Role of Interleukin-10 in Lung Repair During Influenza Infection

by

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Abstract

The mammalian lung is intricately designed for effective gas exchange. However, infections such as influenza can damage the lung tissue and can cause a critical decrease in lung performance.

Influenza is a single stranded RNA virus from the Orthomyxoviridae family. Seasonal influenza outbreaks cause up to 600 deaths in Canada each year, with increased death tolls during years with pandemic strains. Influenza A virus primarily infects the epithelial cells of the respiratory system and leads to acute respiratory infections.

Tissue destruction occurs both as a result of viral-mediated apoptotic pathways, and as a consequence of the cytolytic immune response that is essential to bring the infection under control. The immune system attempts to minimize damage by producing key anti-inflammatory molecules, of which Interleukin-10 (IL-10) is a particularly potent example.

IL-10 is a pleotropic cytokine that not only functions as an anti-inflammatory cytokine, but has been shown to have a role in immune stimulation, increase cytoskeletal repair and aid in the recovery of epithelial integrity. We hypothesized that IL-10 may have an effect on the remodeling and repair of lung epithelium during and after influenza A virus infection. We determined that IL-10 is up-regulated in response to infection. The timing of increased IL-10 also coincided with the height of damage, inflammation and onset of repair during an influenza infection. We used a human bronchial epithelial cell line (16HBE) to develop a model to assess if IL-10 had a direct effect on epithelial repair. Preliminary data suggested that IL-10 may inhibit epithelial cell proliferation, but further research is required.

Our data suggest that IL-10 does not appear to have a role in direct epithelial proliferation but could still have a role in repair. A better understanding of the signals involved in epithelial cell repair could lead to the development of novel therapies that will minimize/mitigate lung damage caused by influenza, as well as other lung diseases.

Preface

For this project I was responsible for experimental design, execution and data analysis for all experiments under the supervision of Dr. Georgia Perona-Wright. Histological preparation was performed by Ingrid Barta from the Histochemistry Service Laboratory at the University of British Columbia Biomedical Research Centre. Histology slides were analyzed with the help of Dr. Ian Welch at the University of British Columbia Centre for Comparative Medicine.
The 16HBE cells line was gift from Dr. Tillie Hackett.

This project required animal ethics approval for the murine influenza model. All experiments were performed in accordance with recommendations from the Canadian council for Animal Care. The Animal Care Committee of the University of British Columbia approved all protocols (A16-0096). Biosafety protocols were approved by the University of British Columbia Biosafety Committee (B16-0018).

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AT I	Alveolar type I
AT II	Alveolar type II
BAL	Bronchial alveolar lavage
BCA	Bicinchoninic acid
CBA	Cytometric bead array
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
EdU	Ethynyl deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
HA	Hemagglutinin
H&E	Hematoxylin and Eosin
IL	Interleukin
IL-10R	Interleukin 10 receptor
IAV	Influenza A virus
IFNγ	Interferon gamma
MCP-1	Macrophage chemoattractant protein 1
NK	Natural killer
PBS	Phosphate buffered Saline
PFA	Paraformaldehyde
PFU	Plaque forming units

PI	Post infection
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SA	Serum albumin
TNF-α	Tumour necrosis factor alpha

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I'd like to start by acknowledging my supervisor and lab mates. Graduate school is made up of many lows and highs. The support, feedback, wealth of knowledge and laughter that I received and shared with Dr. Georgia Perona-Wright, Dr. Stephen Redpath, Nicolette Fonseca and Graham Heieis was paramount to my completion of this degree and helping me stay positive when nothing seemed to be going right. Thank you all for always having my back and being a great lab family.

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Darlene Birkenhead also deserves a huge thank you. I often refer to Darlene as our departmental Wonder Woman. She has helped me with many scholarships, admin paperwork, general advice and has been a kind and supportive listener throughout my time at UBC.

I would like to thank my family for all their support as well. My parents have always stressed the importance of a good education and taught me to run with the opportunities I have been given. They often had no idea what I was talking about when I called them to vent about a failed experiment or problems with an assay, however, they always sat there patiently and let me ramble, while reminding me that it was not the end of the world.

Finally, I would like to thank all of my friends, both old and new. I have received so much support and love from them and it means the world to me. In particular, there are 3 people I would like to give thanks to and who have seen me through lots of laughter and tears in the last few years. Jake Bartsch has been my confidant and best friend for years. He has spent countless hours listening to my rambling about everything under the sun and I wouldn't have made it through graduate school without his support, advice and positive thinking. I would also like to thank Israel Matos. He was one of the first friends I made here in Vancouver and has been there to laugh with me, laugh at me, and to help pick me up and remind me that everything will be okay when I felt lost with worries. Finally, I would like to say thank you to Carlos Diaz-Salazar. His company has helped make late nights at the lab not so bad. He has been there for so many of the smiles and always offered a shoulder and brownies when the tears came.

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Chapter 1: Introduction

1.1 Lung Epithelium

1.1.1 Primary mechanisms of lung protection

The lung is an organ intricately designed to facilitate gas exchange. Large quantities of air are moved in and out of the lung continually and this constant exposure to the outside world puts the lungs at significant risk of infection by pathogens. This high risk area protects itself through multiple layers of defense, such as innate and adaptive immunity, secreted surfactants, and an epithelial barrier.

The lung epithelium acts as a physical barrier between incoming particulates and the blood stream. The cell types found throughout the respiratory tract epithelium undertake a variety of roles that help protect the lungs from infection.

The upper airways are comprised of the nasal passage, the pharynx and the larynx. These sites have the first contact with the outside environment and play a key role in keeping the lower airways clear of harmful debris and pathogens.

The lower airway is important in facilitating the exchange of gases into the blood. It consists of the trachea, bronchi, bronchioles, and alveolar sacs. Collectively these areas form a pulmonary tree-like structure and each site contains specific cell types that serve different functions. In humans, all of the lower airways, excluding the alveolar sacs, are comprised of a pseudostratified epithelium (1). The pseudostratified layer is comprised of ciliated columnar cells, non-ciliated columnar cells, club cells, norendocrine cells, goblet cells and basal cells (1). In the murine airways, only the trachea and main bronchi are pseudostratified and the remaining lower airways are composed of simple columnar and cuboidal cells (1). These many cell types

play an important role in keeping particulate matter and pathogens out of the delicate alveolar sacs.

Ciliated cells form a ciliated elevator that helps move pathogens and other contaminants towards the trachea and out of the lung (2). To facilitate this action, a mucus is secreted from various cells, such as goblet cells. The mucus contains Immunoglobulin A antibodies, lysozyme, lactoferrin and peroxidases, phospholipases and a variety of other proteins (3). The consistency and contents of the mucus act to trap foreign particulates, enhance removal, and act as an extra layer of protection for the epithelial cells. Club cells, which are non-ciliated, non-mucus secreting cells are found in the lower airways as well. Club cells also protect the airway by secreting proteins that aid in detoxifying damaging substances that may enter the lung (4).

In the lower airways within the alveolar sacs, alveolar type I (ATI) and alveolar type 2 (ATII) cells can be found. ATI cells are long flat cells that cover approximately 90-95% of the alveolar surface and are the primary site for gas exchange (5). ATII cells are small cuboidal cells that are also found in the alveolar sacs and are capable of differentiating into ATI cells, making them important in repair after injury.

There are many different types of cells present in the lung epithelium and each type has an important role in lung function and protection from potential risks associated with the outside world.

1.1.2 Lung epithelial cell type differentiation and regeneration

Under homeostatic conditions the lung epithelial layer is relatively stable and has a low turnover rate with less than 2% of epithelial cells proliferating at any given time in both humans and rodents (6-8). Although the rate of proliferation under homeostatic conditions is relatively

low, following an injury to the epithelium, proliferation is increased dramatically in order to repair the delicate tissue (6).

Understanding the differentiation patterns of the different cell types in the lungs is critical in understanding how the lungs are able to remodel and heal after injury or infection. Many lineage-tracing studies have been completed in order to determine the differentiation and regenerative patterns for the different cell types found in the different parts of the pulmonary tract. In the pseudostratified layers of the trachea and main bronchi, basal cells have been shown to give rise to club cells, non-ciliated columnar cells and ciliated columnar cells (9). Club cells have been shown to be self-renewing after injury or ablation and are further capable of giving rise to ciliated cells (10).

When damage to the alveolar epithelium occurs, it can lead to decreased gas exchange and increased risk of secondary infections. When damage occurs here, ATII cells are able to begin proliferating and differentiating into ATI type cells and replace the damaged epithelium (11). They are also able to self-renew to replenish their own population (12).

Recently, a new stem cell population in the lungs has been described that was observed to play a role in lung repair of the alveolar sacs following injury from an influenza infection (13,14). This stem cell population was shown to migrate to the alveoli and help replenish ATI cells, ATII cells and bronchial secretory cells.

The epithelial barrier plays an important role in gas exchange and protection against infection. When damage occurs to this barrier it must be repaired quickly. Progenitor cells and stem cells are capable of proliferation and differentiation into specific cell types that make up the lung epithelium and allow for rapid repair.

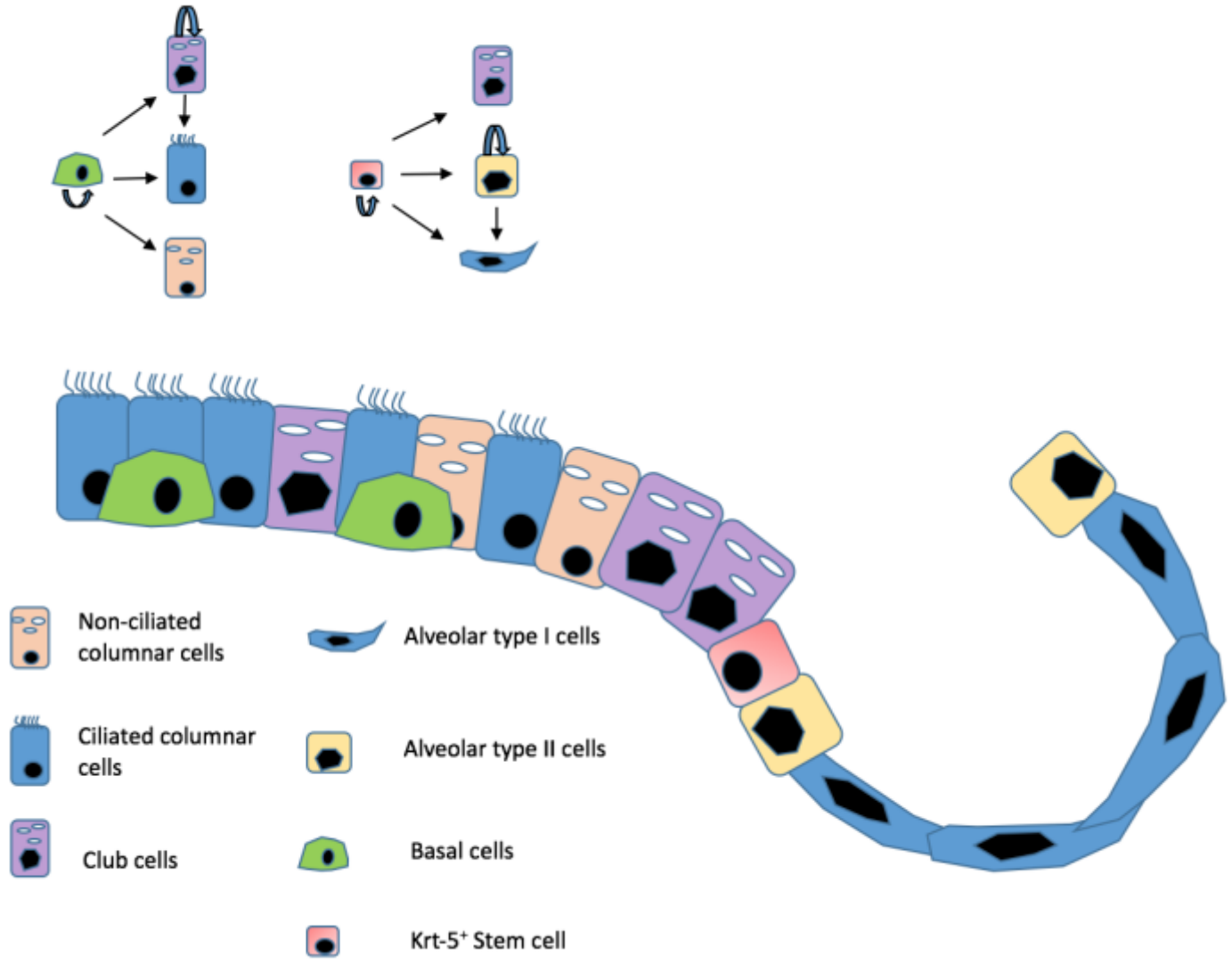


Figure 1-1 Lung epithelial cell differentiation

Many of the major lung epithelial cells have multiple pathways for population regeneration after damage or injury. Basal cells, club cells and ATII cells are all capable of self-renewal. Basal cells are able to give rise to many different epithelial cells within the bronchi and trachea and are found throughout. ATII cells are capable of differentiating into ATI cells and are positioned at the edges of the alveolar sacs. A new type of stem cell has recently been found and is capable of differentiating into club cells, ATII cells and ATI cells in times of injury.

1.2 Influenza A Virus

1.2.1 Pathology and disease

Influenza A virus is an airborne virus that causes severe respiratory infection (15). Influenza is a major health problem in Canada and internationally, with annual outbreaks that result in up to 8,000 hospitalizations and as many as 600 deaths in Canada each year (16) Through rapid replication and budding, the virus is able to spread quickly from cell to cell within the lungs. This form of infection leads to the activation of both the innate and adaptive arms of the immune system, which co-ordinate to reduce viral replication (17).

Damage to the lung epithelium can occur because of two main factors, the influenza virus and the immune response. The viral genome contains instructions for proteins, such as NS1 and PB1-F2, which are capable of manipulating intrinsic and extrinsic epithelial apoptotic pathways and disrupting epithelial tight junction integrity (18-20).

Immune cells such as cytotoxic T cells and NK cells also damage the epithelial barrier through cytotoxic cell killing of infected cells. In addition, exuberant production of pro-inflammatory cytokines by both innate and adaptive immune cells can be toxic to epithelial cells (21).

1.2.2 Influenza A virus and the immune response

The initiation and regulation of an appropriate immune response requires the coordinated action of multiple immune and non-immune cell types. One key mechanism of communication between these diverse cell populations is the use of secreted signaling molecules called cytokines. Specific cytokines are known to be involved in immune responses to different types of pathogens. Influenza is an intracellular viral infection and promotes a "Th1 type response". This

response is pro-inflammatory and is characterized by high expression of the cytokine, interferon gamma (IFNγ) (22). IFNγ is a cytokine that plays a key role in mediating innate and adaptive immunity. It is important in activating macrophages and upregulating major histocompatibility complex II molecules on antigen presenting cells (23).

Influenza A virus enters and infects epithelial cells in the upper and lower respiratory tract. Influenza is a single stranded RNA virus. Therefore, once in the cytoplasm of infected cells, it stimulates pattern recognition receptors such as Toll-like receptors, retinoic acid-inducible gene-I-like receptors and Nod-like receptors on and within the epithelial cells (24,25). Stimulation of these receptors causes epithelial cells to up-regulate major histocompatibility complex class one proteins and secrete Type I interferons and other cytokines to initiate the antiviral response.

Resident macrophages are able to capture virus before it even enters epithelial cells. These macrophages then secrete pro-inflammatory cytokines and chemokines, in order to recruit more immune cell backup. Viral antigen can also be displayed on major histocompatibility complex II molecules, to be presented to T cells later in the response. Other innate immune cells, such as neutrophils, dendritic cells (DC) and monocytes are then recruited to the site of infection to assist in the restriction of viral replication (26). Once present at the site of infection, neutrophils and DCs phagocytose viral particles. DCs process these particles and travel to the lymph nodes where they display the viral antigens via the major histocompatibility class I and class II molecules to T cells. T cells undergo activation and expansion in the lymph node and migrate to the site of infection.

B cells play an important role in helping to clear the infection. They are able to secrete neutralizing antibodies that can bind directly to the Hemagglutinin (HA) protein portion of viral

particles (27). HA is the main protein involved in cell entry for the virus. Neutralizing antibodies help reduce infectivity of extracellular viral particles and allows them to be phagocytosed and destroyed by other immune cells such as macrophages. B cells are also able to assist in antibody-dependent cell-mediated cytotoxicity (28). Antibodies are secreted and bind to viral particles expressed on the surface of infected cells. This allows cytotoxic effector cells to easily recognize infected cells and kill them.

T effector cells are also very important for viral clearance. T cells play many roles in the clearance of IAV. T cells become activated and proliferate in the draining lymph node after they have been presented viral antigen by antigen presenting cells, usually DCs. They then migrate to the area of infection. T cells are able to both kill infected cells as well as produce many important cytokines used for inflammation, inflammatory attenuation, T cell proliferation, cell survival, and differentiation (29). CD8+ T cells are able to kill infected cells that express influenza specific antigen on MHC I molecules on their surface. CD4+ T cells secrete cytokines that increase the proliferation of T cells and B cells, as well as direct the CD8+ T cell response and cause B cells to secrete neutralizing antibodies (30).

Viral clearance is a complex process that involves many cell types and cross communication between these cells. Both the innate and adaptive responses are necessary for clearance of Influenza. Although the immune response is essential, it also can lead to host damage and therefore must be tightly regulated.

1.3 Importance of Interleukin 10

1.3.1 Interleukin 10 as an immune suppressant

IL-10 is a potent immunosuppressive cytokine that is important in maintaining balance during an immune response. Mice deficient in IL-10 expression die of inflammation in the lungs shortly after being infected with influenza (31, 32). Under steady-state conditions, IL-10 is secreted by alveolar macrophages, interstitial dendritic cells and lung epithelial cells into the airways of the lungs to maintain tolerance to environmental antigens and irritants (31, 32). During a viral infection IL-10 is upregulated to help control the inflammatory response. During acute influenza infection, CD4+ and CD8+ cells are the primary producers of IL-10 (33).

1.3.2 IL-10 receptor expression and incorporation

IL-10 signals to its target cells through the IL-10 receptor, which is made up of two molecules each of an alpha and a beta chain (34). The beta chain is incorporated into the IL-10 receptor but is also as part of the receptor for IL-22, IL-26 and IL-28 (35). The alpha chain portion of the IL-10 receptor is only known at present to be incorporated into the receptor for IL-10. IL-10 is a critical cytokine in immune-regulation and the IL-10 receptors are expressed on many cells throughout the body, but there is some debate as to whether or not it is expressed on lung epithelium (36, 37). Our lab has observed IL-10R expression on epithelial cells of C57BL/6 mice (unpublished).

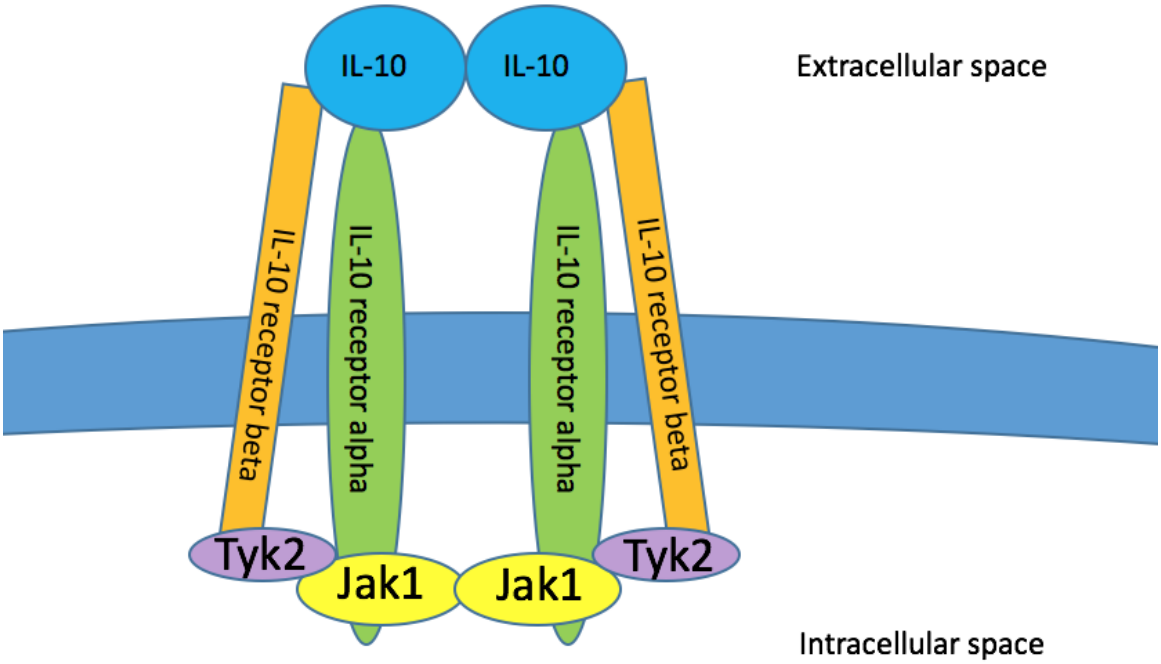


Figure 1-2 Interleukin 10 receptor

The IL-10 receptor is a multi-subunit receptor, consisting of IL-10 receptor alpha chains and IL-10 receptor beta chains. IL-10 receptor alpha chain is only known to be incorporated into the IL-10 receptor, however the beta chains are incorporated into other receptors such as the receptor for IL-22.

1.3.3 IL-10 as a pleotropic cytokine

IL-10 is frequently regarded as an immunosuppressant, but it is actually a pleotropic cytokine with differing function depending on the environment. IL-10 has been shown to have chemoattractant effects for CD8+ cytotoxic T cells and up-regulates E-selectin on endothelial cells (38, 39). IL-10 has been shown to stimulate natural killer (NK) cells to enhance cytotoxic

function (40,41). This stimulating effect on NK cells has been suggested to help decrease tumorigenesis and metastasis (41). IL-10 has also been examined in the context of wound healing. A study by Kieran et al., found that IL-10 was able to reduce scarring and decrease healing time for cutaneous wounds in rats, mice and humans (42). The authors suggested that this was due to both to reduced inflammation at the wounded site, as well as the ability of IL-10 to attenuate fibrosis (43,44).

Obtaining viable lungs for transplantation is a problem in modern healthcare practices. In a study by Cypel *et al.*, damaged human lungs from organ donors were extracted from their original hosts and kept alive using an *ex vivo* lung perfusion method. Ten sets of lungs were used. All lungs underwent the *ex vivo* lung perfusion. Five sets of lungs were not treated with the IL-10 gene therapy and 5 lungs were treated with the IL-10 gene therapy for 12 hours. After 12 hours, Cypel and his colleagues observed reduced inflammation, increased cytoskeletal repair, improved lung function and recovery of alveolar epithelial integrity in the IL-10 gene therapy treated lungs compared to untreated controls (45). Given these studies and the known pleotropic nature of IL-10, it is possible that anti-inflammatory molecules, such as IL-10, can also target cells outside the immune system, including epithelial cells.

1.4 Lung Repair During an Influenza Infection

1.4.1 Repair

As mentioned in section 1.1, damage to the epithelial layer can happen in two main ways during an influenza infection: via virus-mediated pathways or by way of the immune system. In both cases, repair must occur rapidly in order to reduce morbidity and mortality. Repair signals

are tightly regulated and vary depending on the type of damage, where the damage occurs and additional variables such as acute or chronic exposure to inflammation.

1.4.2 Key cytokines involved in lung epithelial proliferation and repair

Several cytokines have already been identified as playing a role in the repair process of lung epithelium, including Interleukin-22 (IL-22), epidermal growth factor (EGF) and amphiregulin.

IL-22 has been shown to induce both proliferation and anti-apoptotic pathways in pulmonary epithelial cells (46). IL-22 is secreted by NK cells and multiple subsets of T helper cells (47). IL-22 is known to act on epithelial cells in the lung and gut, which express the IL-22 receptor (35). The IL-22 receptor is a multi-subunit receptor that is comprised of the IL-10R beta chain and the IL-22 receptor 1 chain.

EGF is another cytokine that is known to promote epithelial repair. EGF has been shown to stimulate proliferation of alveolar type II cells *in vitro* (48). The EGF receptor (EGFR) is expressed on the epithelium at low levels, and after damage the production of EGF has been shown to increase the expression of both EGF and EGFR, initiating a positive feedback loop (49). Sources of EGF include epithelial cells throughout the airways, as well as peribronchiolar interstitial cells and endothelial cells in the blood vessels (49).

In addition to IL-22 and EGF, a lesser-known cytokine, amphiregulin, has recently also been suggested to play a role in the repair of lung tissue after an influenza infection (50). Amphiregulin is secreted by innate lymphoid cells in the lung and, like EGF, it is a ligand of the EGFR. Amphiregulin was shown to restore lung function and promote tissue remodeling after an influenza infection (50).

New functions for different cytokines are constantly being discovered. It is possible that additional cytokines play important roles in epithelial repair but have yet to be explored. The focus of my thesis was to assess whether IL-10 also influences epithelial repair.

1.5 Objectives and Hypothesis

Damage caused during an IAV infection, by the immune system and the virus, can lead to loss of epithelial barrier integrity and consequent secondary infection or decrease in lung function, resulting in morbidity and mortality. Lung repair is essential for survival during and after an influenza infection. Several cytokines necessary for epithelial repair have already been observed but it is likely that others also contribute. IL-10 is a pleotropic cytokine that is known to reduce inflammation and has been seen to improve epithelial barrier integrity after damage (46). I am interested in examining the impact of IL-10 in epithelial repair during an influenza infection. **I hypothesize that the immune signal IL-10 promotes the remodeling and repair of lung epithelium during and after infection with influenza A virus.** The objective of my thesis was to assess the direct impact of IL-10 and related cytokines on lung epithelial tissue in regards to damage and repair during influenza infection.

Many molecules have been examined for a potential role in lung repair, such as IL-22, EGF, and several microRNAs (46, 49, 51), but a role for IL-10 in lung repair has not been thoroughly studied. To test my hypothesis, it was first necessary to establish a working model that allowed me to determine the timeline of damage, inflammation and repair during an IAV infection, and to assess whether IL-10 protein expression coincided with any of these processes. Having established a correlation between IL-10 expression and epithelial damage and repair, I next assessed whether IL-10 had a direct impact on epithelial cell proliferation. Understanding

the impact and role that IL-10 signals have on infection-related damage and lung tissue repair could facilitate the design of future therapies that better balance viral clearance and lung repair during acute influenza infection.

Chapter 2: Methods

2.1 *In Vivo* Influenza Infection Model

2.1.1 Mouse strains and housing conditions

Mice were housed at the University of British Columbia (UBC) Centre for Disease Modeling. C57BL/6 mice used were sex-matched and aged-matched within each experiment, and were used at between 6-10 weeks for all experiments. Experimental protocols were approved by the UBC Animal Care Committee and Biosafety Committee. Mice were kept on a 12-hour day/night cycle and were fed standard lab chow. Dietgel recovery food was provided to all mice on the cage floor for the first 7 days post infection.

2.1.2 Infection conditions

Mice were deeply anesthetized with isoflurane and infected intranasally with 10 plaque-forming units of Influenza Virus A/PR/8/34 (H1N1) in 20µl of sterile PBS. Naïve mice were mock-infected and given 20µl of sterile PBS. Virus was grown in Dr. David Woodland's laboratory at the Trudeau Institute, NY, frozen and transported to UBC under PHAC import permit # N-12-0214. Stock was titrated in the Perona-Wright laboratory at UBC using plaque assays in MDCK cells.

2.1.3 Protein quantification

BAL fluid was collected in 0.5mL of sterile PBS and stored at -80°C. Total protein concentration in BAL fluid was assessed using the Pierce BCA Protein Assay Kit, according to the manufacturer's protocol. Samples were run on a VictorX5 2030 multilabel reader at

wavelength 560nm. Serum albumin concentration in the BAL fluid was assessed using Mouse Albumin Elisa Quantification Set, using the manufacturer's protocol with slight modifications as follows: PBS with 10% newborn calf serum was used as the blocking solution and as sample diluent, and PBS with 0.05%Tween20 was used as wash buffer. Samples were run on a BioRad iMark Microplate reader at wavelength 450nm, using BioRad microplate manager software. The protein concentrations of the inflammatory cytokines TNF-α, IFNγ, MCP-1 and IL-6, and IL-10 protein concentrations, in BAL fluid were assessed using the BD Bioscience Cytometric Bead Array Mouse Inflammation kit. Samples were run on a BD Bioscience FACS Canto and analyzed using FlowJo software. Geometric means were assessed and plotted using GraphPad Prism.

2.1.4 Quantitative RT PCR

Lung tissue was harvested and stored in RNAlater RNA stabilization reagent (Qiagen) at 4°C, before being homogenized in lysis buffer using TissueLyser LT (Qiagen) for 3-5 minutes, and then centrifuged at 13 000 rpm for 5 minutes. and centrifuged. Supernatant was collected and RNA was extracted following the manufacturer's instructions for the RNeasy Mini Kit (Quiagen). cDNA was prepared using the iScript cDNA Synthesis Kit (Bio-Rad). RT-qPCR samples were prepared using Bio-Rad SsoFast EverGreen Supermix and run on Bio-Rad CFX96 Real Time System. The primers used were as follows:

5'- GTGTTCCTACCCCCAATGTG-3' (GAPDH forward),

5'-ATTGTCATACCAGGAAATGA-3' (GAPDH reverse),

5'-ATCGATTTCTCCCCTGTGAA-3' (IL-10 forward),

5'-TTCGGAGAGAGGTACAAAC-3' (IL-10 reverse),

5’-GTAGAGAGGAAGGTGTCTGGG-3’ (EGF forward),

5’-GGATCCACTGCTATATTTGACGG-3’ (EGF reverse),

5’-GCCTTAGCCATCCTGTCCAA-3’ (EGFR forward),

5′-TGCTCGGATGGCTCTGTAAG -3’(EGFR reverse),

5’-GCAGATACATCGAGAACCTGG-3’ (amphiregulin forward),

5’-CGAGGATGATGGCAGAGACA-3’ (amphiregulin reverse).

2.1.5 Histology

Lungs were insufflated using 4% paraformaldehyde and harvested whole. Lungs were stored in 4%PFA for two days and then switched to 70% Ethanol. Lung lobes were embedded in paraffin blocks and sections from each lobe were taken 200-500µm apart. Slides were stained with hematoxylin and eosin. Samples were embedded, sectioned and stained by Ingrid Barta at the Histochemistry Service Laboratory at the University of British Columbia Biomedical Research Centre.

2.2 In Vitro Model and Assays

2.2.1 Cell line

Cell line, 16HBE (Human Bronchial Epithelial cells), were generously gifted from Dr. Tillie Hackett (Centre of Heart Lung Innovation, Vancouver, BC, Canada) and Dr. Bob Hancock (University of British Columbia, Vancouver, BC, Canada). This cell line was originally isolated and transformed using a Simian Vacuolating Virus 40 plasmid by Dr. D.C. Gruenert (University of California, San Francisco, California, USA) (52), and has been used to study forms of lung disease, damage and repair such as those found in cystic fibrosis and chronic obstructive

pulmonary disease and asthma (52-54). Cells were cultured Dulbecco's Modified Eagle Medium (DMEM) with 10% FCS and 1% Penicillin/Streptomycin antibiotics at 37 degrees Celsius with 5% carbon dioxide.

2.2.2 Cell line wounding and stimulation

Cells were plated in 6 well tissue culture plates in DMEM (10% FCS and 1% Penstrep) and grown until 95-100% confluence. Cells were serum starved in DMEM (0.02% FCS and 1% Penstrep) for 18-24 hours and then switched back to media with 10% FCS and left to rest for 1 hour at 37°C. Six wounds were made using a 200µl pipette tip and cells were stimulated with or without recombinant IL-10 (25ng/mL) or IL-22 (10ng/mL) and left to incubate for the indicated amount of time at 37°C with 5% CO_2.

2.2.3 Quantitative RT-PCR

Cells were scraped from 6 well plates and collected in lysis buffer. Cells were mechanically lysed using Qiashredder columns (Qiagen). RNA was then extracted by following the manufacturer's instructions for RNeasy Mini Kit (Quiagen). cDNA was prepared using iScript cDNA synthesis Kit (Bio-Rad). RT-qPCR samples were prepared using Bio-Rad SsoFast EverGreen Supermix and run on Bio-Rad CFX96 Real Time System. IL-10 receptor alpha and beta expression was assessed using the following primers:

5'- GCGAATGACACATATGAAAGCATC -3' (human IL-10Rα forward),

5'- GAACGTGAAGTTTCCCGGC -3' (human IL-10Rα reverse),

5'-TGACTTTCACAGCTCAGTACCT-3' (human IL-10Rβ forward),

5'-AGGTGATGTTTACCCAGTCTGA-3' (human IL-10Rβ reverse),

5’-CGAAAGCATCTTGAGAGGAACA-3’ (human RPS13 forward),

5’-TCGAGCCAAACGGTGAATC-3’ (human RPS13 reverse).

RNA from LPS activated macrophages was included as a positive control in experiments measuring expression of the alpha chain. A non-template control was also used in the PCR reaction, using water instead of any cDNA input.

2.2.4 Proliferation assay

Cells were grown, wounded and stimulated as described in section 2.2.1 and 2.2.2. For EdU assays, EdU was added to the media to a concentration of 10μM with or without cytokine stimulants. After 24 hours the media and cytokine stimulant was removed and the cells were washed in sterile PBS. 0.25% Trypsin 1mM EDTA solution was added to remove the cells from the flask. The cells were then transferred to a 5mL facs tube, washed in media, centrifuged for 5 minutes and supernatant was aspirated. Cells were stained with eBioscience Fixable Viability Dye eFluor® 506. They were then fixed, permeablized and stained using Click-iT® Plus EdU Alexa Fluor® 647 Flow Cytometry Assay Kit or BD Pharmigen® PE Mouse Anti-Human Ki-67 set. Manufacturer’s protocols were followed. Samples were run on BD FACS Canto and analyzed using FlowJo software.

Chapter 3: Results

3.1 Assessing Lung Damage, Inflammation and Repair in an Influenza PR8 Infection

3.1.1 Assessing lung damage

The overall hypothesis of my studies was that the immune signal IL-10 affects the remodeling and repair of lung epithelium during and after infection with influenza A virus. My first aim was to establish an infection model that allowed me to determine the timeline of damage, inflammation and repair during an IAV infection, and to assess whether IL-10 protein expression coincided with any of these processes. My first measure of pathology and disease severity was an assessment of weight loss during infection. C57BL/6 mice were intranasally infected with Influenza A Virus (IAV) PR8 at 10 PFU per mouse and weight changes were recorded as a percentage of each individual's starting weight. The mean weight of the naïve animals remained relatively constant throughout the duration of the experiment with mice remaining at or above their original weight. Weight changes among the infected animals varied but a peak average weight loss was seen at day 7 in both independent experiments. After day 7 post infection (pi) weight began to increase and mice returned to their original weight by day 11 pi (Figure 3-1).

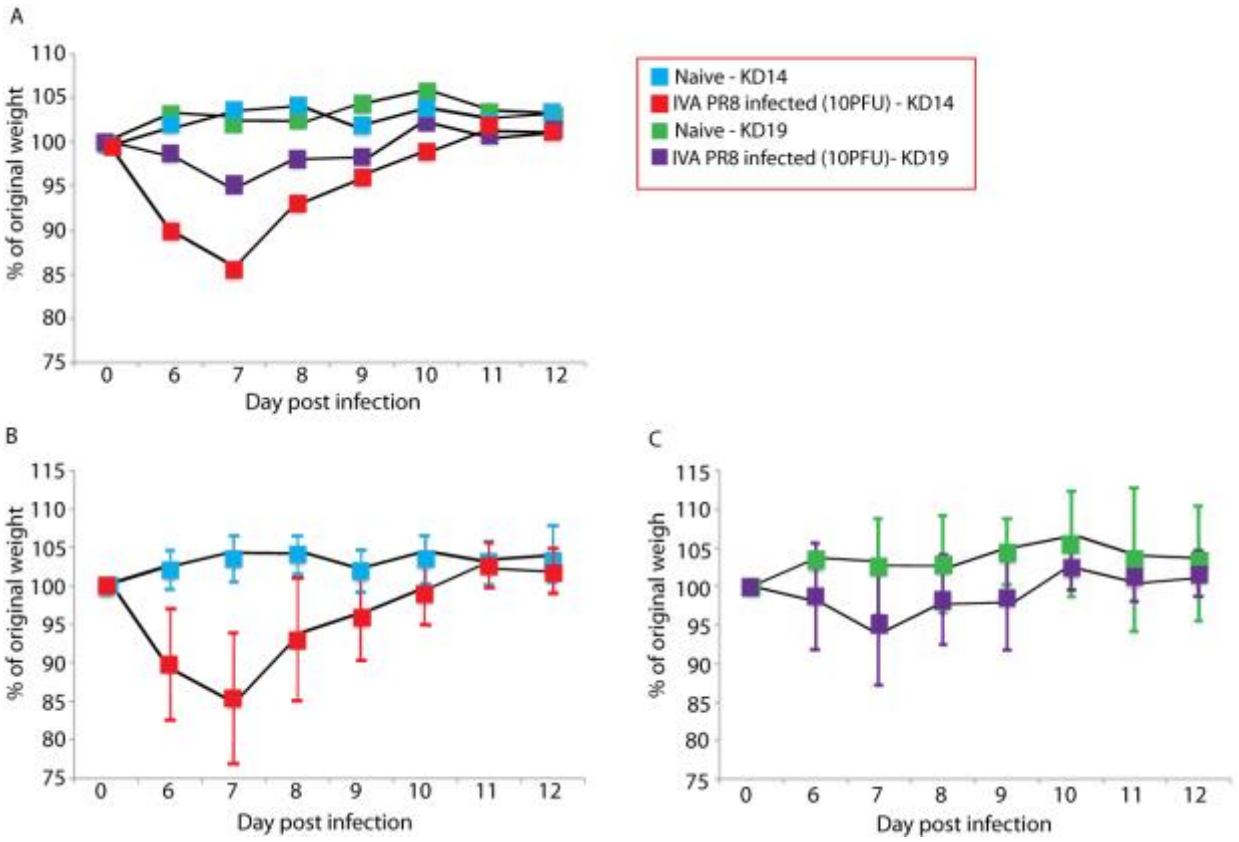


Figure 3-1 Maximum weight loss occurs at day 7 after infection with IAV PR8

Mice were intranasally infected with 10 PFU of Influenza A PR8. Mice were weighed daily from day 6 to day 12 post infection and average weight change was plotted as a percentage of the original weight from day 0. A) Average weight changes of naïve and infected groups of mice for combined data from two independent experiments. B) and C), data from each experiment, illustrated separately B) Average weight changes for naïve and infected groups with standard deviation error bars for experiment number KD14; naïve group n=5, infected group n=25 C) Average weight changes for naïve and infected groups with standard deviation error bars for KD19; naïve group n=5, infected group n=27.

The reduction in mouse weight during influenza suggested that overall health status was most severely affected at day 7 post infection. To assess whether local lung pathology also peaked at the same time, we performed two measures of lung damage: We assessed the concentrations of total protein and a blood specific protein (serum albumin) found in the BAL fluid. An increase in total protein concentration in the BAL fluid suggests damage to the lung

epithelium due to cell lysis and/or leakage of blood proteins into the airways. C57BL/6 mice were intranasally infected with IAV PR8 at 10 PFU per mouse. Mice were sacrificed at various time points post infection and BAL fluid was collected for analysis by bicinchoninic acid (BCA) assay, to measure total protein, and ELISA, to measure serum albumin. Total protein concentrations were significantly increased in the BAL fluid of infected mice at day 7 pi. By day 10 pi the total protein concentration in the BAL fluid had decreased greatly compared to the concentration seen at day 7 pi. By day 14 pi the total protein concentrations had returned to levels equivalent to those observed in naïve controls.

Since total protein concentration could increase due to factors other than just epithelial damage, such as an increased infiltrating cells, we also measured a more direct indicator of damage. Serum albumin (SA) is a blood specific protein and is not expected to be present in the airways of a normal lung. If damage to the lung epithelium had occurred, however, it would be expected that blood and blood-associated proteins such as SA would leak into the airways. Therefore, we measured SA concentrations in the BAL fluid. Within the BAL there was a significant increase in SA concentrations at day 7 pi. and a return to SA concentrations equivalent to naïve animals by day 14 pi (Figure 3-2).

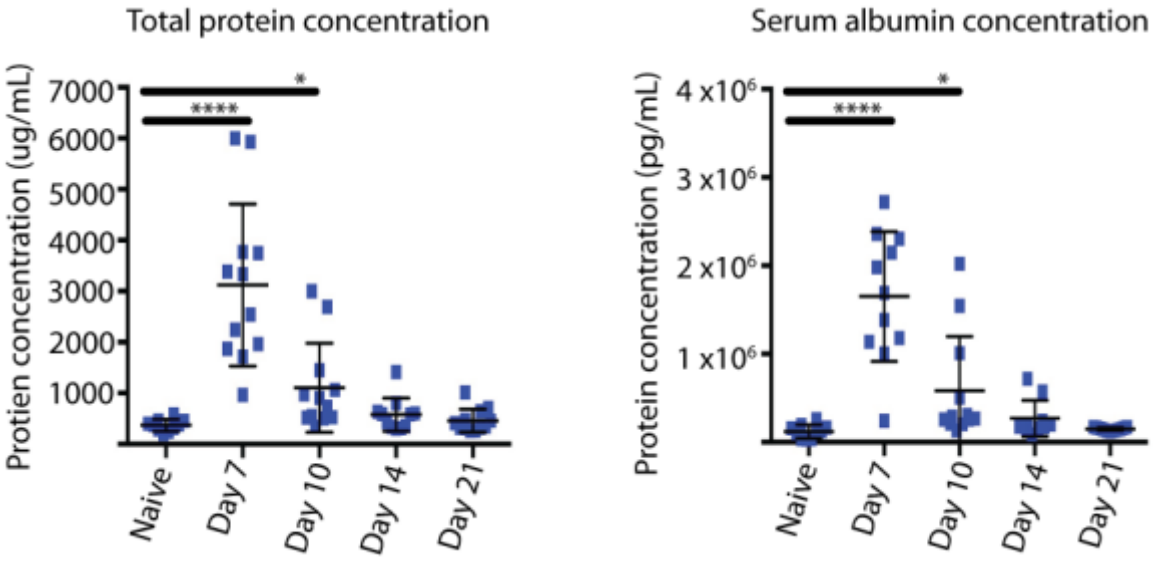


Figure 3-2 Protein concentrations in bronchoalveolar lavage fluid as an indication of pulmonary damage

Mice were intranasally infected with 10 PFU of Influenza A PR8. Mice were sacrificed on day 7 pi, day 10 pi, day 14 pi and day 21 pi. BAL fluid was collected and BCA assay and ELISA assay were used determined protein concentrations. A) Total protein concentrations in the BAL fluid of naïve and infected mice at various time points post infection; compilation of 2 independent experiments. ****$p<0.0001$ and *$p=0.0151$, by unpaired t test with Welch's correction. B) Concentration serum albumin protein found in the BAL fluid of naïve and infect mice at various time points post infection. ****$p<0.0001$ and *$p=0.0248$, by unpaired t test with Welch's correction.

3.1.2 Assessing the inflammatory response to IAV PR8 through cytokine and chemokine expression

Our data so far suggest that peak lung damage occurs around day 7 of influenza infection. To determine how lung damage correlated with lung inflammation in our model, local concentrations of typical Th1 cytokines were assessed. C57BL/6 mice were intranasally infected with IAV PR8 at 10 PFU per mouse. Influenza infection is commonly associated with a Th1-type immune response, which is characterized by the presence of inflammatory cytokines such as Interferon gamma (IFNγ) and Interleukin-6 (22,55,56). We measured the concentration of each of these cytokines by CBA, as well as another pro-inflammatory cytokine TNF-α and the

monocyte chemoattractant protein-1, MCP-1 or CCL2 in the BAL of influenza-infected mice and naïve controls (Figure 3-3). In the case that the concentration level of a sample was below the limit of detection, a value of 0 was given. All of the pro-inflammatory cytokines measured showed significant increases in concentration at day 7 pi compared to naïve mice. There was a dramatic decrease in concentration of each of the cytokines measured between days 7 and 10 pi, suggesting that the majority of the inflammatory response had subsided by day 10 pi. Although significantly decreased, TNF-α concentrations remained significantly increased compared to naïve controls at day 10pi and decreased back to naïve concentrations by day 14 pi.

Therefore, our data suggests that after influenza infection, the height of the inflammatory response, defined by maximum pro-inflammatory cytokine production, correlates with the height of damage at day 7 post infection.

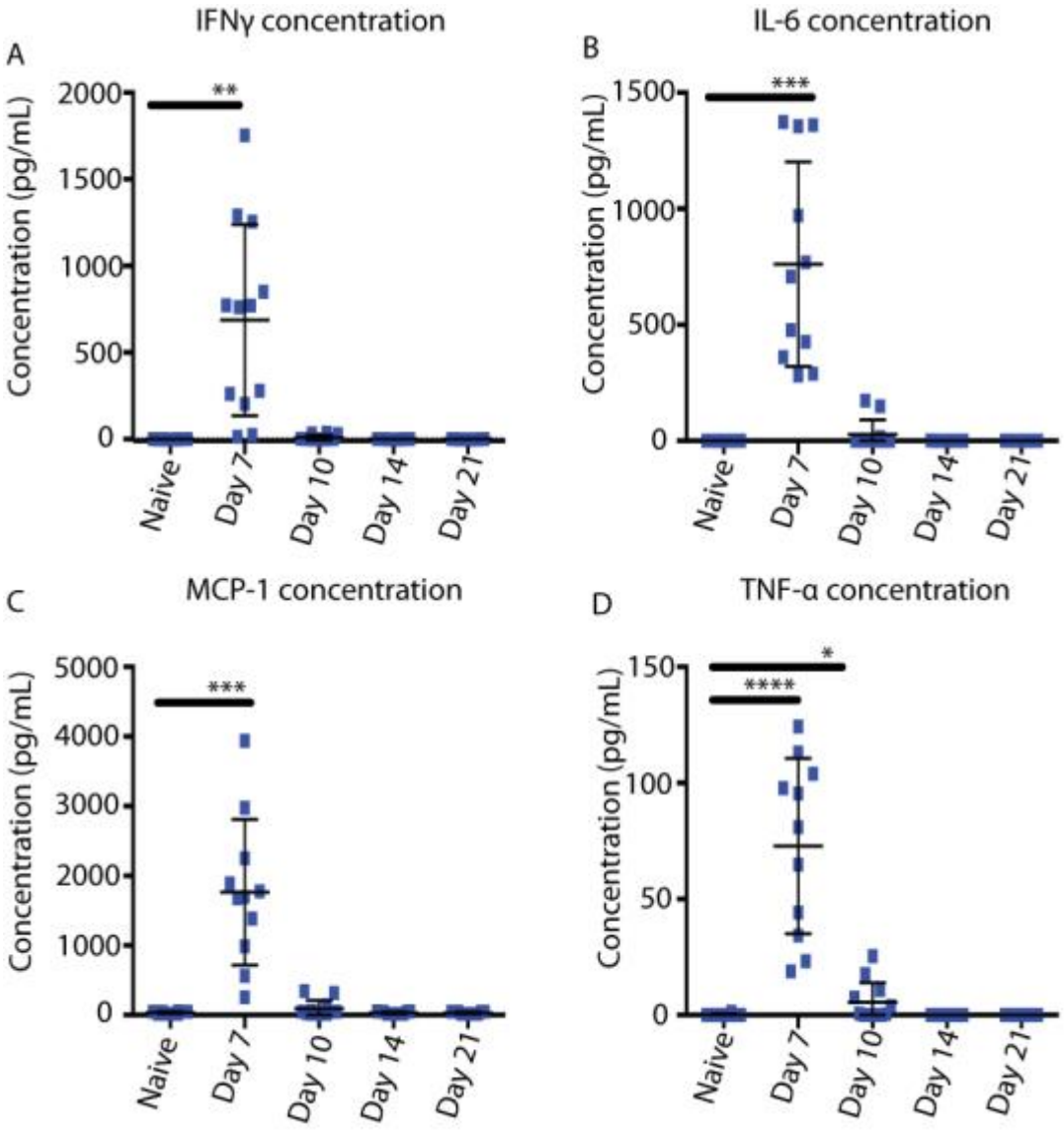


Figure 3-3 Pro-inflammatory cytokine expression in the BAL fluid

Mice were intranasally infected with 10 PFU of Influenza A PR8. Mice were sacrificed on day 7 pi, day 10 pi, day 14 pi and day 21 pi. BAL fluid was collected and expression of various pro-inflammatory cytokines was measured using a cytometric bead array assay. Data is a compilation of 2 independent experiments A) IFNγ protein expression in the BAL fluid of naïve and infected mice. **p=0.0012 by unpaired t test with Welch's correction. B) Interleukin 6 (IL-6) protein expression in the BAL fluid of naïve and infected mice. ***p=0.0002 by unpaired t test with Welch's correction. C) MCP-1 expression in naïve and infected mice. ***p=0.0003 by unpaired t test with Welch's correction. D) TNF-α protein expression in the BAL fluid of naïve and infected mice. ****p<0.0001 and *p=0.0484 by unpaired t test with Welch's correction.

3.1.3 Assessing IL-10 expression in relations to damage and inflammation

Given that I hypothesized that IL-10 will be involved in promoting the repair response, I then assessed the expression of IL-10 in relation to the timing of damage, inflammation, and repair. To do this, IL-10 protein concentrations in the BAL fluid were measured by CBA at day 7 pi, day 10 pi, day 14 pi and day 21 pi, to assess the protein expression in relation to damage and inflammation (Figure 3-4). At day 7 pi, IL-10 expression was significantly increased compared to that of naïve controls. By day 10 pi, IL-10 protein concentrations levels had declined back to that of the naïve controls. This time frame of increased IL-10 expression coincides with the indicators of damage and inflammation measured.

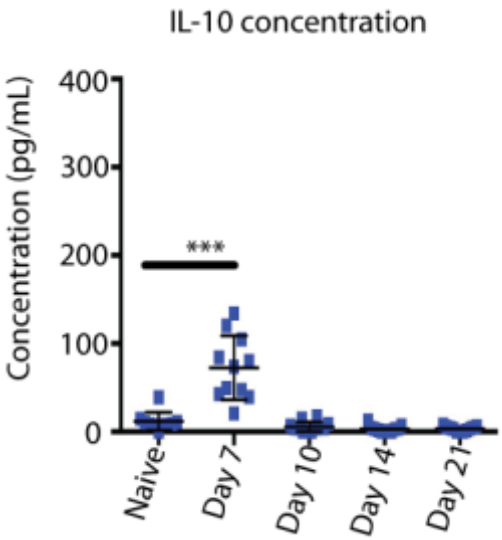


Figure 3-4 IL-10 expression in the BAL fluid

Mice were intranasally infected with 10 PFU of Influenza A PR8. Mice were sacrificed on day 7 pi, day 10 pi, day 14 pi and day 21 pi. BAL fluid was collected and expression of various pro-inflammatory cytokines was measured using a CBA assay. Data was analyzed using Flowjo software. Data is a compilation of 2 independent experiments. ***p= 0.0002 by unpaired t test with Welch's correction.

3.1.4 Assessing indicators of epithelial repair

Thus far we have seen a decrease in total protein and serum albumin concentrations by day 10 pi, which indicates that repair is likely occurring. In order to further assess when repair in the lungs occurs during an IAV infection, we use RT-qPCR to analyze gene expression of amphiregulin, epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR), at various time point throughout an infection (Figure 3-5). EGF and amphiregulin have both been shown to stimulate repair in lung epithelial cells (48,50). Amphiregulin did not show any significant changes in gene expression for infected samples compared to naïve controls at any of the time points. Although there was a trend for an increase in *egf* expression at the later time points, this was not significant or seen consistently between repeat experiments.

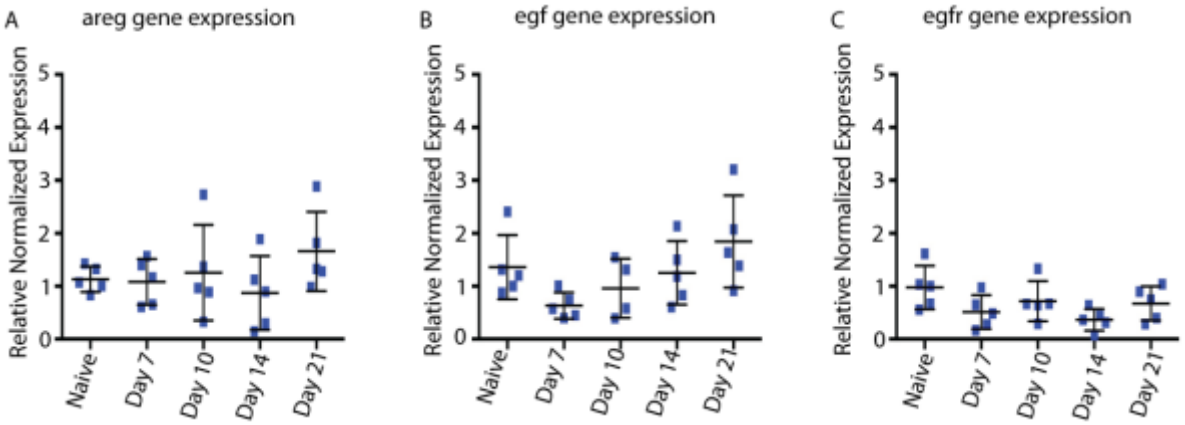
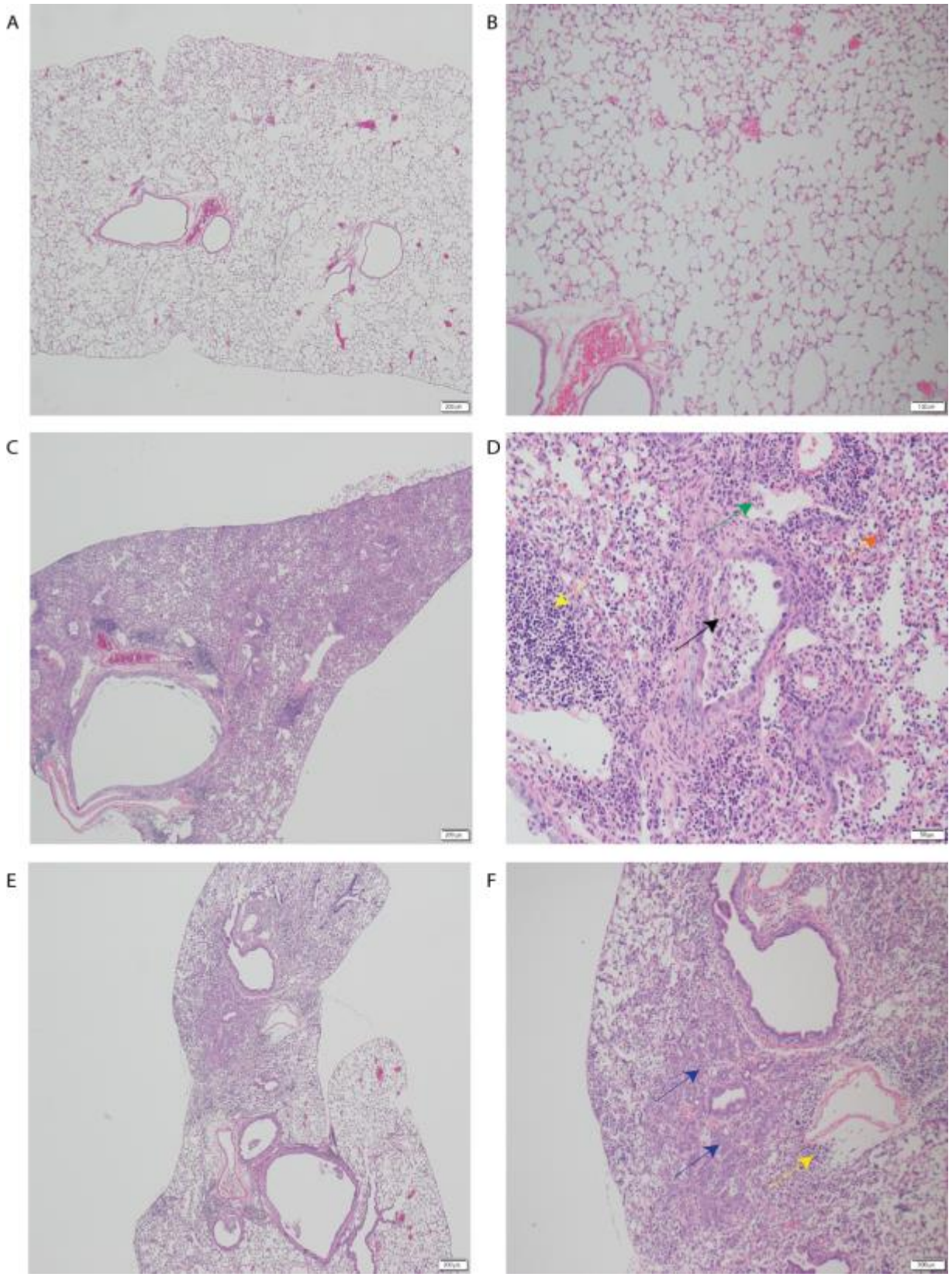


Figure 3-5 Gene expression of known repair markers in the lung during the course of an influenza A PR8 infection.

Mice were intranasally infected with 10 PFU of Influenza A PR8. Mice were sacrificed on day 7 pi, day 10 pi, day 14 pi and day 21 pi. Lung tissue was extracted and stored in RNAlater at 4°C. RNA was later extracted and reversed transcribed to cDNA at concentration of 20ng/µl for all samples. cDNA was used at a 1/10 dilution. Samples are normalized to GAPDH and are presented relative to a naïve control. A) Gene expression for amphiregulin B) Gene expression for egf C) Gene expression for egfr.

3.1.5 Visual profile after IAV infection to observe damage, inflammation and repair

We wished to confirm our results seen biochemically for inflammation, damage and repair through the use of histology. C57BL/6 mice were intranasally infected with IAV PR8 at 10 PFU per mouse and sacrificed at various time points post infection. Lungs were extracted and preserved in 4% PFA, embedded in paraffin, sectioned and H&E stained. Naïve controls samples were observed to have clear airways without inflammation, as would be expected in healthy lung (Figure 3-6 A&B). Observations for infected samples varied between samples but did reveal some repetitive trends. At day 7 pi the lung samples were seen to have infiltrating mononuclear immune cells (yellow arrow), cell apoptosis (green arrow), debris in the airways (black arrow) and red blood cell leakage into the tissue (orange arrow) (Figure 3-6 C&D). Edema can be seen as the light pink staining found in the interstitial space as well. In samples for day 10 pi, these signs of inflammation had begun to subside. Importantly, there was still some localized inflammation and infiltrating mononuclear immune cells present (yellow arrow). There were also some signs of repair to alveolar epithelial cells (blue arrow) (Figure 3-6F), as evidenced by the enlarged alveolar type II cells. When damage to alveolar type I cells occurs, type II alveolar cells divide and differentiate into type I cells. Dividing type II cells are bigger in size than non-dividing type II cells, and also acquire a smooth pale pink/purple colour when H&E stained. We saw evidence of dividing type II cells on day 10 of infection only (Figure 3-6 F). This is direct evidence of pulmonary epithelial repair occurring between days 7 and 10 pi in our model of influenza A infection.


A
B
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D
E
F

Figure 3-6 Histopathology of the lung during IAV PR8 infection

Mice were intranasal infected with 10PFU of Influenza A PR8. Mice were sacrificed on day 7 pi, day 10 pi, day 14 pi and day 21 pi. Lungs were insufflated with and preserved in 4% paraformaldehyde for 2 days and then transferred to 70% ethanol. Lung lobes were imbedded in paraffin, sectioned and hematoxylin and eosin stained. A-B) H&E stained section of a naïve lung lobe. C-D) H&E stained section of lung on day 7 pi of an IAV PR8 infection. At day 7 pi the lung samples were seen to have infiltrating mononuclear immune cells (yellow arrow), cell apoptosis (green arrow), debris in the airways (black arrow) and red blood cell leakage into the tissue (orange arrow) E-F) H&E stained section of lung on day 10 pi of an IAV PR8 infection. Mononuclear cells can still be seen at day 10 pi (yellow arrow), as well as proliferating ATII cells (blue arrows)

3.1.6 Using a lung epithelial cell line to assess the direct impact of IL-10 on epithelial repair

Our data so far shows that, in our model of IAV, damage is present at day 7 pi and that there must be repair occurring between day 7 and day 10 pi, as protein and serum albumin is no longer present in the BAL fluid. High expression of IL-10 is present at day 7 pi, however has decreased by day 10. This could suggest that IL-10 does not play a direct role in epithelial repair. In order to address whether IL-10 has a direct effect on lung epithelial repair, I used an *in vitro* scratch assay model to assess changes in proliferation in the presence or absence of exogenous IL-10.

A human bronchial epithelial cell line (16HBE) was gifted to our lab from Dr. Tillie Hackett at the Centre for Heart Lung Innovation. Cell lines often undergo changes in gene and protein expression, relative to primary cells, once they have been transformed. Our first concern was to determine that the intended 16HBE cell line expressed the IL-10 receptor chains so that it would be able to respond to IL-10. RT-qPCR was performed to assess the expression of the IL-10R alpha and beta chains (Figure 2-7). Gene expression was normalized to the reference gene Ribosomal Subunit Protein 13, and is presented as relative to the unscratched, negative controls.

The IL-10R beta chain is also a component of other cytokine receptors known to be expressed by epithelial cells, such as the IL-22 receptor. The IL-10R alpha chain has only been shown to be incorporated into the receptor for IL-10. We therefore expected higher expression of the IL-10R beta chain than IL-10R alpha chain, even on resting cells. Both genes were detectable, even on resting cells. Absolute quantification was not performed, but the PCR product for the IL-10R alpha chain only became detachable around cycle 34, while IL-10R beta chain became detectable by cycle 23. Thus, in resting cells, *il10rb* was more highly expressed than *il10ra*, but both gene products were detectable.

We next performed a wounding assay to mimic epithelial damage and tested the hypothesis that IL-10R expression would increase in response to damage. We examined gene expression of *il10ra* and *il10rb* at multiple time points after wounding. Expression of both genes remained detectable throughout the time course, but there were no consistent or significant changes in expression in response to wounding (Figure 2-7).

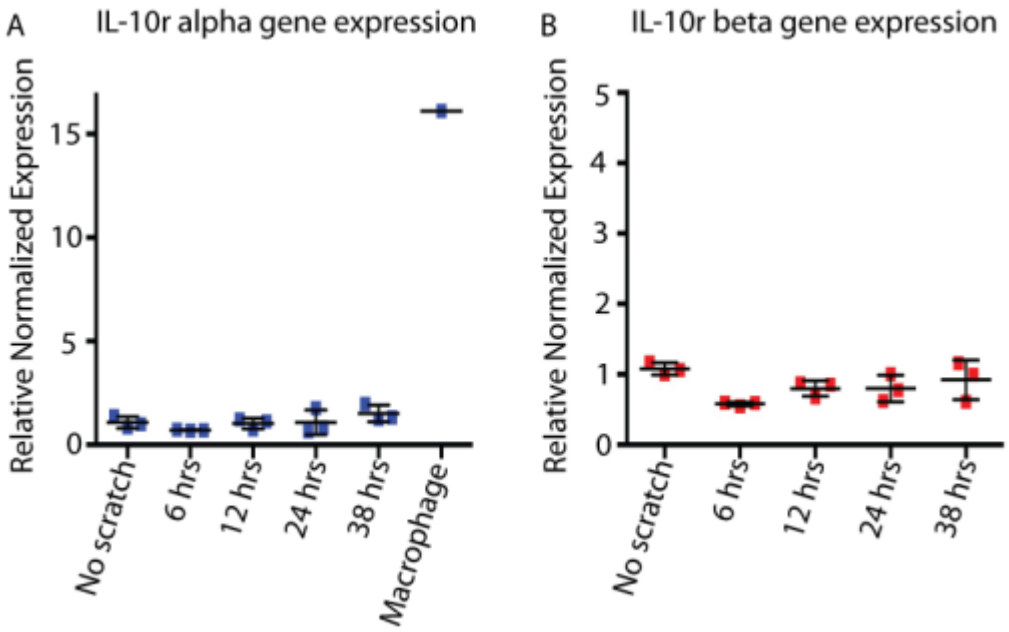

Figure 3-7 IL-10r alpha and beta chain gene expression does not change after wounding 16HBE cells.

Cells were grown until confluent, serum starved for 24hrs, rested for 30-60 minutes and then scratched. RNA samples were taken at 6 hours, 12 hours, 24 hours, and 38hrs. RNA from LPS activated macrophages were used as a positive control and water non template wells were used as negative controls. A) Gene expression for IL-10R alpha chain measured by RT-qPCR B) Gene expression for IL-10R beta chain measured by RT-qPCR.

Having established that the IL-10 receptor was expressed on our cell line, we next wished to determine if IL-10 was able to enhance epithelial cell proliferation after wounding. Ki-67 is an intracellular protein that accumulates in the nucleus when during all active stages of mitosis (G1 phase, S phase, G2 phase and M phase) and can be used as a biomarker of cell proliferation. We chose to examine the presence of this protein in the 16HBE cell line after wounding, in our scratch assay, to determine the levels of epithelial proliferation involved in this type of wound repair. The cells were wounded and then either treated with IL-10 (25ng/mL), IL-22 (10ng/mL) or left untreated in only serum containing media. Scratched and untreated cells were included as a "naïve" control. IL-22 was used as a positive control, as IL-22 has been shown to stimulate

epithelial cell proliferation (46). Unscratched cells were included to assess background expression of Ki-67 for the cell line. We observed similar Ki-67 protein expression in the unscratched control cells as in the scratched and treated cells: in all samples, between 60-70% of cells were Ki-67 positive (Figure 2-8C). This suggests that the majority of the cells are in a state of active cell cycle, which is likely, given that this cell line has been transformed and that Ki-67 expression is not be the best way to measure proliferation in our assay.

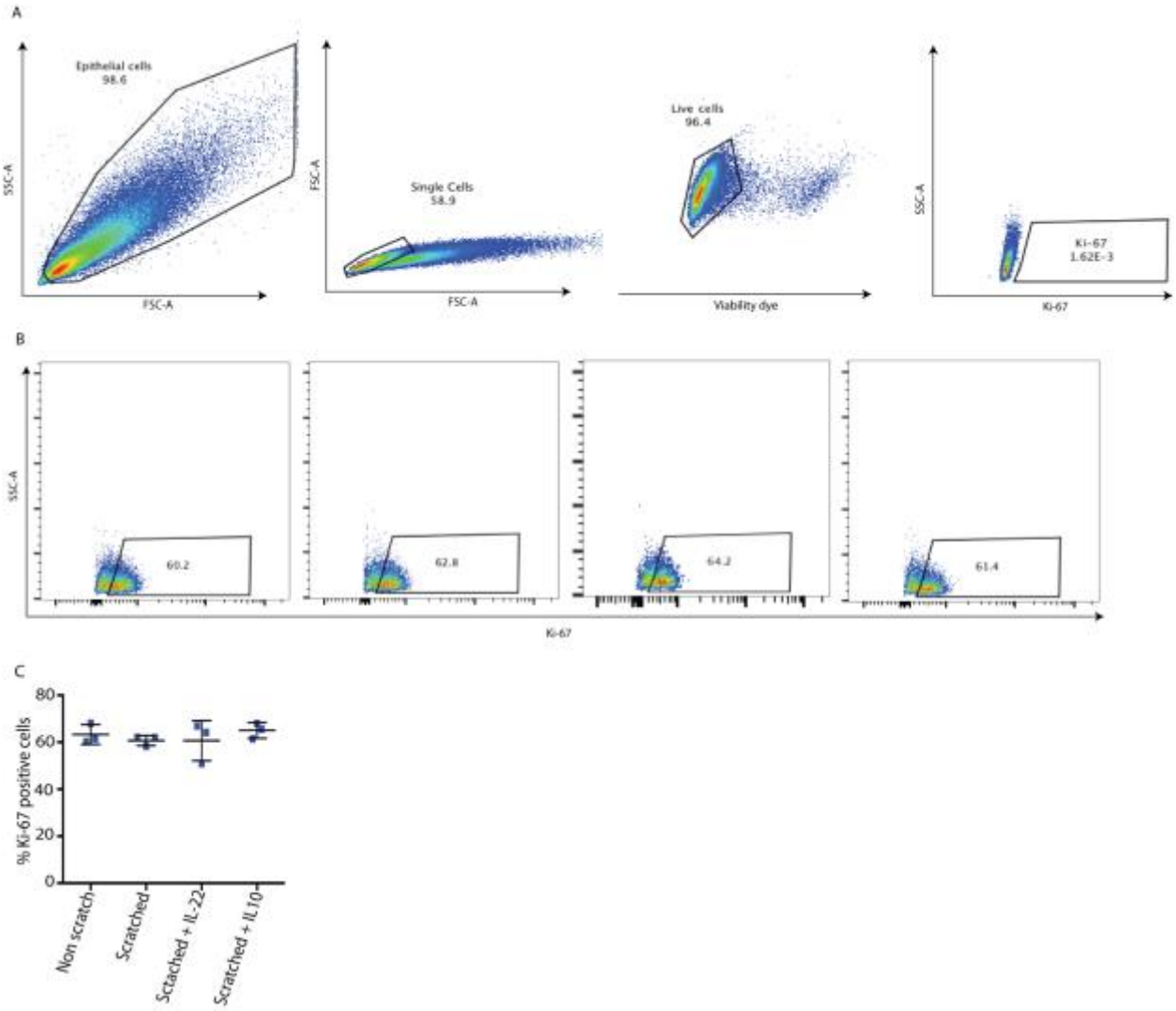

Figure 3-8 Ki-67 expression as an indicator of proliferation and repair

Cells were grown until confluent, serum starved for 18 hours, then scratched and stimulus (IL-10 or IL-22) was added to 10% serum containing media and applied to cells for 24 hours at 37°C. Cells were then stained for Ki-67. Ki-67 expression was assessed by flow cytometry and analysis was performed using FlowJo Software. A) Gating strategy for Ki-67% cells. Debris was first gated out. We then gated on single and live cells. Ki-67 positive populations were then gated on with the help of isotype controls and unstained controls. B) Examples staining for each group. From left to right: unscratched control sample, scratched unstimulated cells, scratched IL-22 (10ng/ml) treated cells and scratched IL-10 (25ng/ml) treated cells. C) Percent of Ki-67 positive cells for each of the 4 groups tested.

EdU is a nucleotide analog that is incorporated into DNA during cellular replication. EdU was chosen to detect epithelial cell proliferation as an alternative to Ki-67. Since EdU is not a natural intracellular protein and rather is only incorporated into cells that have substantially replicated their DNA, we hypothesized that EdU staining would give a more realistic assessment of epithelial proliferation. We found that, in comparison to Ki-67, EdU did show a lower background signal, and scratched and treated cells had higher EdU incorporation compared to the unscratched controls (Figure 3-9).IL-10 stimulated cells were observed to have a significantly decreased EdU incorporation compared to scratched cells treated without any exogenous cytokine.

IL-22 was used as a positive control, as it is known to stimulate epithelial proliferation in vivo (49). Our data showed no increase in EdU incorporation in IL-22-treated cells versus untreated controls. However, this experiment has only been performed once and need to be further optimized and repeated before a conclusive result can be drawn. Therefore, presently we have no evidence to support a direct effect of IL-10 on epithelial cell proliferation after damage.

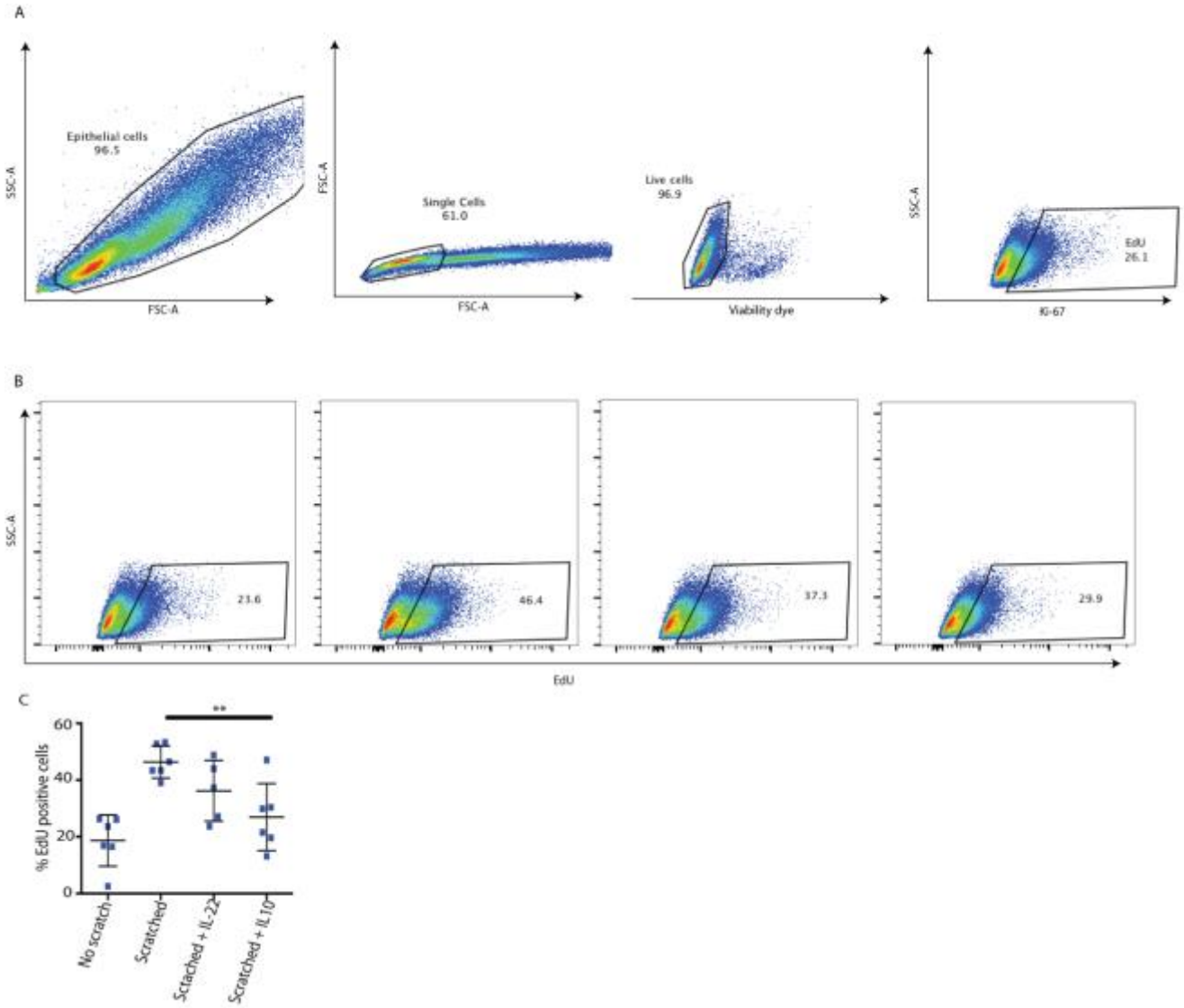

Figure 3-9 EdU incorporation as an indicator of proliferation and repair

Cells were grown till confluent, serum starved for 18 hours, then wounded. Cells were then scratched and stimulus (IL-10 or IL-22) was added to 10% serum containing media and EdU was added at 10um for 24 hours at 37°C. Cells were then stained for EdU. EdU incorporation was assessed by flow cytometry and analysis was performed using FlowJo Software. A) Gating strategy for EdU positive cells. Debris was first gated out. We then gated on single and live cells. EdU positive populations were then gated on. B) Examples staining for each group. From left to right: unscratched control sample, scratched unstimulated cells, scratched IL-22 (10ng/ml) treated cells and scratched IL-10 (25ng/ml) treated cells. C) Percent of EdU positive cells for each of the four conditions tested. **p= 0.0081 by unpaired t test with Welch's correction.

Chapter 4: Discussion

4.1 The Timing of IL-10 Protein Expression Coincides With the Peak of Damage and Inflammation

The overall aim of my thesis was to determine whether the immunosuppressive cytokine IL-10 affected epithelial repair in the lungs during influenza infection. I first compared the timing of pulmonary damage, inflammation and repair with that of IL-10 expression, in a murine model of influenza infection. My data indicate that the peak of IL-10 expression coincides with damage and inflammation. To assess if IL-10 has a direct effect on the proliferation of epithelial cells in response to wounding, I then used a cell line system. The data so far suggest that IL-10 does not directly affect epithelial cell proliferation and may suppress it in this context.

Our first measure of pathology in the murine influenza model was an assessment of weight loss. Tracking weight changes during an IAV infection is a common method used to assess disease severity in murine models (57). We recorded the weight changes of our mice from day 6 to day 12 pi as this time period has been shown in the literature to be the peak of weight loss and recovery (58). In both of my independent *in vivo* experiments, the same protocol for infection was used and day 7 pi was observed to be the peak of weight loss with steady recovery afterwards. Less severe weight was loss observed in the infected mice in my second experiment. The difference between the two experiments is likely due to the increased preemptive health-support measures used in the second experiment, such as providing diet gel (wet food) from the start of the experiment until day 8 pi. Although the severity of weight loss differed, the total protein concentration, SA concentration, and cytokine expression profiles were similar in the two experiments, which suggests similar levels of damage and inflammation occurred in the lungs in both cases. Total protein concentration in the BAL fluid was used as a surrogate measure of

damage because we would expect increased protein concentrations from cell lysis and leakage of blood into the airways after epithelial damage would be expected to deposit multiple proteins in the airways. However, this approach does have some caveats. Since BAL fluid was not centrifuged before testing total protein concentration it is likely the increased total protein concentrations could be due to inflammatory cytokines and other secreted proteins. To address this caveat, a more specific assay was also performed. Serum albumin is a blood specific protein, and thus the detection of SA in the BAL fluid indicates leakage of blood into the airways. The total protein concentrations and SA protein concentration both peaked at day 7pi with a strong decrease by day 10 pi, returning to baseline concentrations. The reduction in both measurements of damage between day 7 and day 10 pi, suggests that an intense repair process is likely occurring within this short time frame. This hypothesis was supported by the damaged epithelium, debris in the airways and the presence of red blood cells in the airway, visible by histology at day 7pi. Sections taken from the lungs of mice at day 10 pi there is reduced debris in the airways, lack of red blood cells in the airway, and notably the first signs of proliferating ATII cells were observed at day 10, indicative of the repair of the alveolar epithelial cells.

In IAV infection, damage to the lung epithelium is caused both by viral replication and by the resulting immune response. We observed that expression of the inflammatory cytokines IFNγ, IL-6, TNF-α and the monocyte chemoattractant MCP-1/CCL2 coincided with our measurements of lung damage: both were significantly increased above baseline at day 7 and had returned to baseline by day 10 pi. In murine models of influenza infection, the innate immune response lasts until approximately day 5pi and the adaptive response occurs between day 5 and day 10 - 11 pi, by which time complete viral clearance is achieved (59,60). By day 7 of an influenza infection mononuclear cells are present at the site of infection and the adaptive immune

response has already been activated. IFNγ acts as a positive-feedback loop for IFNγ expression and aids in the maintenance of commitment to the major Th1 transcription factor, Tbet (61). Both IL-6 and TNF-α are both known to play a role in B cell proliferation, and can also enhance permeability in endothelial vasculature (62-64). MCP-1 is a chemokine that aids in migration and infiltration of monocytes and macrophages. The combination of MCP-1 and IL-6 has also been shown to shift macrophages towards an M2 phenotype, which is important in cleanup and repair processes (65). All of these molecules are important in an adaptive immune response or aid in the restoration and repair processes that are necessary during influenza. Our data confirm that these four inflammatory cytokines peak at day 7 pi, coincident with the measured peak of lung damage, and that they return to baseline by the time that the adaptive immune response is expected to finish.

Our cytokine quantification data for inflammation was also consistent with measures of inflammation used in our histological analysis. Interstitial edema refers to an accumulation of fluid in the interstitial space and is often associated with inflammation. Edema is caused by increased permeability of the endothelial barrier resulting in leakage of fluid from the lymphatic system (63,66). Using hematoxylin and eosin staining, edema can be seen as the light pink staining found in the interstitial space. Since IL-6 was increased at day 7 pi it is not surprising that we also observed interstitial edema at this time as well.

This data, along with the results from our assays measuring lung damage, suggests that the peak of inflammation and damage in this model of influenza occurs by day 7 pi and is greatly resolved, inferring that significant repair has already occurred, by day 10pi. However, it is possible that the peak of inflammation and damage could occur earlier in the infection, but was missed. It would therefore be of interest to examine a time point earlier than day 7pi, such as day

3pi, to examine damage and inflammation as well. First establishing the presence of damage and inflammation was important to ensure that we could later observe resolution of these factors and examine the timeline with regards to IL-10 expression.

We have established that both damage and inflammation were present in our model with a similar timeline to one another. Since we were interested in determining the role of IL-10 on repair, next we assessed the timeline for IL-10 protein expression. IL-10 expression was also seen to peak at day 7 pi. Since IL-10 has already long been established as an important anti-inflammatory molecule, the high protein concentration found in the BAL fluid at day 7 pi, during the peak of inflammation, is expected (67). Given that IL-10 peaks at the height of damage and inflammation it might not be involved directly in the repair process. Instead, IL-10 may aid in the initiation of the repair process as well as contribute to limit the inflammatory response.

Our analyses of lung damage and cytokine protein expression suggested that IL-10 expression may coincide with epithelial damage, inflammation and possibly the initiation of repair. However, our measurements of repair were dominated by assays recording a resolution of damage, rather than a direct measures of repair. This problem is also common in the literature (46,50,68). We made efforts to examine epithelial cell repair more directly, by examining the gene expression of growth factors such as amphiregulin and EGF that have been used by other groups to assess the initiation of epithelial cell repair (50, 69).

Both these molecules are ligands of the EGFR, and both have been shown to initiate proliferation of epithelial cells and aid in restoration of epithelial integrity after damage. While we were able to detect gene expression of *areg*, *egf* and *egfr* in all our samples, there was no significant increase in expression of any of these genes, compared to uninfected mice, at any time point of influenza infection. A paper by Monticelli and colleagues reported an increase in

amphiregulin gene expression at day 10 of influenza infection, compared to naïve mice. However, Monticelli's experiments were performed in rag1-deficient mice, and the difference in mouse strains and influenza PR8 dose could possibly lead to discrepancies between our results and theirs. Rag1-deficient mice lack mature adaptive immune cells (70). T effector cells play an important role in attenuating inflammation and damage during an influenza infection (33). Without the adaptive immune response, the Rag1-deficient mice were unable to clear the viral infection and were unable to fully recover (50). Perhaps the intensified and prolonged damage and inflammation sustained in the Rag1 deficient mice led to the substantial increase in amphiregulin expression that was not seen in our model.

During periods of high inflammation there is a large influx of infiltrating pro-inflammatory cells. It is also possible that the peak expression for amphiregulin, EGF and EGFR may have been missed because RNA for these genes may have been diluted out by infiltrating cells present throughout the infection.

Although we did not detect significant increases in *areg* or *egf* during influenza infection, we were able to observe enlarged cells that were indicative of cellular proliferation by histology at day 10 pi. The proliferation of ATII cells contributes to epithelial repair by providing both new ATII and ATI cells, and is indicated by the emergence of small, rounded ATII intermediates that contrast with the tall, cuboidal structure of mature cells (12,71). Of note, ATII proliferation was not detectable at day 7 pi but was at day 10 pi. This in combination with our other measures of damage resolution, suggests that some aspects of repair occur between day 7 and day 10 pi. In conclusion, my data suggests that IL-10 is present at the peak of damage and inflammation and has decreased by day 10 pi. This could suggest that IL-10 does not have a direct impact on

epithelial cell repair but may still effect lung repair indirectly or affect the initiation of epithelial repair.

4.2 Assessing the Direct Effect of IL-10 on Lung Epithelium

Although IL-10 expression did not appear to coincide with the peak of epithelial repair, I still wanted to further assess if there was any direct impact on epithelial cell proliferation in repair. We used an in vitro assay involving a human bronchial epithelial cell line, 16HBE. Cell lines are a great resource, as they are can be easily grown and are able to simplify complex systems that may be seen in vivo.

We observed ATII proliferation in vivo, however due to necrosis of bronchial epithelial cells observed at day 7 pi and the decrease in airway debris observed by day 10 pi, it is likely that bronchial epithelial cells are proliferating during repair also. Since the ATI and ATII cells make up such a large portion of the lung, it is not unexpected that those cells were easily observed during proliferation. It would be ideal to repeat the proliferation repair assay with other cell types such ATII cells as well.

The 16HBE cells showed measurable expression of both chains of the IL-10 receptor, although the beta chain was more strongly expressed than the alpha chain. These results were perhaps expected, as the beta chain is also a component of the IL-22 and IL-26 receptors, while the alpha chain is unique to the IL-10 R (35). Neither gene was more significantly expressed after influenza infection, compared to uninfected lungs. If IL-10 directly affected epithelial cells in regards to repair, we would expect to see an upregulation of the IL-10R. This could suggest that IL-10 may not have a role directly on lung epithelial cell repair.

However, in order to assess if there is an impact of IL-10 on the proliferation of damaged epithelium, we wounded confluent epithelial sheets mechanically, and measured cell proliferation first by staining for Ki67. Ki-67 is a protein antigen specific to cell cycling and is present in the nucleus of cells in all active phases of the cell cycle except G0, the resting phase (72). We measured Ki-67 protein expression for our wounding assay that had been stimulated with or without IL-10. Negative controls that were not scratched or stimulated were included to determine a baseline level of proliferation for our cell line. We saw high Ki-67 expression in all samples, with 60-70% of cells being Ki-67 positive even in the absence of epithelial wounding. High Ki-67 expression is a prognostic marker for assessing cancer in humans (73). Since 16HBE cells are a transformed cell (52), it is likely that these cells are always in an active cell cycle state and therefore constitutively express Ki-67.

An alternative approach to measuring cell proliferation is to use EdU. EdU is a nucleoside analog that is incorporated into newly synthesized DNA during cellular proliferation (74). Since EdU is not a naturally occurring protein such as Ki-67 is, we would only expect to see EdU in cells that have actually proliferated. This would give a more realistic measurement of background proliferation occurring in the cell line. Using EdU as a measure of proliferation appeared to resolve the high background we observed while using Ki-67.

The scratched unstimulated samples and the IL-22 stimulated samples had significantly different EdU incorporation from each other. In contrast to my hypothesis, IL-10 stimulated cells had decreased EdU incorporation compared to the scratched unstimulated samples. The comparable EdU incorporation between the unstimulated unscratched controls and the IL-22 stimulated samples was slightly unexpected considering IL-22 has been shown to stimulate proliferation in primary human bronchial epithelial cells (75). This data could be due to

differences in performance between primary cells and cell lines or due to differences in IL-22 concentrations used. In our experiments IL-22 was used at a concentration of 10ng/ml, however in this study IL-22 was used at a concentration of 20ng/mL. A titration curve for IL-22 could be performed to determine if the differences are due to differences in concentrations.

In answer to my second aim, IL-10 stimulated cells appeared to have less EdU incorporation compared to control unscratched samples. It is possible that if IL-10 may not enhance epithelial cell proliferation and affect repair in the context of cell migration as opposed to cellular proliferation. Wound healing requires a combination of migration and proliferation. Epithelial cells are capable of collective migration and spread to aid in wound repair (76). It is also possible that IL-10 could actually inhibit proliferation at certain cytokine concentrations. Performing titrations curves for recombinant IL-10 and IL-22 will clarify whether different effects on epithelial proliferation occur at different concentrations of the same cytokine. In addition, the experiment shown in Figure 3-9 has only been performed once and will need to be repeated to ensure reproducibility and statistical significance. However, my initial findings provide no evidence to support my hypothesis that IL-10 can directly affect epithelial cell proliferation. Thus my second aim, in regards to whether IL-10 has a direct effect on lung epithelial repair has not yet been fully addressed and further experiments are required.

4.3 Conclusions

IL-10 is currently known as a potent anti-inflammatory cytokine, but more recently other functions of this pleotropic cytokine have been discovered. Here, I found that the protein expression of IL-10 is increased in response to IAV and the peak of expression coincides with the peak of inflammatory cytokine production. IL-10 expression also coincides with the peak of

alveolar space damage and leakage, but not with resolution of leakage or with other aspects of repair such as the presence of proliferating alveolar type II cells. Our data also suggest that, using influenza PR8 stain in a murine model leads to a repair process that occurs between day 7 and day 10 post infection that resolves and prevents further protein leakage into the alveolar space. Although not many researchers have looked directly at ongoing repair mechanisms, I did this by looking in vivo for evidence of alveolar type II cell proliferation and gene expression of cytokines that stimulate epithelial cell proliferation. Although I did not find a significant increase in *egf*, *egfr* or *areg* gene expression in response to IAV infection, we did see evidence on day 10 for alveolar cell proliferation. This evidence for epithelial cell proliferation occurred at day 10, 3 days after the peak of Il-10 expression. To determine if IL-10 was directly affecting this process, I switched to an in vitro wound healing model but found no evidence to support a direct effect of IL-10 on bronchial epithelial cell proliferation. However, there were some caveats with this approach and these experiments need to be reproduced. Overall, I have shown that IL-10 is produced in response to IAV infection with similar kinetics to inflammatory cytokines and the peak of the response occurs at the peak of lung damage. This is followed quickly by alveolar leakage repair and the initiation of alveolar type II cell proliferation. From my data to date, I conclude that IL-10 may play a key role in wound repair mechanisms although whether this is direct or indirect will require more study.

4.4 Future Directions

We have established that IL-10 protein expression coincides with timing of damage in an *in vivo* mouse model. We have also begun to examine the direct effect of IL-10 on repair pulmonary epithelial cell repair through the use of an *in vitro* model using a bronchial epithelial

cell line. Our data thus far suggest that EdU could offer an effective method to measure changes in epithelial cell proliferation following wounding. This assay could potential aid in answering my second aim, as to whether IL-10 has a direct effect on lung repair. This question has not been completely answered yet and further repetition and optimization of this assay are needed in an attempt reduce variability between technical replicates. Titration curves for IL-22 and IL-10 are also needed to ensure that the optimal concentration of cytokine is being used.

Cell lines that have been immortalized often have mutations or changes to key genes involved in cellular proliferation and apoptotic pathways. The immortalization process can therefore change the way that the cells respond to certain stimuli and conditions compared to primary cells. Therefore, in order to achieve results that are more biologically relevant to what is happening to epithelial cells in vivo, while still maintaining a simplified environment, it would be ideal to repeat an EdU wounding assay using primary bronchial epithelial cells also.

The effects of IL-10 on cellular proliferation could also be explored *in vivo*. Experiments performed in our lab have shown that influenza infection is associated with increased epithelial cell proliferation, relative to uninfected controls. However, when IL-10 signaling was blocked using an IL-10 receptor blocking antibody, no differences were observed between groups treated either with the blocking antibody or an isotype matched control. These experiments have only included small pilots and must be repeated and performed with a larger sample size in order to draw confident conclusions.

Our results so far suggest that, although IL-10 appears to coincident with epithelial damage during influenza infection, is does not directly control epithelial cell proliferation. IL-10 could affect cellular migration instead and it would therefore be interesting to examine the effects of IL-10 on cellular migration for wounded cells. This could be accomplished by

wounding cells using real-time microscope imaging and software to track cell movement over time.

It is also possible that IL-10 does not have a direct impact on lung epithelial repair at all but could still affect repair through indirect mechanisms. Many immune cells are known to express the IL-10 receptor and be able to respond to IL-10 (76,77). It is possible that IL-10 may act on epithelial repair indirectly via immune cells such as alveolar macrophages or innate lymphoid cells during and after an infection. This is another aspect we would like to explore.

By understanding the impact and role that IL-10 signals have on infection-related damage and more importantly on lung tissue repair, this research could facilitate the design of future therapies that better enhance viral clearance and facilitate lung repair during acute influenza infection, and during many other damaging lung diseases, such as tuberculosis, chronic obstructive pulmonary disease (COPD) and asthma.

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