E-11
Ccdc47	1486	1390	2445	2578	1.747	0.805	1.03E-13	1.01E-10
Eprs	4889	4257	7737	7837	1.709	0.773	1.04E-13	1.01E-10
Slc43a3	185	178	492	531	2.807	1.489	1.35E-13	1.20E-10
Atf5	999	726	1866	1467	1.939	0.955	1.53E-13	1.25E-10
Sars	2795	2624	4523	4313	1.631	0.705	3.87E-13	2.94E-10
Psat1	701	618	1358	1203	1.945	0.959	4.34E-13	3.08E-10
Phgdh	617	662	1375	1387	2.161	1.112	1.02E-12	6.79E-10
Pck2	820	733	1531	1702	2.078	1.055	1.52E-12	9.51E-10
Slc7a1	1544	1220	3151	3552	2.46	1.299	8.45E-12	4.74E-09
Atf4	4800	4063	8618	8371	1.928	0.947	1.25E-11	6.66E-09
Aqp9	190	141	478	498	2.984	1.577	2.42E-11	1.22E-08
Nfe2l1	6081	5633	8875	9796	1.594	0.672	2.51E-11	1.22E-08
Ghitm	4411	4246	6850	8433	1.763	0.818	3.52E-11	1.63E-08
Yars	1001	930	1659	1710	1.744	0.802	4.51E-11	1.92E-08
Gtpbp2	1892	1554	3446	3650	2.053	1.038	6.06E-11	2.49E-08
Xpot	1704	1611	2678	2876	1.678	0.747	1.44E-10	5.48E-08
Arsg	848	821	1525	1471	1.794	0.843	1.71E-10	6.29E-08
Nars	4041	3792	5911	5794	1.495	0.58	1.91E-10	6.78E-08
Slc38a2	2837	2460	4214	4576	1.66	0.731	3.74E-10	1.25E-07
Rhbdd1	1134	961	1789	1757	1.696	0.762	4.74E-10	1.49E-07

Eif3c	6684	6272	9554	9781	1.494	0.579	7.98E-10	2.43E-07
Aldh18a1	813	738	1383	1398	1.789	0.839	1.24E-09	3.56E-07
Shmt2	705	652	1209	1251	1.812	0.857	1.08E-08	2.88E-06
Dusp1	438	390	659	850	1.825	0.868	1.36E-08	3.54E-06
Sesn2	756	601	1342	1278	1.938	0.954	1.45E-08	3.67E-06
Cask	716	659	1144	1151	1.672	0.742	2.91E-08	7.20E-06
Cars	1372	1055	2131	2123	1.759	0.815	3.34E-08	8.10E-06
Ppp1r15a	675	536	1234	1339	2.159	1.11	4.88E-08	1.15E-05
Rgl1	3670	3484	5102	6332	1.596	0.674	7.70E-08	1.75E-05
Hid1	100	60	254	198	2.897	1.534	9.19E-08	2.00E-05
Mars	1663	1484	2441	2476	1.561	0.642	1.25E-07	2.66E-05
Ifrd1	1537	1358	2390	3120	1.92	0.941	1.63E-07	3.34E-05
Taf15	1192	1056	1775	1857	1.611	0.688	1.75E-07	3.52E-05
Scpep1	4564	3863	6211	6119	1.467	0.553	2.04E-07	3.95E-05
Sqstm1	15689	13483	24103	23564	1.643	0.717	2.55E-07	4.86E-05
Soat2	84	56	257	223	3.454	1.788	2.98E-07	5.48E-05
Ahcyl1	4889	4474	6591	7990	1.557	0.639	3.25E-07	5.88E-05
Ptgir	3760	3079	5829	5501	1.668	0.739	4.20E-07	7.11E-05
Ero1l	1159	1037	1661	1781	1.573	0.653	7.54E-07	0.00012565
Pim1	1031	850	1395	1567	1.578	0.658	1.59E-06	0.00025269
Iars	1997	1862	2796	2931	1.484	0.569	1.72E-06	0.00026531
Ago2	3556	3262	4922	5301	1.503	0.588	1.75E-06	0.00026683
Gpr141	363	479	559	844	1.65	0.722	1.83E-06	0.00027508
Slc39a2	1523	1173	2060	1886	1.475	0.561	1.94E-06	0.00028685
Maoa	3967	3338	5180	5824	1.512	0.597	2.34E-06	0.00034089
Mtmr3	2754	2641	3649	3749	1.371	0.455	2.37E-06	0.00034089
Hspa1b	58	57	142	183	2.872	1.522	3.06E-06	0.00042397
Ern1	989	865	1526	1468	1.613	0.689	3.59E-06	0.00048416
Rabggtb	525	540	811	864	1.573	0.653	3.54E-06	0.00048416
Dgkg	381	386	755	592	1.752	0.809	3.72E-06	0.00048903

Eif2s2	1211	1149	1786	2142	1.67	0.74	4.05E-06	0.00052605
Cox6a2	31	18	114	138	5.045	2.335	4.36E-06	0.00055673
Gyg	2042	2262	2749	3121	1.364	0.447	5.27E-06	0.00066144
Chac1	143	95	329	349	2.958	1.564	6.61E-06	0.00081975
Mt2	4458	3051	5791	5754	1.573	0.653	6.71E-06	0.00082256
Lonp1	1337	1294	1891	1849	1.421	0.507	8.00E-06	0.00093771
Rora	1053	998	1423	1724	1.533	0.616	9.38E-06	0.00107541
Pfkp	1819	1663	2394	2309	1.352	0.435	9.50E-06	0.00107763
Zbtb18	539	459	793	778	1.581	0.661	9.80E-06	0.00108863
Nfil3	233	193	379	302	1.598	0.676	1.04E-05	0.00114311
Tbpl1	327	343	534	594	1.688	0.755	1.09E-05	0.00118514
Eif4ebp1	511	482	817	762	1.588	0.667	1.29E-05	0.00137874
Ch25h	714	921	993	1521	1.517	0.602	1.33E-05	0.00140107
Cdkn1a	1874	1334	2719	2268	1.575	0.655	1.45E-05	0.00151175
Pogk	1543	1151	2035	2216	1.599	0.677	1.47E-05	0.00151175
Tbc1d31	449	457	709	781	1.642	0.716	1.46E-05	0.00151175
Atp11a	1686	1479	2313	2053	1.38	0.465	1.50E-05	0.00151508
Npc1	11869	11079	14761	15341	1.312	0.392	1.51E-05	0.00151508
2410006H16	69	103	178	216	2.319	1.214	1.73E-05	0.00171193
Trim35	1490	1339	1958	2017	1.407	0.493	2.40E-05	0.00226647
Slamf7	1212	995	1697	2362	1.862	0.896	2.47E-05	0.0023063
Arhgef3	1264	1265	1759	2154	1.533	0.617	3.41E-05	0.00293383
Fyn	692	644	975	1060	1.526	0.61	4.89E-05	0.00395164
Gadd45a	352	285	692	782	2.361	1.24	5.01E-05	0.00398211
Sorbs3	2175	2019	3033	3080	1.454	0.54	5.19E-05	0.00410096
Glrp1	46	49	133	142	2.904	1.538	5.88E-05	0.00457701
Lrp12	6537	6075	8013	8926	1.344	0.426	6.35E-05	0.00483751
Mreg	1138	1091	1696	1859	1.599	0.677	7.65E-05	0.00554989
Sp140	427	473	642	700	1.491	0.577	7.57E-05	0.00554989
Glce	450	445	697	663	1.519	0.603	8.14E-05	0.00582624

Hspa1a	57	44	143	114	2.553	1.352	8.28E-05	0.00588683
Ly9	6446	5751	7944	8259	1.332	0.414	8.35E-05	0.00589861
Slc6a9	256	140	416	376	2.106	1.074	8.57E-05	0.00597242
Lars	1778	1649	2314	2377	1.368	0.452	9.32E-05	0.00637251
Tars	1279	1230	1676	1756	1.367	0.451	9.28E-05	0.00637251
Zc3hav1	1905	1873	2649	2974	1.491	0.576	9.91E-05	0.00672635
Wars	1265	1243	1773	1608	1.347	0.43	0.00011737	0.00782055
Ccbe1	1193	984	1531	1767	1.51	0.595	0.00012029	0.00794028
Zyg11b	1352	1375	1734	2331	1.484	0.569	0.00015046	0.00943591
Rassf8	488	300	738	570	1.699	0.765	0.00015629	0.00974365
Inhba	187	141	383	475	2.766	1.468	0.00020127	0.01198707
Gpt2	447	381	662	718	1.672	0.742	0.00020735	0.01214608
Tmbim1	5059	4358	6479	5673	1.291	0.368	0.00020547	0.01214608
Eif4b	6379	6612	7775	8354	1.241	0.312	0.00022888	0.01311897
Phf10	695	637	955	929	1.414	0.5	0.00023148	0.01319704
Lrrfip2	1819	1610	2295	2376	1.366	0.45	0.00024086	0.01358652
Arpc5l	622	575	830	862	1.415	0.5	0.00025825	0.01411914
Galnt6	4511	4020	5486	5197	1.254	0.326	0.0002651	0.01421725
Mlx	858	811	1153	1147	1.377	0.462	0.00026538	0.01421725
Rbbp8	665	655	950	1135	1.583	0.662	0.0002766	0.01459797
Osbpl9	3567	3416	4371	4745	1.305	0.384	0.00029081	0.01519755
Ctage5	2629	2855	3423	3731	1.305	0.384	0.00029461	0.01532129
Casp4	259	267	399	444	1.608	0.685	0.0003008	0.01545787
Rpl23	5321	5704	6949	7242	1.288	0.365	0.00030119	0.01545787
Setd8	6319	5906	7720	8179	1.302	0.381	0.00031312	0.01596601
Zfand3	2050	1782	2450	2755	1.36	0.444	0.0003145	0.01596601
Vegfa	140	102	239	246	2.072	1.051	0.00035283	0.01765984
Usp1	728	795	1003	1052	1.35	0.433	0.00037514	0.01860177
Nup62	1049	892	1415	1382	1.439	0.525	0.00037832	0.01862811
Mdfic	1395	1227	1690	1971	1.398	0.484	0.00039858	0.01931458

Gars	4895	4744	6274	6033	1.276	0.352	0.00040826	0.01960577
H2-Q4	684	554	886	827	1.39	0.475	0.00042139	0.01996626
Ppapdc1b	320	322	475	489	1.503	0.588	0.00043555	0.02054623
Ubr2	2740	2528	3590	3617	1.366	0.45	0.00044121	0.02072128
Plekhg1	7262	6963	8569	9974	1.3	0.379	0.00046688	0.02173536
Jdp2	206	204	322	357	1.66	0.732	0.00050178	0.0230882
Napepld	442	388	602	644	1.499	0.584	0.00050061	0.0230882
Eif5	3776	3532	4609	6240	1.484	0.57	0.00052428	0.02364686
Il7r	1115	639	1433	1471	1.77	0.824	0.00051981	0.02364686
Slc3a2	10586	8669	14598	14874	1.55	0.632	0.00052272	0.02364686
Ctsl	9389	8612	12454	11375	1.324	0.405	0.00057686	0.02562462
Ubap1	2161	2111	2765	2870	1.319	0.4	0.00060932	0.02651421
Epb4.1	1679	1568	2031	2153	1.29	0.368	0.00062599	0.02710958
Ticam2	1272	1390	1823	2037	1.448	0.534	0.00066248	0.02813817
Pik3ap1	6578	6796	8023	8228	1.215	0.281	0.00074367	0.03109137
Cd83	569	386	735	625	1.45	0.536	0.00076062	0.03143019
Map1b	213	125	325	257	1.764	0.819	0.0008501	0.03459141
Ppp1r15b	3722	3811	4590	4848	1.253	0.325	0.00088049	0.03542221
Tspan5	1198	1206	1501	1618	1.297	0.375	0.00087791	0.03542221
Atp6v1c1	9231	8624	10993	11298	1.25	0.321	0.00090645	0.03619349
Zdhhc18	1871	1436	2223	2476	1.431	0.517	0.00092307	0.03658328
Arl14ep	506	446	666	700	1.435	0.521	0.00093796	0.03703562
Mtbp	126	110	237	198	1.84	0.88	0.00107144	0.04184099
Optn	1543	1512	1981	2262	1.391	0.476	0.00110315	0.04276602
Sun2	3515	3163	4153	4108	1.238	0.308	0.00112419	0.04342382
Metrnl	1359	1537	1927	1853	1.309	0.388	0.00119874	0.04580571
Stxbp5	982	744	1227	1092	1.355	0.438	0.00124465	0.04705402
Mib2	945	798	1275	1253	1.446	0.533	0.00124971	0.0470783
Evi5	795	780	1058	1118	1.381	0.466	0.00125666	0.04717358
Cdk5rap2	1073	953	1342	1333	1.32	0.4	0.00129641	0.04755749

Ffar2	27	32	81	56	2.303	1.203	0.00129221	0.04755749
Lcp2	2779	2815	3329	3707	1.258	0.331	0.00128038	0.04755749
Esyt2	4908	4175	5641	6026	1.288	0.365	0.00130678	0.0476904
Osgin2	213	181	338	393	1.889	0.918	0.00137089	0.0495447
Papd5	1885	1770	2274	2343	1.262	0.336	0.00138408	0.04985017

6. Appendix 2

List of differentially down-regulated genes resulting from a pairwise comparison between cells crosslinked with an anti-Siglec-F antibody and its corresponding isotype negative control (JSSF vs JSIC). The list reports normalised counts, fold change, Log2Fold change, p and adjusted p values for samples labelled as 246 and 266 each corresponding to one independent biological replica.

Id	norm.JSIC246	norm.JSIC266	norm.JSSF246	norm.JSSF266	FoldChange	log2FoldChange	pvalue	padj
Dhcr24	1514	1794	871	871	0.528	-0.922	2.12E-12	1.25E-09
Kctd12	2840	4002	1806	1929	0.551	-0.86	4.45E-11	1.92E-08
Tlr8	2710	3046	1524	1978	0.606	-0.722	8.36E-11	3.30E-08
Ear1	991	1356	648	637	0.552	-0.858	3.58E-10	1.23E-07
Cd180	1133	1405	673	721	0.551	-0.86	4.06E-10	1.31E-07
Alox5	1316	1662	867	764	0.551	-0.86	1.15E-09	3.39E-07
Scd2	3206	3845	1762	2021	0.537	-0.897	3.60E-09	1.01E-06
Kazald1	306	457	175	149	0.431	-1.216	6.16E-09	1.68E-06
Emb	1326	1431	914	719	0.588	-0.765	5.10E-08	1.18E-05
Fasn	3423	3671	2413	2248	0.658	-0.604	8.74E-08	1.94E-05
Sdf2l1	1581	2061	1101	1147	0.62	-0.689	1.51E-07	3.16E-05
P2ry6	704	863	446	404	0.545	-0.875	1.83E-07	3.61E-05
Gusb	6708	7408	5117	4647	0.692	-0.532	2.96E-07	5.48E-05
Siglecf	2108	2739	1501	1018	0.52	-0.944	3.59E-07	6.37E-05
Emilin1	1385	1546	901	962	0.636	-0.653	3.79E-07	6.52E-05
Pros1	924	1268	664	698	0.626	-0.675	3.74E-07	6.52E-05
5031439G07	4449	5071	3396	3415	0.716	-0.481	1.05E-06	0.00017219
Pald1	1127	1376	794	555	0.539	-0.892	1.14E-06	0.00018449
Cyth4	5977	6560	4546	3813	0.667	-0.585	1.72E-06	0.00026531
Crip1	933	769	653	450	0.64	-0.643	2.74E-06	0.00038883
Fscn1	671	340	411	190	0.588	-0.767	3.00E-06	0.00042039
Tuba4a	1822	1731	1374	1104	0.694	-0.527	3.64E-06	0.00048565
Rgs2	1289	1764	945	842	0.585	-0.773	4.39E-06	0.00055673
Myh14	282	162	161	78	0.528	-0.922	6.93E-06	0.0008398
Cx3cl1	625	693	449	319	0.579	-0.788	8.00E-06	0.00093771
Slamf9	445	654	303	361	0.61	-0.714	7.88E-06	0.00093771
Slco2b1	541	621	378	310	0.594	-0.752	8.52E-06	0.00098707
Fam20c	2796	2995	2000	2119	0.712	-0.491	9.73E-06	0.00108863
Clec4a3	2036	2359	1557	1593	0.718	-0.478	1.26E-05	0.00135617

Arsb	207	310	122	152	0.534	-0.904	1.62E-05	0.00161083
Adcy7	6750	7092	5376	5360	0.776	-0.366	1.99E-05	0.00195045
Dbi	2230	2534	1763	1602	0.707	-0.5	2.03E-05	0.00196484
Neurl3	324	473	218	205	0.534	-0.904	2.06E-05	0.00198032
Dock8	6543	7923	5276	5410	0.741	-0.433	2.32E-05	0.00220558
Lrmp	215	309	141	151	0.562	-0.832	2.51E-05	0.00232483
P2ry13	199	349	142	158	0.561	-0.834	2.66E-05	0.00244789
Hp	337	505	190	224	0.493	-1.019	2.76E-05	0.00251155
Itgam	3919	2729	2797	1829	0.691	-0.533	2.78E-05	0.00251155
Frmd4b	466	706	352	322	0.584	-0.776	2.82E-05	0.00252566
Cotl1	12464	11276	9872	8123	0.756	-0.403	2.92E-05	0.00259444
Dok2	467	531	339	234	0.567	-0.819	3.10E-05	0.00273253
Ttc39a	139	169	75	73	0.48	-1.058	3.22E-05	0.00281029
Arhgap4	365	571	232	297	0.573	-0.804	3.29E-05	0.00285479
Calr	36089	38642	28136	29930	0.777	-0.364	3.49E-05	0.00297376
Fdps	1607	1636	1145	1112	0.696	-0.524	4.09E-05	0.00345887
Hspa8	10546	11221	7870	8585	0.756	-0.403	4.20E-05	0.00352554
Ptgs1	51	74	25	16	0.323	-1.629	4.29E-05	0.0035579
Sqle	2564	2502	1752	1818	0.705	-0.504	4.31E-05	0.0035579
Aldh1l1	253	355	149	200	0.576	-0.795	4.72E-05	0.00387446
Pdia3	12419	14795	9100	10748	0.73	-0.455	4.87E-05	0.00395164
Kcne3	103	171	60	70	0.483	-1.051	5.00E-05	0.00398211
Ucp2	22723	25678	18961	17934	0.764	-0.389	5.50E-05	0.00430903
Pdia4	3011	3415	2377	2385	0.742	-0.43	5.98E-05	0.00461714
Celf2	2344	2722	1853	1996	0.761	-0.394	6.09E-05	0.00467201
Pak1	391	521	275	287	0.619	-0.692	6.77E-05	0.00510099
Unc119	1585	1762	1283	1086	0.707	-0.501	6.79E-05	0.00510099
Lair1	1275	1539	833	1127	0.695	-0.525	6.97E-05	0.00519873
Siglece	327	454	193	296	0.627	-0.675	7.13E-05	0.00527759
Krt80	270	309	167	159	0.563	-0.829	7.63E-05	0.00554989

Hmgcs1	3396	3474	2706	2590	0.771	-0.376	7.96E-05	0.0057364
Mmp8	144	172	95	67	0.509	-0.974	8.44E-05	0.00592103
Cxcl14	218	220	139	74	0.469	-1.091	8.79E-05	0.00608441
Pram1	78	114	39	41	0.424	-1.237	0.00010891	0.00734886
Trf	7688	9671	6332	6698	0.755	-0.406	0.00011023	0.00739081
Fes	2061	2284	1577	1246	0.652	-0.618	0.00012115	0.00794028
Gmpr	547	637	423	369	0.669	-0.579	0.0001214	0.00794028
Rnpep	2753	3100	2190	1979	0.714	-0.485	0.0001251	0.00813224
Cd48	3875	4267	3153	3168	0.777	-0.364	0.00013243	0.0085569
Ptprs	873	827	659	448	0.643	-0.636	0.00013755	0.00883366
Rpn1	6477	6447	5216	5002	0.79	-0.339	0.0001411	0.00900765
Glul	9290	10428	7716	7571	0.776	-0.366	0.0001483	0.00941104
Syn1	128	200	88	83	0.525	-0.929	0.00015022	0.00943591
Nedd4	154	160	97	49	0.444	-1.172	0.00016235	0.01002424
Pygl	3781	4647	3170	3125	0.751	-0.413	0.00016267	0.01002424
Klhl6	438	582	313	346	0.65	-0.622	0.00017667	0.01082455
Hsd17b4	5370	6452	4516	4496	0.765	-0.386	0.00018552	0.01130157
Mllt4	638	786	459	387	0.599	-0.74	0.00019291	0.01168558
6430548M08	2459	2355	1926	1718	0.756	-0.403	0.0001948	0.01168921
Deptor	846	1105	679	695	0.708	-0.498	0.00019517	0.01168921
Paqr7	308	369	214	206	0.623	-0.682	0.00020721	0.01214608
Scarb1	2258	2551	1861	1712	0.744	-0.427	0.00021359	0.01244307
Itgax	36320	37340	30225	27717	0.786	-0.348	0.00021722	0.01258576
Cmbl	289	284	192	138	0.57	-0.811	0.00022538	0.0129877
Ikbke	1898	2113	1352	1647	0.747	-0.422	0.0002351	0.01333217
Grap2	406	501	316	283	0.663	-0.593	0.00024539	0.01376869
Cfp	213	460	169	198	0.57	-0.81	0.00025142	0.01385503
Myo7a	5642	5936	4438	3860	0.718	-0.478	0.00025212	0.01385503
Pld4	2243	3536	1835	2137	0.699	-0.517	0.00024993	0.01385503
Vsig8	132	137	64	51	0.424	-1.237	0.00024919	0.01385503

Axl	17901	23475	15216	16051	0.761	-0.393	0.00026418	0.01421725
Lrrc25	141	196	84	85	0.504	-0.989	0.00026224	0.01421725
Rab3d	2507	2796	2063	2007	0.769	-0.38	0.00026941	0.0143609
Vdr	309	327	217	192	0.643	-0.637	0.00027303	0.01448166
Ly6e	5241	6903	4192	4221	0.696	-0.523	0.00028041	0.01472654
Nlrp1a	110	158	64	73	0.517	-0.952	0.00030159	0.01545787
Aldoc	3151	3486	2585	2225	0.725	-0.464	0.00033857	0.01707218
Ptpn18	58	82	23	31	0.388	-1.366	0.00033949	0.01707218
Pdk1	623	791	492	476	0.689	-0.538	0.00037305	0.01858454
Ccnd1	128	158	91	46	0.454	-1.141	0.00037917	0.01862811
Abcd1	1804	2067	1429	1386	0.73	-0.455	0.00039686	0.01931458
Marveld1	1102	1238	818	913	0.74	-0.434	0.00039707	0.01931458
Hsp90b1	21268	23093	15854	18653	0.777	-0.364	0.00040062	0.0193258
Gsn	6808	7306	5621	5372	0.778	-0.362	0.00041229	0.01971056
Siglec1	7703	9884	6503	6922	0.769	-0.378	0.00041682	0.01983792
Pdia6	5999	6592	4835	5144	0.793	-0.335	0.00046298	0.02164816
Cd101	149	242	98	130	0.593	-0.753	0.00050251	0.0230882
Slc18a1	47	47	19	12	0.325	-1.621	0.0005046	0.0230882
Matk	79	96	38	31	0.387	-1.368	0.00052568	0.02364686
Trem2	1739	1927	1480	1147	0.712	-0.49	0.00055852	0.02501831
Sulf2	4400	4851	3722	3172	0.747	-0.422	0.00057387	0.02559848
Fos	645	714	455	509	0.71	-0.494	0.00057934	0.02562817
Ddx58	787	984	621	666	0.73	-0.455	0.00059673	0.02628823
Sdc3	6359	7904	5396	5350	0.757	-0.401	0.00060557	0.02651421
Tm6sf1	1557	1879	1270	1275	0.743	-0.428	0.00060828	0.02651421
Tubb4b	1378	1425	1159	880	0.72	-0.474	0.00062809	0.02710958
Ksr2	58	63	25	16	0.333	-1.587	0.00063412	0.02716693
Snn	812	1024	595	609	0.661	-0.596	0.00063452	0.02716693
Ids	1503	1856	1158	1348	0.748	-0.419	0.00065928	0.02811442
Nbeal2	1197	1078	847	816	0.73	-0.453	0.00068992	0.02918744

Card11	5265	7004	4544	4665	0.76	-0.396	0.00069318	0.02920934
Mlec	7502	8555	6401	6027	0.776	-0.366	0.0007309	0.03067754
Plxdc2	151	271	101	136	0.571	-0.808	0.00074714	0.03111434
Cdh1	1034	856	835	580	0.742	-0.431	0.00075226	0.03120568
Capn5	210	193	108	99	0.514	-0.96	0.00080585	0.0331706
Slc25a13	256	349	171	220	0.649	-0.625	0.00082833	0.03396455
Tmem50b	1045	1165	809	854	0.754	-0.408	0.00084596	0.0345547
Il6ra	599	828	501	470	0.691	-0.534	0.0008723	0.03535976
Gm1966	289	519	249	208	0.592	-0.756	0.00089268	0.03577757
Tbxas1	1592	1934	1379	1209	0.736	-0.442	0.00091023	0.0362089
Epsti1	200	291	143	157	0.618	-0.695	0.00100739	0.03963018
Fn1	10496	11980	8076	8755	0.749	-0.417	0.0010407	0.04079024
Ost4	898	1076	745	701	0.734	-0.446	0.00108629	0.04226625
Ptk2b	4599	5196	3965	3915	0.806	-0.312	0.00115349	0.04439497
Mum1	1009	1127	707	720	0.67	-0.578	0.00116916	0.04483592
Kctd12b	1307	1712	910	1180	0.692	-0.53	0.00121513	0.04626608
Rnase4	264	253	173	175	0.669	-0.579	0.00122079	0.04631634
Abi3	652	757	467	540	0.716	-0.482	0.00126721	0.04740252
Aacs	890	847	628	633	0.724	-0.465	0.00128011	0.04755749
Rin3	1879	2185	1589	1538	0.772	-0.373	0.00129812	0.04755749
Tnc	17	106	7	4	0.128	-2.961	0.00128546	0.04755749
Itga6	463	510	340	362	0.723	-0.468	0.00131069	0.0476904
Pilra	3429	4592	2665	3309	0.747	-0.421	0.00137095	0.0495447