The impact of influenza on the behaviour of lung basal cells

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Abstract

Influenza is the cause of 5 million infections globally, resulting in around 500,000 deaths each year. Despite targeted anti-viral drugs and vaccines, influenza viruses are poorly controlled and pose a particular threat to those who suffer chronic diseases. During infection, virus and immune-mediated damage destroys the airway structure needed for gas exchange and repairing this damage is essential for survival. Lung repair is driven by activation of progenitor cells, but how they carry out this repair is not well understood. Lung progenitors are difficult to study as they are a rare cell type in the lung, and the protocols for lung digest are not optimised for recovery of progenitors. We developed a new protocol for increasing the yield of progenitors from mouse lung tissue and used published transcriptomics data sets to identify progenitor cells *in silico*. We aimed to understand how the composition of the lung was changed during damage, so we infected mice with influenza A virus and studied how the behaviour of the epithelial progenitors changed. We found the composition of the lung changes during the peak and recovery phases of influenza. This change in composition correlates with increased activation and proliferation of progenitors and we show that this is accompanied by a change in cell metabolism, particularly an increase in glycolysis. We found that upper airway basal cells rely on IL-10 signalling to initiate this activation, and we suggest that the immunoregulatory response is necessary to initiate basal cell activation. We considered that lung basal cells might be changed by direct interaction with influenza viruses and using novel, fluorescent influenza A virus, we show that a subset of lung basal cells are directly infected by influenza virus but survive. Direct infection triggers an increase in stress, and further increases in glycolytic metabolism. We suggest this metabolic adaption of epithelial progenitors is a critical step in initiating lung repair after damage, and the stress seen in directly infected cells could result in improper dysplastic repair. Our data support the proposal that new treatments for respiratory diseases could consider targeting mechanisms of repair. Improving how the host repairs damage could not only be effective alongside drugs and vaccines in treating acute infections like influenza, but also in resolving chronic inflammatory such as COPD.

Contents

List of tables

List of figures

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The scientific style dictates that when communicating our work, scientists use the word “we”. Some consider this a stuffy, antiquated tradition. I disagree – there is no science without collaboration, and there is no collaboration without “we”. I’d like to indulge in the opportunity to thank some of the people who made this work possible.

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Publications

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Conference Presentations

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Poster Presentation: "Lung stem cells activate lipid metabolism during early IAV infection"

Author's Declaration

I declare that, except where reference is made to the contribution of others, this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Name: Patrick Douglas Albert Shearer

Signature:

Abbreviations

ARDS	Acute respiratory distress syndrome
ASL	Airway Surface Layer
AT-I	Alveolar Type I
AT-II	Alveolar Type II
BADJ	Bronchoalveolar duct junction
BASC	Bronchioalveolar stem cell
BSA	Bovine serum albumin
BUV	Brilliant ultraviolet
BV	Brilliant Violet
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
COPD	Chronic Obstructive Pulmonary Disorder
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EMT	Epithelial-mesenchyme transition
FACS	Fluorescence activated cell sorting
FGF	Fibroblast Growth Factor
FISH	fluorescence in situ hybridization
FMO	Fluorescence minus one
IAV	Influenza A virus
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILC	Innate lymphoid cell
IPF	Idiopathic pulmonary fibrosis
NHS	National Health Service
NK	Natural Killer
NKT	Natural killer T cell
NP	Nucleoprotein
NS1	Non-structural 1
PBS	Phosphate buffered saline
PNEC	Pulmonary Neuroendocrine cell

PR8	Influenza A/Puerto Rico/8/1934 H1N1
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
SD	Standard Deviation
SLE	Systematic lupus erythematosus
WHO	World Health Organization
WT	Wild type

Chapter-1 Methods

1.1 Mice

C57BL/6 mice were purchased from Envigo (Huntingdon, UK). A mixture of male and female mice was used, but for each experiment mice were sex-matched and used at age 6-12 weeks. Animals were maintained in individually ventilated cages under specific pathogen free (SPF) conditions at the University of Glasgow and procedures were performed under a UK Home Office license in accordance with UK Home Office regulations and following review by the University of Glasgow Ethics Committee. Mice were euthanised by cervical dislocation, or where the trachea was required to be intact were euthanised using an intraperitoneal injection of Dolethal (200mg/ml) (Covertus). Confirmation of death method was using femoral artery cut.

1.2 *Influenza* A virus infections

1.2.1 Virus production and titration

Wild-type *Influenza A/Puerto Rico/8/1934 H1N1* (henceforth PR8) was generated in HEK293t cells using the pDUAL reverse genetics system, a gift of Dr. Edward Hutchinson (MRC-University of Glasgow Centre for Virus Research). Influenza virus was amplified by infection of Madin-Darby canine kidney (MDCK) cells, a gift of Dr. Edward Hutchinson (MRC-University of Glasgow Centre for Virus Research). A T75 of MDCK cells at 80% confluency were infected with PR8 at an MOI of 0.001 pfu/cell and incubated in a 37^{o}C incubator for 48-72hrs until around 10% of cells remain attached. Cell supernatant was pelleted at 1500rpm for 5 mins. 250ul aliquots were prepared containing influenza virus and were stored at -80^{o}C. Virus plaque titres in PFUs per ml (PFU/ml) were obtained in MDCK cells under agarose, following the procedure of Gaush and Smith (Gaush and Smith,1968).

1.2.2 Murine infection

Mice were acclimatised for 1 week after arrival in the animal unit. Mice were briefly anaesthetised using isoflurane, and 20µL PBS or 110pfu/mouse PR8 was administered via the intranasal route using a Gilson pipette tip (size 20ul). Infected mice were monitored daily from the day of infection until day endpoint. All mice were weighed each day from day 4 of infection until endpoint. Weight loss was calculated as a percentage by comparing weight at each timepoint to the weight of mice prior to infection. Endpoints used were 6 days post infection, where the peak of viral infection was thought to be, and 10 days post infection which as previously discussed would be the timepoint of peak repair. Adverse effects were recognised by monitoring animals for various signs of ill health including hunched appearance, staring coat, reluctance to move, isolation, failure to groom and weight loss.

1.2.3 BrightFlu

Experiments using NS1-2A-ZsGreen-2A-NEP (BrightFlu) were carried out by Ryan Devlin (CRUK Beatson Institute for Cancer Research). The sequence of BrightFlu was synthesized (GeneArt, Invitrogen) and cloned into the Bsm BI site of the pHH21 plasmid. Recombinant virus was subsequently generated by reverse genetics as described in (Pirillo *et al*., 2023).

1.2.4 Area Under Curve analysis

To assess changes in data occurring over time, weight loss curve data were statistically assessed using area under curve analysis (GraphPad9). AUC Total area, SEM, and N values were calculated for each sample and then analysed for statistical significance by the methods listed in Column Analyses.

1.2.5 IL-10R monoclonal antibody blockade

For IL-10R1 blockade, purified rat anti-mouse IL-10 receptor (IL-10R) mAb (Clone 1B1.3a BioXcell) was used and where stated a Rat IgG1 isotype (BioXcell) control was given. Both IL-10R1 mAb and isotype control stocks were prepared at 2.5mg/ml in sterile PBS. Each mouse was injected intraperitoneally (i.p.) (26.5G needle) with 200µl (500µg*)* of each antibody. Each treatment was given at D6, D7, and D8 of influenza virus PR8 infection. Mice were weighed and monitored for severity as described above. Injections were given between D6-D8 to allow the antibody to reach the desired concentration by the repair phase, but not from the beginning of the procedure as it was necessary to allow time for the early response to the virus to occur (Sun *et al.*, 2009).

1.2.6 Bronchoalveolar Lavage (BAL)

Where BAL collection was desired, lung dissection was carried out as above, but trachea was exposed before opening pleural cavity. A 20G 1" needle attached to a 2ml syringe loaded with ice cold PBS was inserted into the dissected trachea and clamped in place. 1ml of PBS was injected into lungs until inflated and then was drawn back (~800ul usually recovered) and placed into a 1.5ml Eppendorf tube on ice. BAL samples were centrifuged at 400g for 5mins, and supernatants were removed and stored separately for cytokine analysis. Cell pellets were resuspended in RLT buffer for RNA extraction as described below.

1.2.7 Isolation of cells from lymphoid organs

Whole spleens were harvested and collected in RPMI 1640 (10% FCS, 100 U/ml Penicillin, 100µg/ml Streptomycin and 2mM L-glutamine). Samples were crushed through a 70µm Easystrainer to obtain a single cell suspension. Splenocytes were red blood cell lysed using 1ml of ACK Lysing Buffer (Thermofisher A1049201) for 2 minutes at room temperature, and lysis was stopped by adding 15ml FACS buffer.

1.3 *Isolation of lung epithelial cells*

SOLUTION	INGREDIENTS
DIGESTION MIX	5mg/ml Dispase II (Gibco 17105041) in DMEM/F12 (Gibco 11320033)
STOP SOLUTION	5mM EDTA (Invitrogen AM9261) in DMEM/F12 (Gibco 11320033)
DISPASE WASH	5mg/ml DNAse I (Sigma 4263-1VL) in DMEM/F12(Gibco 11320033)
FACS BUFFER	2% Fetal Bovine Serum (Gibco 26140079), 2mM EDTA (Invitrogen AM9261)

Table 1-1 Ingredients of lung digestion cocktails

The pleural cavity was opened and 10ml of ice-cold PBS (without Ca2+/Mg2+) was injected into the right ventricle of the heart using a 26G needle (lungs should turn white but not expand). The trachea was exposed by cutting away skin around the neck and cutting out salivary glands. A 5ml syringe was loaded with 3ml of ice-cold digestion mix (Table 1) in and injected to inflate lungs (there should be significant increase in lung size). Clamped forceps were used to seal trachea after injection and the trachea and lungs were carefully removed and placed in 3ml ice-cold digestion mix. Lung and trachea samples were incubated at 4oC for 20hrs protected from light, on a gently rocking shaker (15min-1). After incubation, 6ml of stop solution was added to stop digestion.

Unless otherwise stated, centrifugation conditions were 400g for 5min at 4oC. A 70um Easystrainer (Greiner 542070) was placed on a centrifuge tube (Falcon 352070) and was humidified with 2ml of dispase wash. Lung/trachea sample was

poured along with all liquid into the centrifuge tube. The tissue was mashed with the plunger from a sterile syringe (BD, F90518) and another 3ml of dispase wash was added to the filter. A new centrifuge tube was fitted with a 40um EasyStrainer (Greiner 542040) and humidified with 2ml of dispase wash. The contents of the filtered liquid from the first centrifuge tube were poured through the 40um EasyStrainer and a further 3ml of dispase wash was added. The EasyStrainer was discarded leaving a cell suspension that was placed on ice protected from light. The cell suspension was centrifuged, and the supernatant was poured off. Pellets were resuspended in 2ml ACK lysing buffer ACK Lysing Buffer (Thermofisher A1049201) and incubated at RT for 2mins. 10ml FACS buffer was added to quench lysing buffer activity, and cell suspension was centrifuged. Pellets were resuspended in 5ml FACS buffer for cell counting. After counting samples were centrifuged and pellets were resuspended in a volume of FACS buffer to produce a solution of $5x10^6$ cells/ml.

1.4 Intracellular staining, and flow cytometry

Cells were counted and dead cells excluded using trypan blue and a haemocytometer. Fixable Viability Dye eFluor 780/506 (Ebioscience) was used to exclude dead cells. This dye is readily taken up by dead and dying cells, so cells which had high fluorescence in this channel were excluded from analysis using an unstained control. Fc block anti-mouse CD16/32 Antibody (Clone 93, BioLegend) was used to prevent non-specific binding. Cells were stained for surface markers in FACS buffer for 20 min at 4°C. All surface antibodies are listed in Table 2-3. For intracellular cytokine staining, 150µl of BD Cytofix/Cytoperm™ (554714) was added to samples to permeabilise and fix cells for 20 min at 4°C. Samples were then washed in 1ml of BD Perm/Wash™ Buffer (554714) and 50µl of intracellular antibody or appropriate isotype control antibody was added to each sample. Samples were incubated at room temperature for 1 hour (protected from light). Samples were washed as previously and acquired immediately on the BD Fortessa flow cytometer running FACS-Diva software (BD Biosciences). For intracellular staining, cells were surface stained as previously and fixed and permeabilised using the eBioscience™ Foxp3 / Transcription Factor Staining Kit (ThermoFisher 00-5523-00). Cells

were fixed for 1 hour at room temperature (protected from light) and then resuspended in 100µl of intracellular transcription factor stain. Samples were washed as previously and acquired immediately on the BD Fortessa flow cytometer running FACS-Diva software (BD Biosciences). Analysis was performed using FlowJo (Treestar).

Target	Flurochrome	Clone	Dilution	Manufacturer
CD45	eFluor 450	104	1:500	eBioscience
CD45	PE	104	1:500	BioLegend
CD45	BUV395	30-F11	1:200	BDBiosciences
CD45	BV421	30-F11	1:200	eBioscience
CD31	PE-Cy7	390	1:200	BioLegend
EpCAM	APC-eFluor 780	G8.8	1:200	Invitrogen
EpCAM	APC-eFluor 781	G8.9	1:200	eBioscience
EpCAM	BV711	G8.10	1:200	BioLegend
CD49f	PE	g0h31	1:200	BioLegend
CD49f	PerCP-Cy5.5	g0h31	1:200	BioLegend
CD24	APC	M1/69	1:400	BioLegend
CD24	BUV 805	M1/69	1:400	BDBiosciences
Sca-1	PerCP-Cy5.5	d7	1:200	BioLegend
Sca-1	PE	d7	1:200	BioLegend
Sca-1	BV711	d7	1:200	BioLegend
MHC-II (IA/IE)	BUV395	2G9	1:400	BDBiosciences
MHC-II (IA/IE)	eFluor 450	M5/114.15.2	1:200	eBioscience
Podoplanin	BV421	8.1.1	1:200	BioLegend
Ki67	APC	11F6	1:100	BioLegend
Ki67	APC	16A8	1:100	BioLegend
Ki67	AF488	SolA15	1:100	Invitrogen
IAV NP	FITC	D67J	1:50	ThermoFisher
NGFR	APC	ME20.4	1:200	Invitrogen
Fixable Viability Dye	eFluor 506	-	1:1000	eBioscience

Table 1-2 Antibodies used for flow cytometry

1.4.1 Measurement of proliferation by Ki67

Antigen Kiel 67 (ki67) was used as a measure of cell proliferation in flow cytometry analysis. It is present through all active phases of the cell cycle and is absent in resting cells (Starborg *et al.*, 1996). Ki67 was stained as described

above, and quantification was calculated by FlowJo (TreeStar) using an unstained and FMO control.

1.4.2 Fluorescence Assisted Cell Sorting (FACS)

Samples for FACS were stained as described in section 1.4 and were resuspended in FACS buffer for processing. Cells were run through a BD Aria IIu or Aria III cell sorter at a flow rate of 2.8 through a 100µm nozzle. Since the number of cells recovered was small, optimisation experiments were carried out to determine the best method for recovery and RNA extraction of basal cells. We carried out sorting into RNALater and 50% DMEM/50% FCS and found that cells sorted into DMEM supplemented with 10% FCS gave the best recovery of basal cell RNA. Sorted cells were pelleted and RNA extracted as described in section 1.6.

1.5 Molecular Biology

1.5.1 Tissue Homogenisation

Whole spleens were collected and placed in RNA later (Qiagen 76104) and kept in the fridge for up to 1 month. For optimal RNA purity, samples were homogenised in a TissueLyser (Qiagen) (1min 25Hz x2, 1x 5mm steel ball (Qiagen) per sample) in Trizol (ThermoFisher), and centrifuged for 5min at 12,000g for 10 minutes at 4^{o}C. Supernatants were collected, chloroform added, and samples incubated at room temperature for 3min before being centrifuged for 15min at 12,000g at 4^{o}C. The resulting upper aqueous layer was collected and 1.5x 100% ethanol added to each sample. RNA was then purified using the RNEASY Mini Kit (Qiagen 74104), including an on-column DNase digestion (RNase-Free DNase Set Qiagen) and according to manufacturer's guidelines. RNA was eluted in 30-40µl nuclease-free water and RNA concentration determined using a nanodrop 1000. RNA concentration values ranged from 250µg/µl – 3000 µg/µl. Samples which had a low 260/280 consistent with poor purity were discarded.

Where possible, cDNA was generated on the same day as RNA extraction and remaining RNA was stored at -80^{o}C.

1.5.2 Non-tissue extractions

For RNA extraction, cells were pelleted at 400g for 5min and resuspended in RLT buffer per the RNEASY Mini Kit instructions. In samples containing cell numbers less than 100,000 the RNEASY Plus Micro Kit was used (Qiagen 74134), with cell homogenisation carried out using a QIAshredder (Qiagen 79656). The RNeasy kits utilise a specialized high-salt buffer system which allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Samples are lysed and homogenized in the presence of a denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNAses to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water.

1.5.3 cDNA synthesis

cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen 4368814). This kit uses random oligonucleotide primers which are derived from DNase I digestion of calf thymus DNA. A range of 500-2000ng of RNA was transcribed. Cycling parameters were as follows: 25^{o}C for 10 min, 37^{o}C for 120 min, 85^{o}C for 5 min and 4^{o}C. cDNA was diluted 1:20 with nuclease-free water and kept at -20 prior to use.

1.5.4 RT PCR

For real time PCR, PowerUp™ SYBR™ Green Master Mix (Applied Biosystems A25742) and QuantStudio 6 Flex Real-time PCR system (Applied Biosystems) were used. For each gene master mix, 5µl of 2X SYBR master mix was added and 0.5µl of both forward and reverse gene primers (10uM working stocks). Samples were plated in triplicate, and nuclease-free water controls added to ensure no

contamination had occurred. Cycling parameters and dissociation curve conditions are stated in Table 2-1. Ct values were normalised to those for the gene encoding ribosomal protein S29 (RSP29), and expression of genes of interest was determined using the 2−ΔΔC(t) method. See Table 2-2 for primer sequences.

Cycling parameters			
UDG Activation	50˚C	2 min	Hold
Dual-lock DNA polymerase	95˚C	2 min	Hold
Denature	95˚C	15 sec	40 cycles
Anneal/extend	60˚C	1 min	
Dissociation curve conditions (melt curve stage)			
Step	**Ramp rate**	**Temp.**	**Time**
1	1.6˚C/second	95˚C	15 sec
2	1.6˚C/second	60˚C	1 min
3	0.15˚C/second	95˚C	15 sec

Table 1-3 Cycling parameters and dissociation conditions used in RT PCR

Target	Forward Primer	Reverse Primer
IL-10	CTGAAGACCCTCAGGATGCG	TGGCCTTGTAGACACCTTGGTC
IFNγ	TGAGTATTGCCAAGTTTGAG	CTTATTGGGACAATCTCTTCC
RPS29	ACGGTCTGATCCGCAAATAC	CATGATCGGTTCCACTTGGT
P63	CAGGAAGACAGAGTGTGCTGGT	AATTGGACGGCGGTTCATCCCT
KRT5	GAACAGAGGCTGAGTCCTGGTA	TCTCAGCCTCTGGATCATTCGG
CD19	GCCACAGCTTTAGATGAAGGCAC	CATCCACCAGTTCTCAACAGCC
CD45	CTTCAGTGGTCCCATTGTGGTG	TCAGACACCTCTGTCGCCTTAG

Table 1-4 Primers used for targets in RT-PCR

1.6 Cytokine measurement

1.6.1 Acquisition

BAL samples were analysed undiluted. Cytokines were measured using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (AB_2868960) according to

the manufacturer guidelines. This kit provides lyophilised standards of recombinant cytokines that are resuspended to produce a top standard at concentration 5000ng/ml. Two-fold serial dilution are then carried out to a lowest dilution of 1:4096. For a standard curve, a serial dilution was carried out using filtered FACS buffer. The standards, blanks and samples were added to polystyrene tubes and 50µl of capture bead mix (diluted in filtered FACS buffer) was added to each tube and incubated at room temperature for 1 hour (protected from light). Subsequently, 50µl of detection antibody was then added and incubated as previously. Tubes were then washed twice by adding 200µl of filtered FACS buffer to each. For acquisition, samples were resuspended in 300µl of filtered FACS buffer, and 2500 events were recorded per sample. The cytometric bead array was analysed using the BD FACSCanto™ II (BD Biosciences). Analysis was performed using FlowJo (Treestar).

1.6.2 Analysis

For samples, the MFI of PE and APC was calculated. A standard curve was plotted showing the MFI of PE against the known concentration of each cytokine. The unknown samples were calculated by extrapolating from these standard curves. An example of the standards and the resulting standard curve is shown below.

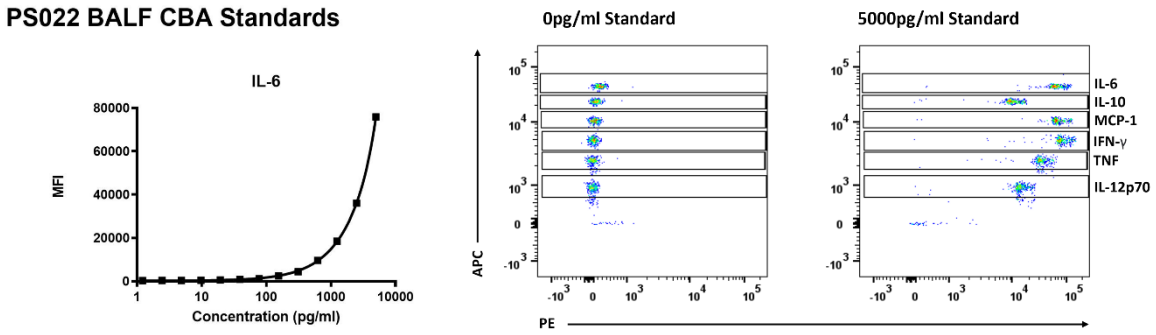


Figure 1-1 Example standard curve extrapolation for CBA

1.7 Albumin ELISA

BAL samples were thawed. To determine the concentration of serum albumin in samples, an ELISA was carried out using Mouse Albumin ELISA Quantitation Set (Bethyl Laboratories) according to manufacturer's guidelines. ELISA wash buffer was made up of 500ml 10X PBS, 2.5ml TWEEN and made up to 5L using distilled water. The albumin detection antibody was conjugated with an HRP enzyme. To quantify the concentration of albumin, TMB substrate was added (Thermo Scientific N301) to initiate a colour change. The reaction was stopped with 0.2M sulphuric acid, and plates were read at 450nm using a VersaMax plate reader. Background was removed by subtracting the blank from all readings. The concentration of albumin was determined by plotting a standard curve and extrapolating values using GraphPad Prism.

1.8 Bioinformatics

1.8.1 Identifying Single Cell RNA-seq data sets

To identify papers which could be used to identify basal cells, a systematic review was performed. First, a question that could be answered using published data sets.

How is lung basal cell transcriptomics changed by influenza virus intracellular infection?

From this question we picked keywords manually, and then using the PubMed Medical Subject Headings (MeSH) database, we expanded our keywords to expand our search.

Keywords from title: Virus, influenza, lung, basal cell, infection

Keywords from MESH search (PubMed)

Influenzavirus A, Negative-Sense RNA Viruses, Bronchioles, Blood-Air Barrier, Virus Diseases, RNA Virus Infections, Respiratory Tract Diseases, Stem Cells, Adult Stem Cells, Wound Healing, Re-epithelializastion

Finally, we used these MeSH terms to write a PubMed Query which provided us with 11 paper results.

((((Lung OR respirat* OR "respiratory tract" OR "respiratory system") AND ("Single-Cell Analysis" OR "Single-Cell Transcriptomics")))) AND (Influenzavirus A)

Published scRNA-seq datasets were accessed through the NCBI Gene Expression Omnibus (GEO). GEO is a public genomics data repository. It is a requirement for many journals that raw data from sequencing experiments is made available here. Where raw fastq files were analysed, CellRanger Count (10X Genomics) was used to align reads to the mouse reference genome (mm10 - C57BL/6J) (NCBI). Cellranger Count provides output files which are compatible with the Seurat pipeline (Hao *et al.*, 2023) which has been used extensively in this project. Seurat was chosen for its compatibility with 10X Genomics technology and its user-friendly design.

1.8.2 Quality control

The following code section includes an example of how quality control metrics were calculated and applied in the analysis of public data sets. The main 3 QC metrics are feature_RNA, count_RNA and % Mitochondrial DNA. The number of unique genes and the total number of molecules detected in a cell are useful for determining cell health. Low quality cells have little to no features, and mulitplets will have a high number of features which deviates from the average. The % of mitochondrial reads is also calculated as dying cells often exhibit high levels of mitochondrial contamination. These statistics are visualised, and then cells falling out with the average are removed.

```
#Bringing raw data into R Studio
x <- read.delim("Flu_treated_CD45n_48h_rep2_umis.txt",
                row.names = 1 ,
                header = TRUE,
                sep = "\t")

# Initialize the Seurat object with the raw (non-normalized data).
CD45n_PBS_ctrl <- CreateSeuratObject(counts = x,
                                     project = "Steurmann",
                                     min.cells = 3,
                                     min.features = 200)

# Calculating the % of features which are mitochondrial
CD45n_PBS_ctrl[["Mitochondrial.DNA"]] <- PercentageFeatureSet(CD45n_PBS_ctrl,
                                                              pattern = "^mt")

#Visualize QC metrics as a violin plot
tiff("QC 3 violins steurmann.tiff", units="in", width=7, height=6, res=600)
VlnPlot(CD45n_PBS_ctrl,
        features = c("nFeature_RNA", "nCount_RNA", "Mitochondrial.DNA"),
        ncol = 3,
        cols = "cornflowerblue",
        pt.size = 0.5,
        )
dev.off()


#Removing cells which had more than 25% of
CD45n_PBS_ctrl <- subset(CD45n_PBS_ctrl,
                         subset = nFeature_RNA > 200 & nFeature_RNA < 1500 & Mitochondrial.DNA < 25)
```

Figure 1-2 Code section for calculating QC metrics

1.8.3 Clustering

1.8.4 Identifying variable features

After quality control the next step is to normalise the reads for each cell. This attempts to account for any variety in number of molecules of RNA in each cell. The “scale.factor” here is an arbitrary constant used to allow log transformation of scaled counts.

```
CD45n_PBS_ctrl <- NormalizeData(CD45n_PBS_ctrl,
                                normalization.method = "LogNormalize",
                                scale.factor = 10000)

CD45n_PBS_ctrl <- FindVariableFeatures(CD45n_PBS_ctrl,
                                       selection.method = "vst",
                                       nfeatures = 2000)

# Identify the 10 most highly variable genes
top10 <- head(variableFeatures(CD45n_PBS_ctrl), 10)

# plot variable features with and without labels
plot1 <- VariableFeaturePlot(CD45n_PBS_ctrl)
plot2 <- LabelPoints(plot = plot1, points = top10, repel = TRUE)
```

Figure 1-3 Code section for normalisation and variable feature identification

Next, we identify the most variable features. This allows identification of some targets for differential expression analysis and these genes are more strongly considered when clustering.

1.8.5 PCA Analysis

Before clustering, data scaling is performed. This adjusts the mean expression and variance of each gene – this prevents highly expressed genes from dominating PCA. Using the previously calculated variable features, PCA is carried out on a variety of dimensions.

The results from this analysis are used to decide the "dimensionality" of the data set for the clustering step. Selecting the dimensionality (dims) is a subjective step and is done by visualising the PCA results by different methods. The most consistent method used in the analysis of this project was the ElbowPlot rather than say the JackStraw method. Though it is important to do this step correctly, it is often the case that biological differences will shine through at most "dims". However, cluster annotation is made more difficult if this step is done improperly.

```
CD45n_PBS_ctrl <- ScaleData(CD45n_PBS_ctrl,
                            features = all.genes)

CD45n_PBS_ctrl <- RunPCA(CD45n_PBS_ctrl,
                         features = VariableFeatures(object = CD45n_PBS_ctrl))

DimPlot(CD45n_PBS_ctrl, reduction = "pca")
DimHeatmap(CD45n_PBS_ctrl, dims = 1, cells = 500, balanced = TRUE)


ElbowPlot(CD45n_PBS_ctrl)

CD45n_PBS_ctrl <- FindNeighbors(CD45n_PBS_ctrl, dims = 1:10)
CD45n_PBS_ctrl <- FindClusters(CD45n_PBS_ctrl, resolution = 0.15)
CD45n_PBS_ctrl <- RunUMAP(CD45n_PBS_ctrl, dims = 1:10)

DimPlot(CD45n_PBS_ctrl)
```

Figure 1-4 Code section for Seurat scaling and cluster identification

As before, the resolution value for clustering is subjective, but can be reached by trial and error, and with a good understanding of the data that is being handled. How many cell types can be expected in this dataset? Are there any clusters that are similar in phenotype? These are all considerations made when deciding how many clusters should be assigned.

1.8.6 Identifying cell types

The final step before differential expression analysis can be carried out is annotation. Here, each cluster is compared to all others and the most differential genes in each cluster are calculated. It is important to remember that these will not necessarily be the most expressed genes, but the "defining" genes of that cluster – i.e. the genes that are unique to each cluster.

```
# find markers for every cluster compared to all remaining cells
epithelial.markers <- FindAllMarkers(CD45n_PBS_ctrl,
                                     only.pos = TRUE,
                                     min.pct = 0.25,
                                     logfc.threshold = 0.25)

filtered.markers <-epithelial.markers %>%
                      group_by(cluster) %>%
                      slice_max(n = 10, order_by = avg_log2FC)


filtered.markers %>%
  group_by(cluster) %>%
  top_n(n = 30, wt = avg_log2FC) -> top10
DoHeatmap(CD45n_PBS_ctrl, features = top10$gene) + NoLegend()

FeaturePlot(CD45n_PBS_ctrl, features = "FluSegment7-p")
```

Figure 1-5 Code section for cell type identification

Once these genes are calculated, the user will then assign each cluster an annotation. Again, this step can be subjective and requires a good understanding of what is expected in the dataset. For well characterised cells whose transcriptional profile is well known this can be simple. However, where cells are sparsely sequenced or their most differential genes not well described, this

can be difficult. For the best results, good surveillance of the literature and use of tools such as the Human Protein Atlas can be used. Although this may be an optimistic view, it is likely that as more sequencing is carried out and as annotation is carried out across the field, this process should become less subjective.

1.8.7 Integration

After carrying out quality control and clustering, integration analysis was performed. To compare different sequencing runs, single-cell sequencing experiments are combined and re-clustered to normalise any batch effects between sequencing runs. The following code shows how this process is carried out, using the integration software “Harmony”.

```
TW_combined <- merge(D0, y = c(D1,D3,D5,D7,D12), add.cell.ids = c("D0","D1","D3","D5","D7","D12"), project = "Two Waves")%>%
Seurat::NormalizeData(verbose = FALSE) %>%
  FindVariableFeatures(selection.method = "vst", nfeatures = 2000) %>%
  ScaleData(verbose = FALSE) %>%
  RunPCA(pc.genes = TW_combined@var.genes, npcs = 30, verbose = FALSE)

TW_combined <- RunHarmony(TW_combined, group.by.vars = "Sample", plot_convergence = TRUE)

TW_combined <- TW_combined %>%
  RunUMAP(reduction = "harmony", dims = 1:dim) %>%
  FindNeighbors(reduction = "harmony", dims = 1:dim) %>%
  FindClusters(resolution = res) %>%
  identity()

DimPlot(TW_combined)
Idents(TW_combined)
```

Figure 1-6 Code section for integration using Harmony

Samples are merged and renormalised as a single Seurat object. After this, Harmony integrates samples into one integrated experiment and the cells are re-clustered. This allows differential expression analysis to be carried out between two Seurat objects as shown in the code block below.

```
#Sub-setting just the basal cells
basal_only <- subset(TW_combined, idents = c("Basal"))
basal_only$celltype.Sample <- paste(Idents(basal_only),
                                    basal_only$Sample, sep = "_")

TW_combined$celltype <- Idents(basal_only)
Idents(basal_only) <- "celltype.Sample"
DimPlot(basal_only)
saveRDS(basal_only,"TW_basal_only_all_TP_harmony_26-07-23.rds")



DE <- FindMarkers(basal_only,
                  ident.1 = "3_D0",
                  ident.2 = "3_D7",
                  verbose = FALSE,
                  min.pct = 0.05,
                  logfc.threshold = 1)
```

Figure 1-7 Code section for differential expression analysis

Seurat *FindMarkers* function is used here to compare the expression of samples from D0 and D7 post infection. Seurat utilises likelihood-ratio testing ("bimod") for single cell gene expression (McDavid *et al.*, 2012).

1.8.8 Preparation of a modified mouse-PR8 genome

To detect IAV features in single cell RNA-seq experiments, a custom mouse reference genome was generated. An example of this procedure is provided by 10X Genomics ("Build a Custom Reference for Cell Ranger (mkref)" Available at: https://www.10xgenomics.com/support/software/cell-ranger/latest/tutorials/cr-tutorial-mr (Accessed: 09/03/2024)). The required GTF files were *IAV PR8* "GCF_000865725.1", and *Mus musculus* GTF "GRCm38.p4". These GTF files were combined and passed into the Cellranger function to produce a custom reference for single cell read mapping.

1.8.9 Bulk RNA-seq

Samples for Bulk RNA-seq were prepared by FACS as described above, and RNA extraction as described above. RNA was eluted in 12ul of nuclease free water

and stored at -80°C for shipping to Novogene Genomics. Samples were sequenced on the Illumina High Throughput Sequencing platform (pe150) following the Novogene pipeline outlined below.

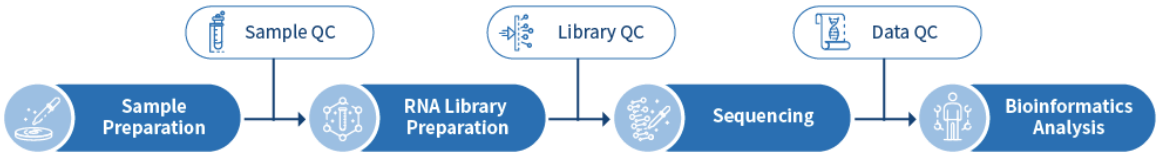


Figure 1-8 Novogene sample pipeline for bulk RNA sequencing

Sequenced reads were mapped to the mouse mm10 reference genome, and differential expression analysis was carried out using DESeq2.

1.9 Wet lab statistics

1.9.1 Normality and Lognormality Testing

When doing pairwise comparisons, data were tested for normal distribution using a Shapiro-Wilk test (normality if alpha >= 0.05). GraphPad Prism (version 9)

1.9.2 Column Analyses

When comparing two groups only, where Gaussian distribution can be assumed, an unpaired t-test was carried out. When data were not normally distributed, a Mann-Whitney non-parametric test was carried out.

When comparing more than two groups with normal distribution, an ordinary one-way ANOVA was carried out. When data were not normally distributed, a Kruskal-Wallis test was carried out. Tukey's multiple comparison correction was carried out for comparisons between 3 or more groups. Data represents mean with standard deviation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant

Chapter-2 Introduction

Influenza is a serious and possibly debilitating disease with conservative estimates suggesting at least 5 million severe cases worldwide each year (Maleki *et al.*, 2023). Current vaccines & treatments tackle the infection, but there is no specific focus on tissue damage. To explore the therapeutic potential of enhancing tissue repair, this thesis focuses on the response and function of epithelial progenitor cells in the lung during influenza. I start by introducing the lung, the importance of its structure and function, and discussing current models of lung epithelial repair. For hundreds of years, physicians have understood that the lung is an organ evolved for breathing. In the 15th Century, Leonardo da Vinci wrote “From the heart, impurities or ‘sooty vapours’ are carried back to the lung by way of the pulmonary artery, to be exhaled to the outer air.”, an incredible observation given the level of medical technology available at the time (Vinci, 1452). His beautiful drawings, preserved in his diaries (Vinci, 1452) (Figure 2-1) are strikingly close to the actual structure of the lung.

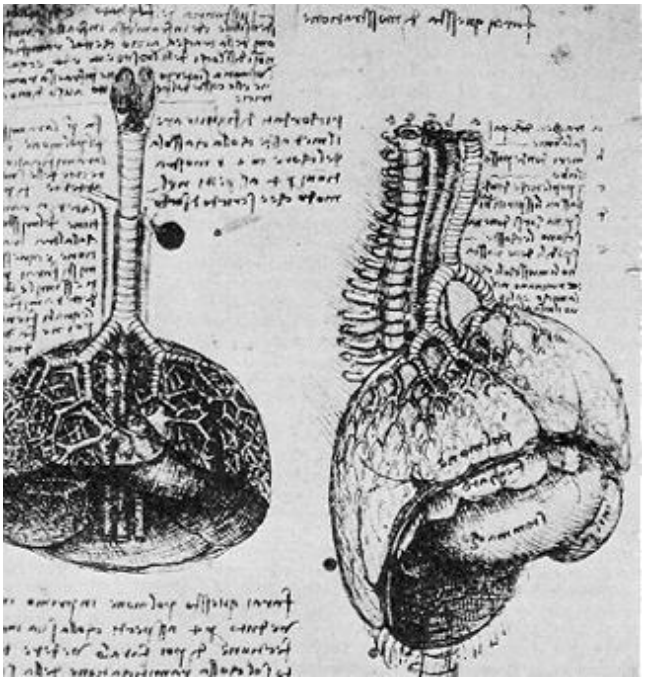


Figure 2-1 Leonardo da Vinci’s drawings of the lung.

The drawings reproduced from his diaries (Vinci, 1452) show remarkable early understanding of lung structure and function. Rear and side views shown.

Thankfully, since the 15th Century, we have come to understand a lot more about the lung, but these early insights laid the foundation for later understanding. The need to breathe has been evolving in animals since we made the leap from sea to land, with what we know as the lung today having roots in the unpaired lung on the bony fish (Osteichthyans) (Cupello *et al.*, 2022). This is the evolutionary route of all vertebrates, and though there are some differences in composition (López and Martinson, 2017), the structure of the lung is similar across most mammals. In humans, evidence of the lung appears around 4-6 weeks of gestation. The respiratory tract starts with formation of bronchioles, and by 20 weeks gestation alveolar ducts and alveolar air sacs can be found (Warburton, 2017). This process is delicately controlled, and in mice, mutations in fibroblast growth factor (FGF) and its receptor can prevent the formation of the trachea and bronchi in development (Warburton *et al.*, 2010). As the alveolar region develops, alveolar type II (AT-II) cells begin to produce surfactant, and at 30 weeks gestation, the surfactant system is developed enough to prevent lung collapse at birth. Alveolar proliferation increases until birth, with the peak at 6 months after birth; though the lungs will not reach maturity until around 8 years of age (Rehman and Bacha, 2023). During development, the placenta allows gas exchange until the umbilical cord is cut and the first breath occurs. This breath pushes the lungs open, and 6 hours later the circulation is well oxygenated (Rehman and Bacha, 2023).

2.1.1 Structure and function of the lung as we understand it.

The lung is a complex organ, made up of several distinct microenvironments, including the tracheal, bronchial, and alveolar environments. The lung is often divided into two zones – the “conducting zone”, the upper which transports gases to and from the lower airway “respiratory zone”, where the gas exchange takes place (Ostrowski 2017). Since the structure of these zones is different, the composition of cells required for them to provide their function is also different. Figure 1-2 shows a schematic of these two zones and the different cell types present in each (Gopallawa *et al.*, 2023). The cells present throughout the

airway are highly specialised for their function. In the upper airway, ciliated cells aid in mucociliary clearance to expel inhaled particles. The airway surface layer (ASL) is a thin layer of liquid which covers the epithelium which allows ciliated cells to beat (Bustamante-Marin and Ostrowski, 2017). On top of the ASL is a layer of mucus which is essential for protection against pathogens and other potentially damaging particles (Lillehoj and Kim, 2002).

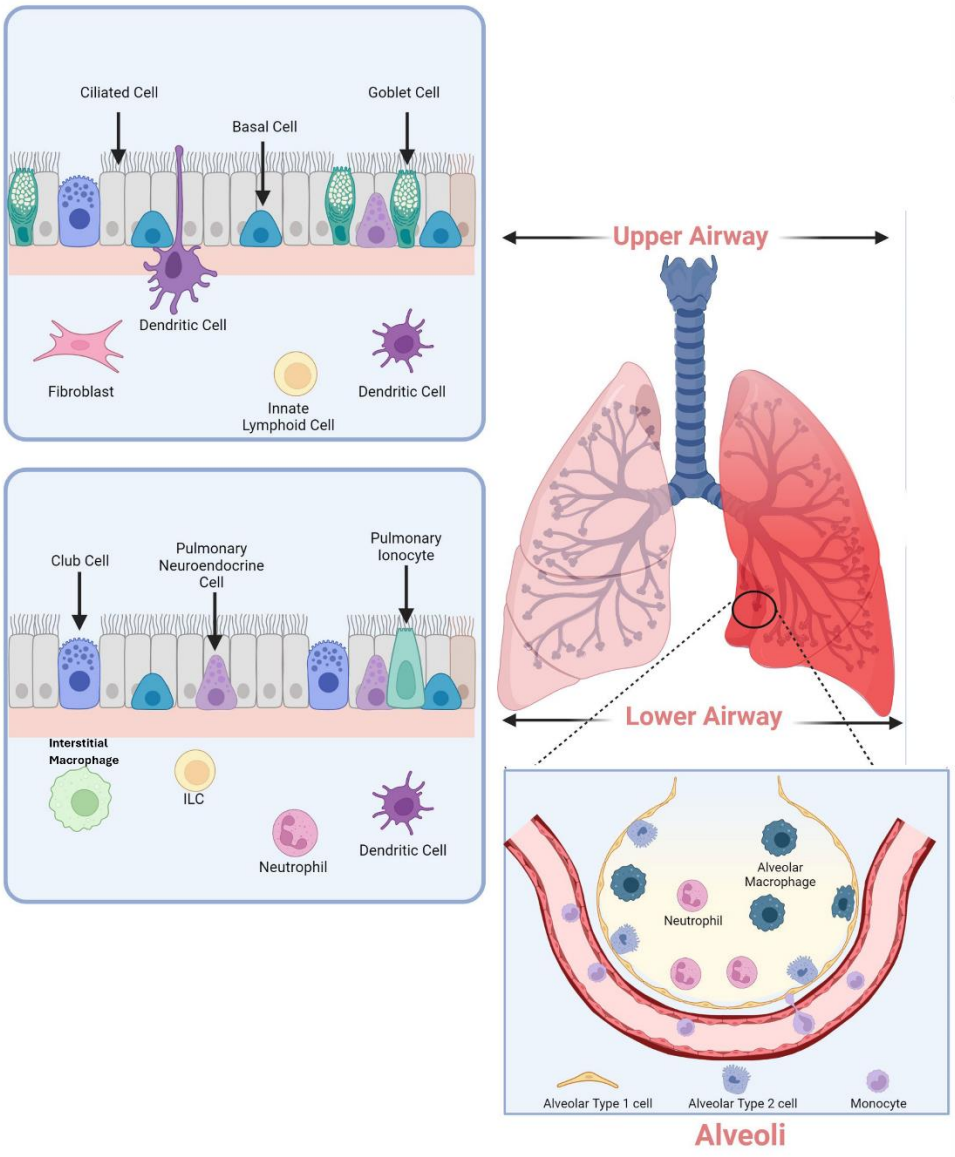


Figure 2-2 Structure of the epithelium of the upper and lower airway.

Adapted from (Lillehoj and Kim, 2002). Upper airway is composed of a variety of columnar epithelial cells, where the lower airways is composed of structural AT-I, and progenitor AT-II cells.

The main producers of mucus are goblet cells (Sleigh *et al.*, 1988) which form 20% of cells in the trachea. Although abundant in the trachea, as bronchioles branch off goblet cells become sparser and make way for club cells (Rokicki *et al.*, 2016). Club cells, which compose up to 22% of the bronchioles (Celli ad Owen, 2013) do not produce mucus, but rather are producers of surfactant and can metabolise harmful compounds from the environment such as naphthalene from cigarette smoke (Harkema at al., 2018) (Zhu *et al.*, 2008). Cells that each form less than 1% of the composition of the lung include pulmonary neuroendocrine cells (PNECs) which are important in sensing physical and chemical stimuli (Noguchi *et al.*, 2020), and pulmonary ionocytes which appear to be involved in fluid release and absorption. This proposed role of ionocytes is controversial though, as they are extremely rare, and have only recently been described (Plasschaert *et al.*, 2018) (Yuan *et al.*, 2023). The cells of the upper airway that have been described so far are produced by differentiation of basal progenitor cells (Hong *et al.*, 2004) (Wu *et al.*, 2022) whose function will be discussed later in this chapter.

Compared to the upper airway with its many diverse cell types, the alveoli are relatively simple. The walls of their balloon shaped structure is composed of long thin alveolar type I (ATI) cells, and cuboidal alveolar type II (ATII) cells (Barkauskas *et al.*, 2013). ATII cells can self-renew, are progenitors of ATI cells and are major producers of surfactant. The surfactant protein family has two main functions – structure and defence. The hydrophobic SP-B and SP-C reduce surface tension within the alveoli (Han *et al.*, 2015) and the hydrophilic surfactant proteins SP-A and SP-D can opsonise bacteria, fungi, and viruses to prevent infection and encourage pathogen clearance.

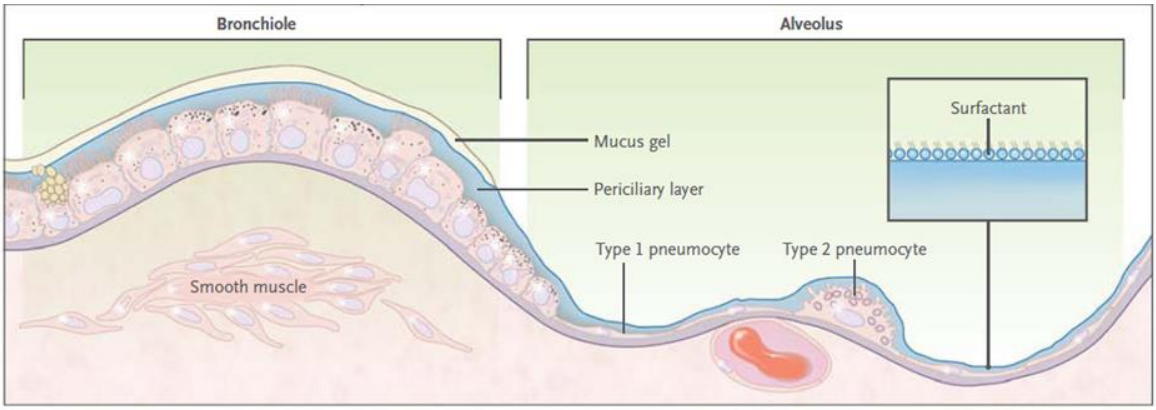


Figure 2-3 The mucus and surfactant layer of the airway.

Adapted from (Fahy and Dickey, 2010) Surfactant coats the respiratory tract and becomes prominent at the bronchoalveolar junction where upper and lower airways converge.

Lung recoil is the process by which the alveoli collapse after inflation due to elastin and collagen surrounding the alveoli. This process is essential for expelling waste gases during breathing and is kept in balance by radial traction which keeps the alveoli open. This traction is provided by SP-B and SP-C, and by the action of extracellular matrix fibroblasts which generate “forces pulling in all directions away from the centre of each alveolus” which keep it open for gas exchange (Seadler *et al.*, 2023).

The most abundant immune cell in the lung is the alveolar macrophage, and although we will discuss their immune function later, they also play a key homeostatic role. Alongside AT-II cells, alveolar macrophages consume and metabolise surfactant to keep the balance of surfactant in check (Garbi and Lambrecht, 2017). Not enough surfactant can lead to respiratory distress syndrome due to high alveolar surface tension, and overproduction of surfactant can lead to pulmonary fibrosis or pulmonary alveolar proteinosis due to the accumulation of surfactant lipid. (Lopez-Rodriguez, 2016) This is thought to be caused by mutations in the GM-CSF receptor of alveolar macrophages, which prevents them from consuming surfactant efficiently (Suzuki *et al.*, 2008).

So far, we have discussed the epithelial structure that allows for efficient gas exchange, but supporting that system is a dense network of vasculature. Gases

for exchange are carried by blood which flows through vessels constructed from endothelial cells. Blood vessel generation is carried out by two kinds of endothelial cell. Tip cells invade and lead the growth and stalk cells fill out the vessel and help generate the lumen (Niethamer *et al.*, 2020). Some epithelial progenitors may take part in homeostasis of the vasculature (Basile *et al.*, 2014), but endothelial cells are the main drivers of neovascularisation after infection and wound healing (Niethamer *et al.*, 2020). The lymphatic system is also derived from endothelial progenitors with Prox1+ lymphatic endothelial cells building the lymphatic structure in utero (Trivedi and Reed, 2023). The lymphatic system plays a key role in drainage: pulmonary capillaries are responsible for carrying most fluid away from the lung, but the lymphatics accommodate any excess, preventing oedema (Pearse *et al.*, 2005). Of course, an essential function of the lymphatic system is coordination of the adaptive immune response in the lung. While the lung contains many resident immune populations (Alveolar macrophages, dendritic cells, innate lymphoid cells etc.), movement of these cells relies on a dense network of lymphatic vessels (Ardain *et al.*, 2020).

2.2 The lung is constantly under challenge.

Humans breathe in and out on average 22,000 times a day and, ideally, we would only breathe in clean air. We live in a messy, polluted world and so our lungs are constantly challenged by the other contents of the air we breathe. The World Health Organization (WHO) estimates that 90% of all people breathe polluted air (World Health Organisation, 2018), with almost half of lung diseases attributed to air pollution (Mannucci *et al.*, 2015). The burning of fossil fuels means that the air is laced with SO_2, NO_2 and CO which have been associated with COPD, Asthma, and pulmonary fibrosis (Bala *et al.*, 2021). These pollutants are only the tip of the iceberg – many respiratory conditions are caused or exacerbated by cigarette smoke, dust, and other carcinogens like asbestos (Kuper *et al.*, 1997). While these aerosol contaminants can cause damage on their own, often the issue is how the lung responds to these contaminants.

Hypersensitivity reactions occur when the immune system overreacts to an allergen or antigen, with common symptoms of wheezing and cough in the mild cases, to the more severe anaphylactic reactions which can be life threatening (Passalacqua and Ciprandi, 2008) (Vaillant *et al.*, 2023). Of course, not all immune responses are inappropriate. As previously described the lung is well evolved for fighting pathogens, and this is out of necessity – the lung is constantly exposed to microorganisms from the air (Segal *et al.*, 2014).

Although it is possible to transmit respiratory viruses by fomites, the most common transmission is by airborne inhalation (Short and Cowling, 2023). Adenovirus, measles virus, SARS-CoV-2, and many others are transmitted this way and infectiousness of exhaled air droplets can be influenced by temperature, humidity, and socioeconomic factors such as overcrowding (Ather *et al.*, 2023). Airborne transmission is also a major infection route for many bacterial pathogens, such as Legionella and M. tuberculosis (L.D. Stetzenbach, 2009). Lung infections are also caused by the fungal pathogens responsible for histoplasmosis and aspergillosis which are particularly urgent as they have recently been shown to be rapidly developing resistance to anti-fungal medications (Fisher *et al.*, 2022).

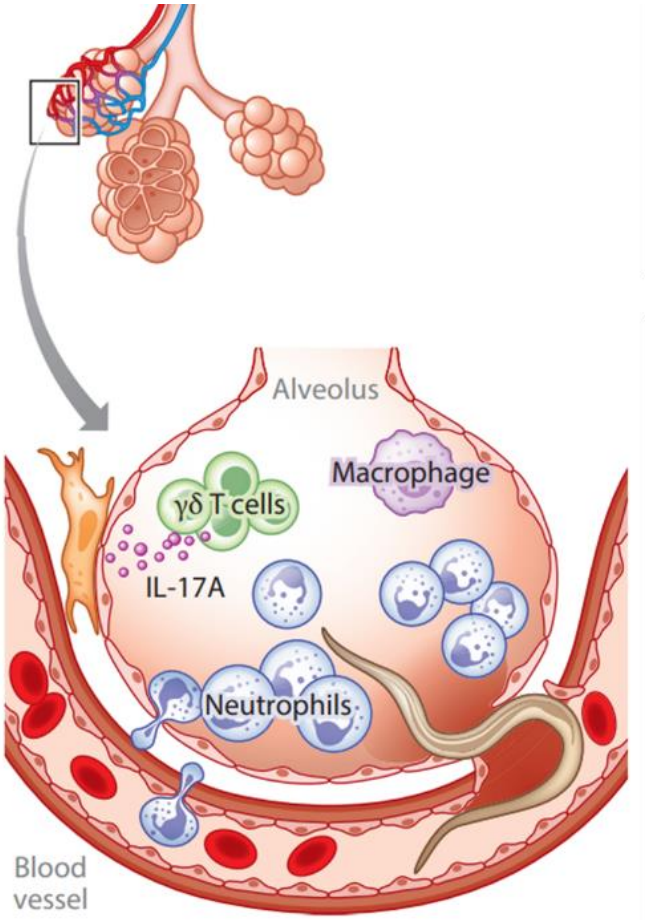


Figure 2-4 Invaders of the alveolar space.

The structure of the alveoli makes it susceptible to non-airborne infections. Adapted from (Allen, 2023).

Finally, there is the burden of parasites in the lung, with many neglected diseases caused by parasites (Leishmaniasis, Strongyloidiasis) are the result of the parasite damaging the lung - though these pathogens are not usually transmitted by the aerosol route (Mehta *et al.*, 2016) (Allen, 2023).

2.2.1 The innate immune response in the lung

If inhaled microorganisms pass the first line of defence (physical barriers, mucus and surfactant), they will next have to contend with the innate immune system. Tolerance or clearance of these microorganisms is required for survival, and the

non-specific innate immune system is responsible for mounting an initial attempt to clear the pathogens, and to buy time for development of a more specific adaptive immune response (Cayrol and Girard, 2014). Epithelial cells of the airway can sense the presence of pathogens through pattern recognition receptors and start to produce pro-inflammatory factors such as IL-6 and interferons (Hartl *et al.*, 2018). This role is complemented by pulmonary macrophages which have a balanced function, both producing inflammatory cytokines and raising the alarm, but also removing debris and initiating tissue remodelling (Byrne *et al.*, 2015).

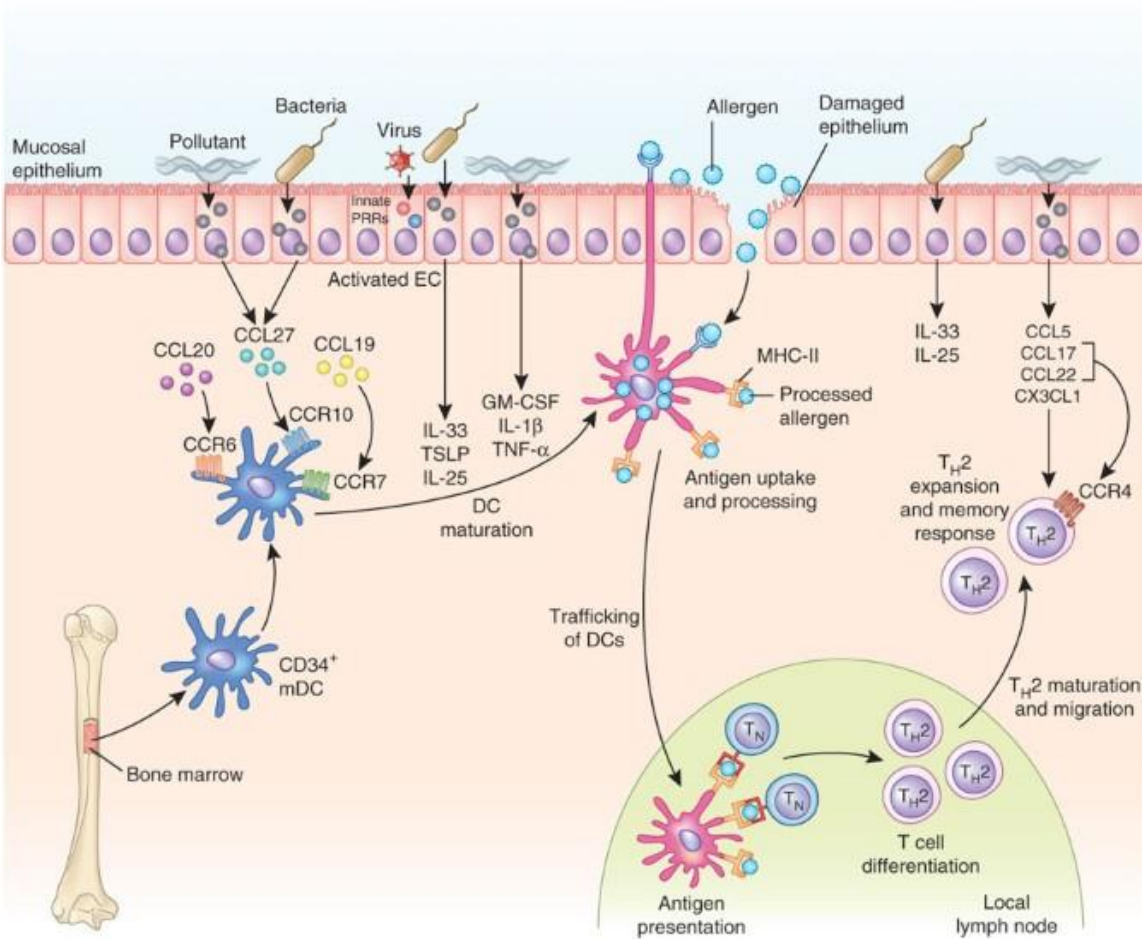


Figure 1.4 Tissue damage and the innate response.

The innate response supports transition to an adaptive immune response. (Holgate, 2012).

The production of these cytokines recruits immune cells and alters tight junctions to warn the surrounding tissue that pathogens are present and draw in

other innate effectors. The first of these to arrive are neutrophils, which can kill by phagocytosis or by degranulation – and uniquely by "NETosis", where neutrophils spill nuclear DNA and myeloperoxidase to form a "net" that immobilises pathogens and amplifies the inflammatory environment (Brinkmann and Zychlinsky, 2007). Mast cells are relevant to the hypersensitivity reaction described in the context of pollutants above but are also active in the absence of allergens. Physical destruction of cells or is another way to trigger the innate response to pathogens, and mast cells destroyed by damage rapidly release their pre-made granules of TNF-α, IFN-γ, and other inflammatory mediators such as IL-33 (Virk *et al.*, 2016). Epithelial cells destroyed by physical damage also release IL-33 which is a crucial part of the innate response to challenge. It is an alarmin which is structurally similar to IL-1 and targets cells expressing the ST2/IL-1RAcP receptor complex (Cayrol and Girard, 2014). IL-33 is useful in viral infections where it induces mucus production and fortifies the airway epithelium and is an excellent example of a cytokine that has key effects on the epithelium and shows that inflammatory mediators are not only designed to target immune cells. IL-33 is also important in parasite infections, where IL-33/ST2 signalling promotes goblet cell hyperplasia which provides protection against parasites such as *Nippostrongylus brasiliensis* (Turner *et al.*, 2013)(Tomita *et al.*, 2000).

Anti-parasitic lung inflammation relies on recruitment of basophils and eosinophils, which require secretion of IL-5, but there is also DAMP and PAMP-driven inflammation which is acutely damaging. Recent studies have shown that a source of these traditionally type 2 cytokines is the innate lymphoid cell (ILC) (Price *et al.*, 2010). ILCs are found in both mouse and human lungs, but the dominant type differs between species. ILC2s are more abundant in mice and ILC3s in humans (Guia and Narni-Mancinelli, 2020), but in both species ILCs play a key role in lung homeostasis. ILC2-derived IL-4, IL-13 and amphiregulin help maintain barrier integrity, and IL-22 produced by ILC3s has an anti-inflammatory effect (Lai *et al.*, 2016) which may be through action on epithelial cells. Il-22 is especially important in influenza as it has been shown to promote epithelial turnover and wound healing (Pociask *et al.*, 2013, Alcorn, 2020). A recent addition to the ILC1 family of cells is the natural killer (NK) cells. These innate lymphocytes are like neutrophils in their ability to degranulate and release

cytotoxic compounds onto pathogens and infected cells; but they are unique in their ability to engage directly with cells through activating and inhibitory receptors. NK cells are particularly important as they can also detect cells lacking receptors, particularly cells that are naked of major histocompatibility complex (MHC) molecules, (Culley, 2009). This makes NK cells experts at detecting and destroying cancer cells, and cells infected by intracellular pathogens (Vivier *et al.*, 2008). NK cells can be thought of as a bridge between the innate and adaptive immune response because of their ability to produce both large amounts of IFN-y, and regulatory cytokines like IL-10 (Sullivan, 2019, Perona-Wright *et al.*, 2009). The adaptive immune response is antigen-specific but requires time to prepare – in this time, dendritic cells (DCs) and other antigen presenting cells (APCs) process and present antigen to the cells of the adaptive immune system – innate immune can stall for time, providing protection while this process occurs. The innate response is also balanced, as if non-specific innate damage runs rampant, there may be no tissue left to protect when the adaptive system arrives.

2.2.2 The adaptive immune response in the lung

Antigen specific, long-lasting immunity to viruses such as IAV is only possible by stimulation of the adaptive immune system. DCs are cells of the innate immune system, but without them it would be impossible to mount an effective adaptive immune response. DCs consume antigen and carry it to naïve immune cells in the lung draining lymph nodes (Kawasaki *et al.*, 2022). In the lymph node, the APC presents its antigen to the cognate T cell, initiating proliferation of antigen-specific T cells. Depending on the antigen, and the signals provided to the APC at the site of inflammation, T cells suited to that challenge will be raised, and migrate to the lung (Cook and MacDonald, 2016). CD8+ T cells that have their cognate antigen presented to them differentiate into cytotoxic T cells, which are particularly helpful at fighting intracellular challenges be they viral or parasitic (Hickman *et al.*, 2008) (John *et al.*, 2009). CD8+ T cells are expert cell killers and produce pro-inflammatory cytokines such as IFN-y; but they have also

been shown to produce regulatory cytokine IL-10, highlighting the balanced nature of the immune response (Zhang and Bevan, 2011). CD8 killing is relatively clean by design, forcing cells to die by apoptosis to prevent excessive damage, but there is collateral damage associated with CD8 killing, and a loss of tissue structure and diversity. This is especially interesting since in the absence of CD8 killing, many cells in the lung can survive IAV infection (Fiege *et al.*, 2019).

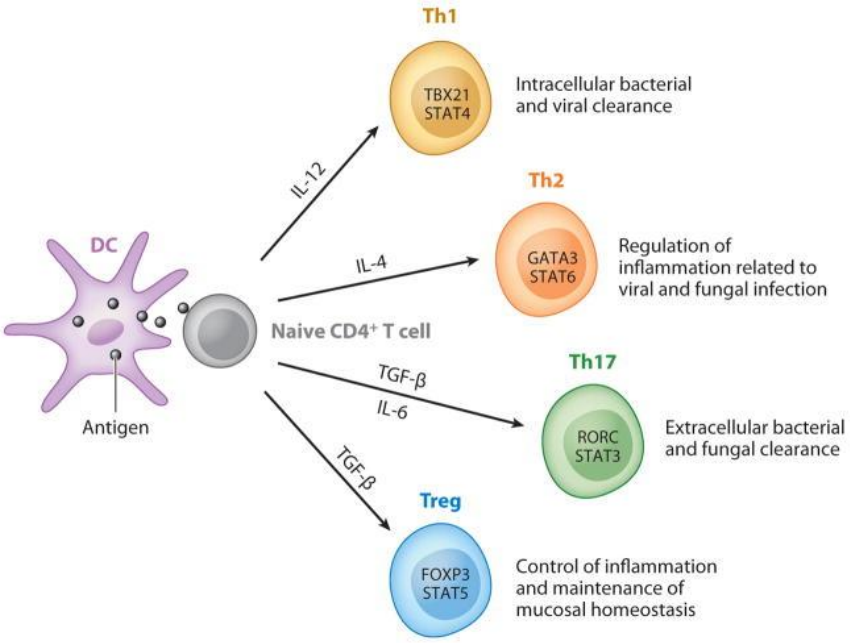


Figure 2-5 The CD4 T cell repertoire.

The adaptive immune response can raise a variety of high specificity immune responses using professional T cell subsets (adapted from Chen and Kolls, 2013).

CD4+ T cells also recognise their antigen in the lymph node but can react to a wider range of challenges which have specific cytokine profiles. These so-called helper T cells have a wide range of functions in host defence, which is summarised in Figure 1-5. This figure shows the how naïve T cells can be skewed to different phenotypes depending on the type of antigen the DC is responding to. The wide functions CD4 T cells can carry out allows our lung (and all our other tissues) to react to a wide range of challenges, this is the advantage of having a flexible adaptive immune system. To go beyond the textbook, there are other subsets of T cells which are still being described. The newly defined Th22 cell, characterised by IL-13 and IL-22 production have been shown to protect the

lung during IAV infection by interacting with epithelial cells. T cell produced IL-22 increases epithelial production of antimicrobial proteins, and strengthens tight junctions (Zhang *et al.*, 2023) (Cipolla and Alcorn, 2020). This shows that T cells not only function to clear pathogens, but also to clear damaged tissue and assist in lung repair.

Another effector arm of the adaptive immune response is antibody, produced by B cells. B cells are rare in the healthy lung, making them a good marker for infection or damage. During inflammation, B cells are activated in the lymph node and begin production of antibodies (Bertrand *et al.*, 2023). B cells can make broadly specific IgM antibodies if they encounter their antigen directly but require T cell help to make highly specific antibody types such as IgG (Polverino *et al.*, 2016). A T cell subset not shown in Figure 1-5 is the T follicular helper (Tfh) cell. Tfh are a recently discovered T cell subset which are key in the formation of germinal centres in lymphoid organs. Germinal centres are secondary lymphoid structures which allow generation of plasma and memory B cells (Stebegg *et al.*, 2018) and are essential for production of highly specific antibodies (Crotty, 2014). There are many ways in which T cells can help B cells carry out their function (seven of which are reviewed here (Crotty, 2015)) but one example is Tfh cells producing IL-21 and CD40L which supports B cell activation and class switching. T cells then encourage hypermutation to occur in activated B cells which allows them to raise the necessary high affinity antigen-specific antibodies (Moore *et al.*, 2001).

Raising a specific response to a new antigen is time consuming, and it is possible that the host may encounter this antigen again. Memory immune responses are faster and qualitatively better than primary responses, which is part of the reason vaccination has been so successful in curbing pathogens. Upregulation of CD69 paired with downregulation of S1PR1 encourages effector T cells to remain in the tissue and become resident memory cells (Humphries *et al.*, 2021). B cells too can have a resident memory phenotype, and tissue resident B cells express CCR6 and CXCR3 (Chen and Laidlaw, 2022). Tissue resident immune cells are long lived and allow rapid responses to previously seen antigen, and in doing so can reduce the amount of damage that occurs on reinfection, reducing

bystander damage (Cipolla and Alcorn, 2020). To go one step further within tissue residency is the formation of tertiary lymphoid structures, particularly the bronchus-Associated Lymphoid Tissue (iBALT) in the lung (Hwang *et al.*, 2016). These structures are normally located close to the basal membrane of the bronchus, but unsurprisingly, iBALT formation in the airway or perivascular space is often associated with conditions such as COPD (Yadava *et al.*, 2016).

Many lung diseases are caused by damage to the lung, and one source of damage that has not been discussed so far is damage caused by the immune system. Previously we discussed the importance of alarmins like IL-33 which are important for responding to tissue damage, but when they are over expressed the resulting hypersensitivity reactions can be damaging to the lung (Jacobs and Liegerman, 2018). Damage causes recruitment of macrophages and neutrophils which can cause incidental tissue damage by releasing cytotoxic cytokines and reactive oxygen intermediates (Sehrawat *et al.*, 2010). and this can lead to a vicious cycle of damage and inflammation thanks to an overreaction of the immune response. In some cases, this a direct response to challenge, but immune-associated damage in the lung can also be a result of other inflammatory conditions. Systematic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are just two examples of how auto-immune conditions can cause lung pathology and fibrosis without the lung responding to an exogenous antigen (Cojocaru *et al.*, 2011). The tendency to have an imbalanced immune response can also be genetically coded. Though rare, there are examples of inborn mutations that prevents the lung from mounting an appropriate response to antigen such as those who generate an auto-immune reaction to type I interferons, which strongly impedes their ability to respond to challenges such as COVID-19 (Casanova and Abel, 2021) (Casanova and Anderson, 2023). This highlights another key aspect of balance in the immune response. If excess inflammation is damaging the tissue and causing inflammation, why not just block the inflammation? If the host does not respond effectively to the insult, they cannot survive the challenge – the immune response is necessarily damaging.

2.3 Influenza is a disease of damage.

2.3.1 It is important to study respiratory viruses.

To this point, we have introduced a broad overview of how the lungs function, and how they respond to challenge. One family of pathogens that are particularly adept in causing damage to the lung are viruses and we have specifically focused on IAV because it is a problematic pathogen, which can cause severe lung damage. Respiratory viruses such as respiratory syncytial virus (RSV), COVID-19 (SARS-CoV-2), and influenza A virus (IAV) can cause bronchiolitis and acute respiratory distress syndrome (ARDS) (Clementi *et al.*, 2021). Respiratory viruses are important because they are seasonal, and unlike viruses like measles virus, do not have a highly effective vaccine (Maliha Moten, 2018), and are circulating due to poor infection control (Chadeau-Hyam *et al.*, 2022). Viruses such as influenza A virus have a high mutation rate meaning that even if we raise a good public health defence using vaccines and anti-viral drugs, these viruses can escape the protection and make these defences less effective (Sanjuán *et al.*, 2016). Influenza viruses have been shown to be the cause of around 28,500 hospitalisations each year in the UK, though due to lack of testing, this number is likely to be higher (Wilkinson *et al.*, 2023). Viruses are a significant burden on healthcare structures, with England spending 136 billion euros (£116BN) on prevention and vaccines in a 2-year period, with 0.47% of national budget spending on vaccines alone (Ethgen *et al.*, 2016).

2.3.2 IAV causes damage to the lung

Viruses are cellular parasites, and their parasitism results in cell death either by apoptosis, lysis, or in some cases necrosis (Atkin-Smith *et al.*, 2018). Whether this cell death is induced by the virus or by the immune response, infection by respiratory viruses results in death of some infected cells. Some respiratory viruses such as RSV (Bedient *et al.*, 2020) and measles virus (Bhattacharjee and Yadava, 2018) cause lytic inflammatory cell death during infection, but IAV is a

budding virus tends that can complete its life cycle without causing lytic cell death Indeed, increases in programmed cell death correlate with suppression of viral replication and survival from influenza (Fujikura and Miyazaki, 2018).

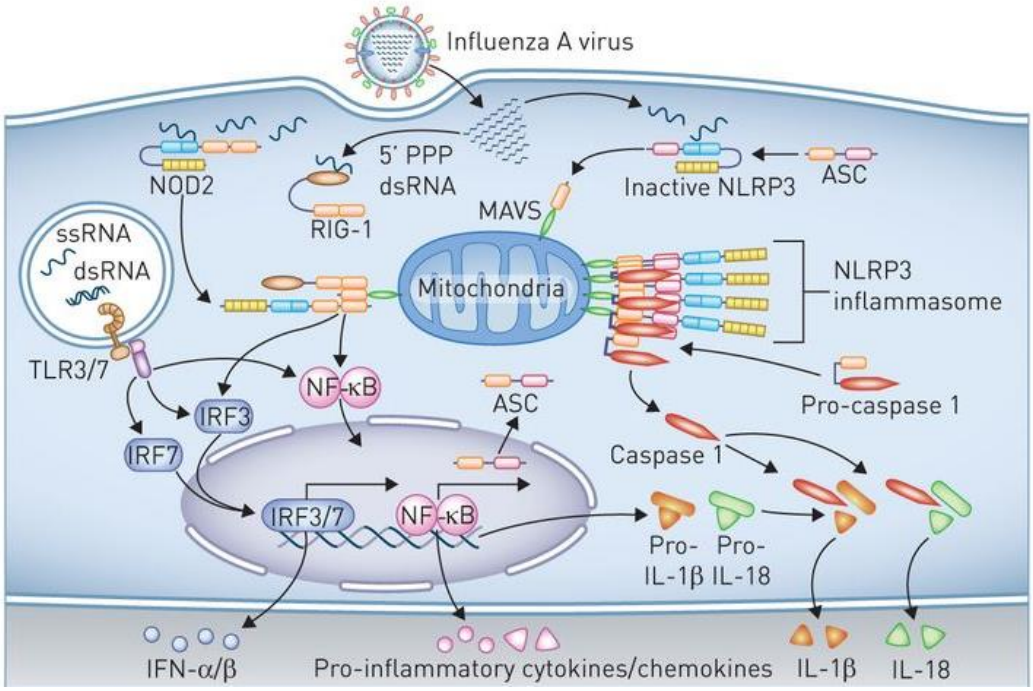


Figure 2-6 Influenza virus has a toolkit for manipulating infected cells.

The inflammatory response to IAV begins inside the cells, but the virus can hold back the alarm (adapted from Herold *et al*., 2015).

When exiting cells, IAV virus buds from the cell membrane to coat the virus particle and this egress is silent as is shown in Figure 2.6. Unlike necrosis, there is little inflammatory response to IAV budding, and so the longer the cell survives, the more virus copies can be generated without triggering the immune response (Herold *et al.*, 2015, Chen *et al.*, 2018, Christian Munz, 2014).

IAV is a family of viruses with a wide variety of hosts and a large diversity of function (Rejmanek *et al.* 2015) (AduBakar *et al.*, 2023). All these viruses share an infection route, entering through the respiratory tract and infecting respiratory epithelial cells, but their tropism depends on the viral subtype. Seasonal, as well as pandemic, strains show specificity for 2,6-linked sialic acids that are predominantly expressed in the human trachea, whereas the avian

viruses preferentially bind to the 2,3-linked sialic acids that are expressed in alveolar type II cells (Herold *et al.*, 2015). This is important as lower respiratory tract infections are associated with more severe pathology and poorer clinical outcomes than upper respiratory tract infections (Short *et al.*, 2014). All IAVs cause some damage to epithelial cells, but some strains have additional mechanisms for inducing damage.

Some strains (H9N2/H5N1) can cause necrotic cell death, which is associated with increased mortality from influenza. Other IAV strains (H3N2) can directly infect alveolar macrophage and neutrophils and kill them, causing further damage (Cline *et al.*, 2017). The IAV PB1-F2 protein can localise to the mitochondria and interact with various mitochondrial membrane proteins inducing apoptosis and exacerbating pathogenesis (Atkin-Smith *et al.*, 2018). IAV has mechanisms for evading both the innate and adaptive immune system (Sandt *et al.*, 2012) but despite these the virus still causes infections which cause damage. The immune system can detect and mount a response to the virus, but the activity of the immune system is also damaging.

2.3.3 The immune response to IAV is damaging.

We have already described immune responses to viruses in general terms, earlier in this chapter, but there are ways in which the response to IAV is unique. The innate response to IAV begins with activation of RIG-I by viral RNA and activation of TLRs 3 and 5 by single or double stranded RNA stimulating production of Type I and III interferons (IFNs). TLR engagement leads to production of pro-inflammatory cytokines like TNF, IL-6 and IL-1β (Chen et at., 2018). These cytokines cause rapid recruitment of neutrophils, monocytes, and natural killer (NK) cells (Zhang *et al*, 2010) which target and destroy infected cells.

This stimulation of the innate immune system is essential for clearance of the virus, but also causes bystander damage to the surrounding tissue as shown in Figure 2.6. As described earlier, the innate immune response is non-specific, and its goal is to provide a broadly effective while the adaptive response develops This figure shows the damage that is caused by IAV, and the immune response to

the virus. A more precise response begins when dendritic cells pick up viral antigen which they present to CD8+ T cells in the mediastinal lymph node.

It may seem obvious that CD8 T cells are required for survival from IAV infection, but recent data is less clear. Historic papers show that transfer of IAV specific CD8 T cells has a protective effect during infection (Wells *et al.*, 1981) (Yap *et al.*, 1978) (Taylor *et al.*, 1986) and that in mice lacking T cells, responses to IAV infection are far worse (reviewed in Schmidt and Varga, 2018). But more recent experiments have shown that this may be more complicated, as CD8+ T cell knockdown resulted in minimal effects on responses to IAV infection (Hao *et al.*, 2022). Cytotoxic T cells can kill infected cells by directly by signalling through FAS, or by releasing perforin, granzyme, and cytotoxic cytokines like IFN-γ (Chen *et al*, 2018) but they do not always hit their target. All of these killing mechanisms should be directional, because the TCR:MHC interaction pulls the two cells together (forms an immune synapse), and the secretion of cytokines and perforin/granzyme is directed across this synapse. Nonetheless, there is some leakage and some bystander damage - perhaps some synapses leak, perhaps some don't form tightly, perhaps some release of cytokines/perforin/granzyme is not fully directional. Perhaps some CD8 cells die and spill their contents. So, while CD8 killing shouldn't affect uninfected cells, bystander damage can be caused by the more non-specific products of CD8 T cells such as perforin (Sandt *et al.*, 2016). There are some examples of experiments where administering virus specific T cells during infection worsened survival from infection (Alwan *et al.*, 1994) which highlights how the precision killers of the adaptive immune system can also be damaging to the lung tissue. So much so that the immune response can be as effective a killer as the virus.

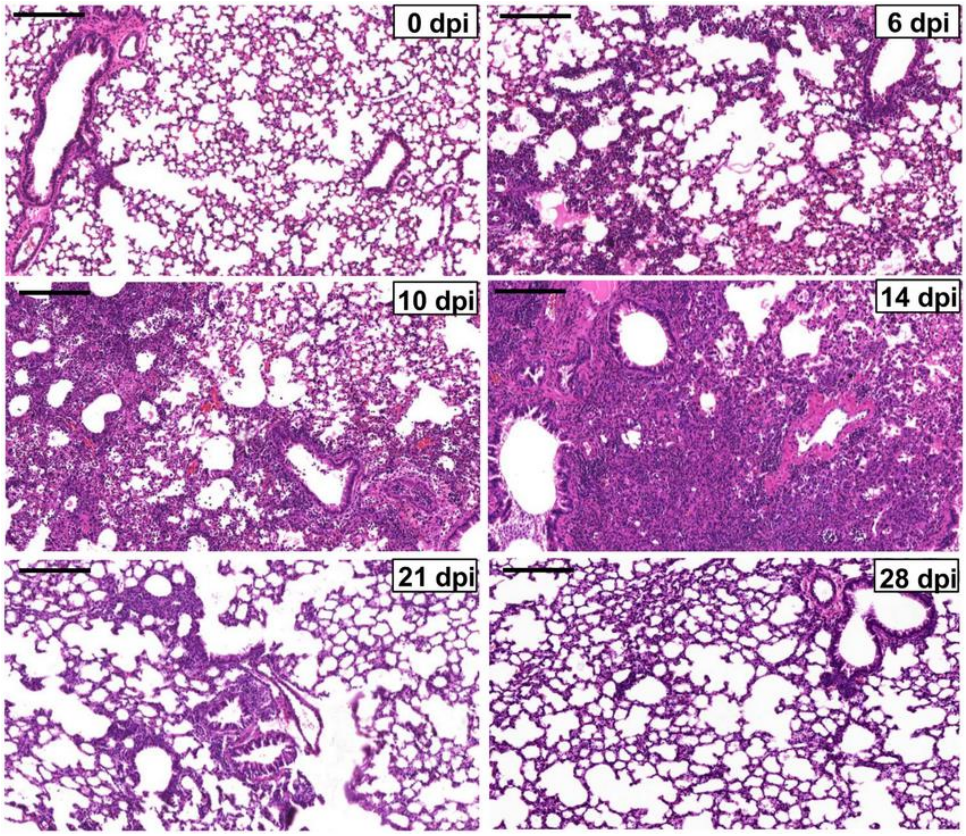


Figure 2-7 Influenza is a disease of damage.

The response to IAV infection is damage, which is recovered after infection (Cui *et al*., 2016).

One of the striking features of the D28 images in Figure 2-6 is that despite huge inflammation and oedema, recovery occurs, and the lung is restored to its original function. Different from how it began, yes, but still as effective in its function. The damage caused by IAV infection (whether caused by the virus or immune response to it) is problematic, as it disrupts the structure of the lung needed for breathing and leaves the lung vulnerable to opportunistic pathogens. 65% lab confirmed cases of flu exhibit secondary infection (Morris *et al*., 2017) suggesting infection associated damage allows pathogens such as Staphylococcus and Streptococcus to expand and cause problematic infections (Melamed *et al*., 2020). This suggests that there is something about influenza that allows opportunistic pathogens to thrive in a way not possible without the virus. This could be the damage caused by the virus and the immune response, or it could be the post-infection immunoregulatory environment which allows these pathogens to thrive.

For example, in clinical diagnosis of COPD, traditional markers of inflammation do not always correlate with patient outcome. Higher fibronectin concentration suggests more fibrosis, and more CRP suggests more inflammation. On their own, these metrics aren't useful in determining whether the patient will recover, but in combination they can be revealing. Patients with a lower ratio of CRP:Fibronectin were more likely to resolve their fibrotic tissue, and this ratio could predict patient recovery more accurately than either metric on its own (Soriano and Agustí, 2008). This shows that recovery from IAV requires both pro and anti-inflammatory stimulation, and so any manipulation of this fine balance may be difficult.

Another example is the balance between IFN-y and IL-10. There are many situations where inflammatory cytokine interferon gamma is expressed alongside anti-inflammatory cytokine IL-10 (Yanagawa *et al.*, 2009) (Marchant *et al.*, 1994). There is also evidence that effector T cells can produce both IFN-y and IL-10 during acute IAV infection in mice (Sun *et al.*, 2009). Blockade of IL-10 signalling causes overstimulated cytotoxic T cell responses and thereby prolonged recovery from to infection, particularly viral infections (Rojas *et al.*, 2017). During IAV infection, IL-10 from macrophages and T cells reduces bystander tissue damage and excessive inflammation by inhibiting proliferation of T cells, while also promoting natural killer (NK) and natural killer T (NKT) cells to kill dendritic cells, further curbing the inflammatory response (Qian *et al.*, 2006).

2.3.4 The future of IAV therapeutics – a balancing act.

We previously described the successes and pitfalls of current influenza vaccines, but they are not the only tool in our arsenal. We also have antivirals, and the extreme specificity of current IAV anti-viral drugs is at the same time a great benefit, and one of their greatest downfalls. Many drugs that are successful at preventing IAV replication have been sidestepped by small mutations. The first IAV anti-viral amantadine blocked the A/M2 proton channel preventing viral replication, which quickly led to the appearance of amantadine resistant strains of IAV (Swierczynska *et al.*, 2022) The most recent drugs in development are cap-dependant endonuclease inhibitors such as baloxavir marboxil, which interferes with IAV cap snatching preventing viral replication and has recently been approved for use in the EU (O'Hanlon and Shaw, 2020). These antivirals are effective but are commonly used for post exposure prophylaxis rather than direct treatment, due to the difficulty to get treatment to a patient during the exponential growth phase of the virus.

Rather than preventing viral expansion, it is also possible to reduce the inflammatory response to the virus. TNF antagonists have been shown to be effective for treatment of rheumatoid arthritis and Chron's disease, and recent studies have shown they can also be effective in treatment of RSV and influenza. Experimental models show that antagonising TNF with an antibody (Hussell *et al.*, 2001) or with a TNF receptor decoy (Shi *et al.*, 2013) reduced number of inflammatory T cells without significant increases in viral clearance time.

So, should we intervene with immune therapies to attempt to limit damage during infection? Modulating the immune response has been effective in treating cancers (Tuttle *et al*, 2023), but interfering with the immune response can have autoinflammatory side effects, for example where checkpoint inhibitor therapies caused immune related adverse events as side effects (Calabrese *et al.*, 2018) which shows that the immune response is carefully balanced. If we let the immune response to activate uninhibited, it is possible to die from immunopathology, but overexpression of anti-inflammatory cytokines TGF-β, IL-

13 and PDGF can be profibrotic, and can leave the lung permanently damaged (TA Wynn, 2007).

Our current therapeutics make no attempt to assist the immune response in maintaining the balance between pro and anti-inflammatory activity. The underlying problem here is that no matter the source of the damage, there is an inability to repair damage caused by infection. As described in the case of CRP:Fibronectin, tailoring the level of tissue damage initiated by infection may be difficult. However, we may be able to improve the way that the damage is repaired. We are beginning to understand the function of tissue remodelling macrophages, and the repair functions of regulatory T cells. Combining this with an understanding of how progenitors recover the tissue may show ways in which we can intervene and produce broadly tissue-repairing therapeutics. If it is tricky to modulate the immune response, can we instead design therapeutics that support the way this damage is repaired?

2.4 Repair or die.

Earlier in this chapter we have described in detail the many ways in which the lung can be damaged. Whether damage is initiated by the immune response, or a pathogen targeted by the immune response, there is a loss of structure and function of the lung, which limits the gas exchange ability of the lung. In development, the branching morphogenesis that creates this structure is governed by tip progenitors, which are the foetal progenitor of the lung, and which give rise to progenitors of the upper and lower airway (Nikolic *et al.*, 2017) (Sun *et al.*, 2022) (Lim *et al.*, 2023). What is not as well studied is how repair occurs in the adult, and specifically how this occurs after infection.

2.4.1 Repair in the lower airway

The alveolar environment is adapted for gas exchange. It is largely composed of long thin alveolar type I (AT-I) cells, providing a layer only one cell thick, ideal for gas exchange (Yang *et al.*, 2016). The alveoli also contain AT2 cells (Seadler *et al.*, 2023) which produce surfactant and act as progenitors of the lower

airway (Ruaro *et al.*, 2021). Experiments from 1954 resulted in the discovery of the AT-II cell, with early tissue damage experiments using osmium tetroxide showing that AT-II cells proliferate in times of damage (Macklin, 1954). Later, Kikkawa and Yoneda would isolate what we now know as the AT-II cell, suggesting they were “defenders of the alveolus” (Kikkawa and Yoneda, 1974) (Mason and Williams, 1977). AT-II cells are long lived, and during damage will proliferate to produce more AT-II cells, which can differentiate into ATI cells (Ruaro *et al.*, 2021). The signals required to carry out this differentiation are still being understood, but recent research has shown that loss of WNT signalling is required for ATII differentiation, and that often this WNT signal is provided by alveolar fibroblasts (Nabhan *et al.*, 2018). There is also suggestion that AT-II cells are activated by TGF-β, but there is also evidence that TGF-β reduces the activation of AT-II cells during lung injury in rats (Khalil *et al.*, 1994).

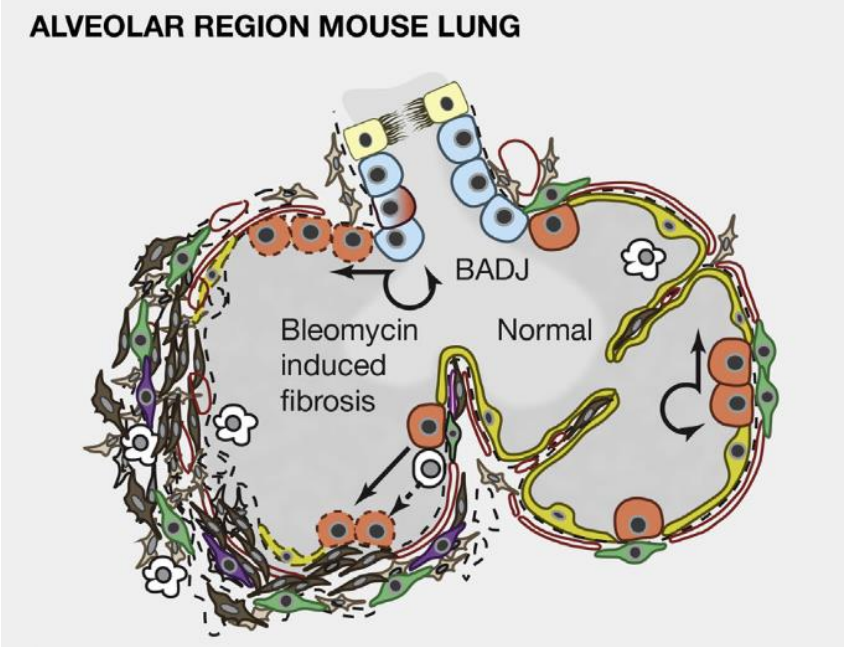


Figure 2-8 Structure of the alveolar space in health and disease.

Damage to the lung has been shown to reduce the number of ATII cells present and cause stem cell exhaustion, which is thought to be one of the contributing factors to idiopathic pulmonary fibrosis (IPF) (Parimon *et al.*, 2020). ATII cells have also been implicated in the development of COPD, lung cancer, and ARDS especially in cases of respiratory viruses such as SARS-CoV-2 (reviewed in Ruaro

et al., 2021) In COVID-19, inflammatory cytokines such as IL-6 have been shown to increase surfactant production leading to further alveolar damage. It is also suggested that since AT-II cells can often have membranes spanning multiple alveoli, they are capable of facilitating the spread of infection. In summary, proliferation, and differentiation of AT2 cells is critical for repair, and at present it is unclear which regulatory factors control this repair.

2.4.2 Repair in the upper airway.

The alveoli are the site of gas exchange in the lung, and these gases arrive through the conducting branches of the upper airway (Plasschaert *et al.*, 2018). The upper airway, from the single trachea down to the thousands of bronchioles that link to the alveoli, is lined with an epithelial layer covered in mucus. The structure of this epithelium has been described in section 1.X. All epithelial cells in this layer are derived from basal cells, which are the progenitors of the upper airway. Basal cells are so named as they reside on the basal layer of the epithelium of the upper airway.

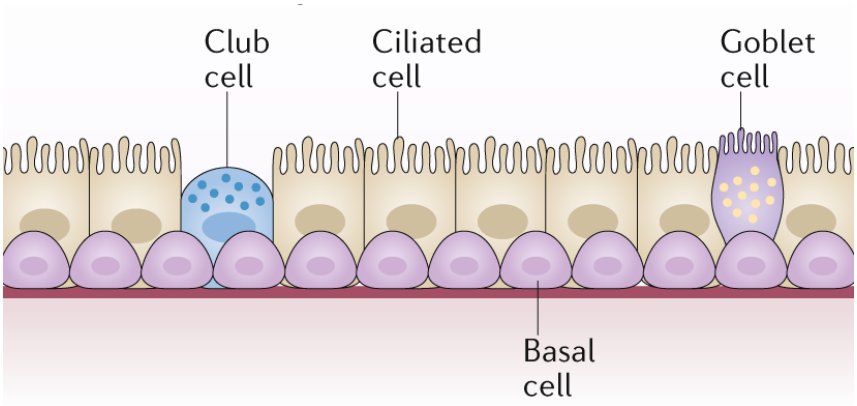


Figure 2-9 Structure of the upper airway.

Basal cells are found in both mice and human lungs, though they are identified by different markers in different species. In the human lung they are mainly characterised by expression of KRT5, KRT15 and a4b6 integrin. While these markers can be found in the mouse lung, Ngfr, Krt14 and p63 are more

commonly used to identify mouse basal cells (Roch *et al.*, 2009). Basal cells were described as progenitors of the trachea by those carrying out lung napthelene injury studies (Cole *et al.*, 2010, Hong *et al.*, 2004). Many studies have since shown this progenitor ability of basal cells in the upper airway (reviewed in Hogan *et al.*, 2014), but recent work has also shown their ability to differentiate out with the conducting airways. The function of basal cells in the lower airway is not currently resolved, but it is a subject of recent investigation (Rout-Pitt *et al.*, 2018). In an experimental influenza A virus infection that caused extensive alveolar damage, basal cells were shown to migrate to the damaged alveolar space and differentiate into secretory cells such as goblet and club cells (Mello Cost *et al.*, 2020, Kumar *et al.*, 2011). At first it was considered that basal cells could be progenitors of the lower airway, particularly in situations of extreme damage, and that they could become ATII cells and repopulate the alveolus (Rawlins *et al.*, 2009). There is also a suggestion that terminally differentiated cells such as club cells can de-differentiate during damage and act as a reservoir for upper airway repair. This function was previously considered to be controversial, but in increasingly becoming more accepted (Tata *et al.*, 2013).

This is a topic of recent study, and some suggest that basal cell repair in the alveolar space (so called “KRT5 pods”) is not considered to be reparative (Kumar *et al.*, 2011, Kim *et al.*, 2021). I hypothesise that this could be neutral repair - a form of triage and that for the lung it is better to have any cell type there to attempt to close the wound. However, there is also suggestion that basal cell proliferation in the alveolar space is part of negative repair - dysplastic basal cell driven repair that can prevent gas exchange from occurring, reducing lung function. For this work we have considered basal cells as progenitors of the upper airway, but have also searched for them in the lower airway, as others have reported they are more abundant out with the trachea during tissue damage.

2.4.3 Repair between the lines

The debate around whether basal cells contribute to repair in the alveolus is further complicated by the hidden third niche of the lung. Connecting the upper and lower airway structure is the bronchoalveolar duct junction (BADJ). This is an area of the lung where the bronchioles transition into the alveoli, creating an environment that contains cells of both upper and lower airway cells (Bender Kim *et al.*, 2005). The discovery of this environment, and the consideration that it is repaired after damage has led to the proposition of another progenitor of the lung, the bronchioalveolar stem cell (BASC) which have markers and functions of both basal and AT-II cells (Kawakita *et al.*, 2020, Freeman and Starkey, 2020).

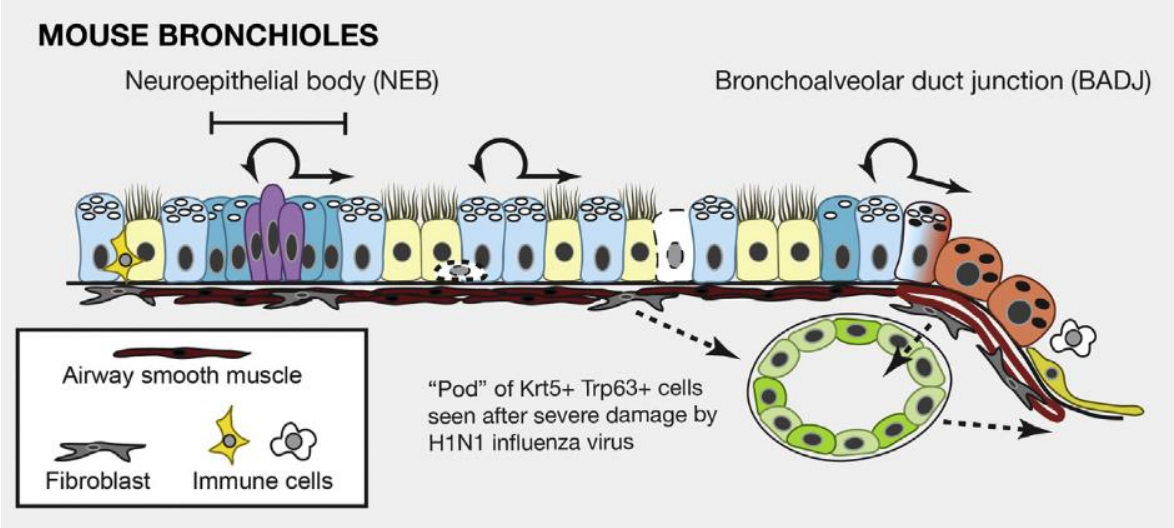


Figure 2-10 The bronchoalveolar duct junction.

The most recent research in basal cell research suggests that basal cell activation is associated with a switch in the metabolic profile of basal cells. It has been shown that ciliated epithelial cells upregulate fatty acid metabolism when compared to basal cells, and the authors suggest this is an essential switch to allow this differentiation to occur (Crotta *et al.*, 2023). This work has been carried out using *in vitro* studies, but it would be interesting to know if these changes could be observed in the more complex in vivo environment. Ischemia and hypoxia are consequences of severe damage in the lung and the changes to

oxygenation that happen in tissue is hard to emulate *in vitro*. As repair begins, there is a rapid remodelling and revascularisation which occurs, created by the hypoxic state of the tissue and this forms a state like that found in the tumour microenvironment (Eming *et al.*, 2021). The change to a low oxygen, tumour cell metabolism reverts to homeostasis during scar formation and long-term healing, but the metabolic profile of the scar is unknown. What is still unknown is how are affected by to a hypoxic environment, and whether reduced access to nutrients and oxygen can prevent basal cells from repairing adequately.

In general, regeneration occurs when a large area of a tissue is destroyed and must be rebuilt from the ground up. Tissue repair is where sections of the tissue have been damaged, but the majority of the tissue is intact. Since most damage caused by viruses doesn't destroy the whole tissue, we focused on repair. Exactly what constitutes effective repair is still poorly understood. The balance between life-saving tissue regeneration, and debilitating fibrosis is maintained in most infections, but when the balance is lost there can be serious long-term consequences. Basal cells take part in good and bad repair and so it is worth trying to understand how they function, so we can prevent the bad and harness the good. It is not clear what the signals are for basal cells to begin repair, or how they are controlled by their environment. This is before considering the variety of challenges the lung can experience, and the fact that the repair response and the response of basal cells could be different in each case. My thesis aims to address these questions, investigating basal cell responses during influenza-induced damage.

2.5 Hypothesis and Aims

This thesis is focused on virus induced damage to the lung, and how progenitor cells recover that damage. It is well understood that lung damage is repaired through the action of basal cells, but there has been limited study into how basal carry out their function, particularly during in vivo infection. Research in this field has been limited by the technical challenge of being able to recover sufficient rare basal cells from lung tissue for thorough analysis. Most previous research in this area has focused on imaging of lung tissue, or in vitro studies. I considered that there are gaps in the understanding of basal cells that could be addressed using flow cytometry and transcriptomic analysis to assess basal cell function in vivo. IAV causes the disease influenza which damages the structure of the lung, making it a good model for studying basal cell response to infection. We hypothesized that influenza would change the behaviour of basal cells and that, either directly or indirectly, IAV would change the way that basal cells repair the lung. To test this hypothesis, we planned 3 main aims:

- To identify and isolate basal cells in mouse lung using flow cytometry, optimising the protocol for yield and specificity; and to perform transcriptional characterisation in health and in influenza.

- To determine the indirect response of basal cells to murine infection with influenza A virus, considering how tissue damage and the inflammatory response change the behaviour of basal cells in the infected environment.

- To determine the direct response of basal cells to influenza A virus infection, asking whether IAV infects basal cells and whether there are consequences of such infection for the functional potential of infected basal cells.

By addressing each of these objectives, we aimed to add to the understanding of basal cells in vivo and specifically how their behaviour changes during a lung-damaging interaction with influenza A virus infection.

Chapter-3 Isolation and identification of lung basal cells

3.1 Introduction

As has been introduced in Chapter 1, there are many ways in which the lung parenchyma can be damaged and no matter the source of the damage, to recover the function of the lung it must be repaired. This repair is carried out by progenitor cells (Alysandratos *et al.,* 2021) which return the original structure of the lung which although different, is now able to carry out its function. The lung is composed of a variety of cell types, and during damage lung progenitors activate and proliferate to restore the lung (Travaglini *et al.,* 2020). The lung has been extensively studied using fluorescence microscopy (Whitsett *et al.,* 2018, Rodero *et al.,* 2015, Lohmann *et al.,* 1990) and the structure of the lung is well characterised (Akram *et al.,* 2019). Fluorescence imaging can be limited is in its ability to observe the variety of functions that individual cells are capable of – microscopy panels are often smaller, and so having enough resolution to identify cells and their functions can be difficult. New technology is allowing these limitations to be overcome, though access to the most cutting edge is not yet widespread (Radtke *et al.,* 2020).

Flow cytometry is a useful technique to use alongside imaging to understand the behaviour of cells in the lung. Cytometers usually have access to a wider array of fluorophores so detailed subsetting of cell types is achievable whilst leaving room for markers of function. In addition, using intracellular staining techniques it is possible to identify markers of proliferation and differentiation in a relatively short time frame after removal from tissue. There are groups who use flow cytometry to generate and study progenitor cells of the lung (Autengruber *et al.,* 2012, Sun *et al.,* 2023) but most current work is currently done using *in vitro* cultures. Epithelial cells are difficult to dissociate from the lung and their size and shape means they are often difficult to work with for flow cytometry. Because of this, epithelial cells (and particularly progenitor cells), are rarely studied by flow cytometry. This may be due to a lack of interest or appreciation

for the key role of progenitors, but it also may be because many lung isolation protocols are not optimised to allow for isolation of epithelial cells (D'Agostine *et al.,* 2022, Atif *et al.,* 2018). Since lung progenitors are rare, no cell can be wasted and so there is a need for a protocol designed for isolation of as many progenitor cells from the lung as is possible. An optimised protocol would allow for a greater yield for cell sorting and downstream analysis, allowing for a refinement in animal experiments.

One of the reasons studies of progenitor cells have been limited is that the enzymes used in many lung digestion methods rely on enzymes such as collagenase and Liberase, which require a warm temperature to be most active (37^0C). These are powerful enzymes, so a short time at this temperature is all that is required to dissociate the tissue (JOVE, 2024) and this thorough digestion needed to dissociate the fibrous lung tissue may destroy epithelial cells. In other tissues such as skin (Liu *et al.,* 2018) and pancreas (Li *et al.,* 2013), slow cold digestion has been used to produce a greater yield of progenitor cells for live cell analysis. We considered that a digest approach like these would allow a greater yield of progenitor cells for our analysis. Another way to answer the unknown questions about progenitor cells is using single cell RNA sequencing. Sequencing of the transcriptome of progenitor allows a much wider range of functions to be interrogated, and one advantage of this technique is that there are many published data-sets available that can be used to answer our questions.

3.2 Aims

This project aims to understand the biological difference between lung progenitors in steady state, and lung progenitors that are active during lung damage. Since isolating enough lung progenitors for functional analysis is currently difficult, we propose these technical aims to allow us to interrogate our biological questions. Our aims are as follows:

- To develop a novel epithelial cell flow cytometry panel to characterise lung epithelial cell subsets and identify as many lung progenitors as possible.

- To optimise our digestion and cell dissociation protocol to increase the yield of lung epithelial progenitor cells for transcriptional analysis by FACS.

- To utilise published single cell RNA sequencing datasets that have not interrogated progenitor cells to determine the transcriptional effect of lung progenitor activation.

3.3 Results

3.3.1 A new lung dissociation protocol optimised for epithelial cell recovery

The first challenge faced in this project was that it was difficult to make statistically strong conclusions about changes to epithelial cells in infection, when we were yielding so few epithelial cells from lung dissociation. Our initial lung digest experiments utilised a "hot digest", the conditions of which are found in table 1 and in Chapter 2. This kind of digest commonly used for lymphocyte isolation (Autengruber *et al.,* 2012) and relies on enzymes such as collagenase used to digest lung tissue for a short time at a warm temperature. A PhD student at the University of Edinburgh suggested a lung digestion method he had been optimising that used a less abrasive enzyme at a lower temperature for up to 20 hours - he referred to this as his "cold digest". As described previously, Piotr was not the first to suggest cold tissue dissociation, but his method for isolating more progenitor cells from lung tissue was effective and has since been published (Janas *et al.,* 2023). Piotr's experiments focussed on increasing yield of progenitors for his *in vitro* cultures, and as part of the paper development, we tested his method for *in vivo* isolation of progenitors for flow cytometry applications.

Hot Digest	Cold Digest
Dispase II (10mg/ml)	Dispase II (5mg/ml)
DMEM/F12	DMEM/F12
37°C 1hr	4°C 20hrs (rocking)

Table 3-1 Conditions for cold and hot lung digest for epithelial cells

We wanted to determine whether a cold digest would allow us to isolate more progenitor cells, and so we carried out an experiment to compare the methods. We compared the digestion methods from Table 1.1, and we included a third condition, a short cold digest (1hr 4^{o}C), to control for the time spent in the

digestion mixture. The cold digest contained a reduced concentration of Dispase to prevent over digestion during the longer 20hr timepoint. The results from these experiments are shown in Figure 3-1.

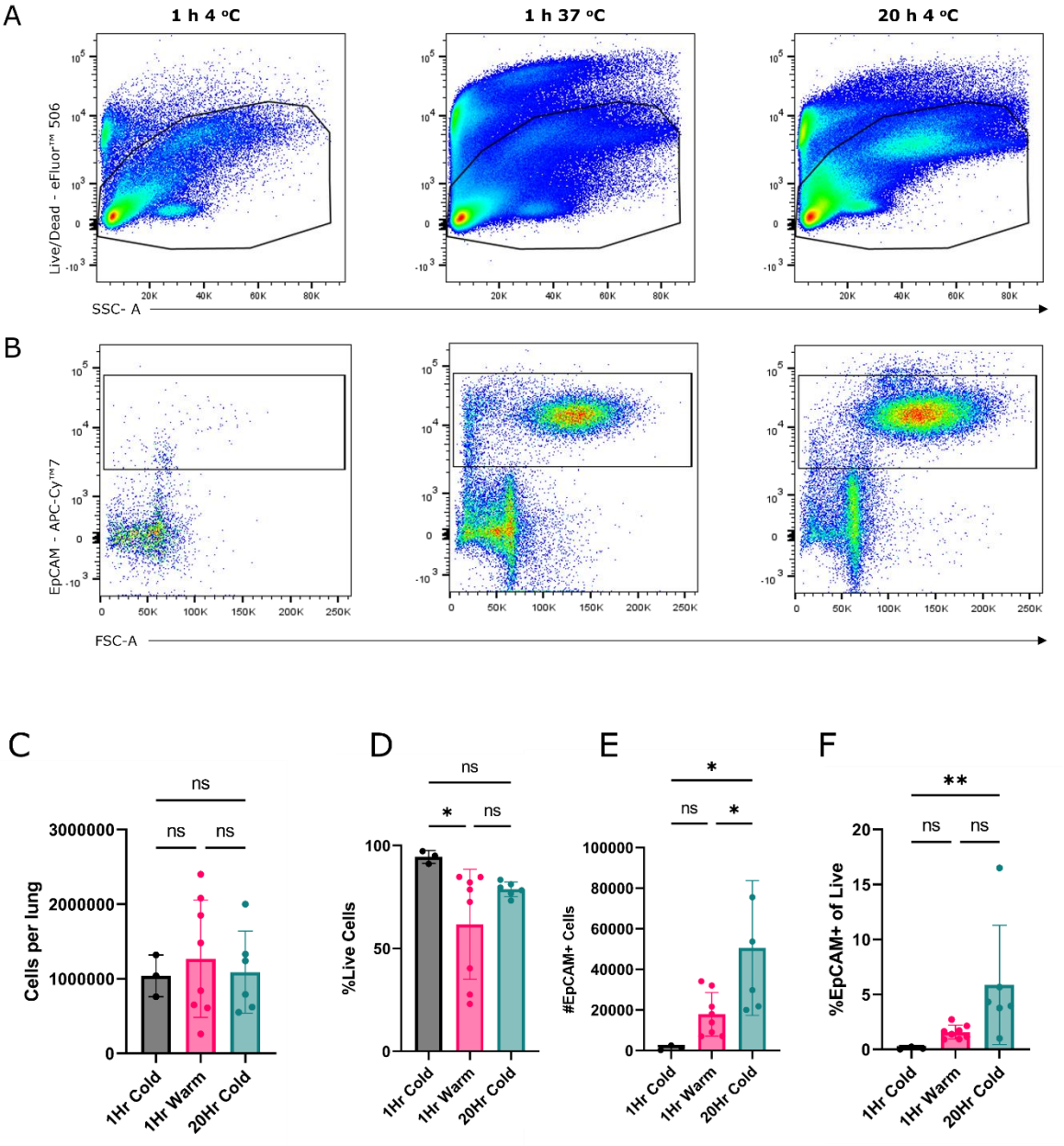


Figure 3-1: Slow, cold digestion provides an increased number of cells with similar viability to other digests.

Representative flow cytometry gating showing (A) Viability of all cells and (B) Epithelial (EpCAM+) cells from each digestion protocol. Percentages of (D) all cells and (F) epithelial cells were used to calculate absolute numbers of (E) epithelial cells. Statistical analyses were performed using one-way

ANOVA with Kruskal-Wallis multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001. n=15, data shown are representative of 4 individual experiments.

We found that the slow cold digest also provided a greater number and viability of epithelial cells compared to a quick hot digest and that the cold digest provided a more consistent viability across all cells. Because our focus was on retrieving the maximum number of progenitor cells so that from each lung so we could observe their behaviour, we wanted to know if this improvement also applied to epithelial progenitor cells.

3.3.2 An extended flow cytometry panel to identify lung progenitors

For our initial experiments, we designed an antibody panel building on the work of Jennifer Quantius and others (Quantius *et al.*, 2016) to measure the yield and quality of progenitor cells isolated by flow cytometry. We chose to do this as this method had yielded a good number of epithelial progenitor cells for *in vitro* cultures, flow cytometry and molecular techniques, all of which we wanted to carry out. Figure 3-2 shows the results for our attempt to replicate this flow cytometry panel.

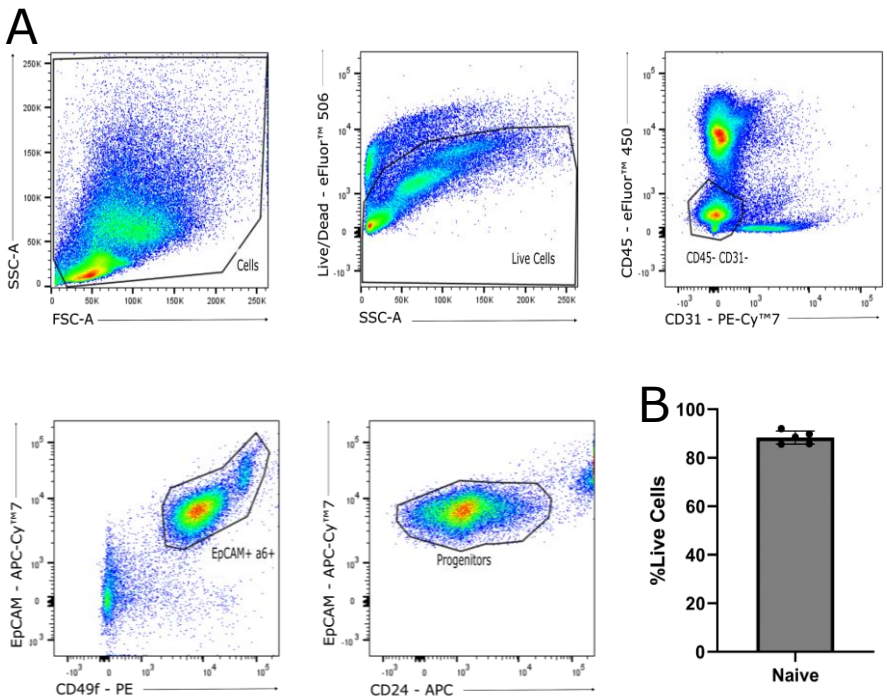


Figure 3-2 Identification of lung progenitor cells by flow cytometry.

(A) Gating strategy for identification of epithelial progenitors from digested lung tissue (B) Viability of all cells using eBioscience™ Fixable Viability Dye eFluor™ 506. Data shown n=5 are representative of 7 experiments.

Flow cytometry gating showing (A) epithelial and (B) progenitor cells from each digestion protocol. Absolute cell numbers and percentages were calculated for (C) epithelial and (D) progenitor cells. Statistical tests were carried out using (C) two-tailed unpaired *t*-test and (D) Mann-Whitney test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $***P < 0.0001$. n=15, data shown are representative of 4 individual experiments.

These results showed that the cold digest allowed us to recover more progenitor cells from the lung when using a cold lung digestion. As previously described, what we have referred to as progenitors so far is a mixture of upper and lower airway progenitors. This flow cytometry panel doesn't discriminate between upper airway basal cells, and lower airway alveolar type II cells. We predicted these two subsets would have different behaviours in damage and so we attempted to design a flow cytometry panel that could separate "progenitors" into basal and AT-II cells. Epithelial cells too are more diverse than just EpCAM+ cells, and so we added markers to identify endothelial cells, and alveolar type I cells (AT-I).

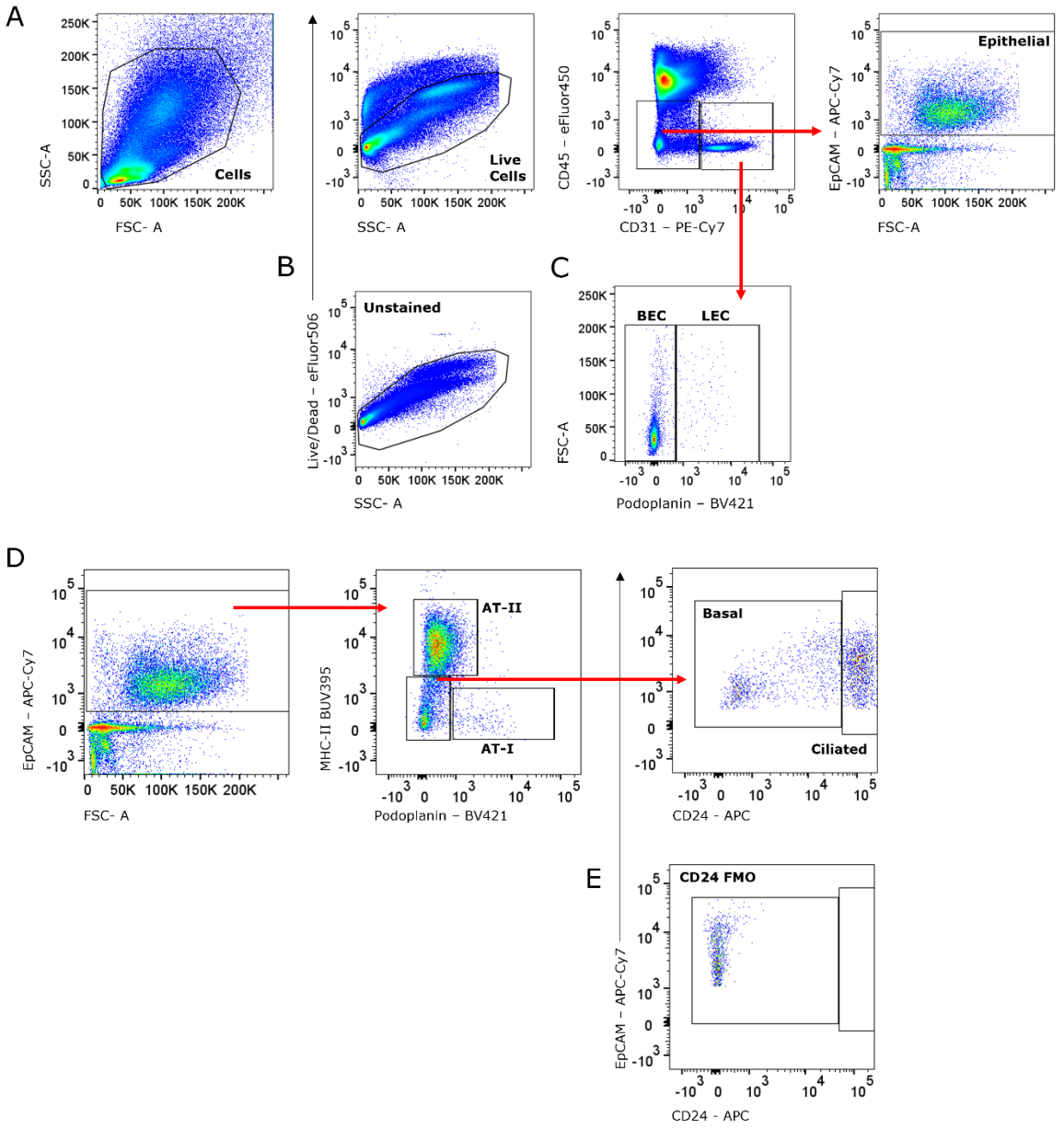


Figure 3-3 An extended flow cytometry panel for detection of lung progenitors.

Representative flow cytometry gating with the addition of MHC-II and Podoplanin to identify further epithelial progenitor subsets. (A) Gating for epithelial cells including (B) Live/Dead unstained control and additional gating for (C) Endothelial cells. (D) Further gating of EpCAM+ cells to include AT-I (MHC-II-, Pdpn+), AT-II (MHC-II+, Pdpn-), Basal (MHC-II-, Pdpn-, CD24lo). (E) Unstained CD24 control.

3.3.3 Identifying basal cells using a bioinformatic approach

These results gave us confidence that we were identifying different epithelial cell subsets, particularly the clear difference between the basal and alveolar progenitor cells. We then FACS sorted both alveolar and basal progenitor cells using this panel to confirm that we had successfully sorted these progenitors. After sorting, we looked for specific basal cell markers, and at PCA to determine if the sorted cells were likely to be basal and alveolar type II cells.

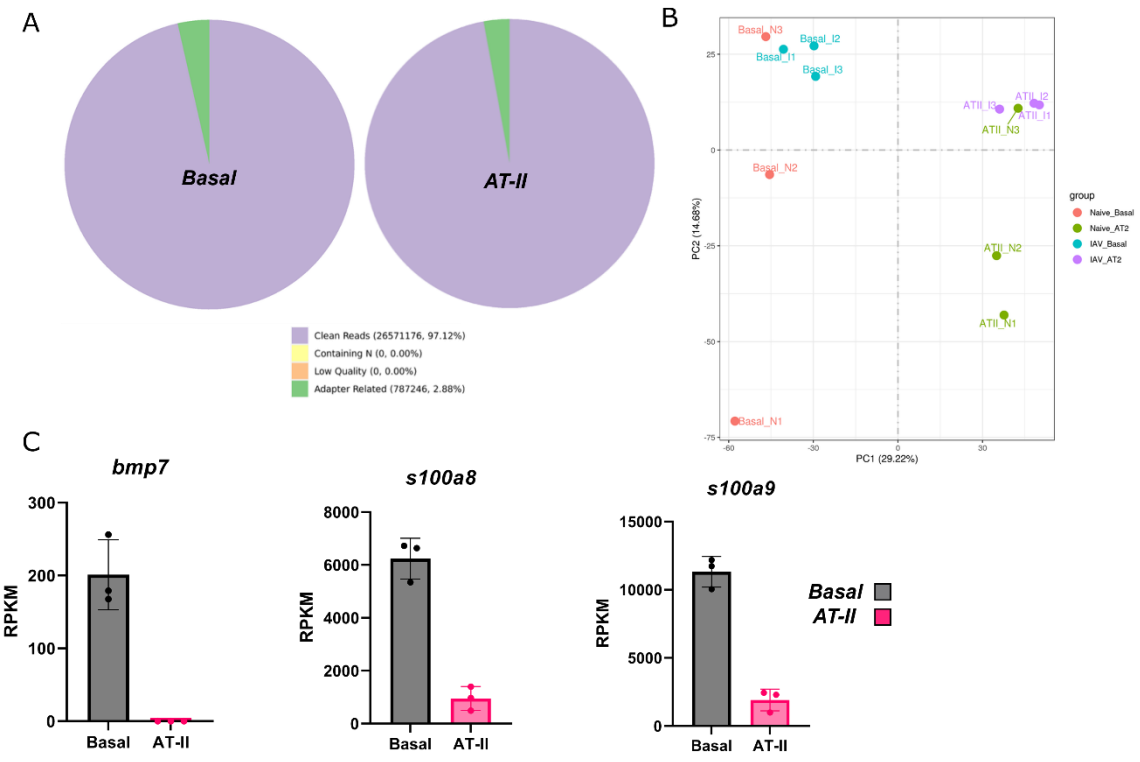


Figure 3-4 Sorting lung epithelial progenitors for bulk RNA-seq.

Results and quality control analysis from bulk RNA-seq carried out by Novogene. (A) Classification of raw reads and (B) Principal Component Analysis (PCA) for each sample. (C) Differential gene expression of known basal cell genes for confirmation of cell sorting.

These results showed that we could use FACS to separate lung progenitor subsets from both naïve and infected mouse lung. We also showed that we could then recover these cells with good enough viability in good enough numbers to allow for transcriptional sequencing. Since our interest was in understanding the differences between the different subsets of lung progenitors, these results were promising. Our particular interest was in the transcriptional differences in infection between naïve and infected basal cells, so this was our next focus. To obtain a more complete understanding of the transcriptome of basal cells, we decided to interrogate single cell RNA-seq data. Since it was difficult to obtain enough cells to do this, and with the cost limitations of this technique, we decided to attempt to identify basal cells from published scRNA-seq datasets. The bulk RNA sequencing we had performed was useful, but it assumes that basal cells are a homogenous population, and single cell sequencing resolution would help us understand if there were different subsets of basal cells that carried out different functions. This was advantageous as other groups had sequenced epithelial cells from the lung but hadn't focused on the basal cells within those data sets. The data sets used were produced as part of mouse studies of infection, with the main data sets coming from influenza infected mice. This approach not only allowed us to mine further data from experiments that were published without the need for additional animals, but it also allowed us to continue to work during the COVID-19 pandemic, where the ability to carry out experiments was limited. Before we could analyse the single cell datasets, we carried out quality control using the protocol in the Seurat processing vignette "Guided Clustering Tutorial" (Hao *et al.*,2023). The procedure was not exactly as described in this vignette, and a full description of our protocol is available in Chapter 2. In short, we attempted to remove dead cells by filtering based on the % of genes that were of mitochondrial origin, and tried to remove doublets by rejecting cells which had a larger than average feature count. An example of these metrics is shown in Figure 3-6.

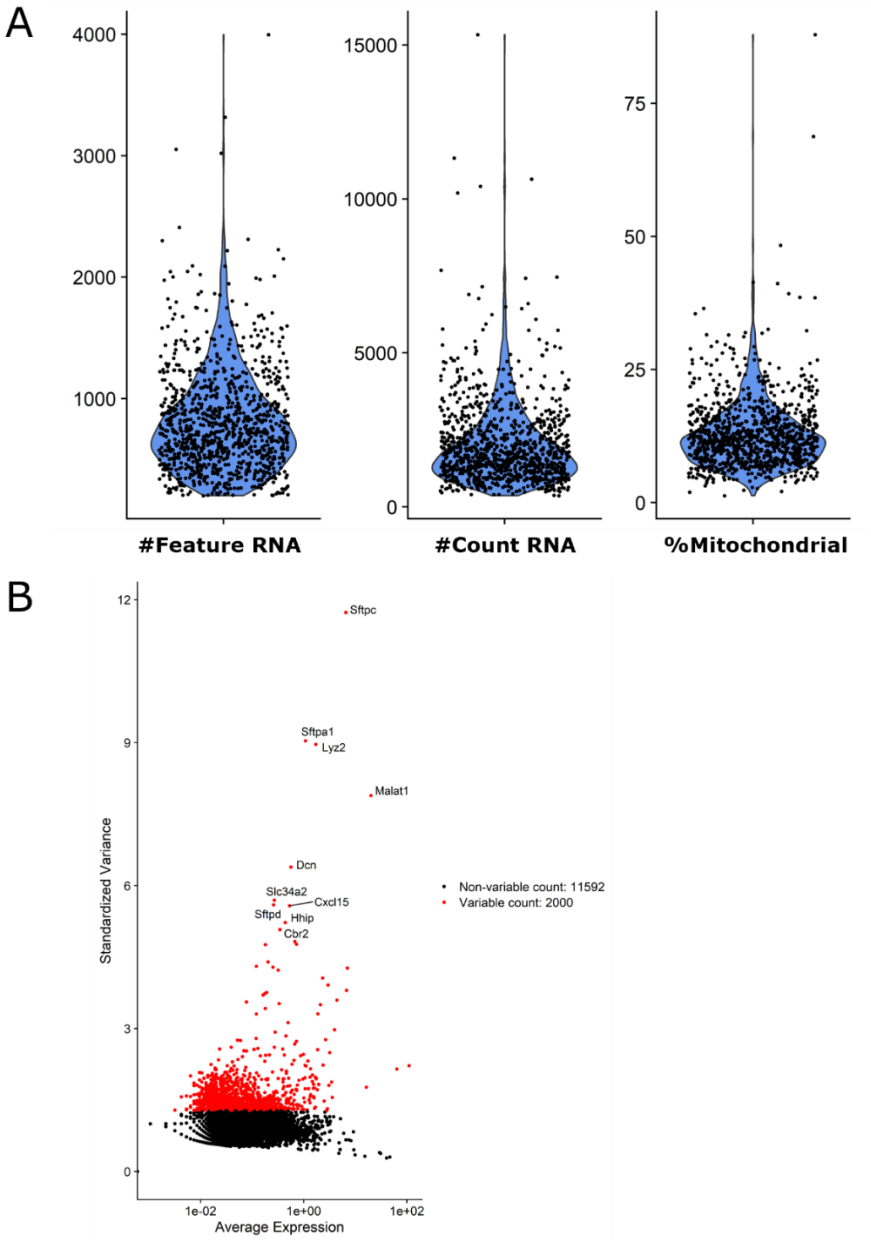
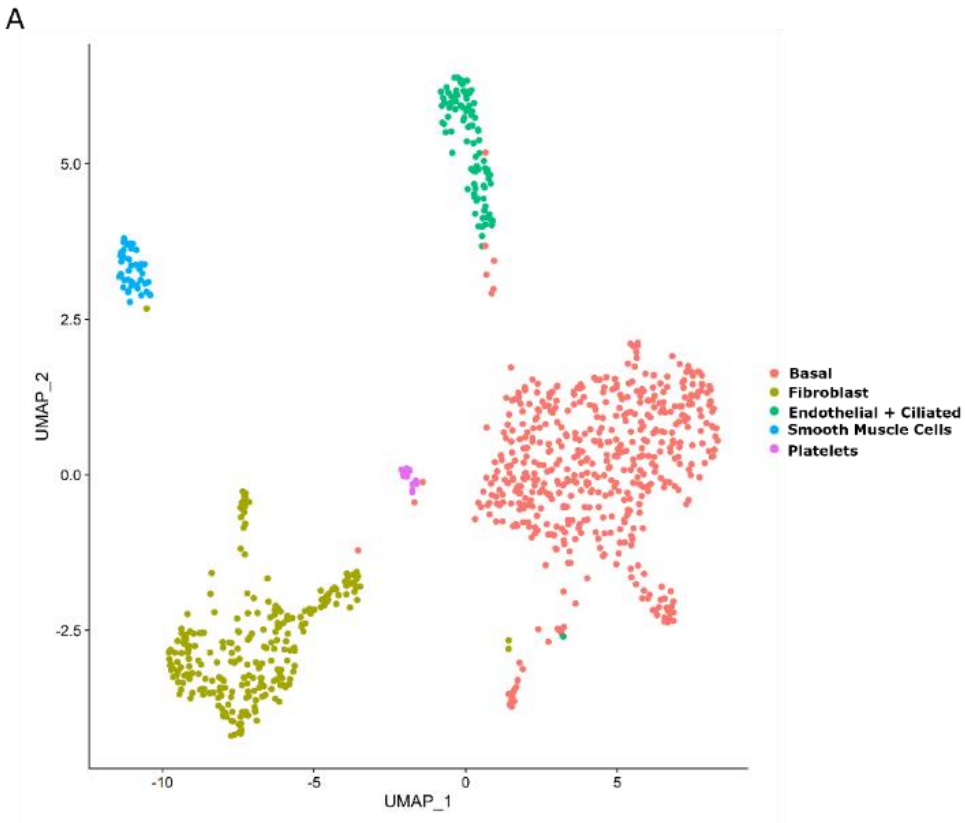


Figure 3-5 scRNA-seq filtering and quality control using Seurat.

Pre-processing of published scRNA-seq datasets using Seurat. (A) QC metrics used for filtering, #Count RNA for low quality cells or empty droplets, #Feature RNA to identify doublets or multiplets and %Mitochondrial genes to identify cells with high mitochondrial contamination (dying). (B) *FindVariableFeatures* function was used to identify the most variable features in the dataset.

The dataset presented in Figure 3-6 is of high quality, with only a few cells being removed due to the quality control metrics. If we decided a dataset was of high enough quality, we then moved on to the clustering step. The full method is listed in Chapter 2, but in brief we determine the correct number of clusters to generate based on statistical tests and what cell types are expected in the data set. Then the variable features across the dataset are calculated, and Seurat segments each cell into a cluster of cells with similar expression. Figure 3-7 shows an example output from this process.



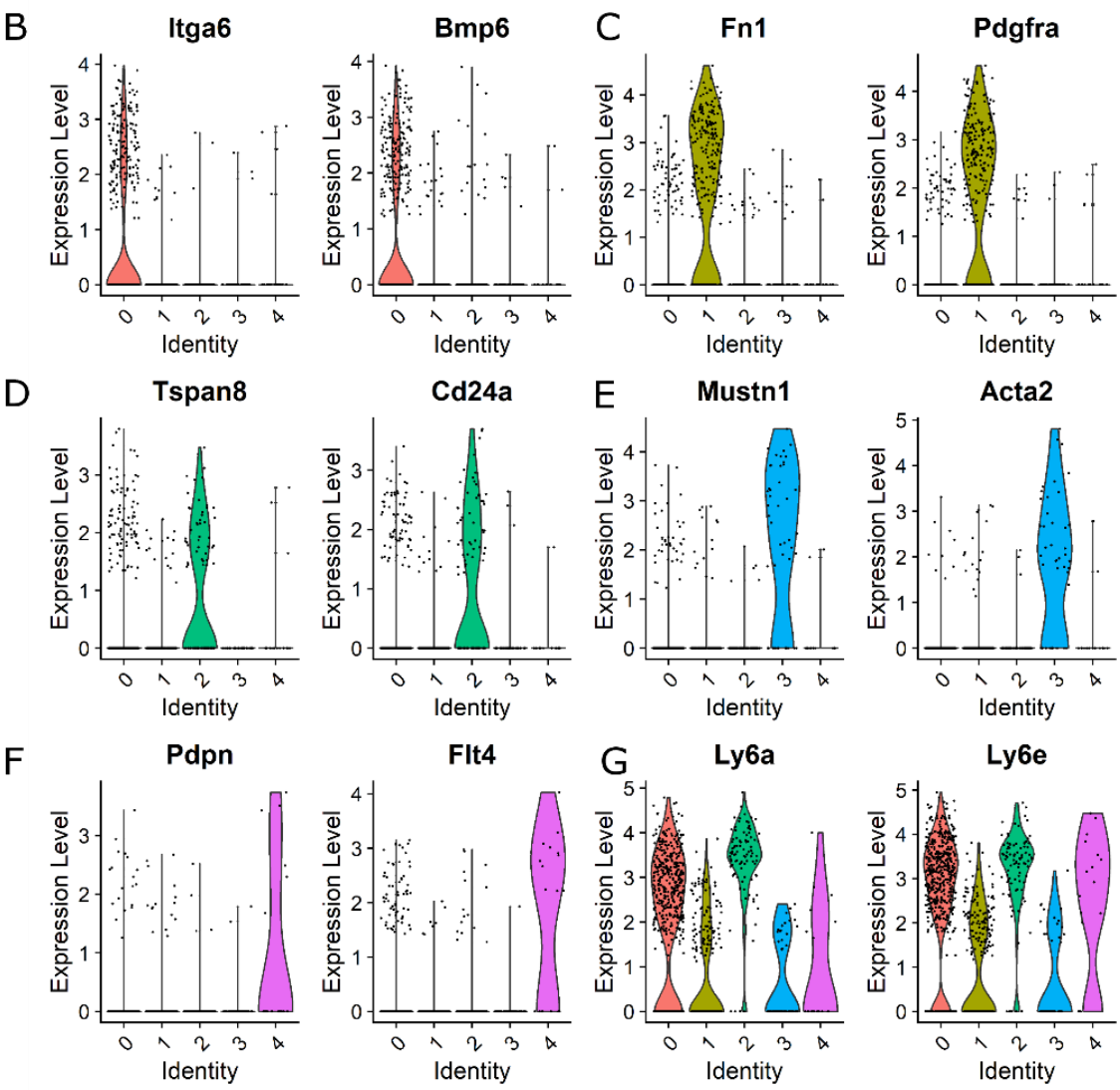


Figure 3-6 Identification of basal progenitor cells by scRNA-seq.

(A) UMAP - Using the *FindNeighbors* and *FindClusters* commands we separated the cells in the Seurat object into 5 clusters (Resolution = 0.15, Dimensions = 10 determined by *ElbowPlot*). We confirmed cluster types using known markers for (B) basal, (C) fibroblast, (D) ciliated, (E) smooth muscle, and (F) platelet cells and (G) Sca-1, a feature expected to be found in all cells.

The clustering and identification steps shown so far were done so that we could identify a cluster of the each cell type from each sample. Our aim was to compare basal cells across different inflammatory conditions, and with these clusters we could then carry out differential expression analysis between basal cells in these different conditions. To do this, individual Seurat objects were integrated into one for downstream analysis. For example if we wished to

compare basal cells from Naïve and D3 post infection, we would integrate those samples to provide more accurate differential expression analysis. There are many different ways to carry out integration (Luecken *et al.,* 2021) and we wanted to decide the method to use for our analysis. We decided to use the dataset which had a timecourse of infection (Zhang *et al.,* 2020). We considered that the timecourse may introduce large batch effects making differential expression analaysis possible, so we used this data set to attempt to gain the most accurate integration to reduce this effect. As methods of single cell integration are becoming more popular, and more methods are being developed (Ryu *et al.,* 2023) we wanted to detemine the most effective method for our analysis. So we carried out integration using some of the popular integration methods and aimed to determine which provided the best overlap in cell clustering. Thanks to Andrew McCluskey, a PhD student collaborator who wrote a Python script which runs identical integrations using a wide variety of integration methods which made the results in Figure 3-8 possible.

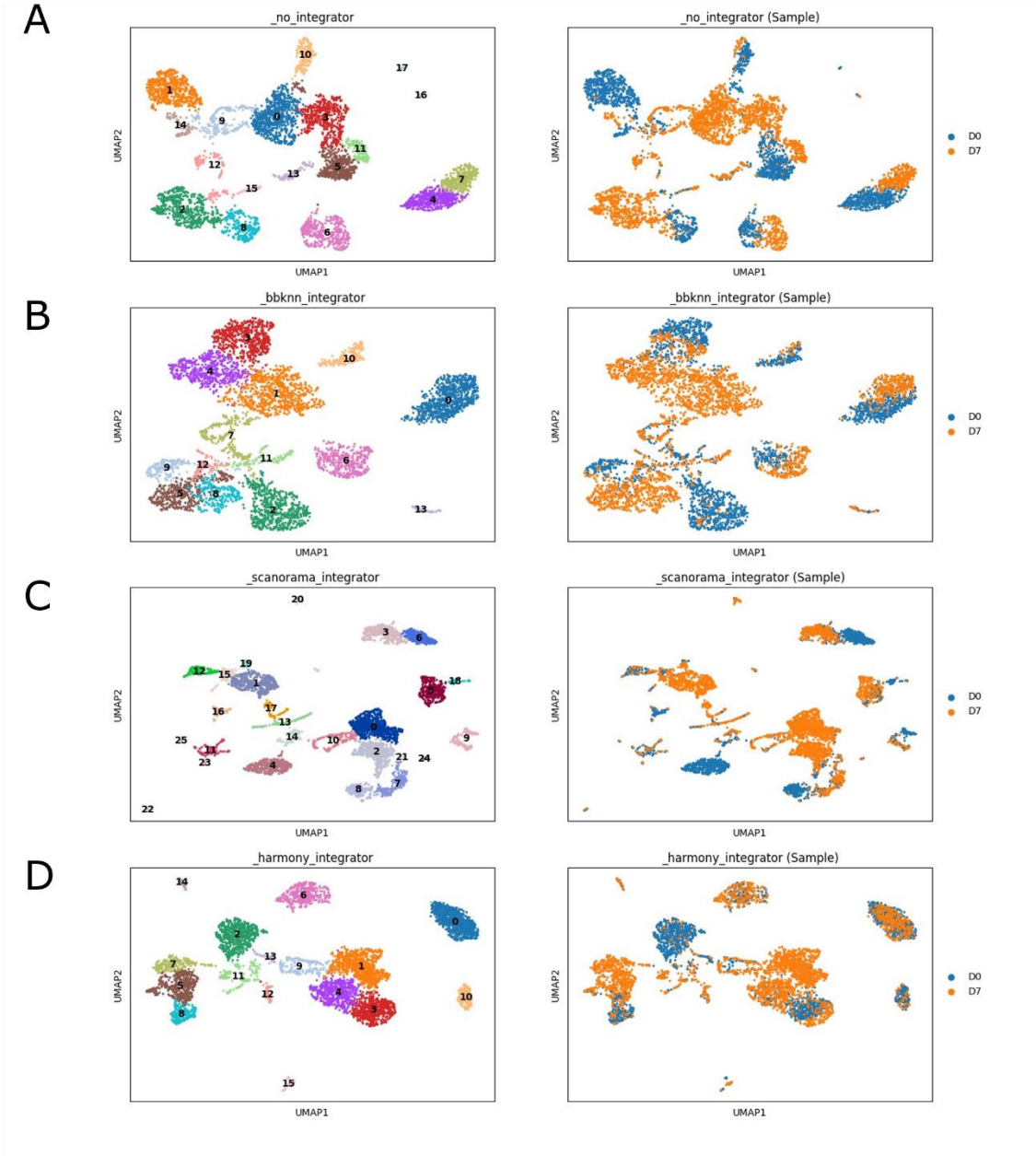


Figure 3-7 Harmony provides the most consistent single cell integration.

UMAPs showing the integrated Seurat objects after *RunHarmony* integration in the R library "harmony". Integration of two Seurat objects using (A) no integration method, (B) "BBKNN", (C) "Scanorama", (D) "harmony" integrator. Using a variety of resolution values and comparing to the no integration controls, harmony was selected as the most reliable integration method which reduced batch effects.

Since we had previously clustered each timepoint, we already had an answer about which cluster we think are basal cells in each timepoint. In theses results, we are looking for the best overlap between the basal cell clusters of each timepoint. This indicated a good integration that has identified similar cell types even though the cells will have different gene expression due to being harvested form different timepoint. In 3-8(A), you can see that with no integration, the two basal cell clusters are considered as separate (cluster 4 and 7). BBKNN and scanorama identify the basal cell clusters and integrate them, but as is clear in (B) and (C), the overall in the basal cell clusters is disparate. However, in (D), the basal cell cluster 0 is well integrated with a near perfect overlap. These results of our integration comparison led us to choose the package *Harmony* for integration of our Seurat objects going forward, allowing us to compare different conditions within scRNA-seq experiments.

The results shown in this chapter lay the foundations for the work shown in Chapter 4 and Chapter 5. In those chapters we attempt to interrogate the changes conferred on basal cells by damage and inflammation. To answer those questions, we need robust systems to identify basal cells. This chapter shows how we were able to improve the yield of progenitor cells from lung digestion preparations, and how that ability allowed us to carry out functional and transcriptional analysis of progenitors. We also show here that basal cells can be identified from published single cell RNA-seq datasets, and that that published data can be used to answer further the questions left unanswered in the current basal cell literature. Without the work carried out and shown in this chapter, we could not have generated the data shown in Chapter 4 and Chapter 5.

3.4 Conclusions

- We aimed to develop a procedure for isolating rare progenitor cells from whole lung tissue and we discovered that a slow, cold tissue dissociation provides a greater yield of lung epithelial cells.

- We aimed to harvest enough basal progenitors from mouse lung tissue to be able to carry out bulk RNA-seq and compare their transcriptome during infection. We showed that our cold digest and extended flow cytometry panel allow us to do this and that we can extract high quality RNA for sequencing using this method.

- We hypothesised that there was untapped data showing the transcriptional character of basal cells in published single cell RNA-seq datasets, and we showed that it was possible to extract that data. We also showed that Harmony provides a more reliable integration of time course single cell experiments than Seurat, BBKNN and scanorama integrators.

3.5 Discussion

3.5.1 Lung digestion optimisation

The overall aim of this project was to increase the availability of basal cell data that we had access to. Basal cells are a rare cell type and are not widely studied especially in an infection setting. Part of the reason for this is their rarity, and the fact that they are difficult to isolate from the mouse lung. There are groups who study basal cells (Rock *et al.,* 2009, Nikolic and Rawlins, 2017), but largely this is done with in vitro cultures, or by using human primary stem cells. Previously, isolation of epithelial or immune cells from whole lung tissue relied on enzymatic digestion with a Collagenase, Liberase, or with commercially available kits such as Miltenyi Biotec's Lung Dissociation Kit. Collagenase enzymes are effective at digesting collagen (Elvin Harper, 1980), but rely on a high concentration of enzyme for a short period at high temperature and are usually combined with a method of physical dissociation (chopping or shaking) during the digestion (Singer *et al.,* 2016, Mariano *et al.,* 2023). For our initial experiments, we used this method and were able to recover some epithelial progenitors. However, when attempting to sort progenitors from the lung after this method, we could only recover 500-1000 cells per lung by FACS

Piotr Janas, a collaborator at Edinburgh University introduced us to a protocol using Dispase II at a lower concentration for a slow, cold digestion. Dispase is unique in that it targets the basal membrane on many tissues as it specifically targets fibronectin and type IV collagen (Priore *et al.,* 2008). We had success isolating progenitors for cell culture and have since published this method (Janas *et al.,* 2023). The concept of a slow cold digest is not new (Liu *et al.,* 2018, Li *et al.,* 2013) but Piotr's was the first protocol do this with lung tissue. We found this method produced a higher concentration cell suspension after dissociation and improved the consistency of our cell viability in our experiments. This not only allowed us to obtain more progenitor cells by FACS, but also allowed us to reduce the number of mice used in our experiments. Our animal experiments comply to the Animals in Scientific Procedures act. Established in this act are the 3Rs in research. One of these aims is to "Improve standards where animal

use is necessary, optimising model selection and study design and minimising suffering as far as possible" We believe our new protocol not only improves the amount of data generated by our studies, but also reduces the number of animals required to answer our questions.

3.5.2 Basal cell cytometry

Previously, we had followed the gating strategy of lung progenitors discussed by Jennifer Quantius which found a population of EpCAM^{high} CD49f^{high} CD24^{low} Sca-1+ cells which once sorted could differentiate into both upper and lower airway epithelial cells (Quantius *et al.*, 2016). We suspected this population contained basal progenitor cells but that all these cells could not be basal, as basal cells are a rare cell population in the lung. Quantius' strategy builds on work by Carla F Bender Kim which shows that Sca-1 is constitutively expressed across many cell types in the lung (Kim *et al.*, 2005). Kim's work was reviewed by Holmes and Stanford who describe Sca-1 as an "enigma" because it is expressed across many murine tissues and cell types. However, they also describe that in many tissues expression of Sca-1 is correlated with termination of cell cycle and the upregulation of differentiation (Holmes and Stanford, 2007), which led us to consider Sca-1 as a marker of activation, rather than of stemness. The abundance of EpCAM^{high} CD49f^{high} CD24^{low} cells suggested to us that this population included a mix of both bronchial and alveolar progenitors, basal and AT-II cells respectively. To further refine our flow cytometry panel, we included MHC-II to identify AT-II cells (Toulmin *et al.*, 2021), and Podoplanin (Pdpn) to identify AT-I cells (Quantius *et al.*, 2016). This brought us to the gating strategy shown in Figure 3-5, which also identifies blood and lymphatic endothelial cells. The strength of this approach is that it allows separation between upper and airway cell types, allowing separation of basal and alveolar type II cells. One limitation of this gating strategy is that the use of CD24 could mean that some cells identified as basal cells may be upper airway epithelial cells. Since basal cells are the progenitors of these cells, it is difficult to concretely separate them, though we think our gating on CD24 is a reasonable approach.

Another marker suggested for identification of basal cells from mouse tissue is Nerve Growth Factor Receptor (NGFR , *Tnfrsf16*). NGFR has been used to identify basal cells from differentiated epithelial cultures (Crotta *et al.,* 2023) and from murine tracheal digests (Rock *et al.,* 2009), though the latter also relied on a KRT5-GFP transgenic mouse to identify Keratin 5, a canonical marker of basal cells in microscopy (Hewitt *et al.,* 2023). We were not able to reproduce staining with NGFR in our experiments, using neurospheres as a positive control which are strongly NGFR positive (Sandberg *et al.,* 2014). We also attempted to replicate basal cell staining use Griffonia simplicifolia isolectin A3B (GSI-A3B) (Rock *et al.,* 2009) but found that this lectin bound strongly and non-specifically to all epithelial subsets. We suggest that these markers are useful when working with pre-enriched or cultured basal cells, but that for whole lung digest preparations our panel produces a more consistent identification of basal precursors. Our experiments combine methods from several other approaches to produce a method for identification of basal cells by flow cytometry using only 6 commercially available antibodies, many of which are routinely used in mouse immunology experiments.

3.5.3 Basal cell transcriptomics

Our initial experiments attempting to sort basal cells using flow cytometry yielded very low cell numbers. This combined with the fact our extended panel left little room for functional readouts, we decided to try to identify basal cells from published scRNA-seq datasets. We hypothesised that published data sets contained basal cells that had been sequenced but had not been analysed. We selected the dataset published by (Steuerman *et al.,* 2018) because they had carried out the sequencing on CD45- cells, giving greater resolution within the epithelial compartment and the data set produced by (Zhang *et al.,* 2020) because it was a timecourse of influenza virus infection, which was directly relevant to our questions. These datasets are of high quality and contain enough basal cells to make inferences of basal cell behaviour, but both have only sequenced a low number of basal cells, making concrete answers difficult. However, they are useful for identifying key markers and pathways that can then be targeted by methods such as flow cytometry and bulk RNA-seq. Some further

reading into different methods of single cell integration and some comparisons of our own datasets (Figure 3-9) lead us to the decision to choose Harmony for our integration method. Particularly the suggestion that Seurat v3's batch effect removal properties often masked significant biological differences in mouse datasets (Luecken *et al.*, 2021).

Overall, we suggest that the techniques we have used may have limitations in isolation, but when used together can work together to answer biological questions relating to basal cells. We have shown an improved method for isolating progenitor cells and shown how we can identify basal cells in published single cell RNA-seq experiments. These techniques can be used synergistically to confirm results and discover targets for future analysis. The next two chapters attempt to answer biological questions relating to basal cell behaviour during infection, and without these optimisations, these experiments would have been more difficult, and produced less reliable answers.

Chapter-4 Influenza virus infection changes the composition of the lung and alters the behaviour of basal progenitor cells.

4.1 Introduction

In the previous chapter, we discussed the variety of cell types that make up the lung, and how we could identify these in our experiments. We next wanted to understand how these cell types were affected by influenza virus induced damage. Turnover of epithelial cells in the lung is normal, and essential to maintain the structure of the lung. Turnover is low during homeostasis but increases dramatically during lung damage (H Spencer, 1962) (Schilders et al., 2016). Whether lung damage is caused by a virus (Clementi et al., 2021), a bacterium (or its toxin) (Kitur et al., 2015) or a lung therapy (Valliere and Barker 2004) (CH Collis, 1980), all sources of damage cause loss of structure and diversity of epithelial cells which must be recovered to survive the challenge. Lung damage results in haemorrhage, and loss of the gas exchange capability of the lung. This damage is not only of concern for the organism, but it also may change the access that cells of the lung have to nutrients, and gases such as oxygen. The damaged lung is a different environment to steady state, and it's not clear how the metabolic profile of the cells of the lung changes during this challenge.

Influenza is a disease of damage, (Kalil et al., 2019, Taubenberger and Morens, 2008) with damage caused by the virus and the inflammatory response that attempts to clear the virus. The inflammatory response is essential for surviving infection, though the immune response can also be damaging and is thought by some to interfere with optimal repair. Defining optimal repair is difficult, and there are different suggestions as to what that repair looks like. It's not exactly clear how cytokines and other damage induced factors affect repair, and understanding this could be useful in the development of new therapies for treating respiratory viruses. Some suggest that blocking of damage associated

molecular patterns (DAMPs) such as IL-1 could be effective in preventing overactivity of the innate immune response; limiting damage and therefore limiting opportunity for poor repair (Hernandez et al., 2014). While others suggest that blocking activity of effector Th17 cells reduces lung fibrosis after acute injury (Cipolla et al., 2017). In general, immune blockade experiments have been shown to be promising *in vivo*, though their effectiveness in human clinical trials has been found to be mild (Lucas et al., 2020). One target of recent interest in lung repair is IL-10, a cytokine which is traditionally considered to only be anti-inflammatory. IL-10 has been the focus of recent repair research due to work showing the importance of IL-10 in repair of the gut (Quiros et al., 2017) (Pull et al., 2004), and some promising studies showing the pro-repair effects of IL-10 in the lung (Shamskhou et al, 2019) (Cypel et al., 2009).

Lung repair may seem like a simple process - clear the source of the damage and close the wound – but if repair is done improperly it can lead to infection (Ruuskanen et al, 2011) or fibrosis (Robert M Strieter 2007). The composition of the lung is tightly controlled, and over or underrepresentation of cell types can exacerbate diseases of the airway such as asthma and COPD (Hicks-Berthet et al.,2021), (Fahy and Dickey., 2010). There are many similarities between how the lung repairs itself after damage and how the lung is constructed during development. The process of lung development in early life is well studied (Rawlins et al, 2007) (Cardoso and Lu 2006), but optimal repair after damage is still not fully understood.

During an influenza virus challenge, basal cells are most visible around the time of peak damage (11 days post infection) and ciliated epithelial cells stain positively for bromodeoxyuridine (BrdU), suggesting they are undergoing cell division (McKeon et al, 2011). The function of basal cells in steady state and in other sources of damage has been discussed in Chapter 2, but very little research has been carried out to understand how basal cells react to the damage induced by influenza virus infection. To interrogate this effect, we utilised a mouse model for IAV infection that would allow us to isolate and analyse basal progenitor cells. This system was flexible and allowed us to investigate basal cell

behaviour in response to different viruses (PR8, BrightFlu), and under cytokine blockades (IL-10R). In this chapter we tried to understand how the lung is changed by IAV infection, and what effect this infection had on the cells of the lung. We attempted to understand how progenitor cells contribute to the repair of the lung, in particular how progenitor's transcriptional changes were accompanied by a change in their metabolism. Since IL-10 had been shown to be pro-repair in other tissues, we tried to understand whether IL-10 had a similar pro-repair function in the lung and whether it could directly act upon basal cells during an acute injury.

4.2 Aims

- To understand how the lung epithelial compartment is changed by IAV infection.
- To determine how the behaviour of progenitor cells changes during IAV infection.
- To understand how IL-10 signalling contributes to lung repair.
- To determine how basal cell activation relies on a change in metabolic consumption.

4.3 Results

4.3.1 Influenza is a disease of damage.

To assess the effect of damage caused by an Influenza virus infection on the epithelial cells of the lung, C57BL/6 wild-type mice were infected with 100pfu influenza A/PR/8/34 (H1N1) (IAV) for 10 days (Figure 4-1A). This timepoint was chosen because others have shown that the peak of lung damage and the peak of lung epithelial cell proliferation are around 10 days post influenza virus infection (Kumar et al., 2011) (Major et al., 2020). To track the success and the severity of the infections, weight change of mice was tracked throughout infection. Infected mice lost weight during IAV infection and peak loss was at day 8 (Figure 4-1B). To confirm that infection was successful, qPCR was used to determine the amount of IFNγ present in the spleen of experimental mice (Figure 4-1C). Increased expression of IFNγ transcript in infected mice compared to the uninfected mice suggested that mice had been successfully infected.

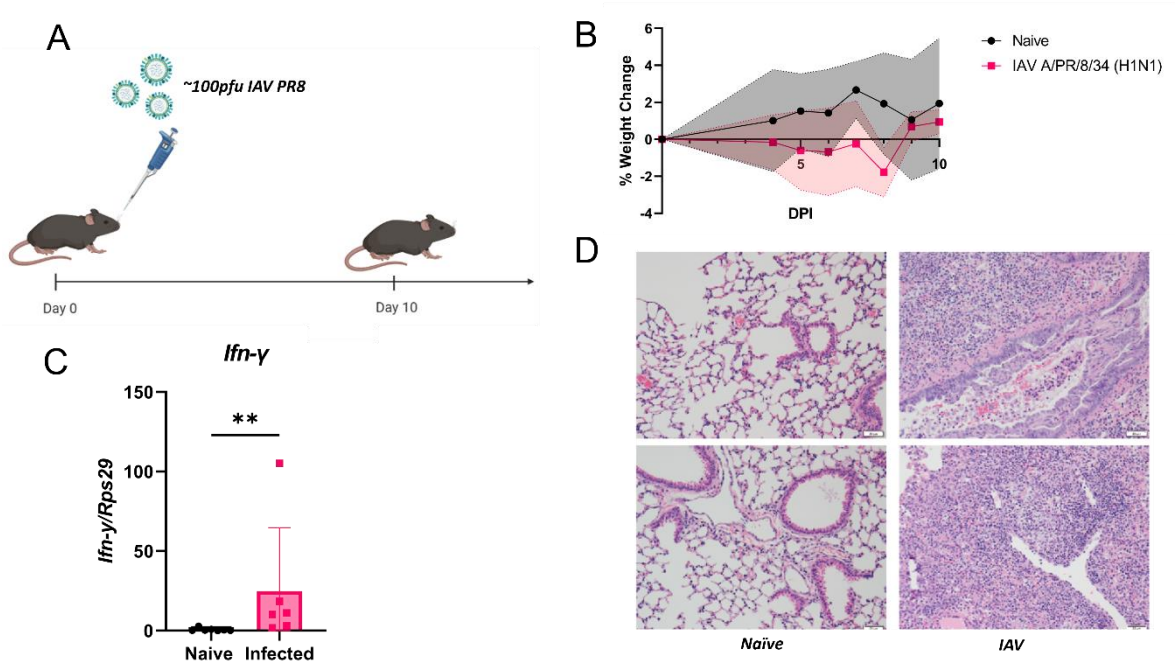


Figure 4-1 Influenza A virus as a model of lung damage in C57BL/6 mice.

(A) Age and sex matched C57BL/6 mice were infected with 100pfu of influenza A virus and were weighed daily. After 10 days, mice were culled and lung, spleen and BAL samples were collected. (B) To determine success of infection weight loss percentages were calculated and tracked across infection. Data shown in solid lines is average weight change of whole group, shaded areas show individual values with error bars showing variability of weight loss in infection model. Transcripts of IFN-y were measured by qRT-PCR in the spleens of experimental mice (C). Data shown n=6 are representative of 9 experiments. (D) H&E images showing damage caused by IAV infection in nacie and Vertex reporter mice. Images taken by Dr. Nicolette Fonseca. Statistical tests were carried out using (B) area under curve with a two-tailed unpaired t-test and Mann-Whitney test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ***$P < 0.0001$. Weight loss data shown in average per group, shading shows 1SD.

After confirming the experimental model was working as intended, lungs were dissected from experimental animals and used for flow cytometry to identify the epithelial cells and to determine if the populations of epithelial cells present after infection were different to those found in naïve mice. Lung cells were isolated using the protocol developed in Chapter 3, stained with CD45, CD31 and EpCAM antibody and the CD45- CD31- cells were identified as all epithelial cells (Figure 4-2A). The CD45+ cells were considered of immune origin (Koretzky et al., 1993) and the CD31+ cells are likely endothelial cells (Lertkiatmongkol et al., 2016) and so both were removed from analysis. The ratio and absolute number of epithelial cells was calculated across a time course of IAV infection, and a loss in both number and percentage of epithelial cells was observed during early infection, with this loss being recovered by 10 days post infection (Figure 4-2B-C). Epithelial cell ratios at 28 days post infection were provided by a colleague, Ryan Devlin (PhD student, Ed Roberts group, Beatson Institute), who showed that the levels of epithelial cells are returned to baseline long after infection. Loss of epithelial cells during influenza virus infection has been shown before (Major et al., 2020) and this data along with our results suggests there is an activation of progenitor cells during IAV induced damage which we went on to interrogate.

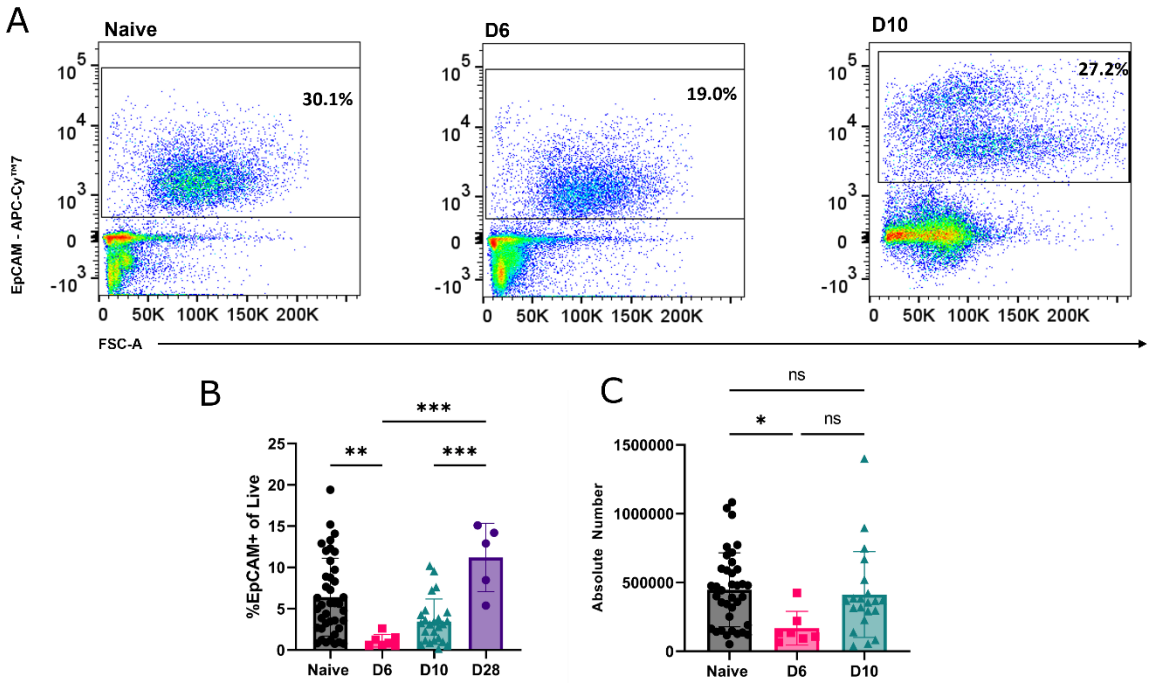


Figure 4-2 Influenza A virus changes the epithelial composition of the lung

Lung cell preparations were stained for flow cytometry and lymphocyte (CD45+) and endothelial (CD31+) cells were excluded. Epithelial cells were quantified by expression of EpCAM (A) across the time course. The proportion (B) and number (C) of epithelial cells was calculated using live cell counts. Images shown in A are representative of 9 experiments. D28 data were produced by Ryan Devlin, a PhD student colleague and show the return of epithelial cells to baseline long after infection is cleared. Statistical tests were carried out using one-way ANOVA with Kruskal-Wallis multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

4.3.2 Repairing the damage requires activation of progenitors

The epithelial cell compartment of the lung contains a variety of different cell types (Evans et al., 2017), and our previous experiments showed there is a loss of number and diversity of these epithelial cells after influenza virus infection. Since progenitor cells are responsible for restoring lost epithelial cells, we considered that they may be activated by influenza virus infection. We designed an experiment to understand the effects of influenza virus on those progenitor cells - specifically the AT-II (Figure 4-3) and basal cells (Figure 4-4).

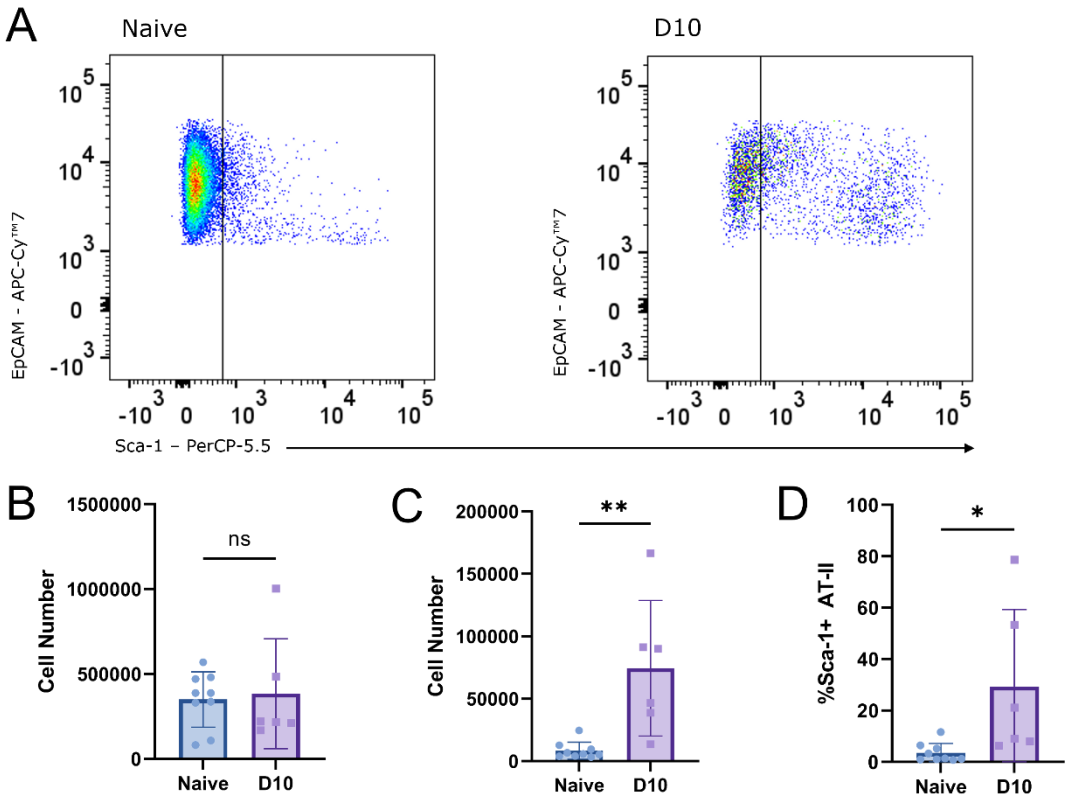


Figure 4-3 Alveolar type II cells are activated by influenza virus infection

Epithelial cells were gated by expression of EpCAM across the time course. The (A) number of ATII cells was quantified by the number of EpCAM+ PdPn- MHC-II+ events. The (B) absolute number pf AT-II cells, and the (C) absolute number of activated AT-II cells was determined by expression of Sca-1. (D) The proportion of AT-II cells that are positive for Sca-1. Data shown n=15 are representative of 9 experiments. Statistical tests were carried out using a two-tailed unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

Since damage during viral infections occurs in both the upper and lower airway, and this damage is recovered by different progenitor cells, we wanted to understand if progenitor cell restriction was seen throughout the lung. First, we analysed the AT-II cells, which are the progenitor cells of the alveolus. We found that at 10 days post IAV infection, there was no change in the number of AT-II cells (Figure 4-3B), but that the number (Figure 4-3C) and percentage (Figure 4-3D) of activated AT-II cells (measured by Sca-1) was increased. We discussed in Chapter 3 Sca-1 as a marker of activation, but in this context, where data suggests expression is induced in the earliest stages of differentiation, we think it is a robust marker.

We then identified basal progenitor cells in our lung samples to determine how they respond in the context of influenza virus infection (Figure 4-4). Basal cells were increased in number (Figure 4-4B) and increased their expression of activation marker Sca-1 both in number of Sca-1+ cells (Figure 4-4C) and percentage of Sca-1+ cells of all basal cells compared to the basal cells from uninfected mice (Figure 4-4D). These results showed us that both alveolar and tracheal progenitor cells are activated during IAV infection and that basal progenitors increase in number. Our hypothesis is that this is necessary to repair the damage caused by the infection.

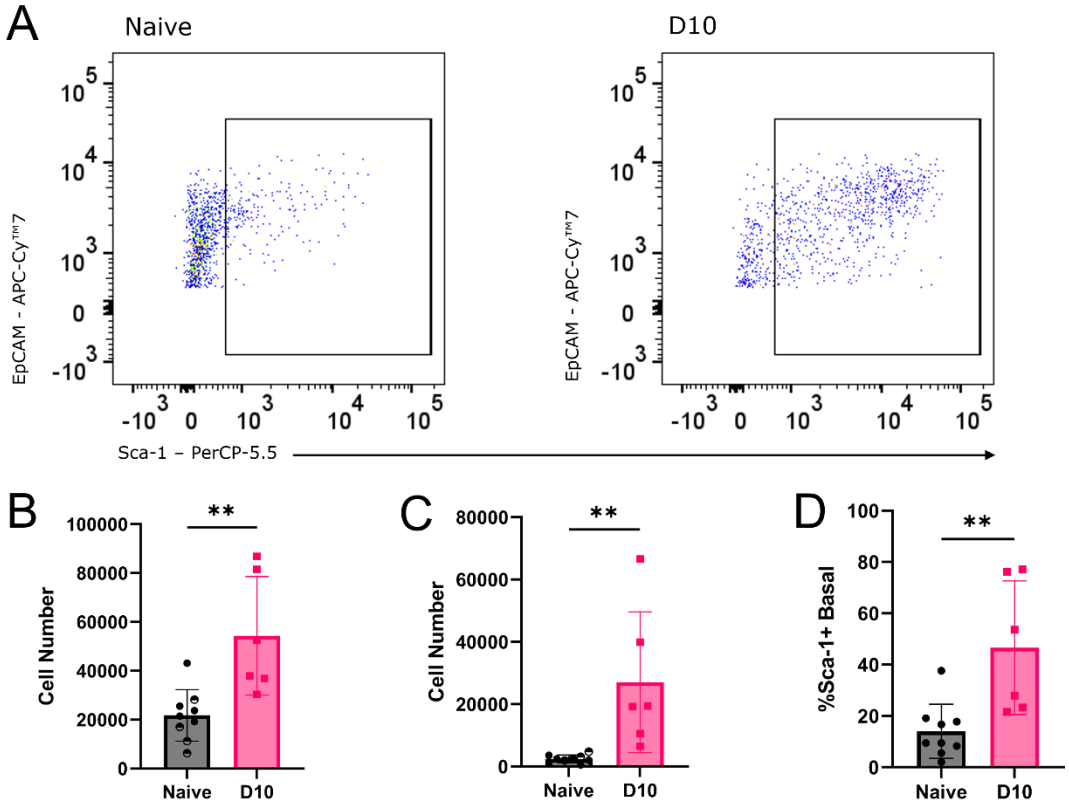


Figure 4-4 Basal cells are activated by influenza virus infection

Epithelial cells were gated by expression of EpCAM across the time course. The (A) number of basal cells was quantified by the number of EpCAM+ PdPn- MHC-II- CD24lo events. The (B) absolute number of basal cells and (C) absolute number of activated basal cells was determined by expression of Sca-1. (D) The proportion of basal cells that are positive for Sca-1. Data shown n=3-5 are representative of 9 experiments. Statistical tests were carried out using a two-tailed unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

The results in Figure 4-3 and Figure 4-4 showed that basal and alveolar progenitors activate during infection, but we wanted to further characterise their function. We had predicted that increase in basal cell numbers and activation was a function of lung repair, and so we next wanted to compare the gene expression of basal cells during infection to those from a naïve animal. To do this we FACS sorted basal and alveolar progenitors from naïve and IAV infected mice and carried out bulk RNA-seq to assess transcriptional changes during infection. Figure 4-4 showed that basal and alveolar progenitors activate during infection, and we first wanted to determine if this was replicated in our RNA-seq results. We did not detect sca-1 in our bulk RNA-seq results, and so to assess activation we targeted genes involved in cell cycle maintenance. We considered that a reduction in these genes would be indicative of an arrest of homeostatic turnover in favour of differentiation. We found these genes were decreased in basal cells from infected mice (Fig 4-5C), giving further backing to our conclusion that basal cells activate during infection. From this point, our analysis focused on basal cells. This is because most respiratory viral infections are active only in the upper airway of the lung, with only the most severe infections causing damage in the lower airway.

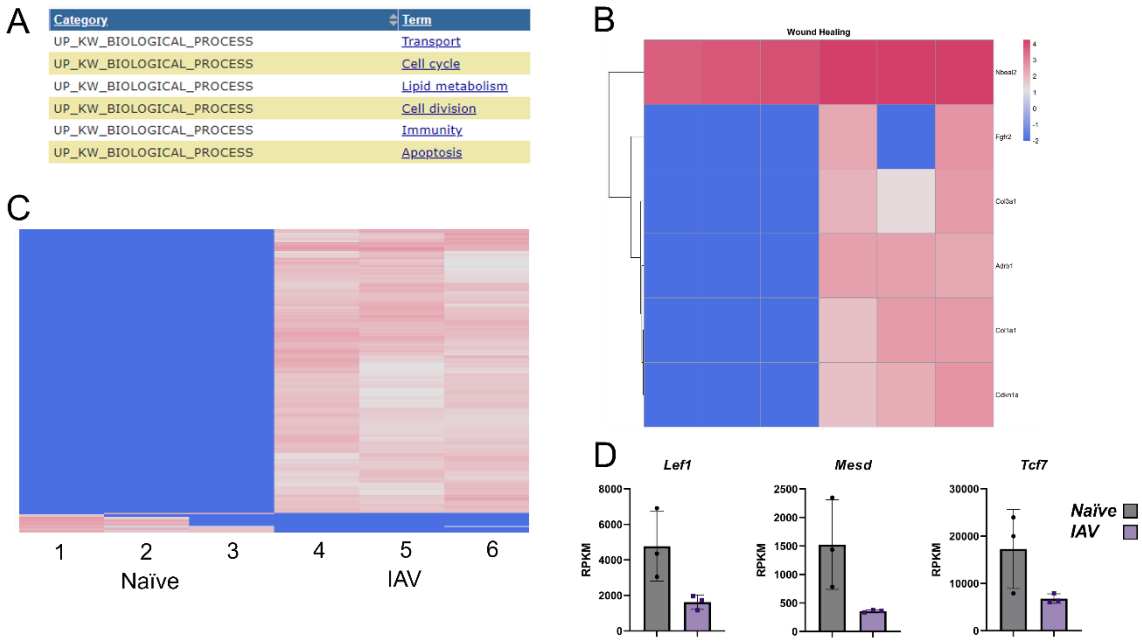


Figure 4-5 Basal cells are activated and show a pro-repair phenotype after influenza virus infection.

50-100 thousand basal cells were FACS sorted on the staining profile CD45- CD31- EpCAM+ PdPn- MHC-II- CD24lo. After sorting they were pelleted and lysed for RNA extraction. Basal cell RNA samples were sequenced and mapped to the mm19 Mus musculus genome, and differential gene expression was calculated. (A) All differentially expressed genes that were significantly different between naïve and infected groups. (B)Pathway analysis was used to identify differentially expressed pathways. The most relevant pathways were selected, and expression of known cell activation markers was carried out (C). Data represent n=6 samples. There are 3 replicates in each condition – these replicates are comprised on Each sample is comprised of cells pooled from 5 mice naive, and 3 mice (infected). Significant results where adjusted pval < 0.05.

We then wanted to characterise the functional response of basal cells during influenza virus infection, so we determined the significantly differentially expressed genes (adjusted p-value < 0.05) (Figure 4-5A) and carried out pathway analysis using the database for annotation, visualization, and integrated discovery (DAVID) tool. DAVID is a bioinformatics resource developed and maintained by Laboratory of Human Retrovirology and Immunoinformatics (NIH). We selected DAVID for our functional analysis because it is free, user-friendly and because it draws from a wide variety of annotation databases. Not only does DAVID source from the most common protein databases (Entrez Gene and Uniprot), it also draws from many secondary sources (KEGG, Human Protein Atlas, Reactome) and many historical functional annotation sources (Affymetrix, Biocarta, Agilent). This gives access to a breadth of pathways for functional annotation and gives us confidence that results are not biased due to only accessing one database. One of the most differential pathways from this analysis was the wound healing pathway (GO:0042060) which had many significantly different genes increased after infection including collagen genes (Col1a1, Col3a1) (Figure 4-5B). While production of collagen is essential for remodelling of damaged tissue, overproduction of collagen is also associated with fibrosis (Liu et al., 2021). These results alongside the results that basal cells are activated during infection and there are clear signals of differentiation in basal cells that have experienced infection. We suggest that basal cell activity after infection is an essential part of the repair process, and that while this activation may be pro-repair, the activation of progenitors must be tightly controlled to prevent fibrotic repair.

4.3.3 Basal cells fuel their activation with an increase in glycolytic metabolism

It was clear that basal cells played an active role in the resolution of infection, and we wanted to further understand how basal cells changed during IAV infection. We had access to a published scRNA-seq data set which included epithelial cells that had been exposed to influenza virus over a time-course (Zhang et al., 2020) and since the authors' analysis had not included basal cells, we decided to use their published data to determine how the basal cells were changed. We were also interested in this data set because these experiments were using single cell RNA-seq, which has a greater transcriptional resolution. Additionally, isolating basal cells from mouse lungs in sufficient numbers for our analysis required many mice – performing analysis on previously generated data allowed for a reduction in the number of mice used in this project, consistent with principles of the NC3Rs. The NC3Rs are a list of aims outlined by the UK Home Office to refine animal use for scientific procedures in the UK. Since we and others (Major et al., 2020, Kumar et al., 2011) had identified that basal cells were most active at 10-12 days post infection, we carried out our first analysis at day 12 (Figure 4-6A).

We hypothesised that a change in activation by basal cells might be driven by a change in their cell metabolism, and by carrying out differential gene expression and pathway analysis we focused on pathways associated with metabolism. We identified a significant change in the expression of genes involved in mitochondrial respiration (GO:0022904) in infected basal cells at 12 days post infection compared to naïve (Figure 4-6B). We were initially surprised to see that basal cells reduced their mitochondrial respiration as we assumed that activation would be an energy intensive process. To see if other aspects of cell metabolism were affected, we analysed genes associated with glycolytic cell metabolism (GO:0006096) at the same timepoint (Figure 4-7A).

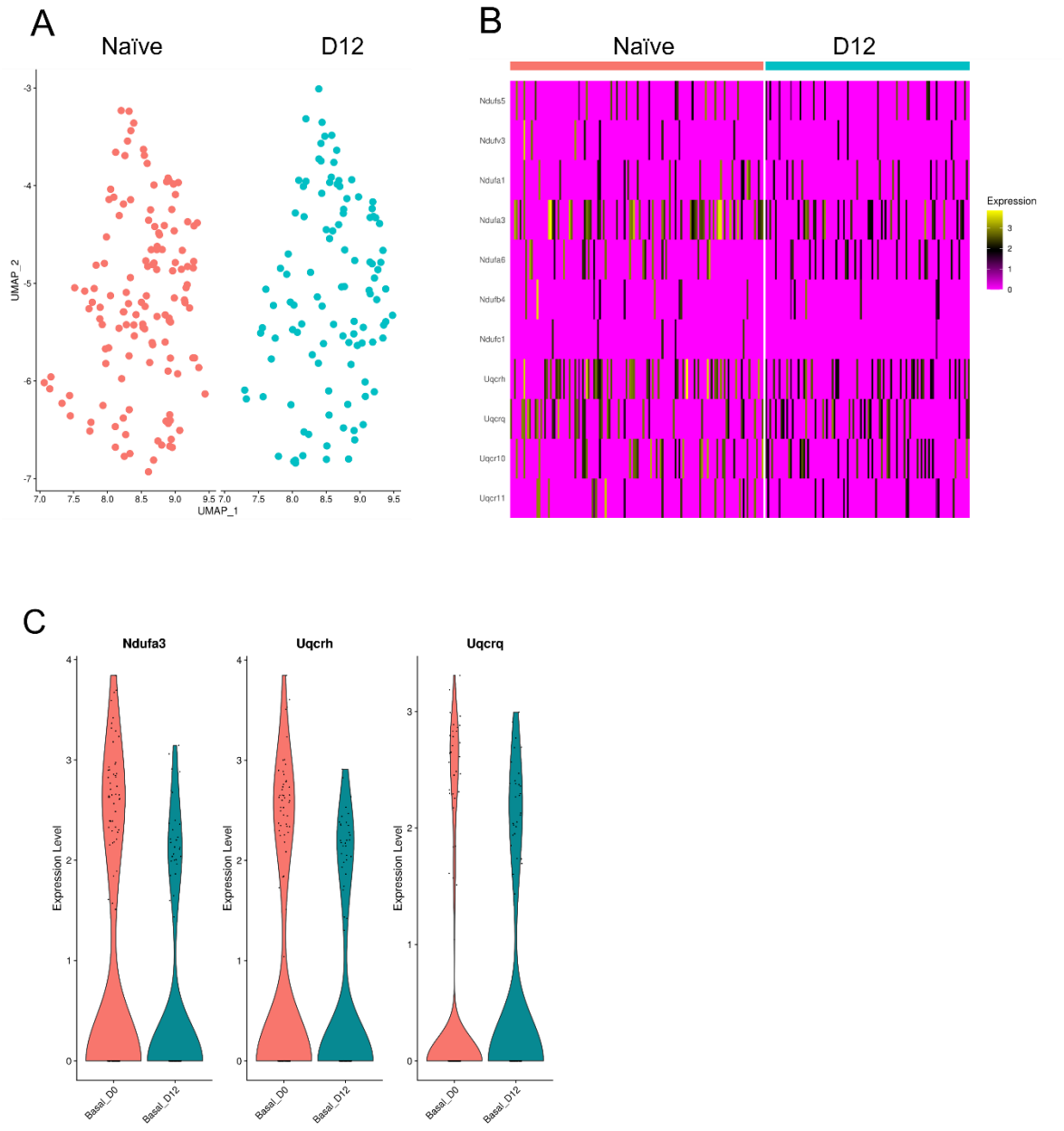


Figure 4-6 Basal cells downregulate mitochondrial respiration during the repair phase of IAV infection.

Lung cells were sequenced by 10X Genomics (Zhang et al., 2020) and we analysed the data that were produced as described in Chapter 2. Sequencing reads were aligned to the mm10 Mus musculus genome using CellRanger. Cells were normalised and clustered using Seurat as described in Chapter 2. Differential expression of cells from D0 and D12 days post IAV infection was calculated (A) and the most differential pathways were calculated using EnrichR. Respiratory chain, one of the most differentially expressed pathways was discovered (B) and several genes involved in mitochondrial respiration were found to be reduced in infected basal cells (C).

Here we found that glycolytic genes were strongly upregulated in the basal cells of D12 post infection mice compared to uninfected. Indeed, this activation did appear to be energy intensive, and we concluded that basal cells were relying on glycolysis, which is a less efficient way to consume glucose. Again we were surprised by this result – we have shown that basal cells activate early in infection and maintain this activation long after the challenge has passed. One reason basal cells might do this is because glycolysis is 100x faster than oxidative phosphorylation (Melkonian and Schury, 2023) and if basal cells are under stress, they may need to access the fastest source of energy that is available. This result made us consider that basal cells may be under stressed conditions – either starved of oxygen or energy. As this is consistent with similar results in cancer (Tameemi et al., 2019) and immunology (Phan et al., 2015) research we next targeted pathways involved in cell stress or apoptosis.

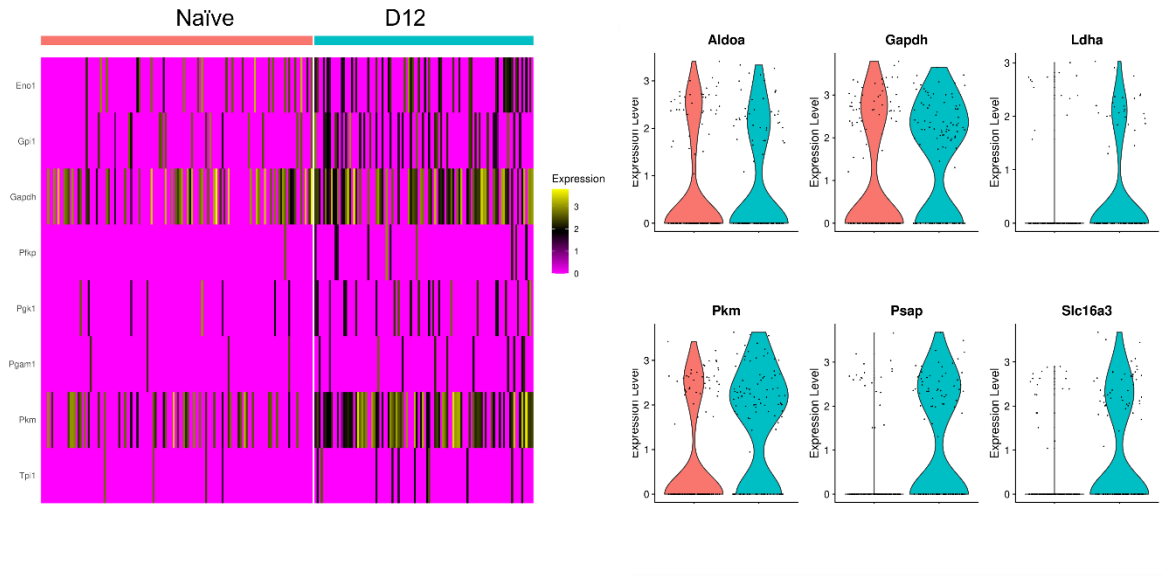


Figure 4-7 Basal cells are more glycolytic during the repair phase of IAV infection.

Lung cells were sequenced by 10X Genomics as described here (Zhang et al., 2020). Sequencing reads were aligned to the mm10 Mus musculus genome using CellRanger. Cells were normalised and clustered using Seurat as described in Chapter 2. Differential expression of basal cells from D0 and D12 days was calculated, and pathway analysis was carried out on the significantly different genes using EnrichR. There was a significant decrease in production of genes involved in the glycolytic metabolism pathway (A) and several genes involved in glycolysis were found to be increased in infected basal cells (C).

While searching for signs of cell stress in basal cells from infected animals we found the upregulation of genes involved in ferroptosis (GO:0097707); many of these genes were strongly expressed in naïve mice but were also upregulated during the repair timepoint of influenza in basal cells from infected mice (Figure 4-8). This increase in ferroptosis could be a reaction to reactive oxygen species (ROS) production due to increased glucose metabolism (Liemburg-Apers et al, 2015), or it could be an attempt for basal cells to die by an inflammatory form of cell death rather than an influenza virus favoured apoptotic cell death (Tang et al., 2021). We suggest that basal cells are acutely stressed by the need for rapid activation and proliferation, and this may reduce their ability to carry out efficient repair of the lung. This is consistent with other results that will be discussed in Chapter 5.

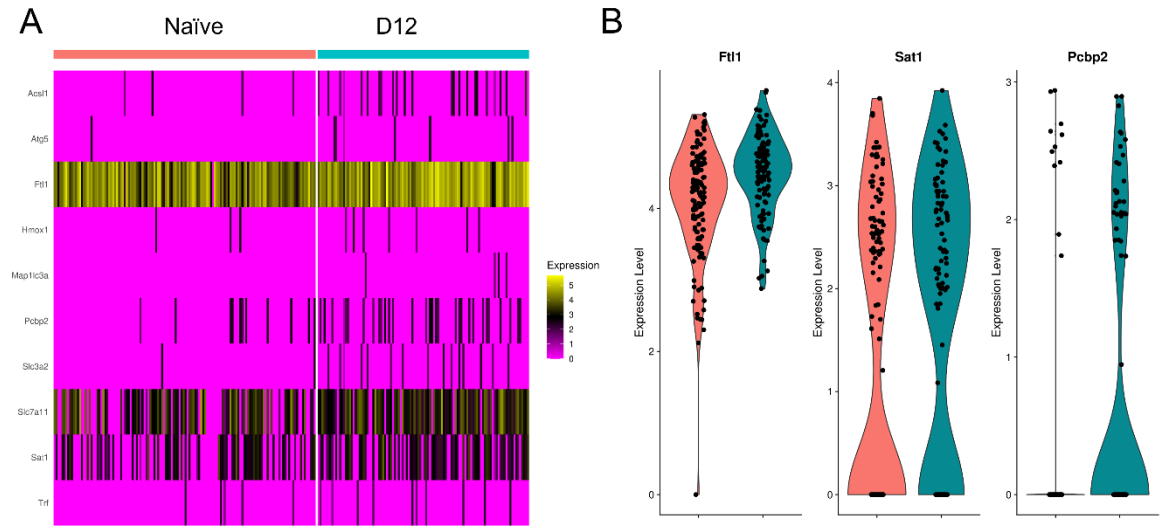


Figure 4-8 Basal cells increase genes involved with ferroptosis in the repair phase of IAV infection.

Lung cells were sequenced by 10X Genomics as described here (Zhang et al., 2020). Sequencing reads were aligned to the mm10 Mus musculus genome using CellRanger. Cells were normalised and clustered using Seurat as described in Chapter 2. Differential expression of cells from D0 and D12 days post IAV infection was calculated (A) and the most differential pathways were calculated using EnrichR. Ferroptosis, one of the most differentially expressed pathways was discovered (B) and several genes involved in ferroptosis were found to be increased in infected basal cells (C).

4.3.4 Basal cells engage in immunosuppression during peak viral damage.

In Chapter 5 we will discuss whether basal cells can be infected by IAV, and the implications of that question; but in this part of the project, I focused on how basal cells engage with the immune system regardless of whether they are directly infected. These experiments consider that during influenza, basal cells are in an environment soaked in cytokines and other damage factors. In our analysis of published single cell RNA-seq experiments, we found that at D12 post infection, the pathway regulation of immune response (GO:0050776) was differentially expressed, with many anti-inflammatory genes specifically upregulated in basal cells from infected mice such as Socs3 and Stat2 compared to naive (Figure 4-9 A-C). In our bulk RNA-seq results we also found a strong increase in the number of IL-10 transcripts by basal cells, and we wanted to explore this further in the published scRNA-seq dataset. We found no difference in IL-10 expression in the infected basal cells compared to naïve, but we observed increased expression of the IL-10rα, and genes associated with the downstream signalling of IL-10, during the day 12 post infection timepoint (Figure 4-9 E). In combination, these data suggest that basal cells may be involved of the calming of the immune response after damage, and that their ability to respond to and produce IL-10 may mean they have an anti-inflammatory role in the post-infection lung.

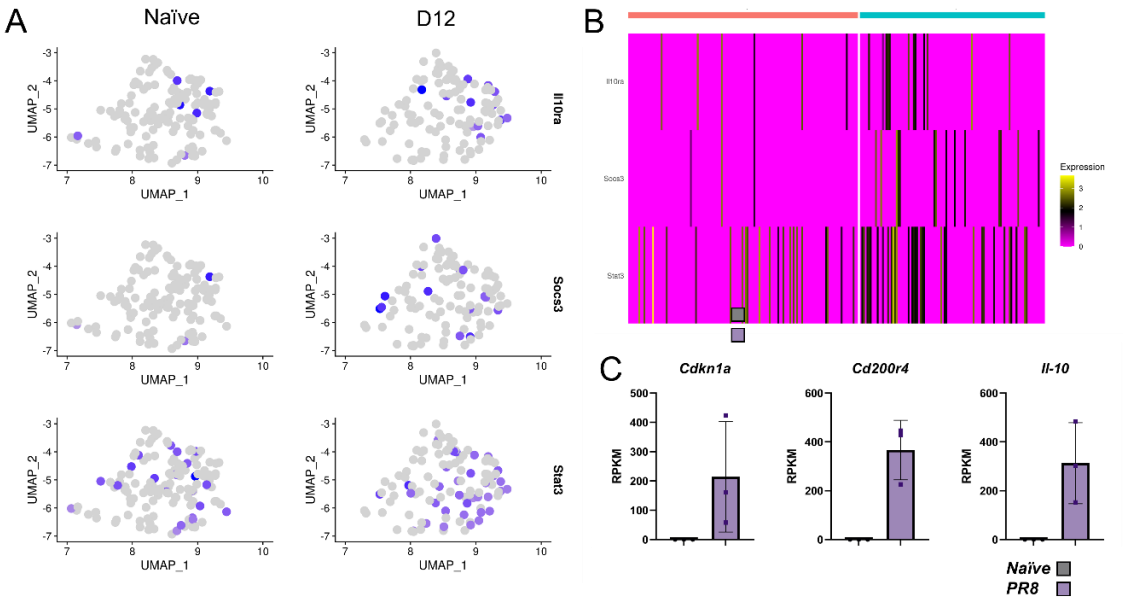


Figure 4-9 Basal cells activity in recovery from IAV infection has an immunosuppressive character.

Differential expression of basal cells from D0 and D12 days post IAV infection was calculated, and the most differential pathways were calculated using EnrichR. Immune regulation, one of the most differentially expressed pathways was identified and several genes involved in immune suppression were found to be increased in infected basal cells. The IL-10 receptor alpha chain was found to be increased in infected basal cells, as well as genes downstream of the IL-10 receptor (A-B). Immunosuppressive genes were also found to be increased in basal cells from an infected mouse from bulk RNA-seq analysis (C).

4.3.5 Il-10 signalling is needed for efficient repair after influenza virus infection

In Chapter 1 we discuss the role of IL-10 in influenza virus infection, and its controversial role in tissue repair. Interestingly, we show that basal cells can produce IL-10 and our single cell data suggests that basal cells respond to IL-10. We wanted to further explore the impact of IL-10 on basal cells during IAV infection. We designed an experiment to block the signalling of IL-10 during the repair stage of infection (Figure 4-10 A). IL-10rα was blocked from 6-10 days post infection, and the effectiveness of this blockade was measured by qPCR for IL-10 from the spleen of infected animals (Figure 4-10 B). IL-10 transcript was

detected in the spleen of infected mice, but not in those that had the IL-10 receptor blockade suggesting the blockade was successful.

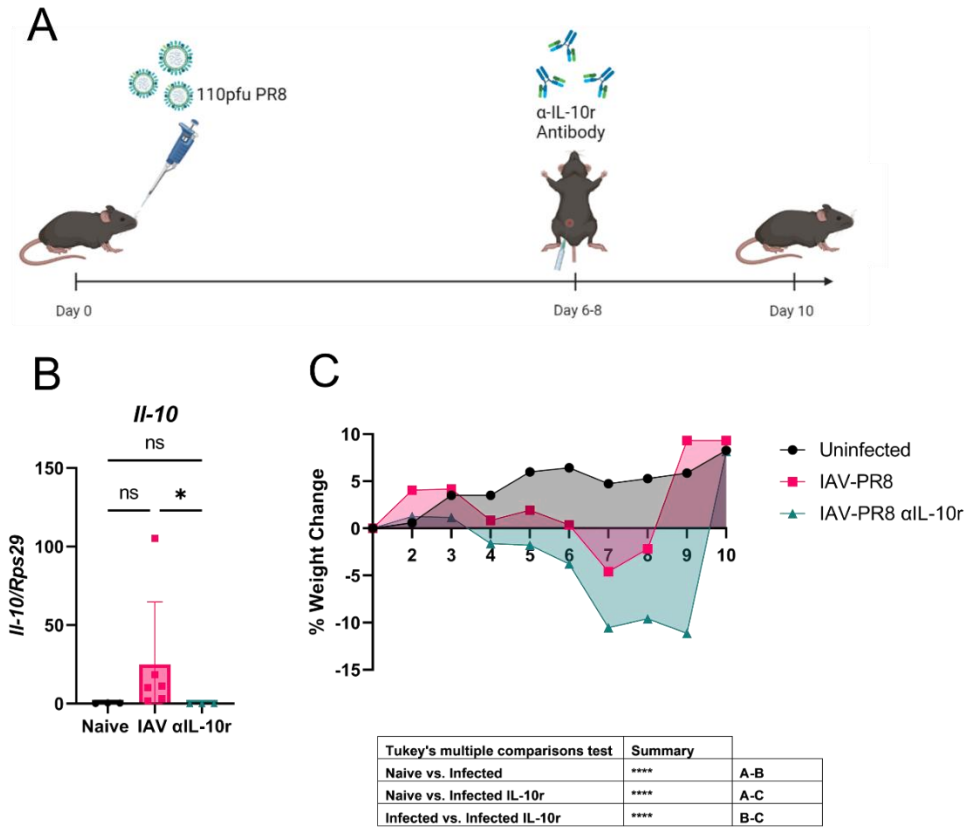


Figure 4-10 IL-10 signalling is required for effective repair during IAV infection.

To determine the importance of IL-10 during IAV infection, IL-10ra signalling was blocked during the repair phase of IAV infection (A). To ensure the blockade was effective, IL-10 transcripts were measured in the spleen of experimental mice (B). Weight change of experimental mice was measured throughout infection to determine whether the loss of IL-10 signalling affected the recovery from infection (C). Statistical tests were carried out using (C) area under curve with Tukey's multiple comparisons test(C) and (B) one-way ANOVA with Kruskal-Wallis multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

The weight loss of all experimental mice was measured throughout, and mice that experienced IAV infection and isotype antibody injection suffered expected weight loss (Figure 4-10 C). Mice who were infected with IAV and experienced IL-10 receptor blockade lost significantly more weight and took longer to recover their lost weight. We concluded that this inability to recover the lost weight was a sign that there was a defect in the repair process in the receptor deficient

mice. We hypothesised that the reduced ability of these mice to recover from infection involved their lung progenitor cell activity, and so again we looked at the activation of basal cells during IAV infection.

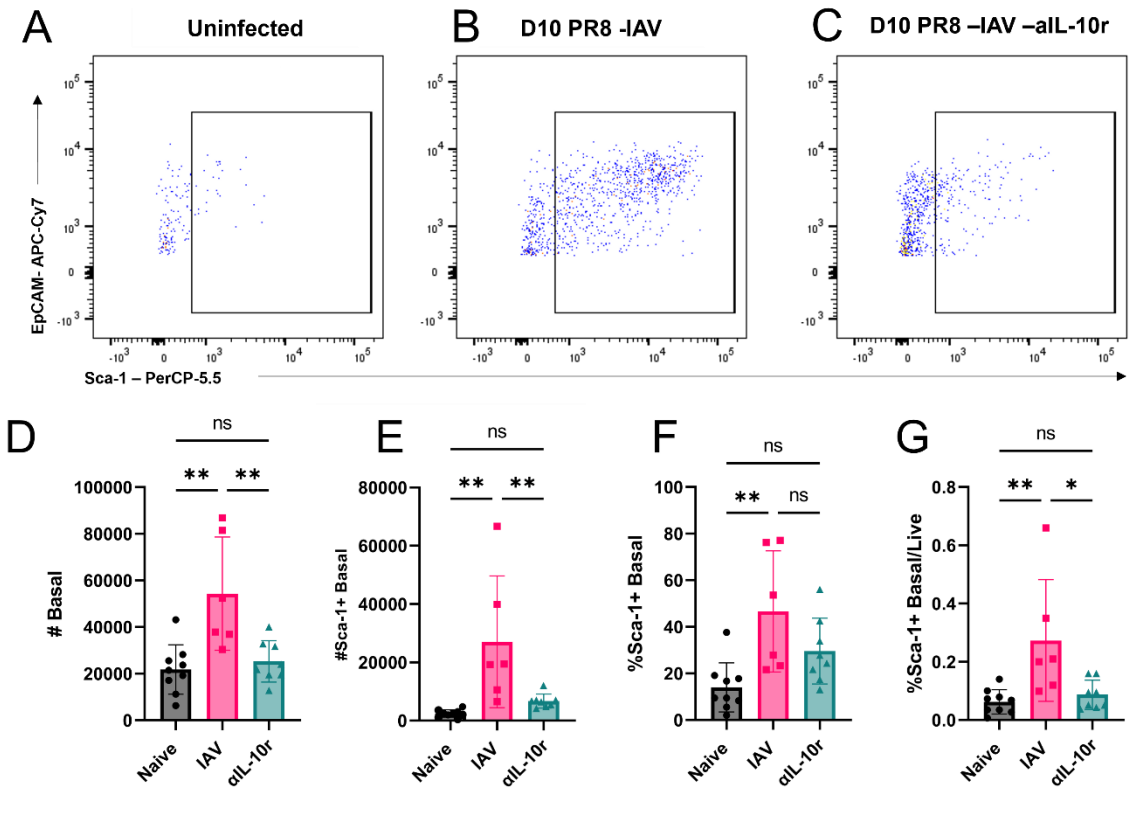


Figure 4-11 Blocking IL-10 receptor during IAV infection reduces basal cell activation

To determine the effect of blocking IL-10 during IAV infection on basal cells, basal cell activation was measured using Sca-1. Basal cells from (A) uninfected, (B) IAV infected with isotype control antibody injection and (C) IL-10 receptor blockade mice were analysed. The number of basal cells (D), the number of Sca-1+ basal cells and the proportion of basal cells as a percentage of (F) all basal cells and of (G) live cells was calculated. Data n=3-5 are representative of 4 experiments. Statistical tests were carried out using a one-way ANOVA with Kruskal-Wallis multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

We observed an increased activation in basal cells of mice from the mice infected with IAV with an isotype antibody injection (Figure 4-11A-B); but this activation was not mirrored in the IAV IL-10 receptor blockade mice, which had basal cell activation at the same level as naive (Fig 4-11C). We have shown previously that to recover from influenza virus infection, progenitor cells

activate and proliferate to repair the damage to the lung. A reduction in the activity of basal cells, and a delayed recovery from IAV induced weight loss in IL-10 signal deficient mice brought us to conclude that IL-10 signalling is key to basal cell activation, and that it may play a role in basal cell repair function.

4.4 Discussion

The aim of this chapter of work was to determine whether the composition of the lung was changed during and after IAV infection and we found this to be the case. We hypothesised that to restore the lung to its homeostatic state there would be activation and proliferation of progenitor cells and we have also shown this to be the case. We understood that repair was the role of progenitors, but we didn't understand what transcriptional changes progenitor cells underwent during repair. We designed experiments to understand the transcriptional changes between basal cells from naïve and infected mice and interrogated those differences. We found many differences between naïve and infected basal cells and showed that basal cell activation was accompanied by a switch in cell metabolism, and an increase in cell stress. We also found that basal cell activation relied on IL-10 signalling, and that when the IL-10 receptor was blocked, basal cell activation was reduced. Many strains of influenza have been shown to destroy epithelia in vitro (Essaidi-Laziosi et al., 2018) and in vivo (Kumar et al., 2011). Our results in Figure 4-2 showing a loss of epithelial cells post influenza complement these results. This reduction in percentage could reflect an influx of other cells, such as immune cells, but we also see that total number is reduced which suggests an actual depletion of cells.

Depletion of cells after IAV infection was expected, and we considered that to restore those lost cells, progenitor cells would activate and proliferate. Others have shown that alveolar progenitors are more active during IAV infection due to IAV or immune system related damage (Major et al., 2020) which we also found (Figure 4-3). In cell culture systems basal cells have been shown to proliferate after exposure to influenza virus (Kumar et al., 2011) (Quantius et al., 2016) and we showed that basal progenitors activate and proliferate at the same time in vivo (Figure 4-4A, Figure 4-5C). What remains unknown is the mechanism of this

activation, or how the delicate balance between over and under-proliferation is managed. One suggestion is that this activation is a direct response to the virus or the innate response to the virus, but it has been shown that alveolar epithelial progenitor proliferation is inhibited by primary interferons (Major et al., 2020). Another suggestion is that a signal to begin proliferation may come from immune cells in the form of Fgf10 or Il-10 from fibroblasts or macrophages (Quantius et al., 2016). Our results show that basal cells upregulate the receptor for Fgf10 (Figure 4-5B) and IL-10 (Figure 4-9 A,C) suggesting that basal cells look to the immune system for their cue to activate. These experiments highlight the balancing act at play, suggesting that high levels of interferons during early infection may halt proliferation to protect the stem cells and clear the virus; then, as these early anti-viral signals subside, basal cells receive dominant signals to activate and repair the damage. This balancing act is clearly important to effective repair since when this system goes wrong it can lead to an inability to clear the virus (Mauad et al., 2009) or a fibrotic repair response (Stancil et al., 2021). Once it had become clear to us that basal cells were strongly activated during IAV-induced damage, we then focused on characterising that activation transcriptionally. We considered that if we could understand what effective repair looked like, future therapeutics could be designed with consideration of basal cell activation in mind. Thanks to complementary techniques of single cell RNA-seq and bulk RNA-seq we were able to gain new insight into basal cell function during infection (Figure 4-5-4-9). A recurring theme in our results was that basal cells changed their metabolic profile during infection. It was recently shown in vitro that lung epithelial progenitors not only could change their energy source during activation, but that this was an essential step in moving from steady state stemness to a differentiating phenotype (Crotta et al., 2023). We found that at D12 post infection, basal cells reduce expression of genes associated with mitochondrial metabolism and increase their expression of glycolysis mediating genes (Figure 4-6, Figure 4-7). This acts in slight contrast to the work of Crotta et al. where they show the most striking differences between basal and differentiated cells are in the increase in fatty acid oxidation (FAO). However, this study shows that the switch to FAO is linked to mucus production and may be a function of terminally differentiated epithelial cells. We suggest that these differentiated airway cultures may

exaggerate the differences between basal cells and ciliated cells, and that this might account for some differences in our results. Other in vitro cultures of airway progenitor cells (Li et al., 2019) have shown that reduction in glucose availability leads to reduced proliferation and cell death and so we suggest that an increase in uptake and consumption of glucose is essential for basal cell activation in repair. The wound environment is often ischemic (Eming et al., 2021) it may not always be possible for basal cells to access the amount of glucose they require to perform their repair function adequately. It isn't clear exactly how oxygenated an area of lung damaged by flu is, but disrupted blood supply could lead to oxygen or glucose not being delivered to the site of infection. While the 16th century medical adage "Fasting is a great remedie of feuer" (John Withals, 1574) has been shown to be largely myth, some reputable studies show that survival from viral infection can be improved by access to a high glucose diet (Wang et al., 2019). "Feed a cold, starve a fever" aside, it could be considered that maintaining a high blood-glucose level during infection could allow progenitor cells to access the quick-burning fuel they require for their activation. Interestingly, studies on the different macrophage subsets in the lung show that alveolar macrophages have reduced glycolytic capability, and this reduces their ability to respond to a type 2 immune challenge (Svedberg et al., 2019).

As well as showing the need for a change in metabolism, Stefania Crotta's work also showed that activated bronchial progenitors increase their lipid consumption. We did not find a significant difference in lipid metabolism in our data sets but did see an increase in iron scavenging and ferroptosis pathways (Figure 4-8) – particularly Ftl the secreted form of the ferritin protein (Kernan and Carcillo, 2017). Ferritin is produced as part of the innate acute phase response and is known to be essential when fighting bacterial infections – the limiting factor of proliferation of many invasive bacteria is access to host iron (Parrow et al., 2013). The importance of iron availability in viral infections is less obvious, there is some evidence that influenza can increase ferritin production in humans (Soepandi et al., 2010) and interestingly, high levels of ferritin resulted in less effective influenza virus vaccine responses as haemodialyzed patients produced fewer anti-HA neutralising antibodies (Eiselt et

al., 2010). It stands to reason that basal cells might induce iron-scavenging genes during infection as part of the acute response to viral infection, but why then are these genes still upregulated 12 days post infection? One answer could be that as previously discussed, the peak damage stage of influenza (D11) occurs after the immune response has cleared the virus (Kumar et al, 2011) and that iron scavenging becomes more important at this later timepoint. This is especially pertinent given that 65% of hospitalisations with influenza result in a bacterial secondary infection (Morris et al., 2017). If the repair environment is flooded with iron-rich blood, it may be part of the role of basal cells to scavenge that iron and prevent it from being accessed by opportunistic pathogens. One way to improve basal cells in their repair function during infection would be to assist their iron scavenging ability – if they are dying by ferroptosis during infection they are likely being overwhelmed by iron (Li et al., 2020) and some level of chelation during recovery from influenza could be effective.

So far, we have discussed the relationship between basal cells and iron from an anti-pathogen standpoint. But what if this attempt to reduce iron availability is part of a repair mechanism? Data connecting iron scavenging to the adaptive immune system is limited and dated (Fargion et al., 1991), but some have suggested that part of the Th2 response could be involved in limiting the availability of iron (Tilg et al., 2002) and that high levels of IL-10 can even induce anaemia (Choucair et al., 2019). This was of particular interest because we had found that post infection, basal cells were found to respond to IL-10 (Fig 4-9 A) and produce IL-10 transcript (Figure 4-9 C). Given the link between IL-10 and Fgf10 and our previous results (Figure 4-5B), we were particularly excited by the concept that part of basal cell activation was iron scavenging and that this could be partially IL-10 mediated. As previously mentioned, Il-10 is not considered to be a pro-repair cytokine but there is growing evidence that “anti-inflammatory” and “pro-repair” may be two sides of the same immunological coin (Quiros et al., 2017)(King et al., 2014). After results which suggest that IL-10 production may be a part of basal cell activation (Figure 4-9) we wanted to understand how IL-10 was involved in repair after influenza. To do this we blocked signalling of IL-10 which allows cells to continue producing IL-10, but they should not be able to receive an IL-10 signal (Figure 4-10A). Strikingly we

found mice that had experienced an influenza virus infection under IL-10Ra blockade to suffer worse weight loss during infection, and that these mice took longer to recover from that increased weight loss (Figure 4-10C). In PR8 infected C57/Bl6 mice, increased weight loss is correlated with sickness score (Toapant and Ross, 2009) and so we saw this delayed recovery as a sign that the repair process was slowed in our IL-10 receptor blocked mice. One interpretation of these results is that a viral infection such as influenza induces a strong IFN-γ response and an aggressive Th1 cytotoxic T cell response (Graham et al., 1993)(Price et al., 2000) for which we have removed the counterbalancing IL-10. This would mean that there would be an increase in immunopathology and more damage to repair, hence the slower recovery. However, as in some contexts IL-10 has been shown to be promote growth and cytotoxicity of CD8+ and NK cells (Hurme et al., 1994) (Jinquan et al., 1993) (Moore et al., 2001), hence the suggestion that IL-10 can have a pro-repair function in the lung. As mentioned previously, IL-10 is involved in a delicate balance during repair and the effects of disturbing this balance go beyond just increasing or decreasing the activity of one cell type.

We found that the activation of basal cells was once again induced by IAV infection, but that this activation was reduced to baseline by blocking IL-10 receptor signalling. The IL-10 that basal cells are responding could come from many sources. IL-10 in the lung is sourced from epithelial cells (Bonfield et al., 1995, Lim et al., 2004) and CD8 T cells (Sun et al., 2009), but also alveolar macrophages and Foxp3+ Treg cells (Kawano et al., 2016) (Hawrylowicz and O'Garra, 2005). By blocking IL-10 signalling, basal cells may not get the cue from the adaptive immune system to begin repairing the lung. Our hypothesis is that basal cells are activated early in infection, though primary interferons may dampen their activation during early viral pathogenesis. Then once the virus is being cleared, signals from effector T cells or Il-10 producing B cells (Sun et al., 2023) encourage basal cells to activate and begin their repair function. We suggest that part of this repair function is constitutive release of IL-10 which amplifies the pro-repair signal to other epithelial cells, and to other damaging immune cells such as CD8 T cells. Production of IL-10 by lung basal cells has not been reported before, but there are examples of epithelial cells in other tissues

producing IL-10 to modulate the immune response (Hyun et al., 2014) (Nguyen et al., 2021).

So far, we have only discussed basal cell activation considering that all activation of basal cells is pro repair, and beneficial to the host. We have already described that basal cell activation is necessary to recover damaged epithelium, but basal cell activation is not necessary always beneficial. Following damage, basal cells have been shown to become involved in repair in the alveolar space (Kumar et al., 2011, Ray et al., 2016). One suggestion for this function is that it is essential for repair, allowing emergency recovery during severe damage (Vaughan et al., 2014, Zuo et al., 2014). However, there is also evidence that during this function, basal cells can also differentiate in inappropriate locations, causing dysplastic, fibrotic repair (Mello Costa et al., 2020). Our data fit with the idea that basal cells are upregulated during infection and suggests that IL-10 is important in the activation of basal cells. We suggest that proper repair depends on the timing of basal cell activation, and that the over-activation seen in others work (Mello Costa et al., 2020) could be caused by misbalancing of pro-repair cytokines such as IL-13 and IL-10. We suggest that the reduction in activation seen by blocking IL-10 may lead to less dysplastic repair, but also may lead to a slower recovery from influenza. This is based on our data showing that IL-10 signal blocked mice had a higher mortality, and slower recovery than IL-10 competent mice. The key questions in this chapter concerned how the composition of the lung changed during influenza, and to what extent that change in composition was driven by the activation or progenitor cells. We have shown some of the changes that occur during lung damage and shown some of the mechanism around basal cell activation. We also show how basal cells change transcriptionally during lung damage, and how we can interfere with the activation of basal cells by restricting cytokines such as IL-10. In this chapter we have not considered how basal cell activation and behaviour is changed directly by IAV, but since IAV is an intracellular pathogen, we considered that the virus may have direct effects on the behaviour of basal cells. In the next chapter, we attempt to identify directly infected basal cells, and show how IAV can directly change the behaviour of basal cells.

Chapter-5 Direct infection by influenza A virus changes the behaviour of mouse basal cells.

5.1 Introduction

The primary function of the lung is to facilitate gas exchange. Through pathogenesis, trauma or allergy, the lung parenchyma becomes damaged, and our lungs react to this damage. Influenza viruses are among the most destructive of these insults: many pandemic influenza strains can cause rapid progression to acute respiratory distress syndrome (ARDS). H7N9 and H5N1 avian influenza viruses have been shown to be some of the most damaging (Gao et al., 2013), but H1N1 strains have also been shown to contribute to ARDS and pulmonary fibrosis (Nakajima et al., 2012). In Chapter 4 we attempted to understand how this kind of damage changes the behaviour or basal cells, and we showed that basal cells are activated during IAV induced damage. We showed that that basal cells require anti-inflammatory cytokine signal for their activation, and that a hallmark of their activation is a change in their metabolism.

We are fortunate to have influenza virus vaccines that are extremely effective in generating neutralising antibody, but the high mutation rate of influenza viruses mean that our seasonal vaccines are not robust (Trombetta et al., 2022). This means that cells are infected, and so either by pressure from the virus, or cytopathic effect of the immune system, damage is caused. It is worth preventing viruses from accessing our cells not only to prevent viral replication, but to prevent those viruses from manipulating our cells and tissues. RSV owes its name to its ability to cause fusion of cells into syncytia (Morris et al., 1956), a behaviour that has been shown in other respiratory viruses such as SARS-CoV-2 (Rajah et al., 2021). What is unique about influenza virus induced damage is that fibrosis caused after this damage seems to be reversible in a way that fibrosis caused by viruses like Cytomegalovirus (HCMV) is not. This may be due to how CMV induces TGF-B signalling (Wen et al., 2011) or the latent function of herpesviruses (Mineo et al., 2011, Huang and Tang, 2021). Huang and Tang discuss that some patients with IAV induced fibrosis have high levels of TGF-β

and that the virus may be stimulating the TGF-ß/Smad pathway to induce pulmonary fibrosis (Huang and Tang, 2021). Influenza viruses are not commonly thought to be inducers of fibrosis, but all sources of damage carry that risk. This project hasn't attempted to assess fibrosis, but the fact that different viruses induce different kinds of damage suggests that the fibrotic process is not just governed by the immune response to challenge, but that the virus itself can affect this process. RSV has been shown to infect mouse basal cells, and when it does it pushes basal cell differentiation away from ciliated cells, and towards secretory cell types (Persson et al., 2014) and so we hypothesised that IAV may be able to directly infect basal cells and change their behaviour. Recent work has shown how histone modifications affect the way basal cells carry out their repair function (Weiner et al., 2022) and some work showing that influenza virus is able to epigenetically change epithelial cells by altering histone methylation (Marcos-Villar et al., 2018). We suggested that if IAV was capable of infecting basal cells, that direct infection might impair their ability to activate, or induce an antiviral response.

5.2 Aims

- To determine if mouse basal cells can be infected by IAV.
- To determine the effect of direct infection by IAV on basal cells
- To precisely define the indirect effect on repair of IAV infection by studying the behaviour of "bystander" basal cells

5.3 Results

5.3.1 Basal cells are infected by IAV

In Chapter 4, we showed that basal cells activate during IAV infection, and that their behaviour is changed during the response to infection. We hypothesized that many of these changes were caused by the immune response to the virus, but we also considered that the virus may be directly changing the behaviour of basal cells. To determine whether basal cells were infected, we targeted the viral nucleoprotein (NP) with a fluorescent intracellular antibody. We selected NP as it is the second most abundant IAV protein produced during replication (Kummer et al., 2014) (Ka-Leung et al., 2009) and there was a conjugated antibody which had been validated for flow cytometry (Martínez-Colón et al., 2019). Previous studies showed that peak viral load during PR8 infection of mice was around 4 days post infection (Kumar et al., 2011) (Blazejewska et al., 2011) – we considered that since basal cells are situated on the basal membrane of the trachea, their peak infection time would be later than other epithelial cells.

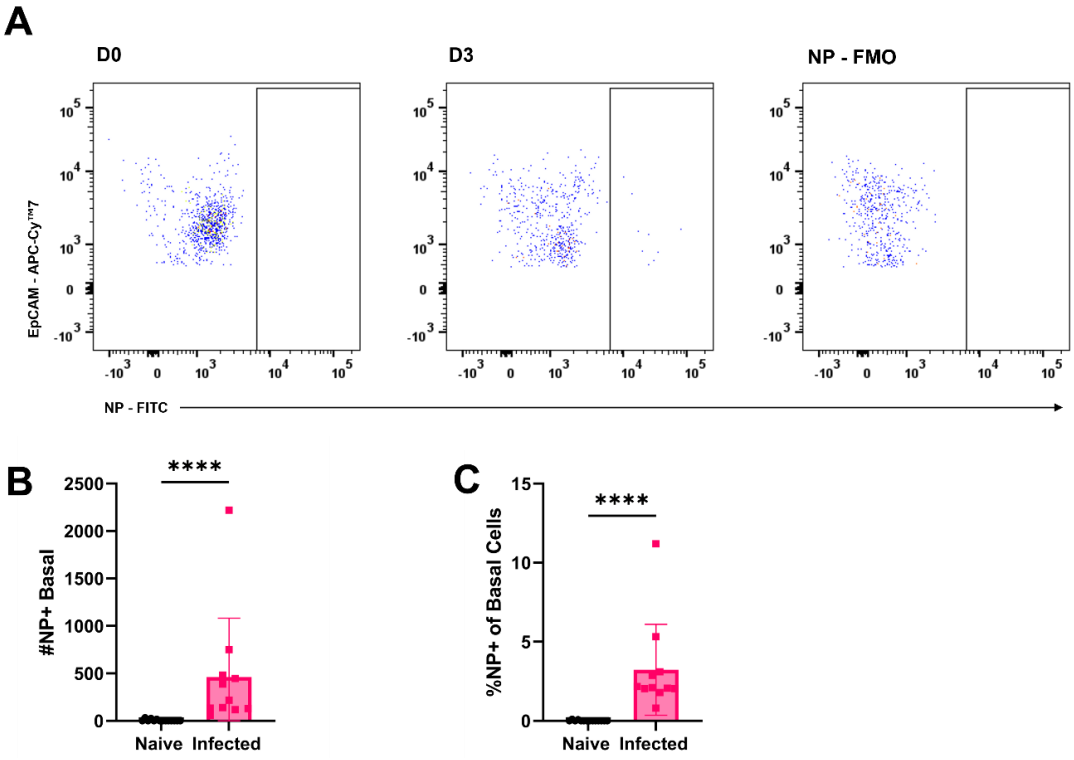


Figure 5-1 Mouse basal cells are positive for IAV PR8 NP protein at D6 post infection.

Age and sex matched C57BL/6 mice were infected with 100pfu of influenza A virus and were weighed daily. After 6 days, mice were culled and lung, spleen and BAL samples were collected. (A) Lung cells were stained and analysed by flow cytometry using a fluorescently labelled antibody against the NP protein. Absolute number (B) and percentage (C) of NP+ basal cells were calculated. Data shown n = 3-5 mice are representative of 3 experiments. Statistical tests were carried out using a two-tailed unpaired t-test and Mann-Whitney test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

We stained for viral NP 6 days post infection and found basal cells that were positive for NP (Figure 5-1 A-C) and no staining in our naïve and FMO samples. To strengthen this finding with a complementary technique, we worked with colleagues in Ed W. Roberts lab to optimise the detection of a novel, fluorescent transgenic influenza virus named “BrightFlu” (Pirillo et al., 2023). The virus was designed to be brighter than previous fluorescent influenza viruses, such as Color-flu (Fukuyuma et al., 2015). BrightFlu expresses zsGreen downstream of the NS1 protein, and we showed that the virus infected epithelial cells, can penetrate deeply into the lung, and remains infective after retrieval from an infected host (Pirillo et al., 2023). We also found that basal cells were positive for BrightFlu, with peak infection having around 10% infected basal cells (Figure 5-2). We found infected basal cells during the peak infection timepoints, but in contrast to other epithelial cells, basal cells were most infected at D3 post infection where epithelial cell infectiousness peaked at D6.

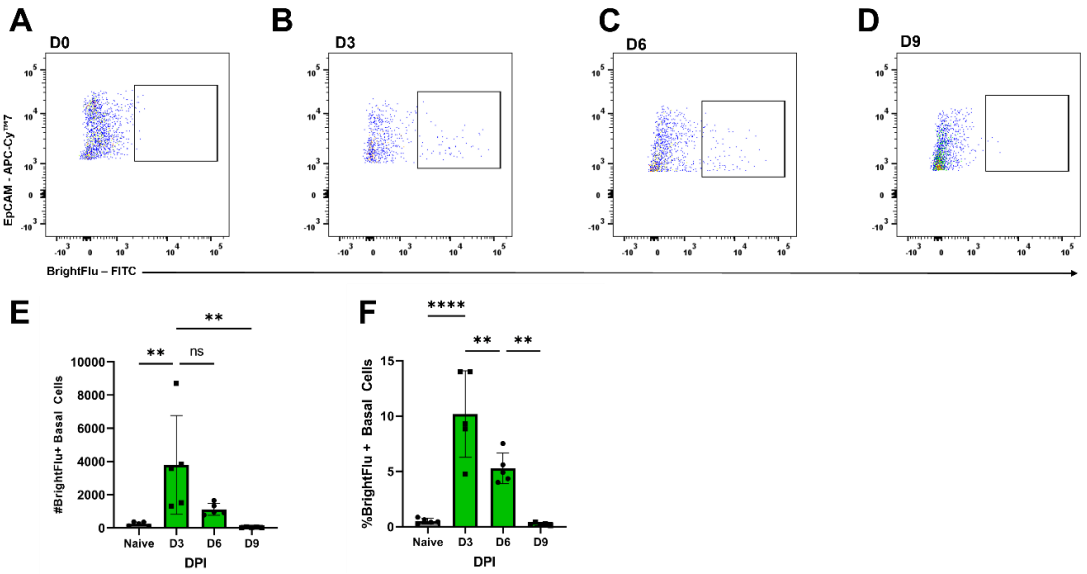


Figure 5-2 Mouse basal cells are infected with IAV across a time course

Age and sex matched C57BL/6 mice were infected with 100pfu of influenza A virus “BrightFlu” and were weighed daily. (A) Naïve and infected mice were culled at (B) D3 “early”, (C) D6 “peak”, and (D) D9 “recovery” timepoints, where lungs were dissected for flow cytometry. Basal cells were stained and analysed by flow and the (E) Absolute number (F) and percentage of BrightFlu+ basal cells was calculated. Data shown n=20 are representative of 1 experiment. Statistical tests were carried out using one-way ANOVA with Kruskal-Wallis multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.001 and ***P < 0.0001.

Together, the NP and BrightFlu data show that basal cells are infected during IAV infection, and we suggest that IAV may be able to directly change the behavior of basal cells. We tried to identify if this was the case by interrogating published single cell sequencing experiments.

5.3.2 Basal cells change their behaviour in response to early IAV infection

In Chapter 4 we studied the behaviour of basal cells during a timepoint of peak damage and repair (D10-12 post IAV infection). Since our BrightFlu time course suggested peak infection of BCs at day 3, we investigated the transcriptional differences during the peak viral replication phase (D3 post IAV infection). We used the D3 timepoint from the published scRNA-seq data set described in Chapter 2 to determine if there were any signs of early basal cell activation, that may be induced by the virus. Carrying out differential expression analysis between the naïve and D3 infected mouse basal cell transcriptomes showed strong expression of Sca-1 in basal cells from infected mice, which was consistent with our results in Chapter 4. We also found differential expression of interferon-stimulated and anti-viral genes such as *Irf7* and viperin. Pathway analysis revealed significantly different expression of GO:0051607 (defence response to virus) and the KEGG term map05164 (Influenza A). Figure 5-3 A shows that basal cells are involved in the response to infection, and this gives further confidence that basal cells are infected directly. The results in Figure 5-3C show that basal cells are activated during infection, and that this is true as early as 3 days post infection.

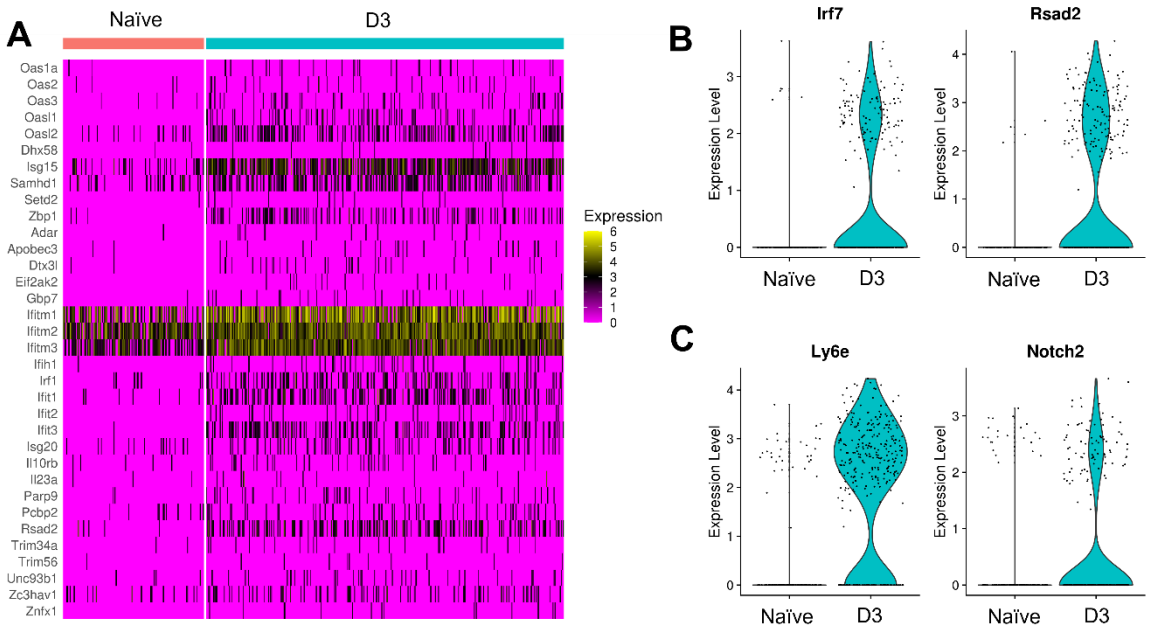


Figure 5-3 Mouse basal cells mount an antiviral response to IAV

Lung cells were sequenced by 10X Genomics as described here (Zhang et al., 2020) – we utilized this published data set to extract further information about the behavior of basal cells during infection. Sequencing reads were aligned to the mm10 Mus musculus genome using CellRanger. Cells were normalized and clustered using Seurat as described in Chapter 2. Differential expression of cells from D0 and D3 days post IAV infection was calculated and the most differentially expressed genes plotted (A). Genes associated with (B) antiviral response and (C) basal cell activation were identified.

Our previous analysis in Chapter 4 showed that basal cell activation was linked to metabolic changes, so we continued our pathway analysis with a focus on terms associated with cell metabolism. We found a decrease in GO:0022904 (respiratory chain) and the genes expressed in that pathway are shown in Figure 5-4A. We also show an increase in GO:0006869 (lipid transport) in Figure 5-4C. In Chapter 4, we discussed how our data complement the recent proposal that progenitors switch energy sources during activation; here, at day 3, our data also show increases in lipid metabolism which is consistent with other published work (Crotta et al., 2023), and additionally we show that mitochondrial metabolism is also linked to basal cell activation. We conclude that these changes at day 3 are a signal of early basal activation, and they are likely the beginning of the striking changes in basal cell metabolism shown at D12 in data shown in Chapter 4.

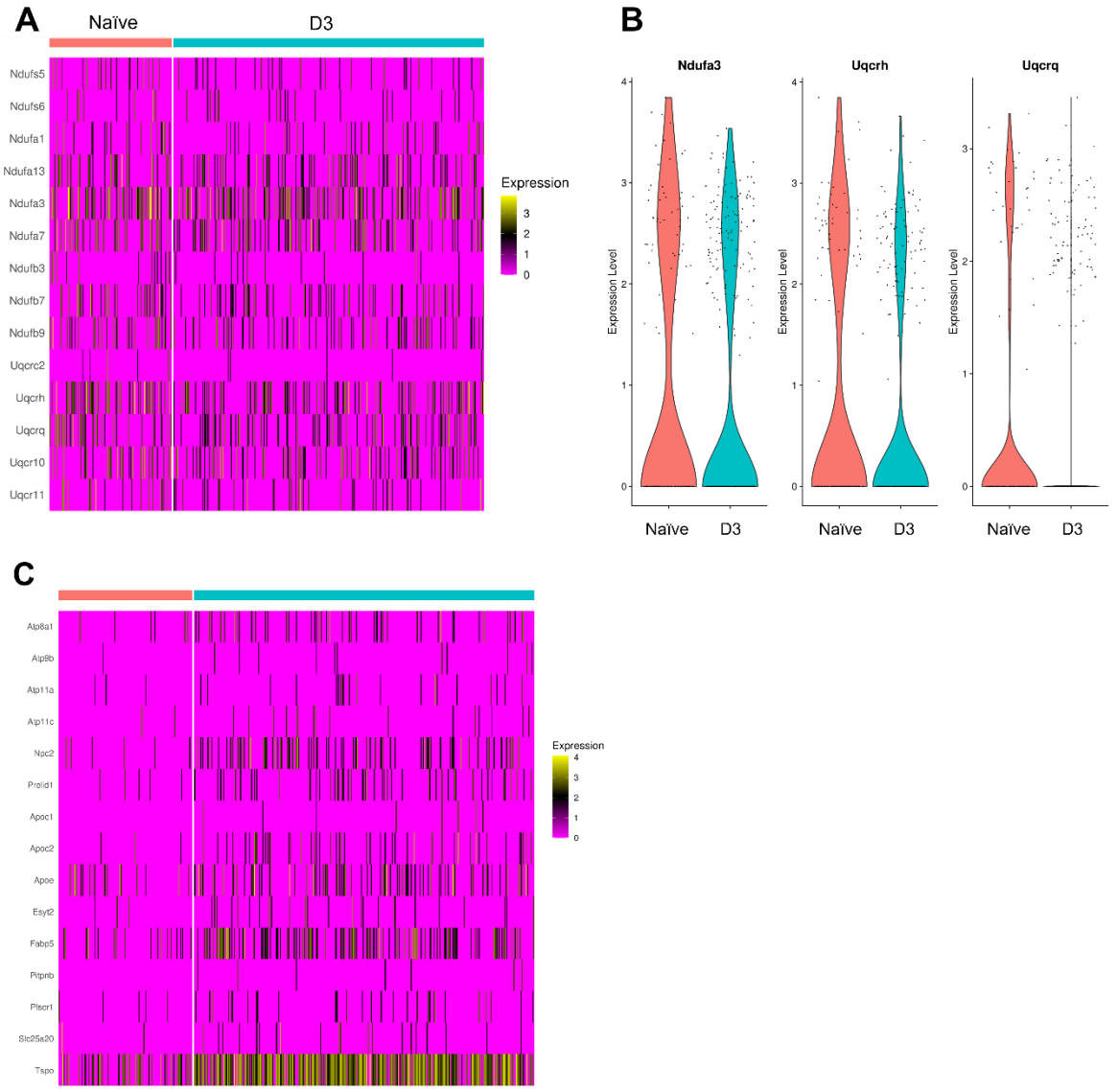


Figure 5-4 Basal cells show metabolic changes during early IAV infection

To determine how basal cells were different during early infection, irrespective of direct infection, basal cells from D0 and D3 IAV infection were compared. Differential expression of basal cells from D0 and D3 days post IAV infection was calculated and the most differentially expressed genes (logFC > 0.5) were identified. Pathway analysis was carried out using DAVID and pathways associated with (A,B) Respiratory Chain and (C) Lipid Transport were found to be differentially expressed.

Since our previous analysis had shown that basal cells may be engaged in anti-inflammatory behaviour, we looked at these pathways in the early timepoint. In our D12 analysis, we found that IL-10 receptor genes were upregulated, and we

found this to be changed at D3 also (Figure 5-5A). The anti-inflammatory response is controlled by other cytokines that IL-10, and so we looked in our data for pathways involved in immune regulation. We found that basal cells were strongly upregulating Il-4 (Figure 5-5B) and Il-13 (Figure 5-5C) receptors during peak IAV infection. Il-4 and IL-13 have been shown to be tissue protective during acute lung injury (Chenery et al., 2021) which led us to believe that basal cells exhibit a pro-repair phenotype very early in infection.

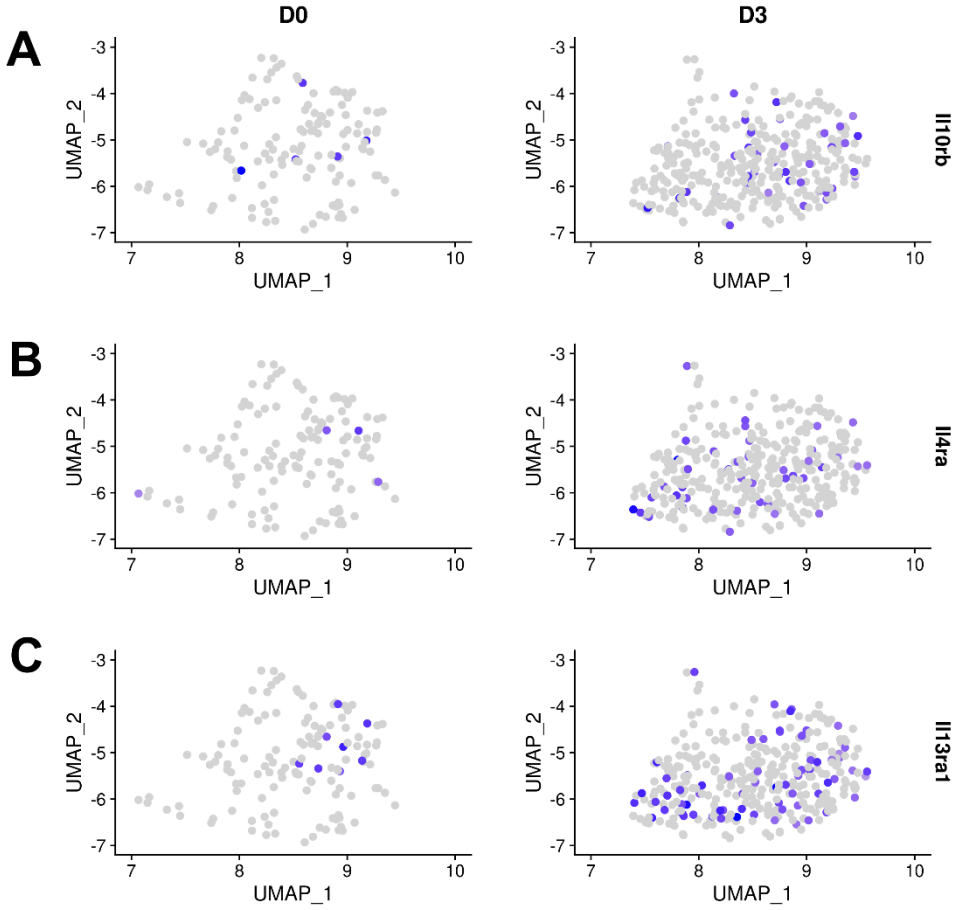


Figure 5-5 Basal cells look for a Th2 signal during IAV infection

Differential expression of basal cells from D0 and D3 days post IAV infection was calculated and the most differentially expressed genes were calculated. Pathway analysis was carried out using DAVID and pathways associated immune suppression were identified to be differentially expressed. Further analysis of these pathways found the (A) Il-10, (B) Il-4, and (C) IL-13 receptor genes to be strongly upregulated in D3 infected basal cells.

5.3.3 Identifying directly infected basal cells

So far, we have been able to compare basal cells from naïve and basal cells in infected mice, and although we have shown that basal cells can be infected, we have also shown that not all basal cells are infected (Figure 5-1, Figure 5-2). While this analysis gives us a broad understanding of how basal cells react to IAV infection, we also wanted to understand how direct infection affects basal cells. We considered but discounted sort-purifying infected cells to perform a bulk RNA-seq experiment, as only ~3% of basal cells are infected and the experiment would require many mice to obtain sufficient material. Fortunately, IAV is an RNA virus, and so 10X Genomics single-cell sequencing will amplify influenza viral elements as well as host cell RNA. To detect these viral elements, we used Cellranger "mkref" (make reference) function (10X Genomics) to combine the mouse and influenza virus genomes for the alignment step of the single cell analysis.

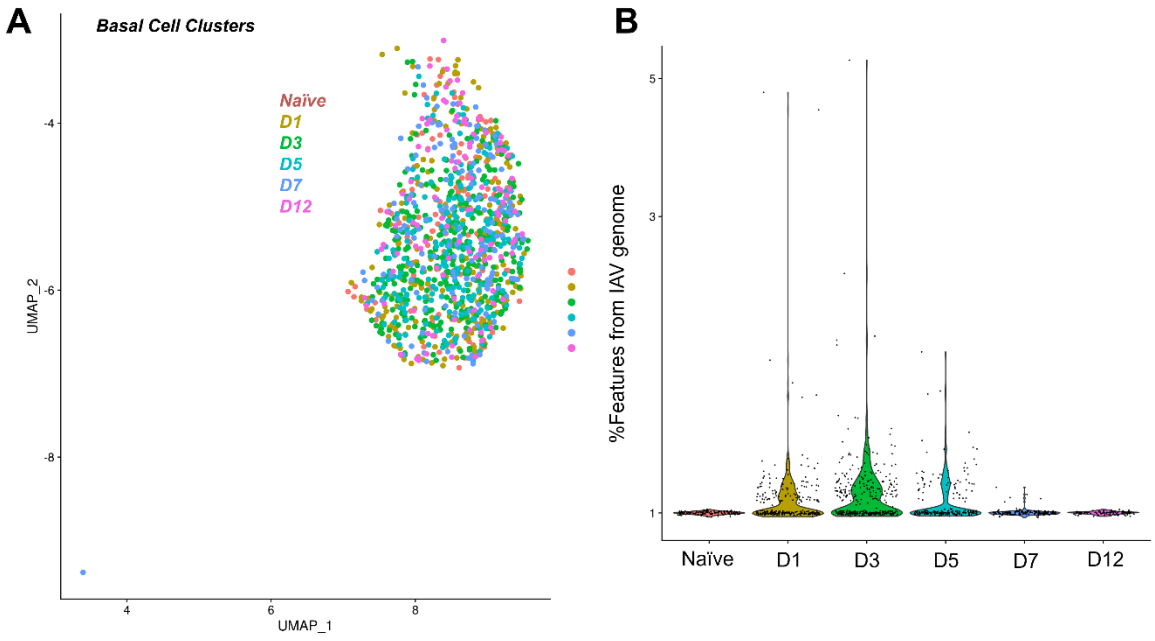
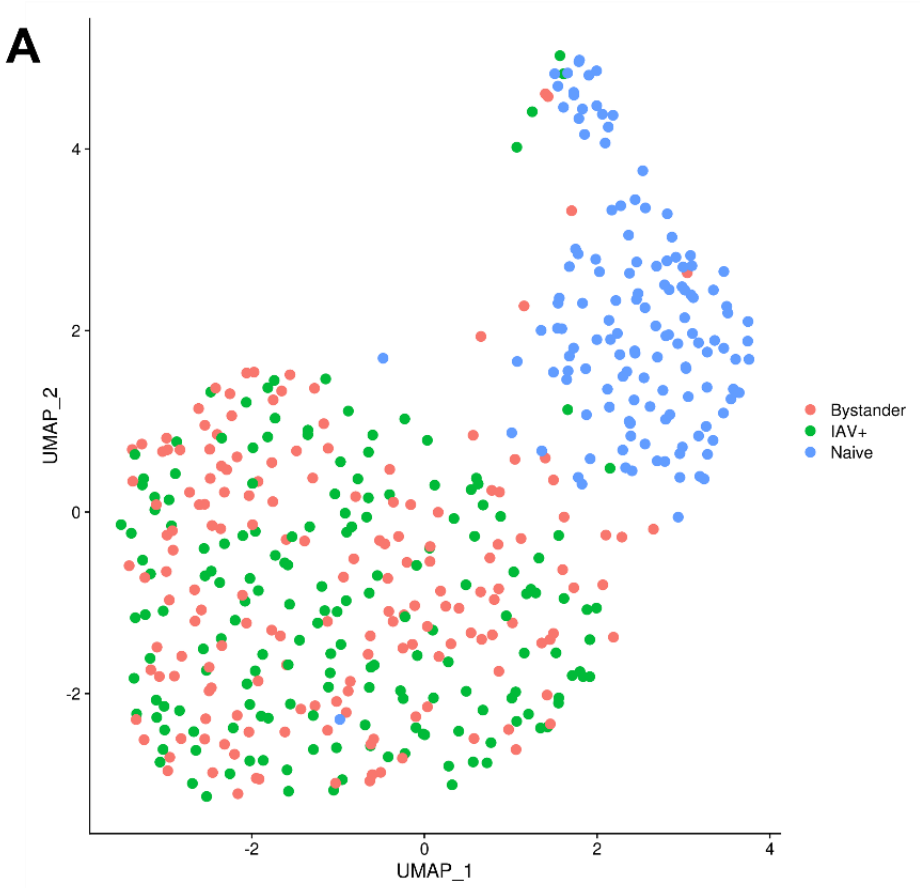


Figure 5-6 Basal cells express IAV features during in vivo IAV infection

This figure contains only basal cells that were sequenced by 10X Genomics. Sequencing reads were aligned to the *mm10.PR8* genome using *CellRanger* and basal cells were identified. Cells were normalized and clustered using Seurat as described in Chapter 2 (A). Expression of IAV features by basal cells was calculated across a time course of infection (D0 – D12 post IAV infection) (B).

After alignment, Cellranger produced output files which contained cells which have reads from both mouse mm10 (NCBI: GCF_000001635.20) and IAV PR8 (NCBI: GCF_000865725.1) genomes. FASTQ files from (Zhang et al., 2020) were then aligned using this new genome (hereafter referred to as *mm10.PR8*). After alignment, cells went through quality control and clustering as described in Chapter 2 and basal cells were identified (Figure 5-6A). The percentage of features from the IAV genome was then calculated for all basal cells across the time course, and the peak of viral infection was again found to be D3 (Figure 5-6B).

5.3.4 How do directly infected and bystander basal cells differ?



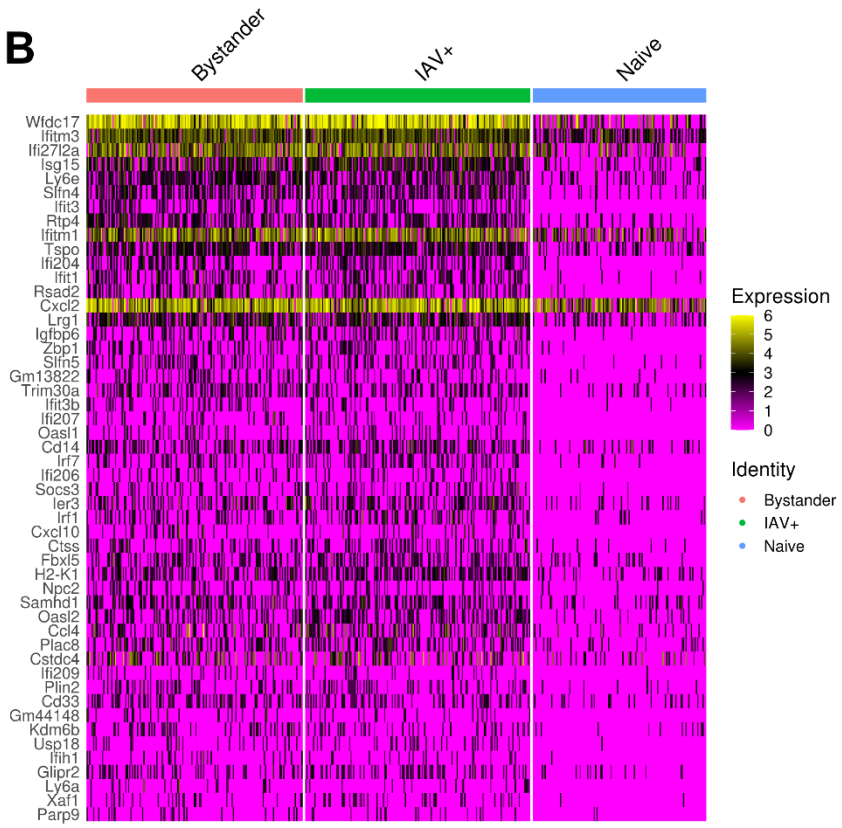


Figure 5-7 Both directly infected, and bystander basal cells respond to infection with transcriptomic changes.

We accessed data produced by (Zhang et al., 2020) as described previously. We then aligned sequencing reads to the *mm10.PR8* genome using *CellRanger*. We normalized and clustered cells using Seurat as described in Chapter 2. After identification, basal cells were classified as uninfected, infected bystander, or directly infected (IAV+) based on their expression of IAV features. Basal cells from naïve and infected samples were integrated and cells which had a %IAV features >0 were classified as IAV+. (A). Differential expression was carried out between the three populations, and the most differentially expressed genes were calculated (B).

Our first question was whether there was evidence of viral infection in basal cells. We identified IAV+ basal cells which complemented our earlier data showing basal cell infection with antibody and fluorescent detection of influenza virions (Figure 5-1 and Figure 5-2). Detecting virus in basal cells raised the possibility that the virus might be able to alter basal cell function, and the process of lung repair, by manipulating BC gene expression. Because this and previous analysis showed that basal cells were most infected at D3, we selected this timepoint for our next analysis. Using the percentage of IAV features to

determine IAV+ cells (%IAV features > 0), we were able to cluster cells into naïve, infected bystander and infected IAV+ clusters and carry out differential expression analysis between the groups.

We found that many innate immune and anti-viral genes were activated in IAV+ cells (Figure 5-3A-B), likely a response to direct infection by the virus. Surprisingly we also saw increases in these genes in the bystander cells, and this could be a result of the release of interferon, or other inflammatory signals released by nearby infected cells or immune cells. We then considered whether direct infection induces basal cell activation, and whether the active basal cells that we saw were the directly infected ones. Both clusters of IAV+ and bystander cells showed similar activation by measurement of Sca-1 (Figure 5-3C). Using these data, we could conclude that basal cells did not have to be directly infected to activate during infection. Equally, it does not appear that direct infection reduces the activation of basal cells. We followed on this question to consider whether the quality of basal cell activation was changed during direct IAV infection.

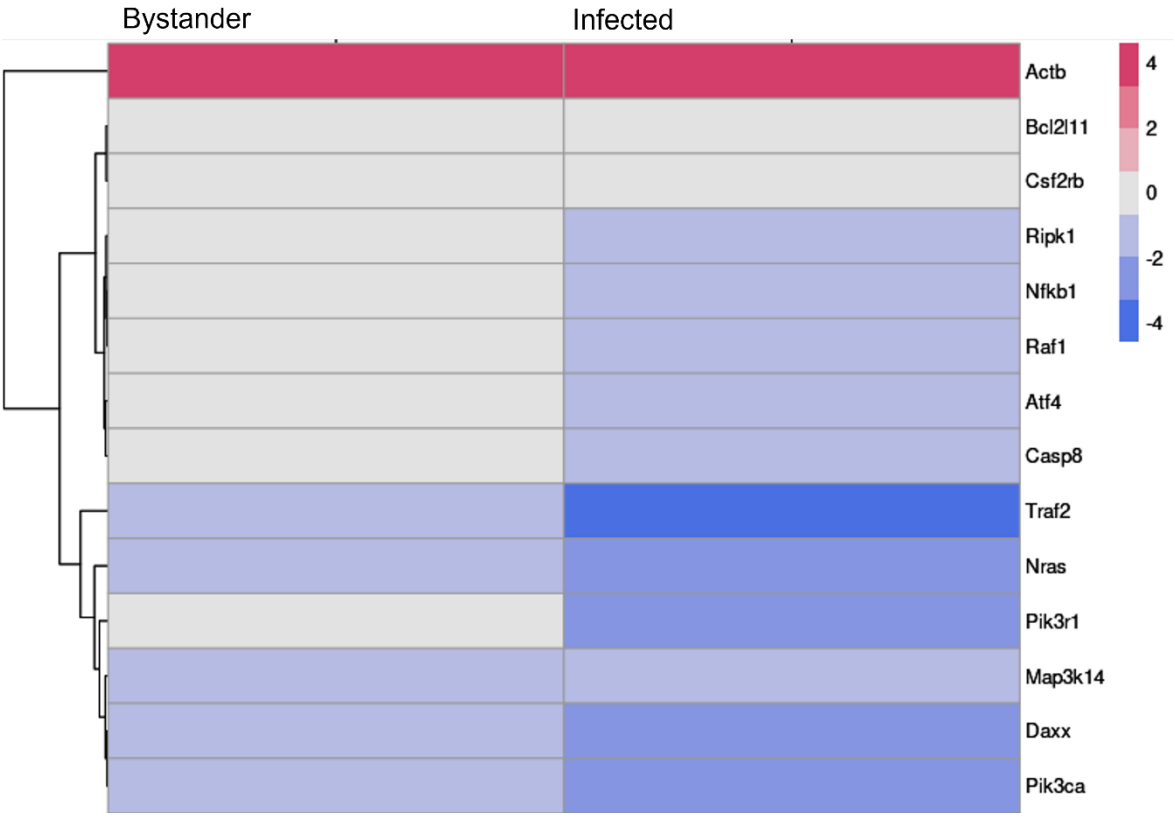


Figure 5-8 Directly infected basal cells are not more apoptotic than bystanders.

Differential expression analysis was carried out between IAV+ and bystander cells to identify genes associated with direct IAV infection. To determine if IAV+ basal cells were more apoptotic than bystander basal cells, genes associated with gene ontology term apoptotic process (GO:0006915). In the genes expressed from that pathway, there was no significant difference in expression between IAV+ and bystander cells, with most genes being downregulated in IAV+ basal cells.

We considered that the stress caused by the virus may push basal cells to be more apoptotic, that the strain of the virus production would lead the basal cells to die. This could be a survival function, since cells dying through non-apoptotic pathways can bring attention to the immune system, and a dead cell cannot produce more copies of the virus. Despite this, when we looked at genes associated with apoptosis (Figure 5-8), we found no significant difference in the production of genes associated with term apoptotic process (GO:0006915). We suggest that since progenitor cells are more resistant to apoptosis than other epithelial cells (Quantius et al., 2016), the effects of stress on cell death may be masked. It could also be a function of the immunosuppressive capabilities of IAV

– the NS1 protein of IAV has been shown to have many mechanisms to suppress host cell apoptosis (Robert M. Krug, 2015, Zhirnov et al., 2002).

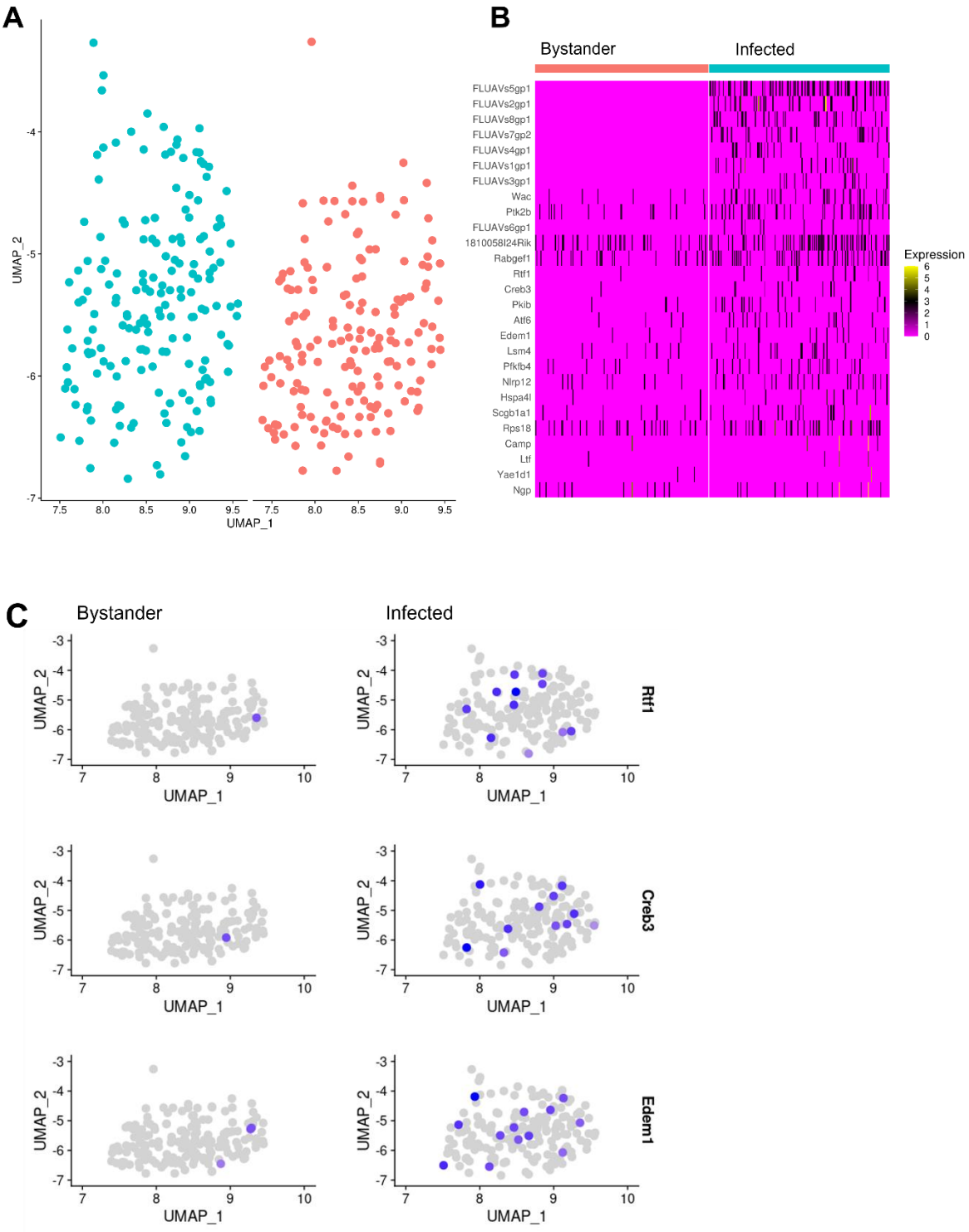


Figure 5-9 Directly infected basal cells exhibit signs of stress.

Cells were normalized and clustered using Seurat as described in Chapter 2. After identification, basal cells were classified as naïve, bystander, or IAV+ based on their expression of IAV features. Naïve basal cells were then removed, and clustering was performed again to compare directly infected and bystander cells (A). Differential expression was carried out between IAV+ and bystander cells to identify genes associated with direct IAV infection (B). One of the most strongly upregulated pathways was Golgi stress, with many genes associated with endoplasmic reticulum production strongly upregulated in IAV+ basal cells.

Now that we had seen the similarities between the IAV+ and bystander cells, we turned our attention to the differences between these two cell types (Figure 5-9A). We found a difference in the expression of goblet cell marker *Scgb1a1* suggesting that infected basal cells may be more skewed to goblet cell differentiation compared to bystander basal cells, but the most striking differences were in the Golgi and endoplasmic reticular stress pathways with genes such as *Creb3* and *Edem1* strongly upregulated in infected basal cells (Figure 5-9C). We hypothesised that this change in directly infected basal cells was due to the pressure of producing new copies of influenza virus. We propose this increase in cell stress is a direct effect of the virus changing basal cell behaviour; perhaps the virus forces the basal cell to prioritise production of virus over other homeostatic functions. This might reduce the effectiveness of the basal cell and prevent proper activation and differentiation. It is possible that D3 is too early for a sufficient answer to this question, and that cell fate decisions may be made later, after the viral challenge has been removed. In further analysis, we could look at how these cell fate markers change throughout the time course experiments we have been analysing to determine if these decisions are affected by direct IAV infection.

The results in this chapter show strong evidence that basal progenitor cells are directly infected by IAV; we have shown that with two different viruses, and several different viral identification methods. We also used transcriptional data to show that directly infected basal cells have a distinct transcriptional profile to bystander cells. Interestingly, our data also show infection of basal cells as early as 24 hours post infection, which would have been difficult to detect without our transcriptomic approach. In Chapter 4 we showed that basal cells had anti-inflammatory properties and relied on this for their activation, but here

we show that early basal activation has a pro-inflammatory anti-viral flavour. We suggest that basal cells are plastic and can drive an inflammatory response during infection; but then receive an immunoregulatory signal which begins their repair function. The host balances pro and anti-inflammatory measures during infection, and we suggest that basal cells are a part of that balance.

5.4 Discussion

We first aimed to determine whether basal cells were infected with IAV, and we showed that they were by several methods. As discussed before, Quantius and colleagues showed infection of upper airway epithelial cells which would include basal cells, and by staining for the IAV nucleoprotein (NP), found these to be infected (Quantius et al., 2016). There are also beautiful images showing viral proliferation in canine epithelial cells including in the basal layer, suggesting that basal cells can be directly infected in a natural canine infection. In Figure 5-1 and 5-2 we show that mouse basal cells are infected by IAV in vivo by observing both NP and NS1 inside basal cells with intracellular staining. We have also confirmed this with a bioinformatic approach in two single cell sequencing experiments. Basal cells are a logical target for viruses such as IAV, as they are more resistant to apoptotic death (Erb et al., 2005) and have been shown to be more proliferative when cells around them are undergoing apoptosis (Fujino et al., 2019).

To produce progeny, viruses access and hijack host cellular machinery. The infection of mature epithelial cells is well known (Webster and Beare, 1991, Matrosovich et al., 2004), and the infection of the different subtypes of these cells such as AT-II (Weinheimer et al., 2012) and club (Heaton et al., 2014) cells has been well characterized. Direct infection of basal cells has not been conclusively shown, and this may be that the rarity and difficulty of studying basal cells has led to a gap in research. Why does it matter whether specific cell types are infected? Viruses from the Herpesviridae can change the behaviour of cells they directly infect, an ability which has also been shown for RNA viruses such as SARS-CoV-2 and IAV (Soto et al., 2022). Mechanistically, IAV made changes to histones leading to decreased anti-viral responses and changes in cell

cycle regulation (Marcos-Villar et al., 2018). Viruses such as respiratory syncytial virus (RSV) have also been shown to directly infect basal cells and alter their ability to repair the lung properly (Persson et al., 2014). We suggested that IAV may have similar deleterious effects on repair when the virus directly infects basal cells.

In previous figures we showed that basal cells contained IAV proteins, but we were curious about how this direct infection changed the behaviour of basal cells. Since IAV is an RNA virus, we were able to detect IAV products in our scRNA-seq data sets by modifying our reference genome. In Figure 5-2 we found the peak infection of basal cells occurred at D6, but this is at the protein level. In Figure 5-5 we looked at the RNA level and found the peak to be at D3, but we also found expression of IAV features as early as D1 post infection. This was helpful as it allowed us to determine cells which were present in an infected mouse, but not directly infected (bystanders); and cells that were replicating IAV features (directly infected). Fortunately, IAV transcripts are polyadenylated and so can be detected by 10X sequencing, where viruses such as Zika virus cannot (Ratnasiri et al., 2022).

In Chapter 4 we described how basal cells were altered by influenza at a repair timepoint (D12), when most of the virus should have been cleared. Basal cells get their name from their location on the basal lamina, and we considered that basal cells may not experience many of the effects of early IAV infection due to this sheltered location. However, in Figure 5-3 we see that basal cells are activated at this timepoint (D3), change their metabolic profile, and raise a non-specific antiviral response like that of other epithelial cells.

When we interrogated the difference between bystander and directly infected cells, we noticed clear differences. Directly infected cells showed increases in ER/Golgi stress and seemed to be less apoptotic than bystander cells. This was surprising to us since we expected directly infected cells to be more apoptotic and show more signals of cell death. However, it is possible that the lack of difference seen in apoptotic pathway genes is not the full picture. Influenza is a budding virus and so it is advantageous for the virus to keep host cells alive as

long as possible (Christian Munz., 2014). The influenza virus NS1 protein has many anti-inflammatory and anti-apoptotic effects used to keep viral replication undisturbed (Zhirnov et al., 2002), so it is possible that any changes in the number of basal cells dying by apoptosis are masked by the virus. It is possible that infected basal cells are more apoptotic than bystander cells, but transcriptionally they appear to be not changed from bystanders. Since basal cells are progenitors, it is possible that their natural resistance to apoptosis is compensating for the stress they are experiencing. This is an important consideration as in the intestine, ER stress has been shown to induce a loss of stemness in intestinal stem cells, leading to increased differentiation (Meijer et al., 2021, Turischeva et al., 2022). Progenitor cells undergoing prolonged stress may lead to dysplastic differentiation, or production of cancer cells (Bergmann and Steller, 2010). Classical signs of basal cell activation and tissue regeneration are strongly associated with cancer in humans (Reya et al., 2001). This is interesting to consider alongside our other results since tumours have been described as "Wounds That Do Not Heal" (Harold F. Dvorak, 1986) (Balkwill and Mantovani, 2001). Although only an association study, influenza infection has been associated with an increased risk of developing lung cancer in humans (Weng et al., 2019) – this association is backed by animal studies of IAV infection which also show that IAV infection worsens tumour growth in mice with lung cancers (Garmendia et al., 2023). This is of particular interest to us as these studies also showed that lung tumour cells are directly infected by IAV. We speculate that if IAV was able to infect basal cells, these basal cells could become oncogenic, or through increased rapid activation, the tissue repaired by those infected basal cells could be repaired incorrectly.

Survival from influenza is often viewed through the lens of clearing the virus with a rapid innate response that allows time for the host to mount an effective, adaptive response. What is often not considered is that the host not only has to survive virus, but it also must survive the immune response it has raised to fight the infection (Rouse and Sehrawat, 2010, Kaufmann et al., 2010). As previously discussed, the Th2 response raises an anti-inflammatory pro-repair response, but thus far there has been no suggestion that basal cells take part in this role.

Indeed, the results in Figure 5-4 showing that basal cells upregulated receptors for classical Th2 cytokines (IL-4, IL-13) was initially surprising.

One of the tools in the immunological arsenal against IAV is neutralising antibody; it has been shown that IL-4 is necessary for production of antibodies against a wider range of IAV epitopes (Miyauchi et al., 2021). This source of IL-4 acts mainly in the lymph node, and the story in the lung parenchyma is not as clear. There is some promising data showing that IL-4 is protective during IAV infection (Bot et al., 1999), and that IL-4 is necessary to survive viral/bacterial co-infection (Peng et al., 2021). The latter study discusses that loss of IL-4 during co-infection disrupts the process by which alveolar macrophages are replaced by inflammatory macrophages, and the lack of inflammatory cell infiltration leads to a poor response to the subsequent challenge. Conversely, if the IAV challenge occurs after an allergic sensitisation, IL-4 has been shown to be damaging, with allergic mice recovering more quickly after IL-4 receptor blockade (Shahangian et al., 2021). These results seem contradictory at first, but there is a common link. It may not be as simple as more or less IL-4, but that IL-4 is important in tailoring the balance between pro and anti-inflammatory recruitment. Our data suggest that basal cells play a role in this balance: they could be looking for a signal from the anti-inflammatory immune response to begin the repair process. Models of allergic airway inflammation have been shown to induce epithelial-mesenchymal transition (EMT), where mature epithelial cells de-differentiate to change their cell type (Gandhi et al., 2013). This gives strength to our conclusion that epithelial cells can react to cytokines such as IL-4 and IL-13, and that challenges to the immune system confer changes to the lung that go beyond the training of immune cells. While the imprinting by an asthmatic challenge may have left the lung susceptible to viral infection, it may be better prepared for a bacterial or parasitic infection (Zuyderduyn et al., 2011). We speculate that this means these cytokines can act in a “pro-repair” manner by acting on progenitor cells.

We have shown that basal cells are infected by IAV, and that directly infected basal cells are transcriptionally different to bystander basal cells. Though there is no difference between apoptosis or activation between IAV+ and bystander

cells, infected basal cells show signs of productive stress and a stronger anti-viral response to bystander cells. While it is well understood that viruses infect epithelial cells, we do not fully understand what changes these viruses can confer on these cells. Since most mature epithelial cells are killed by IAV infection, though dying cells may affect the inflammatory environment, the effect of dead epithelial cells on long term function of the tissue may be minimal. Dead epithelial cells may not persist after infection, but progenitors do. What is not clear is the long-term effect of infection of long-lived progenitor cells. Others have shown that basal cells can be directly infected by viruses such as Rhinovirus and RSV, and that they can change the kind of repair those infected basal cells carry out (Jakiela et al., 2020, Persson et al., 2014). We have shown conclusively that basal cells are infected in Influenza A virus infection in vivo, both at the protein and RNA level, and have shown that direct infection of basal cells changes their transcriptome. We have made some interesting discoveries about how basal cells interact with the immune system, and we show that basal cells produce receptors of immunosuppressive cytokines. We also suggest that direct infection of progenitor cells may have deleterious effects, and that improvement of anti-viral and vaccine therapies could prevent these cells being infected. Perhaps preventing direct infection of basal cells will limit dysplastic repair carried out, and lead to a more effective repair response. We suggest that direct IAV infection of basal cells should be prevented, and that once infected these basal cells perform a sup-optimal repair function.

Chapter-6 Discussion

The purpose of this project was to provide additional information on the function of lung basal cells. Basal cells are known to be important in both homeostasis, and in the response to lung damage. The work shown in chapter 3 shows how we tried to improve our techniques for studying basal cells, so that we could answer the questions posed in chapters 4 and 5. In chapters 4 and 5, we attempted to understand how basal cells changed transcriptionally during lung damage, and we tried to understand the timing around basal cell activations. Our overall aim was to understand how acute inflammation activated basal cells, and whether influenza virus, or the immune response to the virus, changed how basal cells behaved and we have shown that basal cells respond to cytokines, that they are metabolically regulated, that they are infected by IAV, and that that infection changes their behaviour.

6.1 Transcriptomics of progenitors – the needle in the haystack

Basal cells have been known to function as progenitors of the lung for over 30 years, yet extensive understanding of their behaviour is not understood in the same way that it is for tissues such as the gut. Part of this is that recent advances in technology have allowed lineage tracing and cell fate experiments to be done, so we can now understand better what basal cells do in development. These techniques are useful for understanding lung development but are limited as they usually don't contain a significant immune component. While they can be useful in developmental biology, they are limited in usefulness when asking complex immunological questions. What is not known however, is functionally how basal cells respond to different challenges in the lung. Part of this limitation is that basal cells are a rare cell type, and isolating enough of them to do functional experiments has so far been difficult. Our results in chapters 3 - 5 show how we have tried to overcome this limitation by studying basal cells in published single cell RNA-seq datasets. In doing this, we have not only produced further results from data that had not been discovered,

but we were able to answer questions about a rare cell type in a real *in vivo* infection model. Another way we have contributed to the field is by developing a different digestion method which yields a greater number of progenitor cells of greater viability than what was previously available which we showed data on in Chapter 3. This new digestion method allowed us to yield enough cells to allow us to carry out bulk RNA sequencing on basal cells and answer transcriptomic questions even more specifically than with the single cell RNA-seq experiments. These advances allowed us to ask interesting biological questions about how basal cell behaviour changes over the time course of infection, and how the metabolic profile of basal cells changes over this time.

Single cell RNA-seq was first performed in 2009 on a mouse blastomere, with the first commercially available platforms becoming available later in 2014 (Wu et al., 2018). Despite being less than 10 years old, single cell sequencing technology has become ubiquitous and in the time since its creation there has already been significant advances in sequencing technology. This is in part because single cell sequencing as it stands is limited. Lähnemann and colleagues comprehensively review what they consider to be the eleven grand challenges of single-cell data and they highlight some key issues, such as the difficulty in sequencing rare cells, and the comparative lack of sequencing depth when compared to bulk RNA-seq (Lähnemann et al., 2020).

These and other limitations have been found in our work - we are attempting to analyse a rare population, and there is only so much that can be answered with the limited sequencing depth that is affordable in single-cell RNA sequencing (Stark et al., 2019). Because the time course experiment contained all lung cells, sequencing depth in the basal cells was low, and so some genes for cytokines and their receptors were difficult to detect. In the time since the invention of this technology there has already been significant advances in sequencing technology. One of the limitations of single cell is a loss of tissue structure and a loss of how cells interact with each other in tissue, especially since we are only observing the RNA component of cells. A recent focus of sequencing has been on attempting to sequence cells without disrupting their tissue structure using techniques such as fluorescence in situ hybridization (FISH)

(Abdul Shakoori, 2017) or CODEX (Black et al., 2021). More popular are techniques that combine these methods, providing histological, spatial, cellular, and molecular technologies to allow for extraordinary amounts of biological information to be gathered on small neighbourhoods of cells in tissues such as the lymph node and brain (Radtke et al., 2024, Zhao and Germain, 2023).

We predict that the future of sequencing will be in tissue, and we are excited that some of the questions left unanswered by this work could be answered using these technologies. In this project we attempt to understand the difference between basal cells that experience an infection environment, and those that don't. However, all our analysis utilises cells that were broken into a single cell suspension, and all the architecture of the tissue is lost. We can make inferences based on cell interactions with virus, and cytokines that cells produce, but it is difficult to know if a cell analysed this way was anywhere near the area of the infection, or if it was interacting with other cell types. This makes it difficult to suggest that changes we observe are directly related to the virus. We have advanced what is known about basal cells during infection, especially since much prior work was carried out with microscopy. Spatial technologies becoming more available and affordable might allow these questions to be answered more specifically.

6.2 Basal cells are activated by infection and remain active after the infection is cleared.

One recurrent theme throughout these infections is that basal cells are active early in infection and remain active long after the virus is cleared. Markers of activation and progenitor proliferation are increased as early as 24 hrs post infection, and we have seen this activation remain 28 days after IAV infection. As discussed in chapter 4, activation and proliferation during repair is energy intense, and basal cells may simply be using any energy source they can lay hands on. Originally the results showing sustained basal cell activation after infection surprised us. We could understand that it was necessary for basal cells

to activate during infection to repair the damage done by the virus. But why then, were basal cells still activated long after infection?

One reason for this could be that during IAV infection there is a rapid damaging response where the main aim is surviving the infection (Ferreira et al., 2023). This then transitions into a wound environment, where the tissue can then be remodelled (Jiang and Zhang, 2023). The wound environment provides an opportunity for the proper architecture of the lung to be restored, rather than the lesion being quickly covered. This triage response is essential we expect for preventing secondary infection and immediate death, but after the infection is cleared, there is an opportunity for a more reasoned repair, and this is why we think basal cells remain active so long after challenge. It is also possible that long-term basal cell activation could be a defence mechanism against subsequent infection. If the lung has recently experienced a challenge, it is possible that it may soon experience another challenge. Organisms like *Aspergillus* and *Staphylococcus* (Rynda-Apple et al., 2015) not only take advantage of the physical damage caused by the antiviral response, but this damage allows access to additional nutrients and a change in the oxygen availability which may be favourable to these pathogens (Obat et al., 2023). Perhaps it is worthwhile to keep a pool of activated basal cells after damage to allow a better response to the next challenge the lung will face. Work in muscle stem cells has shown that progenitor activation results in a loss in quiescence, but that stem cells will still produce cell cycle genes (Zeng et al., 2022). This suggests that once activated, stem cells can remain cycling with some cells going on to become differentiated.

However, prolonged activation can run the risk of stem cells becoming malignant, though this may be a risk worth taking in the face of a secondary infection. In chapter 4 we see that basal cells are activated during infection, and this activation is sustained long past viral infection. In chapter 5 we see further activation across a time course, which coincides with an increase in ribosomal and nuclear protein production pathways. We also see an increase in ER stress, induced by direct infection of basal cells by IAV. This increased productivity could simply be a result of proliferation, and this may be a

necessary step in basal cells fuelling their tissue repair role. However, this is a function that we suggest must also be tightly balanced. Due to their productive ability, progenitor cells are particularly vulnerable to becoming oncogenic – many cancer cells share surface marker expression with progenitor cells (Yin et al., 2021, Dika et al., 2020). The most common cutaneous malignancy is basal cell carcinoma, where basal cells of the skin aberrantly divide causing an invasive cancer (Kasper et al., 2012).

6.3 Basal cells are changed by direct IAV infection.

Throughout IAV infection we have seen changes in the metabolic profile of progenitor cells. These results may be a hint to how progenitors respond to different types of challenge, and that each kind of insult may drive a different energy source to be used. The repair process of activation and proliferation is an already productive phenotype for basal cells, and this is before considering that these basal cells can be directly infected by IAV. We show in chapter 5 that basal cells can be directly infected by IAV, and we suggest this direct infection is a further productive stress. Not only does producing viruses cost energy and cell resources (Richard Compans, 2007, Brett and Holtzman, 2015), but IAV manipulation of the mitochondria also may put basal cells under further oxidative stress (Castellanos et al., 2021). We suggest that a combination of stress from activation needed to repopulate the damaged lung, in addition to the stress caused by the virus might lead to dysplastic repair of the lung. Either through damaging necrotic cell death, or by improper differentiation in the wrong location.

We have also shown that basal cells not only contain virus material, but that they can actively produce IAV vRNA. By using a published data set with multiple timepoints (Zhang et al., 2020) we were able to assess the progression of changes over the full-time course of infection. We were able to use those timepoints to answer specific questions about how basal cell behaviour changed

during different timepoints of IAV infection. Considering all these timepoints together, there are some clear themes that persist throughout.

Previous experiments have shown that direct infection of basal cells can skew which cell type they differentiate into (Persson et al., 2014), and some of our results in chapter 5 showing increases in goblet cell markers on directly infected cells suggests that this could be a consequence of IAV cell manipulation. A recent study showed that after IAV infection, mice experience an increase in the number of tuft cells present in their lungs (Barr et al., 2022), which is suggested to be a part of pathogen clearance and prevention of commensals from expanding which has been shown in the gut (Moltke et al., 2015). This appears to be a non-specific response to damage, which then protects those mice from subsequent parasite infections. However, it also leads to "bronchiolised" regions of the lung that were once alveolar and sites of gas exchange, that are now composed of upper airway like basal cells and their progeny (Vaugahn et al., unpublished). This could reduce the gas exchange capability for the purpose of protecting against future pathogens.

6.4 Basal cell activation relies on IL-10 signalling.

Another argument as to why basal cells remain activated after infection could be that activated basal cells during infection do not receive a "turn-off" signal after they challenge has receded. It is not clear exactly where basal cells get this stop signal, but some have suggested it is regulated by *Fgf10* or that the signal to stop proliferation is governed by a reduction in oxygen availability (Emma L. Rawlins, 2008). In the brain, neural stem cells have been shown to reduce activation based on their access to certain metabolites required for lipogenesis (Homem et al., 2015) which ties in nicely with recent work in lung progenitors (Crotta et al., 2023). We suggest that another stop signal could come from the immune response. We have discussed in chapter 4 that basal cells upregulate *Il10ra* and receptors such as *Il4rl1* and *Il13r1* in what we suggest is a signal from the type 2 response. This signal to "turn off" could be a result of IL-4

from mast cells (Jain-Vora et al., 1998) or effector T cells (Park et al., 2023), or through IL-10 signalling from macrophages, effector T cells (Cheung et al., 2002) or even potentially neutrophils (Kang et al., 2020). Lung epithelial cells have also been shown to contain IL-10, which is released upon damage. We hypothesise that the type 2 immune response can be pro-repair, and that basal cells receive a signal from the type 2 arm of the immune response to begin their activation. This idea was strengthened by our results in chapter 5 showing that basal cell activation relies on IL-10, and by blocking access to IL-10, basal cell activation is ablated.

Wound healing can be a long-term process and we suggest that basal cell expression of IL-10 can further the wound healing environment by amplifying the anti-inflammatory IL-10 signal bringing in other regulatory immune cells, or tissue remodelling macrophages and fibroblasts. Some research suggests that blocking or knocking out of IL-10 has little effect on recovery from influenza (McKinstry et al., 2009), but this work was done with a very low dose of PR8 influenza virus. Even with a low dose, blocking IL-10 produced a stronger Th17 response than without the blockade, which could be damaging in a more severe infection. Indeed, Jie Sun and colleagues showed that IL-10 blockade during IAV infection led to worsened pathology in mice (Sun et al., 2009) and there is a wealth of work showing that without IL-10, the host is killed by type 1 and type 17 immunopathology (Couper et al., 2008, Redpath et al., 2014). These disagreements could be a case of differing study design, but it is worth remembering that these cytokines act in delicate balance. There are examples of cases where over production of IL-10, or too early production of IL-10 can lead to worse disease outcomes, and a worse outcome for infected mice (Keer Sun et al., 2010). It has been shown in the intestine that epithelial cells can produce IL-10 (Nguyen et al., 2021, Hyun et al., 2014) and our results also show that activated basal cells can produce IL-10. We suggest that basal cell response to IL-10 is not just activation, but that basal cells can produce a positive feedback loop which generates a repairing environment.

Effective wound healing is in a constant balance between fibrotic repair (overexaggerated repair), and pathology (under-exaggerated repair) and that IL-

10 plays a part in this. Others have suggested that IL-10 produced by interstitial macrophages is necessary for maintaining barrier integrity by acting on epithelial cells (Bain and MacDonald, 2022) and that macrophage sourced fibronectin induces proliferation of bronchial epithelial cells (Han and Roman, 2006). We take this one step further and suggest that this IL-10 may be acting directly on basal cells, inducing their activation. Further evidence to this point is during severe lung damage, resident alveolar macrophages are repopulated by monocyte derived ones, which remodel the lung in a fibrotic manner (Misharin et al., 2017). We often think of primary interferons being responsible for inducing an "antiviral state" in epithelial cells (Katze et al., 2002). We suggest that IL-10 signalling to epithelial cells may be initiating a "pro-repair" state. This might reduce their production of anti-viral and pro-inflammatory factors in favour of homeostatic genes. This could reduce further damage from the immune system and allow other epithelial cells to go back to their homeostatic functions (e.g. production of surfactant or mucus). If we can understand how to exploit the functions of IL-10 during infection, we could base future therapies on the idea of fortifying wound repair by modulating expression of IL-10 or indeed, the targets of IL-10.

6.5 Therapeutics for inflammatory lung conditions

One of the reasons that this project was relevant is that there is a huge burden of lung disease in the UK and around the world. In England, respiratory disease affects one in 5 people, and costs the UK NHS an estimated £11BN annually (NHS England, 2024.). Part of this burden is caused by respiratory viruses such as IAV and we need to reduce the burden of this disease. One way to reduce the burden of respiratory viral infections is with vaccines. We have previously discussed the limited efficacy of the current IAV vaccines, but there has been promising progress made towards the development of vaccines against influenza, RSV, and coronaviruses (Priya Venkatesan, 2023). This is in large part thanks to the success of mRNA vaccines which showed striking ability to reduce the hospitalisation rates of those infected with SARS-CoV-2 during the 2019

pandemic (Whitaker et al., 2023). Vaccination is an excellent way to slow the spread of infection, but vaccines are pathogen specific therapies. There is a growing desire to design therapies that can broadly protect against many respiratory pathogens, by treating the symptoms common to multiple lung diseases. This could by development of new vaccines that aren't restricted to one pathogen – there is current research into vaccines that could protect against all influenza viruses (Xiong et al., 2023), or all herpesviruses (Tan et al., 2023) and with vaccines that are broadly protective against families of viruses by inducing T cells (Sarah Gilbert, 2012, Xing et al., 2023). Vaccines are an inexpensive, effective, and low-risk way to prevent infection but there is a growing desire to add different therapies to the repertoire of respiratory therapeutics. One way to do this is to administer antibodies, rather than to rely on the vaccinated individual producing their own. Monoclonal antibodies have been shown to be effective in treating conditions such as COPD and asthma, with the greatest success using IL-4 blocking therapies (Plichta et al., 2023). Our results show that activation of basal cells relies on signalling through IL-10, and we suggest that this is part of a signal from the anti-inflammatory immune response to begin proliferation.

Though we have a strong repertoire of vaccines and anti-virals designed to prevent death from respiratory viruses, treatments for those with fibrotic lung diseases are limited. Since anti-fibrotic drugs such as pirfenidone are deigned to slow rather than cure fibrosis by reducing collagen production, corticosteroids are still regularly used to treat conditions like COPD and IPF (Liu et al., 2022, American Lung Association, 2024.). At Guy's and St Thomas' hospital an experimental technique was used to block vagus nerve activity in the lung of a patient with severe COPD to attempt to reduce tightness of the airway and reduce mucus production (NHS England, 2024). This is an impressive translational therapy and was a great success for the patient, but it highlights the extremes we go to in attempting to treat severe lung fibrosis. Fibrosis is caused by improper repair of damage, and our work further highlights that the repair process can be manipulated. We often think of fibroblasts and macrophages as being the cells most associated with tissue remodelling, but here we suggest that basal cells are capable of "pitching in" on the repair process. Basal cell

sources of IL-10 and *fgf10* may support the function of other tissue remodelling cells. For example, basal cell produced IL-10 could confer an anti-inflammatory phenotype on a monocyte derived macrophage, preventing that monocyte from becoming inflammatory. We know that we can intervene with immunological therapies, but if we learn how (and more importantly when) to intervene during the damage that can lead to dysplastic repair, we could prevent fibrotic repair before it happens.

6.6 The future of basal cell research

I have been fortunate to be able to draw on extensive research on progenitors in the lung which has allowed me to ask targeted questions during this project. Much of the published work studying basal cells has been done to understand the development of the early lung, and to unpick the variety of progenitor cells the lung can generate. This is interesting and important research, particularly considering that the goal of some of that research is the generation of transplantable lung tissue (Demchenko et al., 2022, Tsuchiya et al., 2014). However, research into the behaviour of epithelial progenitors during infection is still limited. I believe that this project has addressed a small part of this field, by showing that basal cells are activated during IAV, and that this activation relies on IL-10. However, I haven't shown any evidence of the source of the IL-10 basal cells react to. Nor have we shown that the loss in basal cell activation found by blocking IL-10 is a result of direct action on basal cells, or whether we are blocking another cell type which then acts on basal cells. We have shown that basal cells produce IL-10, and we infer that this is evidence it is a direct action, but to show this more clearly, a basal cell only inducible IL-10 knockout could provide a more concrete answer.

If the goal is to manipulate the balance between damage and repair, the consequences of getting the timing wrong could be disastrous. The main question I feel that is still unanswered in this field is how basal cells that survive direct influenza virus infection are changed by that challenge. Is there an

imprinting on basal cells that experience an infection that changes their response to future challenges? My hypothesis would be that a basal cell that has survived an IAV infection would be better prepared to respond to that infection in the future, but this project has not addressed this. It is also possible that the tissue that is repaired by a basal cell that has survived infection may be composed of different cell types, and therefore differently prepared for future challenge. If directly infected basal cells are more productively stressed, those basal cells may differentiate into the cell type that is the least complex in order to carry out emergency repair. This may be the reason that others have shown that viruses skew basal cells to a certain cell type. I would like to interrogate this by assessing differences in tissue repaired by basal cells that were directly infected by IAV and tissue repaired by uninfected basal cells.

Our work interrogated the transcriptional changes in basal cells directly infected by IAV, but as previously discussed the sequencing methods used had their own limitations. One experiment I would like to do is to carry out bulk sequencing on IAV+ and IAV- basal cells, to compare their transcriptome. Our current analysis shows some difference in the transcriptome of directly infected cells, but there are very few basal cells in our analysis, with even fewer directly infected. I would expect IAV to change the behaviour of basal cells it infects, and I would predict IAV would hamper their repair capabilities. I have made this comparison using published single cell data sets, but for the reasons discussed above, the number of cells and sequencing depth of those experiments is limited. Carrying out this experiment with bulk RNA-seq would provide a clearer answer, since the sequencing available in bulk RNA-seq experiments is deeper and much more affordable. The reason this wasn't carried out so far is that only around 3% of basal cells are infected at peak timepoint, and to sort enough IAV+ basal cells even with our current digestion method would require a large number of experimental animals.

6.7 Final Summary

The hygiene hypothesis is a strongly debated immunological phenomenon, with some suggesting that regular exposure to a wide variety of pathogens is essential for the development of a well-balanced immune system (Pfefferle et al., 2021, David P Strachan, 1989). A core theme in this work has been that experiencing infection isn't always a beneficial training exercise, but that it can cause lasting changes to tissues. It isn't clear yet whether IAV infection has a strictly beneficial or detrimental effect on the long-term health of the lung, but it is clear that the post-IAV lung is different. Does subsequent exposure to viral infections change the structure of our lung, making it less effective at its role in gas exchange? Is the constant exposure to seasonal respiratory viruses slowly degrading the ability of our lung to function, leading to fibrotic and chronic inflammatory lung conditions? We have attempted to do work that can be built on to answer some of these questions, but also, we have suggested that this is a different lens through which to look at infection. Our work suggests that there is another consideration to be made when calculating the burden of respiratory infections. It's not simply the cost of the vaccine or respiratory, but also the cost (social and economic) of a person walking out of hospital with reduced lung function.

6.8 Graphical Abstract

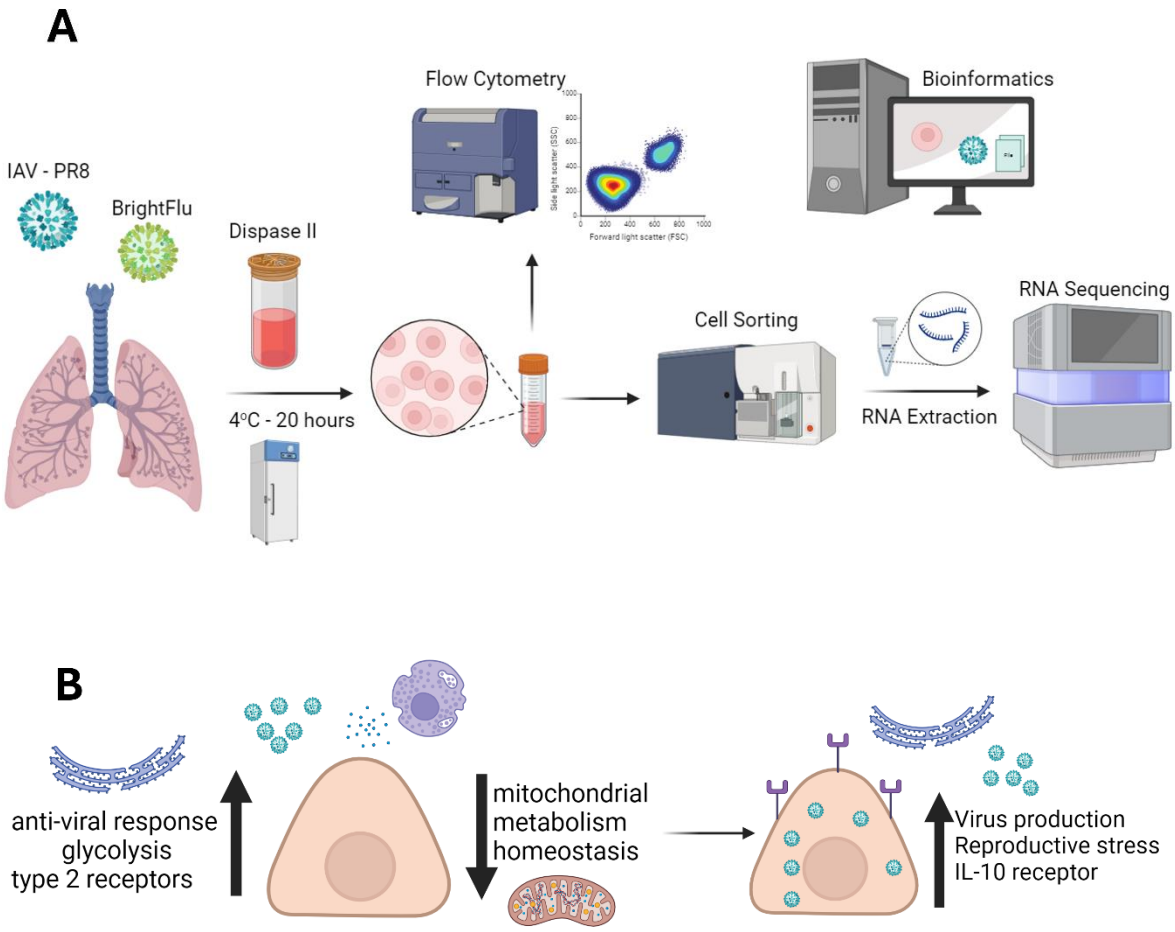


Figure 6-1 Graphical Abstract

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