CHARACTERIZATION OF PRIMARY MURINE ALVEOLAR EPITHELIAL CELLS AND THEIR RESPONSES TO RESPIRATORY VIRAL INFECTIONS

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Abstract

Alveolar epithelial cells are important targets of respiratory viral infections that cause severe diseases in the lung. The alveolar epithelium, which covers more than 99% of the large internal surface area of the lung, is composed of two morphologically distinct cell types. The alveolar type I (ATI) cells are large thin cells that function in gas exchange. The alveolar type II (ATII) cells are cuboidal and produce surfactant that is required to prevent collapse of the air spaces. ATII cells divide and trans-differentiate into ATI cells to repair damaged epithelium. Both ATI and ATII cells are infected by viruses and contribute to cytokine production during infection. The goal of this dissertation was to establish an *in vitro* model of murine ATI and ATII cells and determine their susceptibility and responses to infection by respiratory viruses used in murine models: influenza A virus strain PR8 and murine coronavirus, MHV-1. Isolated ATII cells were cultured to maintain an ATII phenotype as determined by expression of LBP180 or trans-differentiate into an ATI-like cell phenotype determined by expression of T1α. Primary cultures of either phenotype supported replication of PR8 and MHV-1. Further, both viruses induced cytokine expression by ATI cells. Expression of several cytokines and chemokines were induced by both viruses, while MHV-1 infection resulted in expression of an additional set of cytokines that were not expressed by PR8-infected cells. To help better understand how PR8 infection alters cellular protein levels, proteomics analysis using liquid chromatography-mass spectrometry was examined in ATII cells. Protein profiles in PR8-infected and uninfected ATII cells were compared. PR8 reduced expression of various functional classes of proteins that may enhance viral replication and alter the homeostatic functions of ATII cells. These data will help identify cellular proteins whose functions in the virus life cycle could be targeted for

antiviral strategies. Moreover, differentiated cultures of murine alveolar epithelial cells will be critical for identifying mechanisms of viral damage to the alveolar epithelium during respiratory infections, by providing an *in vitro* model to perform mechanistic studies that can be correlated with *in vivo* studies in well-established murine models.

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β	beta
α	alpha
γ	gamma
ABCF1	ATP-binding cassette, subfamily F, member 1
AIMP1	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1
ATP	Adenosine triphosphate
BCL6	B-cell lymphoma 6
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
Cl	Chlorine
CXCL	Chemokine (C-X-C motif) ligand
FGL2	Fibrinogen-like protein 2
IL	Interleukin
IL-R	Interleukin receptor
IL-ST	Interleukin signal transducer
IP	Inducible protein
IRF	Interferon regulatory factor
ISGF3	Interferon stimulated gene factor 3
LPS	Lipopolysaccharide
LT	Lymphotoxin alpha
MCP	Monocyte chemoattaractant protein
MHC	Major Histocompatibility Complex

MIP	Macrophage inflammatory protein
Na	Sodium
NF-κB	Nuclear factor-kappa B
RNase L	Ribonuclease L
STAT	Signal transducer and activator of transcription
TGF	Tumor growth factor
Th	T helper
TNFRII	Tumor necrosis factor receptor type II
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
TOLLIP	Toll interaction protein

Chapter 1

Literature Review

1.1 Importance of the alveolar epithelium in the lungs

The respiratory epithelium is constantly exposed to airborne particles including microbial pathogens. It consists of the upper and lower respiratory tracts. The upper respiratory tract includes nasal and sinusoidal epithelia. The lower respiratory tract, including the lungs, consists of the conducting airways, trachea, and bronchial epithelia. The bronchiole tubes carry air into the lungs where it ends deep into the alveolar epithelium, which lines the alveoli. The alveolar epithelium plays a role as a protective physical barrier between the external environment and the lung tissues and as an initiator and regulator of innate and adaptive immune responses in the lung. Since it is constantly exposed to invading particles, it can be injured and consequently, structural integrity and protection can be jeopardized. Respiratory virus infections result in the most severe disease when the alveolar epithelium is infected compared to the rest of the respiratory tract. This is because of the critical role of the alveolar epithelium in respiration.

1.1.1 The structure and functions of the alveolar epithelial cells

The alveolar epithelium covers 99% of the internal surface area of the lung (Dobbs *et al.*, 2010) and is composed of two types of cells, the alveolar type I (ATI) and type II (ATII) cells (Figure 1.1). The ATI cells are flat, have thin cytoplasmic extensions (50-100 nm) and cover more than 95% of the basement membrane of alveolar epithelium and make up 8% of total cells in the normal adult lung (Crapo *et al.*, 1982; Dobbs *et al.*, 2010).

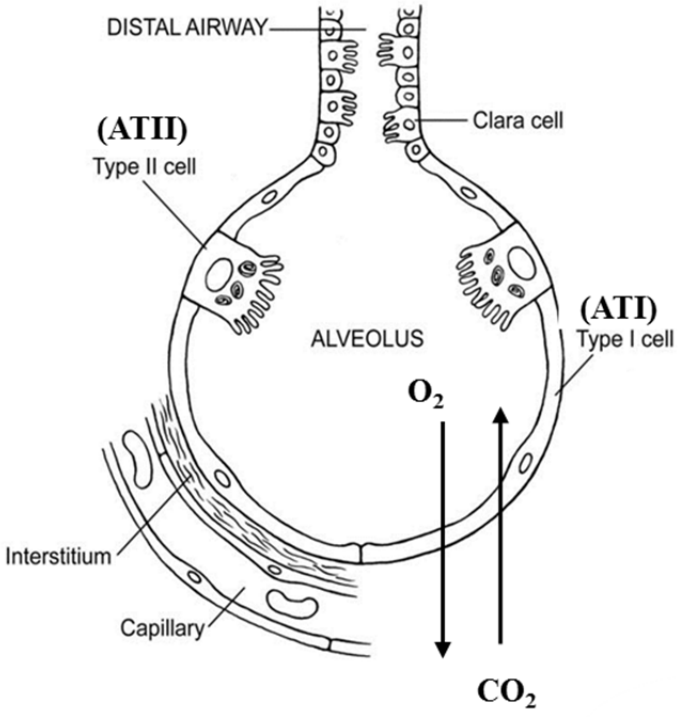

Figure 1.1 Cross-section of the alveolus showing ATI and ATII cells. ATI cells are flat and involved in gas exchange. ATII cells are cuboidal, produce surfactant and are involved in trans-differentiation into ATI cells during lung injury. Modified from (Zemans & Matthay, 2004), BioMed Central is the original publisher.

These morphological characteristics allow for free diffusion of gases at the alveolar surface. ATI cells express a large number of proteins among them are T1α and aquaporin. T1α is required for regulating differentiation of epithelial cells towards the ATI cell phenotype (Ramirez *et al.*, 2003). Mice with a null mutation of the *T1α* gene have narrower than normal alveolar sacs that fail to expand at birth and deficient differentiation of ATI cells (Millien *et al.*, 2006). Furthermore, T1α directs the ATII-ATI signalling pathway that inhibits proliferation once ATI cells are differentiated (Ramirez *et al.*, 2003). ATI cells contain molecular machinery for active ion transalveolar transport. They have Na^+, K^+ and Cl^- ATPase activity and express channels to transport these ions across cells (Johnson *et al.*, 2002). In addition, they also contribute to maintaining the alveolar fluid balance and resolving airspace edema (Johnson *et al.*, 2002). Aquaporin 5 is a water channel protein that transports fluid across the alveolar epithelium (Ma *et al.*, 2000; Williams, 2003).

The ATII cells are small, cuboidal and represent 5% of the alveolar surface area while accounting for approximately 15% of total cells in the healthy human lung (Crapo *et al.*, 1982). These are positioned at the corners of the alveoli so that their morphology does not hinder the gas exchange by the ATI cells (Figure 1.1). ATII cells are considered the stem cells for ATI cells in response to ATI cell injury; the hyperplastic ATII cells cover the basement membrane and then trans-differentiate into an ATI phenotype (Herzog *et al.*, 2008). However, ATII cells are primarily known for synthesizing, secreting and recycling the pulmonary surfactant. A thin layer of phospholipid, pulmonary surfactant maintains the structural integrity of the alveolus (Hawgood, 2004). It functions to prevent collapse of the alveolus during gas exchange by lowering the surface tension during end-expiration (Griese, 1999). It also maintains the volume of fluid lining the alveoli and size of the alveoli in

different phases of the respiratory cycle (Akella & Deshpande, 2013). In addition, it acts as a defense to airborne pathogens. The pulmonary surfactant is a surface-active, complex mixture of about 90% phospholipids and 10% proteins (Griese, 1999; Perez-Gil, 2008).The phospholipids of the surfactant have a characteristic composition in that 40-45% is fully saturated dipalmitoylphosphatidylcholine that is mostly responsible for the high surface activity of the pulmonary surfactant (Rooney *et al.*, 1994). Other lipids, which include the unsaturated phosphatidylcholines, phosphatidylglycerol, phosphatidylinosotol and cholesterol, play an important role in surfactant metabolism (Hawgood, 2004). The surfactant phospholipids and proteins are synthesized separately and packaged into lamellar bodies, where synthesis and metabolism of surfactant also occur (Zen *et al.*, 1998). The lamellar bodies then undergo exocytosis to secrete surfactant components, forming a lattice structure called tubular myelin (Rooney *et al.*, 1994; Suzuki *et al.*, 1989). The tubular myelin then forms the surfactant monolayer by adsorbing the lipid components into the air-liquid interface of the alveoli (Perez-Gil, 2008).

Surfactant also contains four surfactant-associated proteins synthesized by the ATII cells, surfactant protein (SP)-A, B, C and D. SP-B and C are hydrophobic proteins (Voorhout *et al.*, 1992) and play a role in structural organization of the surfactant, hence are critical for phospholipid production. Processing and proteolytic cleavage of SP-B occurs in the multivesicular body before fusion of this compartment with the lamellar bodies, followed by secretion of the mature protein into the alveolar space with other surfactant components (Weaver & Conkright, 2001). Functions of SP-B are to increase the rate of adsorption of phospholipids at the air-liquid interface and to replenish the pre-formed surfactant film during respiration (Hawgood, 2004). Similar to SP-B, SP-C is also secreted

into the alveolar space after post-translational modifications and proteolytic cleavage where it stabilizes the alveolar surfactant film by enhancing the adsorption rate of phospholipids and by increasing the resistance of surfactant against inhibition by serum proteins (Griese, 1999).

On the other hand, SP-A and SP-D are hydrophilic proteins (McCormack & Whitsett, 2002),that are responsible for host defense, enhancing clearance of various microbes from the lungs. SP-A is enriched in tubular myelin and at surfactant membrane interfaces where it intercepts inhaled microbes whereas SP-D largely exists in the fluid phase of the lung alveolar lining. These proteins are members of the mannose-binding proteins subgroup of C-type lectin, and have a role in inhibiting infectivity of various organisms directly and by enhancing phagocyte activation (van Iwaarden *et al.*, 1992). They bind to carbohydrates such as mannose and fucose, which are found on glycolipids and glycoproteins on surfaces of pathogens. Through their carbohydrate recognition domains, SP-A and D bind to viral carbohydrate moieties in respiratory syncytial virus (RSV) and influenza A virus (IAV) to block access of cell surface receptors to the receptor binding site (Hartshorn *et al.*, 1994; van Iwaarden *et al.*, 1992). This binding aggregates the viral particles and enhances virus removal from the lung by mucociliary mechanisms and activation of the phagocytic cells (Hartshorn *et al.*, 1994). SP-$D^{-/-}$ mice infected with IAV (LeVine *et al.*, 2001) and SP-$A^{-/-}$ mice infected with RSV (LeVine *et al.*, 1999) have severe lung inflammation and increased proinflammatory cytokine production that correlates with impaired viral clearance. Additionally, SP-A has a role in regulating surfactant homeostasis by controlling the secretion and uptake of the surfactant by the ATII cells (Dobbs *et al.*, 1987; Rice *et al.*, 1987).

1.1.2 Respiratory viral infections of alveolar epithelial cells

As the primary target of respiratory viruses such as IAV and severe acute respiratory syndrome coronavirus (SARS-CoV), infection of the alveolar epithelial cells could result in severe consequences. As mentioned before, the alveolar epithelial cells are vital for the maintenance of lung function. Infection of ATI cells could impair gas exchange and ion fluids transport across cells in the alveoli. Infection of ATII cells could compromise innate defense of the alveoli. In addition, it could interfere with transition of the ATII cells to ATI cells to reform the injured alveolar epithelium. Thus, infection of these cells could lead to significant lung pathology and ultimately death to the host.

Studies highlight viral infection in these cell types and the correlation with severe disease and mortality. Patients with severe cases of H5N1 and 2009 pandemic (H1N1) virus infections had viral pneumonia and respiratory distress syndrome with diffuse alveolar damage in their lungs (Esteban *et al.*, 2004; Nakajima *et al.*, 2010; Tran *et al.*, 2004). The diffuse alveolar damage occurred due to formation of hyaline membrane lining the alveoli, inflammatory cell accumulation in the lungs and pulmonary edema. Further, autopsies of patients who died from H5N1 and H1N1 revealed that viral replication is localized to the lungs where the ATII cells are the major site of virus replication (Nakajima *et al.*, 2010; Uiprasertkul *et al.*, 2005). Comparison of the high pathogenic IAV strains, 1918 H1N1 and H5N1 viruses with low pathogenic seasonal H1N1 isolate shows that high pathogenic viruses increase macrophages and neutrophils early on following infection and the inflammatory cells have sustained presence in the lungs of mice compared to low pathogenic virus, which is cleared quickly (Perrone *et al.*, 2008). Moreover, the high pathogenic viruses cause elevated levels of pro-inflammatory cytokines in the lungs of mice at day 4 post

infection, a time point which correlate with rising viral titers and increased disease severity. H5N1/97 antigens were detected in ATI and ATII cells of primates, which were euthanized at 4 days post infection, at the time when there was flooding of alveoli with fibrinous exudate containing mononuclear cells (Kuiken *et al.*, 2003b).

The autopsies of patients infected with SARS show viral RNA and proteins in ATII cells and the presence of this correlates with collapsed lung and alveoli filled with fluid (Chow *et al.*, 2004; Ding *et al.*, 2003; Tse *et al.*, 2004; Ye *et al.*, 2007). As in human cases, lethal infection in mice infected with mouse-adapted SARS-CoV show viral antigens in alveolar epithelial cells with infiltration of mononuclear cells and cellular necrosis (Roberts *et al.*, 2007). In primates infected with SARS-CoV the alveolar epithelial cells are infected and the lungs of severely affected animals show diffuse alveolar damage and infiltration of macrophages, monocytes and neutrophil infiltrates (Kuiken *et al.*, 2003a; McAuliffe *et al.*, 2004; Smits *et al.*, 2010). Similarly, patients with the Middle East respiratory syndrome coronavirus (MERS-CoV) had lesions throughout the lower respiratory tract indicating possible infection of the alveolar epithelial cells and infiltrations of neutrophils and macrophages (Drosten *et al.*, 2003; Guery *et al.*, 2013). These studies highlight the consequence of infection of the alveolar epithelial cells by the respiratory viruses.

1.1.3 Culture of primary ATI and ATII cells

Early alveolar epithelial cell responses may play critical roles in exacerbating pulmonary inflammation during respiratory viral infection by IAV and SARS-CoV. However, these responses can be difficult to detect using *in vivo* models. Although several lung epithelial cell lines obtained from bronchoalveolar carcinoma or immortalized by

transfection have been used to dissect the roles of the alveolar epithelial cells *in vitro*, their characteristics share only limited similarity to the alveolar epithelium *in vivo* (Driscoll *et al.*, 1995; Kathuria *et al.*, 2007b; Koslowski *et al.*, 2004; Wikenheiser *et al.*, 1993). Existing lung epithelial cell lines, for example, fail to produce surfactant that is characteristic of ATII cells in the lung (Mason & Williams, 1980). These differences limit the usefulness of continuous lines in identifying physiologically relevant mechanisms of viral pathogenesis.

Cell culture models of differentiated primary alveolar epithelial cell in rats and humans have been well-established and used to study different parameters in the lung. Primary alveolar epithelial cells from human lung tissues have been used to study respiratory viral infection *in vitro* (Dominguez *et al.*, 2013; Qian *et al.*, 2013; Wang *et al.*, 2011; Yu *et al.*, 2011), phospholipid production (Ballard *et al.*, 1986; Robinson *et al.*, 1984; Shapiro *et al.*, 1989), interactions with extracellular matrix components (Papadopoulos *et al.*, 1990), and morphology in mixed cultures (Bingle *et al.*, 1990). However, human lungs are not readily available and genetic variability limits their usefulness for mechanistic studies. Distinct phenotypes of rat alveolar epithelial cells have also been widely used as *in vitro* models for studies of lung physiology (Borok *et al.*, 2002; Gonzalez *et al.*, 2005; Qiao *et al.*, 2003; Sugahara *et al.*, 2006), and viral infection (Miura *et al.*, 2007; Miura *et al.*, 2006). In order to maintain the rat ATI and ATII cells in culture, ATII cells are isolated from lung tissue and maintained in their ATII phenotype or cultured under specific conditions to trans-differentiate into ATI cells. This is due to challenges in harvesting and maintaining the ATI cells with sufficient purity in primary culture and the fragility of these cells during isolation (Borok *et al.*, 1998; Chen *et al.*, 2004). When cultured on matrigel/collagen and DMEM containing rat serum and keratinocyte growth factor ATII cells maintain their

cuboidal shape (Mason, 2002). They express lamellar bodies and surfactant proteins, which are also expressed by the ATII cells *in vivo* as already mentioned (section 1.1.1). On the other hand to acquire the ATI phenotype, ATII cells are plated on tissue culture plastic or collagen/fibronectin coated plates in DMEM without keratinocyte growth factor for 5 days. Culturing ATII cells for this time period allows them to lose the ATII characteristics and gain ATI phenotype (Ramirez *et al.*, 2003). The ATI cell characteristics are the appearance of the large flat morphology and expression of T1α, aquaporin 5 and caveolin-1 (Williams, 2003). Limitations of rat model systems include the very few genetic knock-out lines and established models of respiratory viral infections (Funk *et al.*, 2009; Miura *et al.*, 2007; Rzepka *et al.*, 2012).

On the other hand, the *in vitro* model of murine alveolar epithelial cells has not yet been well-characterized. Studies have optimized the isolation of ATII cells (Corti *et al.*, 1996; Herold *et al.*, 2006; Marsh *et al.*, 2009; Rice *et al.*, 2002); however, these studies have conflicting reports on the *in vitro* phenotypes of these cells cultured in same conditions. Unlike in other *in vitro* systems fewer studies have used murine primary alveolar cells to study lung physiology or viral pathogenesis. Murine ATI cells that were derived from isolated ATII cells have been used to study influenza virus infection *in vitro* (Herold *et al.*, 2008; Herold *et al.*, 2006; Tate *et al.*, 2011). Based on the availability of well-characterized genetic lines and established models of respiratory viral pathogenesis, it is highly desirable to fully characterize murine ATI and ATII cell models to study virus-host interactions in the differentiated cell types of the alveolar epithelium.

1.2 Overview of influenza virus

Influenza viruses belong to the family *Orthomyxoviridae.* These viruses are responsible for recurrent epidemics and are a major threat to public health as they occasionally cause pandemics. Influenza outbreaks cause high mortality in the elderly, infants and patients with chronic diseases. There are three types of influenza viruses, A, B and C, distinguishable on the basis of antigenic differences between their matrix and nucleoproteins (Lamb & Krug, 2001). These viruses also differ with respect to host range, variability of the surface glycoproteins, and genome organization (Lamb & Krug, 2001). Phylogenetic analyses of nucleoproteins and polymerase proteins (discussed below, section 1.2.1) indicates that influenza A and B viruses are closely related to each other than to influenza C (Gammelin *et al.*, 1990; Krossoy *et al.*, 1999). Influenza C virus infects humans and pigs and causes local epidemics and is less common. Influenza B virus infects humans and seals (Osterhaus *et al.*, 2000), and this limited host range is responsible for the lack of influenza B pandemics in contrast to IAV. On the other hand, IAV infects a broad number of animals, humans and birds (avian), with birds being reservoirs for the virus. The wide spectrum of animals infected by IAV often results in zoonotic infection through host switch and is responsible for the generation of IAV pandemics. The worst pandemic was recorded in 1918 and killed up to 50 million people worldwide (Johnson & Mueller, 2002). Recently, the 2009 (H1N1) pandemic resulted in over 15,000 deaths worldwide (Dawood *et al.*, 2009; Perez-Padilla *et al.*, 2009; Shieh *et al.*, 2010). IAVs are also responsible for the large flu epidemics, in the United States alone deaths due to seasonal flu are estimated to range about 3,000-49,000 a year (Thompson et al., 2009; Thompson et al., 2010). Therefore, IAV is the

most intensively studied and mainly the focus of efforts to control influenza in humans. The discussion in this work will be limited primarily to IAV.

1.2.1 Biology of influenza A virus

IAV has a segmented, single-stranded, negative-sense RNA genome (Hale *et al.*, 2010). It is enveloped with helical nucleocapsid morphology. Each of the 8 segments encodes for one or two of the 10-11 proteins that the virus expresses (Figure 1. 2). IAV particles are covered with two major surface glycoprotein projections: receptor binding protein hemagglutinin (HA) and sialic acid cleaving enzyme neuraminidase (NA). On the basis of antigenicity of the HA and NA surface glycoproteins, IAV are classified into currently at least 17 HA subtypes (H1-H17) and 10 NA types (N1-N10) (Yen and Webster, 2009). Mutations sometimes occur in the HA and NA genes producing advantages for viral strains and allowing them to evade pre-existing immunity, which is often a cause of seasonal influenza outbreaks. The HA protein binds to sialic acid residues of galactose sugars on the tips of host cell glycoproteins (Taubenberger & Kash, 2010). The specificity of sialic acid α2-3 or α2-6 linkage binding by a particular strain of influenza virus is an important determinant for species specific restriction of the virus (Matrosovich *et al.*, 2004). Human IAV strains attach to host cells with α2-6 linkages that are a major type of sialic acid present in human respiratory epithelial cells, whereas avian IAV binds to α2-3 linkages (Gagneux *et al.*, 2003; Matrosovich *et al.*, 1999). However, α2-3 linkages are also found in human ciliated epithelial cells and epithelial cells in the lower respiratory tract along the alveolar walls (van Riel *et al.*, 2007). This has implications for human infections with avian influenza

virus strains, which has been the basis for all of the pandemic strains (Childs *et al.*, 2009; Matrosovich *et al.*, 1999; Neumann *et al.*, 2009).

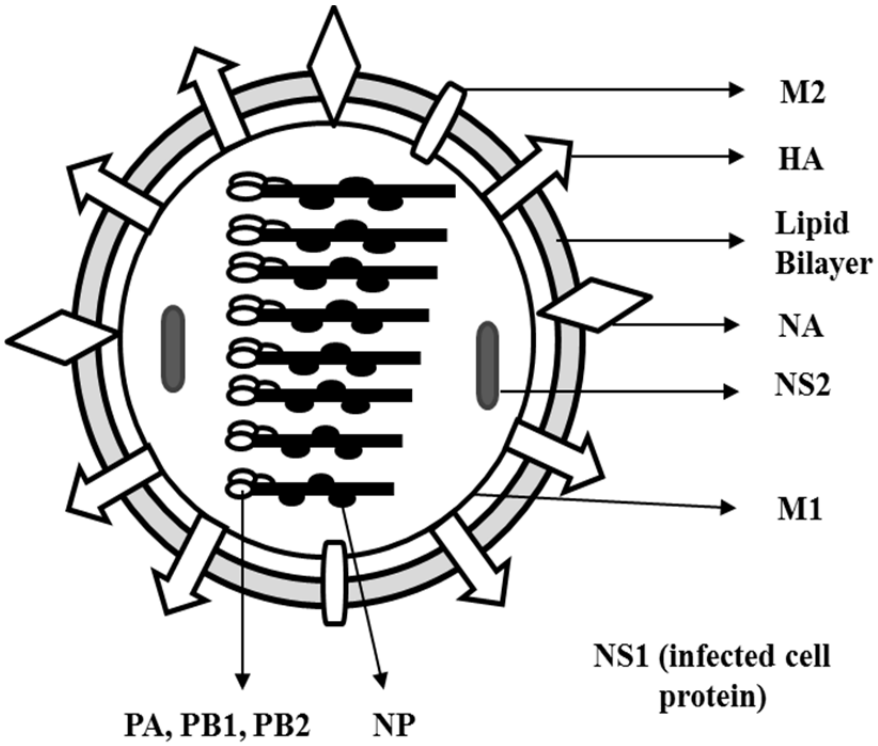

Figure 1.2 Diagram representation of IAV virion. HA and NA are surface glycoproteins projecting from the virus particle. The small numbers of M2 protein are embedded in the lipid bilayer. M1 is underneath the viral envelope and interacts with surface proteins and ribonucleoproteins. The eight RNA segments are covered by NP and contain viral polymerase complex (PB1, PB2 and PA). NS2 is contained within the virion whereas NS1 is only found in infected cells.

Once influenza binds to the cell via HA, the viral particles are internalized by receptor-mediated endocytosis. The M2 protein makes a pore on the viral particle to facilitate uncoating by allowing acidification of the interior of the virion. HA is cleaved into two subunits, HA1 and HA2, to promote fusion of the viral envelope with the endosomal membrane (Klenk & Garten, 1994). The transcription and replication of IAV occurs in the host nucleus and is carried out by the three viral polymerase proteins, which make up the RNA-dependent RNA polymerase (RdRp) of IAV: polymerase basic 1, 2 (PB1, PB2) and polymerase acidic (PA) proteins (Engelhardt & Fodor, 2006; Taubenberger & Kash, 2010). The RdRp are part of the ribonucleoprotein complex consisting of genomic viral RNA, minor amounts of the non-structural protein 2 (NS2) and nucleoprotein (NP), which wraps around the viral trimeric RdRp complex per RNA segment (Engelhardt & Fodor, 2006). The newly synthesized viral ribonucleoprotein complexes are exported from the nucleus to the cytoplasm by the NS2 and M1 proteins where they are assembled into virions together with other viral proteins at the plasma membrane. Once at the plasma membrane, lipid rafts serve as platforms for virus budding (Nayak *et al.*, 2004). Outward bending of the plasma membrane due to clustering of HA and NA in lipid rafts initiates the bud formation (Chen *et al.*, 2007; Lai *et al.*, 2010). The newly produced virions are released after NA cleaves the sialic acids from the viral glycoproteins, HA and NA, to prevent aggregation of viral particles at the plasma membrane. The non-structural protein 1 (NS1) is only expressed in infected cells (Julkunen *et al.*, 2001). It has multiple functional domains, which include dsRNA binding, nuclear localization and nuclear export signalling (Hale *et al.*, 2008). Furthermore, it has multiple mechanisms by which it functions and contributes to IAV pathogenesis, including inhibition of host mRNA processing to enhance viral RNA

translation (Bergmann *et al.*, 2000; Geiss *et al.*, 2002; Salvatore *et al.*, 2002), and type I interferon antagonism (Billharz *et al.*, 2009; Kochs *et al.*, 2007; Opitz *et al.*, 2007; Wang *et al.*, 2000), to intercept the host immune responses.

The segmented nature of IAV genes makes it easier for the virus to generate diversity through genetic reassortments. Thus, co-infection of one host cell with 2 different IAVs can result in virions containing gene segments of both parental viruses as was the case with the 2009 (H1N1) pandemic (Dunham *et al.*, 2009; Garten *et al.*, 2009). Pandemics of 1957 and 1968 arose due to the human H1N1 influenza virus acquiring a novel HA, NA and the PB1 gene from avian source, generating virus subtype H2N2 in 1957 and H3N2 in 1968 (Webster *et al.*, 1992; Webster *et al.*, 2007). In addition, the RdRp is error-prone and responsible for point mutations that result in seasonal variability in IAV and influenza B virus (Boivin *et al.*, 2010). The constant evolution of IAV thus requires more studies to be undertaken to understand its pathogenesis.

1.2.2 Host responses to influenza A virus

The initial infection by IAV occurs in the epithelial cells of the upper respiratory tract before the virus gets disseminated to the lower respiratory tract. Infection of the lower respiratory tract often results in viral pneumonia with diffuse alveolar damage as was seen in the severe pandemic of 1918 in patients who died and also in the 1957 pandemic, virus antigens were detected in alveolar epithelial cells. The infected cells recognize IAV through pathogen recognition receptors, which include toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG-I). IAV components are detected by TLR3 (Le Goffic *et al.*, 2007) and TLR7/8 (Blasius & Beutler, 2010; Lund *et al.*, 2004). The signalling by these receptors

leads to production of proinflammatory cytokines, chemokines and type I interferon responses (Alexopoulou *et al.*, 2001; Heil *et al.*, 2004).

The release of proinflammatory cytokines and chemokines results in localized inflammatory activation and recruitment of immune cells (Leon *et al.*, 2013; Sanders *et al.*, 2011; Teijaro *et al.*, 2011). Following infection with H5N1, patients that died had higher serum levels of macrophage and neutrophil chemoattractants (CXCL10, CXCL2, IL-8) and pro- and anti-inflammatory cytokines (IL-6, IL-10) (de Jong *et al.*, 2006; Peiris *et al.*, 2004). Though the recruitment of these innate immune cells to the IAV-infected lungs plays a role in clearance of viral infection, they can also become activated and produce nitric oxide synthetase 2 (NOS 2) and tumor necrosis factor (TNF-α), contributing to the pathology often seen in infected lungs (Jayasekera *et al.*, 2006; Lin *et al.*, 2008). Infection of human lung organ culture model with H3N2 and H1N1 viruses shows induction of IL-6, MCP-1, MIP-1α/β, IL-8 and IP-10 (Wu *et al.*, 2010). In addition, immunohistochemistry staining of virus and cytokines, MIP-α and IP-10 show that the alveolar epithelial cells are the source of these inflammatory molecules. Furthermore, IAV stimulates the NLRP3 inflammasome-cytoplasmic complex, which leads to IL-1β production; a cytokine involved in induction of Th1/17 cells and expansion of antigen-specific $CD4^+$ T cells (Ichinohe *et al.*, 2010). Hence the innate immune response also plays a crucial role in subsequent activation of adaptive immune responses, which eventually clear the viral infection.

Type I interferons (IFN) such as IFNα and IFNβ are involved in the immune responses of IAV-infected alveolar epithelial cells (Chan *et al.*, 2005; Wang *et al.*, 2011; Yoneyama *et al.*, 2004). These inhibit viral replication and contribute to the initiation of more specific adaptive immune responses. As IAV virions have to go through the cytoplasm

on the way to the nucleus, it is during this period that the RIG-I receptor detects the viral RNA and in turn induces a cascade by producing IFN-α/β (Kato *et al.*, 2006; Loo *et al.*, 2008; Nakhaei *et al.*, 2009; Yoneyama & Fujita, 2009). IFN-α/β are released from the infected cells and bind to their receptors on neighboring cells. This results in subsequent activation of the nuclear IFN gene transcription factor complex, ISGF3, which comprises of STAT1/2 complexed with IRF-9 (Randall & Goodbourn, 2008). The activated ISGF3 then stimulates transcription of several hundred genes downstream of IFN-stimulated response elements (Der *et al.*, 1998; Sadler & Williams, 2008). The induction of these genes will prime the cell to block viral replication and induce antiviral proteins in response to secreted IFN. Some of the proteins include expression of ds-RNA-dependent protein kinase (PKR), RNase L/ 2'-5' oligoadenylate synthetase (OAS), Mx and IFITM. Interferon inducible transmembrane (IFITM) genes restrict viral entry as they act on viruses that enter through the endosomal pathway (Brass *et al.*, 2009). They also affect the function of viral proteins involved in viral fusion in the endosome and fusion activity of the HA protein (Huang *et al.*, 2011b). PKR is activated by dsRNA and cellular stress (Stark, 2007). Activated PKR phosphorylates eukaryotic initiation factor 2α (eIF2α), which reduces translation initiation and synthesis of proteins (Julkunen *et al.*, 2001), to limit viral replication. OAS requires binding of dsRNA for activation to enzymatic activity and once it is activated it generates the 2'-5' oligoadenylate, which activates the latent cytoplasmic RNase L (Garcia-Sastre, 2011). The activated RNase L cleaves viral and cellular RNA to prevent viral replication (Chakrabarti *et al.*, 2011). The orthomyxovirus resistance gene or *Mx* encodes for the Mx protein that inhibits IAV replication (Haller *et al.*, 1998; Haller & Kochs, 2002). Mx proteins are GTPases that exist as oligomers and, depending on animal species, reside either

in the cytoplasm or nucleus of the IFN-α/β activated cells (Haller *et al.*, 1998; Melen & Julkunen, 1994; Pavlovic *et al.*, 1990). Mx oligomerizes to form a ring around the ribonucleoproteins of IAV to block viral replication (von der Malsburg *et al.*, 2011). In addition to having direct antiviral properties, IFN-α/β activation also modifies the host response. It activates dendritic cells resulting in the up-regulation of MHC genes thus antigen presentation to $CD4^+$ and $CD8^+$ T cells, contributing to initiation of the adaptive immune response (Santini *et al.*, 2000).

1.2.3 Mouse models of influenza A virus

The mouse is the most common model for studying influenza pneumonia and evaluation of antiviral therapy to treat IAV infections. Many reagents are available to study the effects of virus replication and treatment on the murine immune system (Barnard, 2009). Furthermore, the host factors that contribute to pathogenesis of IAV could be validated by using gene knockout mouse models. Additionally, mice have been used in preclinical studies to evaluate efficacy of vaccines/drugs (Bodewes *et al.*, 2010). Hence, the immunization mechanisms identified in mice can help in designing vaccines that would induce robust immunogenicity against IAV. The inexpensiveness of the animals and the general fidelity of illness in mice to the human disease (Barnard, 2009), and the well-characterized genetics also make it easier to study IAV mouse models.

Mice are not naturally infected with human or other strains of influenza virus and thus human viruses like seasonal IAV have to be adapted to replicate in the lungs of mice. This is performed by serial passage of human virus in lungs of mice to increase virulence and pathogenicity. The mouse-adapted IAV usually induce pathologies in the bronchi or

lungs of mice (Hirst, 1947; Raut *et al.*, 1975). Furthermore, the mouse-adapted IAV possesses an increased ability to be able to infect alveolar epithelial cells, and can cause lethal pneumonia (Hirst, 1947; Raut *et al.*, 1975; Wyde *et al.*, 1977). The mouse-adapted human influenza A/Puerto Rico/8/34/(H1N1(PR8)) virus has been adapted as such (Mori *et al.*, 1995). After intranasal inoculation with PR8, mice develop a lower respiratory tract infection with lung consolidation and interstitial pneumonia (Blazejewska *et al.*, 2011; Fukushi *et al.*, 2011; Garigliany *et al.*, 2010). Several studies document induction of IFNα, TNFα, IL-α/β and IL-6 in bronchoalveolar lavage fluid or lung homogenates in association with lung pathology in PR8-infected mice (Conn *et al.*, 1995; Kurokawa *et al.*, 1996; Peper & Van Campen, 1995). These observations are similar to those observed in humans (Sweet & Smith, 1980). As in seasonal IAV infection in humans, onset of symptoms, lung pathology and cytokine production in mice are temporally related to virus replication (Vacheron *et al.*, 1990). Similarly, low pathogenic infections are non-lethal and restricted to the upper respiratory tract and cleared within a week, while high pathogenic infections affect the lower respiratory tract (Perrone *et al.*, 2008). Hence, the changes that occur in mice during IAV infection may provide insight into factors that affect the development of disease in humans. The PR8 strain was used in this study.

1.3 Overview of coronaviruses

Coronaviruses (CoVs) are a genus belonging to the family *Coronaviridae*. CoVs infections are associated with respiratory and enteric disease in humans and animals. They are classified into four genera, *Betacoronavirus, Alphacoronavirus, Gammacoronavirus* and *Deltacoronavirus* (de Groot *et al.*, 2013; Woo *et al.*, 2009). Human coronaviruses (HCoVs)

such as HCoV-229E and OC43 have been known to cause mild upper respiratory diseases (Hamre & Procknow, 1966; McIntosh *et al.*, 1967; Patrick *et al.*, 2006). However, the outbreak of the highly pathogenic SARS-CoV in 2003 with a lethality rate of about 10% revealed the potential lethal consequence of CoV-induced disease in humans (Drosten *et al.*, 2003; Ksiazek *et al.*, 2003). Following identification of SARS-CoV, two additional HCoVs were identified, NL63 and HKU1, which produce lower respiratory infections in infants, young children and elderly (Fouchier *et al.*, 2004; Pyrc *et al.*, 2004; van der Hoek *et al.*, 2004; Woo *et al.*, 2005). The high frequency of recombination and rate of mutation are reported to be responsible for the ability of CoVs to be able to adapt to new hosts (Herrewegh *et al.*, 1998; Lai & Cavanagh, 1997; Lau *et al.*, 2011; Zeng *et al.*, 2008). The recent outbreak of a novel coronavirus in 2012, MERS-CoV (Zaki *et al.*, 2012), put the spotlight back on CoVs and their potential to become highly pathogenic. The emergence of MERS-CoV is similar to SARS-CoV in causing severe acute respiratory disease and results in a mortality rate of approximately 60% (de Groot *et al.*, 2013), which is higher than that of SARS-CoV. Both the severity of MERS-CoV respiratory disease and genetic similarity to betacoronavirus are reminiscent of the emergence of SARS-CoV in 2003 (de Groot *et al.*, 2013; Peiris *et al.*, 2003).

Mouse hepatitis virus (MHV) is a coronavirus that is a natural pathogen of mice, has different strains, which, depending on the primary target tissue, can cause hepatitis, gastroenteritis or demyelinating encephalitis (Bergmann *et al.*, 2006; Perlman *et al.*, 1986; Weiss & Navas-Martin, 2005). However, studies have demonstrated that intranasal infection of mice with the MHV-1 strain induces pathologies and pulmonary injuries that are similar to characteristics observed in lungs of patients infected with SARS-CoV (De Albuquerque *et*

al., 2006; Franks *et al.*, 2003; Khanolkar *et al.*, 2009a; Leibowitz *et al.*, 2010; Tse *et al.*, 2004). MHV, SARS-CoV and MERS-CoV all belong to the same genera, betacoronavirus, which makes them share similarities in their replication, transcription mechanisms and genome organization (Snijder *et al.*, 2003; Thiel *et al.*, 2003; van Boheemen *et al.*, 2012). Hence, MHV-1 has been used as a clinically relevant model to study SARS-CoV pathogenesis. The following review will be focused on MHV-1 and its pathogenesis.

1.3.1 Biology of mouse hepatitis virus

MHV is enveloped, with a positive sense single-stranded RNA genome of about 30 kb in size (Sawicki *et al.*, 2005). Virions are 90 to 120 nm in diameter and contain a lipid bilayer surrounding a helical nucleocapsid to protect the genome (Sawicki et al., 2007). The large genomes of these viruses encode for three broad classes of proteins. The structural proteins include the spike (S), nucleocapsid (N), membrane (M), hemagglutinin esterase (HE) and envelope (E) proteins. The second class of proteins is the accessory proteins that are group-specific and differ among MHV, SARS-CoV (Figure 1.3) and other CoVs. These are non-essential for virus replication *in vitro* but may influence viral pathogenesis and disease outcomes *in vivo* (Sawicki *et al.*, 2005). In addition, they regulate virus-host interactions and promote development of an intracellular environment for viral growth (Frieman & Baric, 2008). The third class of proteins is the non-structural proteins that are encoded in the 5'-most two-thirds of the coronavirus genome (ORF1ab) and are essential for polyprotein processing and viral RNA synthesis (Brian & Baric, 2005; Sawicki *et al.*, 2005).

MHV infects host cells that display the carcinoembryonic antigen 1a receptor, CEACAM-1a, on their surface for attachment by the S glycoprotein and entry into the cell

(Nedellec *et al.*, 1994; Williams *et al.*, 1991). The virus entry into the cell occurs through fusion of viral and host plasma or endosomal membranes and subsequent release of genomic RNA into the cytoplasm, where replication and protein synthesis take place (Bergmann *et al.*, 2006; Weiss & Navas-Martin, 2005). Translation of the ORF1ab is initiated to generate mature replicase proteins and the RdRp. The RdRp drives replication of the genome through synthesis of full length negative strand RNA that serves as a template for full length genomic RNA and subgenomic mRNAs. The subgenomic mRNAs serve as templates for viral protein synthesis. After translation, S, M, E and HE proteins are then localized to the Golgi intracellular membranes where assembly occurs (Klumperman *et al.*, 1994). The N protein makes complexes with genome RNA, to form helical structures. It further interacts with the M protein (Kuo & Masters, 2002), just before budding into vesicles occurs. Virus release occurs through fusion of virion-containing vesicles with the plasma membrane. The S glycoprotein mediates cell to cell fusion, promoting syncytia formation and viral spread (Weiss & Navas-Martin, 2005).

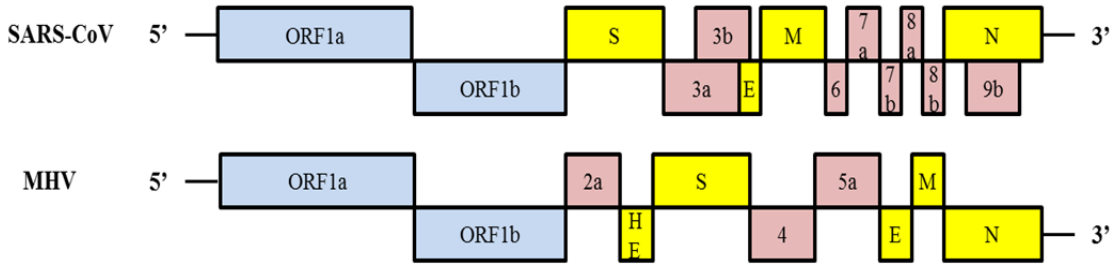

Figure 1.3 Diagram of representative MHV and SARS-CoV genomes. CoVs share a conserved organization of the RNA genome. The 5' two-thirds of genome contains large opening reading frames (ORF1ab) encoding non-structural proteins, in blue. The 3' one-third encodes for structural proteins (S, M, E, N and HE), in yellow along with accessory proteins unique to each virus group, in pink.

1.3.2 Mouse hepatitis virus-1 as an animal model for SARS-CoV

Intranasal inoculation of MHV-1 produces SARS-like clinical and pathological features in mice. This, however, varies among the mouse strains and the A/J mice has been found to be more susceptible to intranasal MHV-1 infection than other strains (De Albuquerque *et al.*, 2006). All A/J mice infected with MHV-1 develop severe respiratory symptoms including increased respiratory rate, decreased activity and eventual death (De Albuquerque *et al.*, 2006; Khanolkar *et al.*, 2009a; Leibowitz *et al.*, 2010). The histopathological examination of the lungs reveal a severe pneumonitis similar to that observed in SARS patients (Ding *et al.*, 2003; Nicholls *et al.*, 2003; Tse *et al.*, 2004). This includes intrapulmonary cell infiltrates such as macrophages, neutrophils, hyaline membrane, giant cells, congestion and hemorrhage (De Albuquerque *et al.*, 2006; Leibowitz *et al.*, 2010). Furthermore, the lung tissues show the presence of fibroleukin mRNA transcripts and protein (De Albuquerque *et al.*, 2006). Expression of fibroleukin is associated with pulmonary fibrin deposits, which is also a prominent feature in patients with SARS (Farcas *et al.*, 2005; Hwang *et al.*, 2005). The fibroleukin is expressed by inflammatory cells and ATI cells. The bronchial epithelial cells are fused, form giant cells, which is also a hallmark of SARS infected patients (De Albuquerque *et al.*, 2006). The potential benefit of IFN treatment was reported in SARS-CoV infected patients. Patients given IFN alfacon-1 displayed more rapid recovery than patients given other drugs (Lucchiari *et al.*, 1993). Similarly, mice that are given IFN treatment post-MHV-1 infection also exhibit a delayed on-set of disease (De Albuquerque *et al.*, 2006).

1.3.3 Mouse hepatitis virus-1 and the innate immune response

The pathological features seen in SARS are thought to be mediated by robust increases in proinflammatory cytokines in response to viral replication in the lungs (Chien *et al.*, 2006; Jiang *et al.*, 2005). In mice infected with MHV-1, there are higher levels of mRNA transcripts for IL-6, IFN-γ, IFN-β, IFN-α2, IFN-α4 and CCL2 at early time points (De Albuquerque *et al.*, 2006; Leibowitz *et al.*, 2010). More moderate induction of TNFα, FGL2, IFN-α1, IFN-α5, CCL3, CCL5 and CXCL2 is also observed (Leibowitz *et al.*, 2010). The early activation of these inflammatory mediators increases trafficking of immune cells such as monocytes and macrophages into the lungs very early in infection. The inflammatory mediators correlate with the observation seen in SARS patients (Ward *et al.*, 2005; Wong *et al.*, 2004). Furthermore, the levels of IFN-γ, TNF-α, IL-6, FGL2 and CCL2 are reported to increase over the course of infection and are associated with more severe pulmonary pathology (De Albuquerque *et al.*, 2006; Leibowitz *et al.*, 2010). These inflammatory cytokines and chemokines are also shown to be important in determining disease severity and host outcome in SARS patients (Wong *et al.*, 2004). It is important to determine the cell type responsible for the production of these cytokines *in vitro*.

1.4 Research objectives

The alveolar epithelial cells are the primary target of respiratory virus infections in the lung. Respiratory virus infections have been found to result in severe cases when the alveolar epithelial cells are infected hence it is important to study the role of these cells during viral pathogenesis. The recent emergence of MERS-CoV, which causes severe respiratory disease, and the occurrence of seasonal influenza outbreaks are a cause for

concern and highlight the need to better understand the pathogenesis of respiratory viruses. ATI and ATII cells are physiologically relevant cell types in which interactions of the respiratory viruses with these cells can be studied *in vitro*.

The overall objective of this work was to determine how the alveolar epithelial cells regulate immune response to infection by MHV-1 and PR8. In particular, to achieve this we optimized the isolation and culture of mouse ATI and ATII cells to use as an *in vitro* model. Next, we examined the susceptibility of these cultures to PR8 and MHV-1 and compared the innate immune responses of these cells during respiratory viral infections; this is discussed in Chapter 2. In Chapter 3, we used proteomic analysis of ATII cultures infected with IAV to identify potential mechanisms that contribute to viral pathogenesis in the lungs. The findings from this study will complement existing data in murine models of viral pathogenesis. This will improve our understanding of the virus/host interactions in the lung, which is needed for developing strategies of control and prevention of emerging coronaviruses and influenza viruses.

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Chapter 2

Differentiated phenotypes of primary murine alveolar epithelial cells and their susceptibility and response to infection by respiratory viruses

2.1 Abstract

Severe respiratory viral infections are associated with spread to the alveoli of the lungs. There are multiple murine models of severe respiratory viral infections that have been used to identify viral and host factors that contribute to disease severity. Primary cultures of murine alveolar epithelial cells provide a robust *in vitro* model to perform mechanistic studies that can be correlated with *in vivo* studies to identify cell type-specific factors that contribute to pathology within the alveoli of the lung during viral infection. In this study, we established an *in vitro* model to compare the responses of type I (ATI) and type II (ATII) alveolar epithelial cells to infection by respiratory viruses used in murine models: murine coronavirus MHV-1 and mouse adapted influenza A (H1N1) virus, strain PR8. Primary ATII cells were isolated from mouse lungs and cultured to maintain an ATII phenotype as determined by expression of LBP180 or trans-differentiate into an ATI cell phenotype determined by expression of T1α. Cells of both ATI and ATII phenotypes were susceptible to MHV-1 and PR8. Epithelial cells produce cytokines in response to viral infection, thereby activating immune responses. Thus, virus-induced cytokine expression was quantified in ATI and ATII cells. Both cell types had increased expression of IL-1β mRNA upon viral infection, though at different levels. MHV-1 and PR8 induced expression of a number of shared cytokines by ATI cells; however there were several cytokines whose expression was induced uniquely by MHV-1 infection. This *in vitro* model will be critical

for future studies to determine the roles of these specialized cell types in the pathogenesis of respiratory viral infections.

2.2 Introduction

Respiratory viral pathogens from several different families are a major source of morbidity and mortality worldwide. While infection of the upper respiratory tract is common and results in subclinical or mild disease, infection of the alveolar epithelium in the lungs can result in severe, potentially lethal diseases, including viral pneumonia, acute respiratory distress syndrome (ARDS) and severe acute respiratory syndrome (SARS). These severe diseases can result from infection by currently circulating viruses, including influenza viruses and respiratory syncytial virus (RSV), or by new viruses that emerge in the human population from animal reservoirs, such as new strains of influenza A virus (IAV) and severe acute respiratory syndrome-coronavirus (SARS-CoV). Murine models have been invaluable in the identification of virus and host determinants of disease pathogenesis during respiratory viral infections. While these models provide a complex view of host/pathogen interactions, there is a critical need to have physiologically relevant *in vitro* models that can be used to delineate cell type-specific mechanisms that contribute to disease pathogenesis in the lung. The goal of this study was to develop such an *in vitro* model, from which data can be correlated to well-established *in vivo* models of respiratory viral pathogenesis.

The extensive surface area of the alveolar epithelium is composed of two morphologically and functionally distinct cell types. ATI cells, which cover 95% of the surface area of the epithelium, are large thin cells that function in gas and ion exchange and fluid transport (Williams, 2003). ATII cells produce pulmonary surfactant that is required to

prevent alveolar collapse and proteins that participate in innate defense of the lung (Mason, 2006). As the dividing cells of the alveolar epithelium, ATII cells serve as progenitors to repair damaged epithelium. Research studies have taken advantage of this to establish ATI and ATII cell cultures *in vitro*. Isolation of the ATII cells and maintaining them in culture with either an ATI or ATII phenotype has been well-established in the rat model (Dobbs, 1990; Dobbs *et al.*, 2010), while few studies on these cultures have been done in mice (Corti *et al.*, 1996; DeMaio *et al.*, 2009; Marsh *et al.*, 2009; Rice *et al.*, 2002). The rat model has thus been used as a prototype for generating the murine ATI and ATII cell cultures *in vitro*. The differentiated phenotype of ATII cells and conversion of the ATII phenotype to an ATI phenotype is dependent on the extracellular matrix used to culture the cells and addition of factors in the medium to promote trans-differentiation. To maintain cells with ATII phenotype, cells are cultured on a collagen/matrigel matrix in the presence of keratinocyte growth factor (KGF) and fetal bovine serum (FBS) (Mason, 2002). Under these conditions, the ATII cells maintain lamellar bodies and surfactant production (Suwabe *et al.*, 1991; Xu *et al.*, 1998). To achieve an ATI phenotype, ATII cells are cultured on coverslips with fibronectin and medium containing FBS without KGF. These cultures have decreased expression of ATII-specific proteins and surfactant production by day 5, and have increased expression of ATI-specific proteins (Dobbs *et al.*, 1988; Ramirez *et al.*, 2003). As in the rat model, when cultured *in vitro*, mouse ATII cells readily lose expression of ATII-specific proteins, which suggests trans-differentiation to an ATI phenotype (Corti *et al.*, 1996; DeMaio *et al.*, 2009; Marsh *et al.*, 2009; Rice *et al.*, 2002). Though these studies describe a method for isolation and culture of mouse ATII cells, the transition to an ATI cell phenotype

was not characterized. Studies that fully characterize the ATI and ATII phenotypes of primary murine alveolar epithelial cells are still lacking.

The alveolar epithelium is a critical target for severe respiratory virus infections. Infection of ATI or ATII alveolar epithelial cells of the distal lung has been detected in fatal cases of avian H5N1 and 2009 pandemic (pH1N1) influenza viruses, RSV, and SARS-CoV (Johnson *et al.*, 2007; Korteweg & Gu, 2008; Nicholls *et al.*, 2006; Shieh *et al.*, 2010; Shieh *et al.*, 2005; Uiprasertkul *et al.*, 2007). Infection of alveolar epithelial cells is also associated with severe disease in murine models of respiratory viral infections, including mouse-adapted IAV and SARS-CoV (Blazejewska *et al.*, 2011; Hrincius *et al.*, 2012; Roberts *et al.*, 2007). Viral infection of these physiologically critical cell types causes direct damage to the alveolar epithelium and also immune-mediated pathology, both of which will impair respiration and/or lead to lung collapse due to impaired surfactant production. Alveolar epithelial cells produce inflammatory cytokines and chemokines in response to viral infection and thereby may elicit responses that contribute to both viral clearance and immune-mediated pathology.

Primary cultures of differentiated alveolar epithelial cells are a valuable model to study virus-host interactions in physiologically relevant cell types *in vitro*. The goals of this study were to optimize the culture of primary murine ATII cells to maintain an ATII cell phenotype or trans-differentiate into an ATI cell phenotype, then compare the susceptibility of ATI and ATII cultures to infection by respiratory viruses that cause severe disease in mice: PR8 and MHV-1. MHV-1 is a murine coronavirus that causes severe disease and pathology in the lungs of specific genetic lines of mice (De Albuquerque *et al.*, 2006; Khanolkar *et al.*, 2009b). The pathology of MHV-1 infection in A/J mice resembles that

seen in fatal SARS cases, thus MHV-1 is studied as a model of SARS pulmonary pathogenesis (De Albuquerque *et al.*, 2006). PR8 is a mouse-adapted IAV model for influenza infection in mice. The spread of PR8 to the alveoli results in increased disease severity in mice similar to influenza disease in humans (Blazejewska *et al.*, 2011; Shieh *et al.*, 2010). We further evaluated expression of inflammatory cytokines by ATI and ATII cultures in response to infection by these viruses. Based on their susceptibility and response to infection by respiratory viral pathogens, these cultures will be valuable in future studies to characterize the differential responses of ATI and ATII cells to viral infection and to identify the pathological mechanisms associated with viral infection in these biologically relevant cell types.

2.3 Materials and Methods

2.3.1 Cell lines and viruses

Madin-Darby canine kidney (MDCK; ATCC: CCL-34) cells were cultured in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) containing 10% FBS (Atlanta Biologicals, Norcross, GA) and 1% antibiotic-antimycotic (Invitrogen). 17Cl-1, a spontaneously transformed clone of BALB/c 3T3 cells (provided by Dr. Kathryn Holmes, University of Colorado Denver School of Medicine), were cultured in Dulbecco's modified eagle's medium (DMEM) (Invitrogen) with 10% FBS and 1% antibiotic-antimycotic. The murine lung epithelial cell lines used for identification of antibodies to distinguish ATI and ATII phenotypes were E10 and MLE-15, respectively. E10 is a spontaneously immortalized cell line derived from adult mouse lung ATII pneumocytes as described by Smith and colleagues (Smith *et al.*, 1984; Smith & Lykke, 1985). The cell line expresses 13 mRNAs

specific for ATI cells including T1α and aquaporin 5 but not ATII cell markers (Kathuria *et al.*, 2007a). E10 cells were provided by Dr. Maria Ramirez, Boston University School of Medicine. Cells were maintained in CMRL 1066 medium (Invitrogen), 10% FBS, 0.5 mM glutamine (Invitrogen) and 1% antibiotic-antimycotic. The MLE-15 cells are derived from pulmonary tumors in transgenic mice expressing SV40 large T antigen under transcriptional control of the human SP-C promoter (Wikenheiser *et al.*, 1993). They express SP-B and SP-C and have lamellar bodies. MLE-15 cells were provided by Dr. Beth Vordestrasse, Washington State University with permission from Dr. Jeff Whitsett, Cincinnati Children's Hospital Medical Center. The cells were cultured in HITES medium (RPMI 1640 with 1 X final mixture of insulin-transferrin-selenite (Invitrogen), 100 uM hydrocortisone (Sigma - Aldrich, St Louis, MO), 100 uM β-estradiol (Sigma-Aldrich) and 10 mM HEPES (EMD, Gibbstown, NY) supplemented with 2 mM L-glutamine, 2% FBS and 1% antibiotic-antimycotic. Cells were maintained at 37°C in 5% CO_2.

MHV-1 and IAV (A/Puerto Rico/8/1934/H1N1) were obtained from the American Type Culture Collection and BEI Resources, respectively. MHV-1 was propagated in 17Cl-1 cells, purified by sucrose gradient centrifugation, and titrated by plaque assay on 17Cl-1 cells, as previously described (Frana *et al.*, 1985; Sturman *et al.*, 1980). PR8 was propagated and titrated by plaque assay in MDCK cells in media containing 0.1% bovine serum albumin (BSA) (EMD) and TPCK-trypsin (Invitrogen; 1 ug/ml).

2.3.2 Primary mouse alveolar epithelial cells isolation and culture

Animal protocols were approved by the University of Idaho Animal Care and Use Committee according to the National Research Council Guide for the Care and Use of

Laboratory Animals. C57BL/6 mice from ages 6-8 weeks (16-21 g) were obtained from the Center for Reproductive Biology at Washington State University (Pullman, WA). ATII cells were isolated from mice using a previously published protocol (Corti *et al.*, 1996). ATII cells were dissociated from lung tissues by incubation in dispase (BD Biosciences, San Jose, CA), followed by mechanical disruption of the alveoli in DMEM with 0.01% DNase (Sigma-Aldrich). The cells were filtered and negative selection was performed by incubation with biotinylated monoclonal antibodies to CD16/32 and CD45 (Southern Biotechnology, Birmingham, AL) at 37°C for 30 min. After centrifugation, cells were incubated with Streptavidin-coated Dynabeads (Invitrogen) at 4°C for 30 min. This was followed by magnetic selection for 15 min to remove hematopoietic cells. Where indicated, we utilized cis-4-hydroxy-L-proline (cis-OH proline) (Sigma, St. Louis, MO) in our cultures at 100 ug/ml (Kao & Prockop, 1977). Alternatively, after negative selection cells were incubated three times for 40 min at 37°C on tissue culture treated dishes and non-adherent cells were plated as described below. To maintain an ATII cell phenotype, the ATII cells were cultured on millicell inserts (EMD Millipore Corp., Billerica, MA) coated with different ratios of rat tail collagen and BD Matrigel (BD Biosciences), in DMEM or bronchial epithelial cell growth medium (BEGM) (Lonza, Walkersville, MD) supplemented with KGF; 10 ng/ml (ProSpec, Rehovot, Israel) for 5 days. Final conditions to maintain a differentiated ATII cell phenotype included plating cells on 70% collagen and 30% matrigel with DMEM/10% FBS/KGF. To promote trans-differentiation into an ATI cell phenotype, ATII cells were cultured on fibronectin (Sigma-Aldrich; 5 ug/ml) in DMEM/10% FBS for five days. The cultures were maintained at 37°C and 10% CO_2.

2.3.3 Indirect immunofluorescence assay

Expression of ATI and ATII phenotypic markers on cell lines or primary cells and viral proteins was analyzed by immunofluorescence assay (IFA). Cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100 for 15 min at room temperature (RT). The following steps took place in a humidity chamber at 37°C. To block, cells were incubated in either 2% serum diluted in phosphate buffered saline (PBS) /0.1% BSA or 7% serum in PBS for 30 min. The cells were incubated in primary antibodies diluted in the blocking buffer for 1 hr. The primary antibodies used were mouse monoclonal antibody to LBP180 (Abcam, Cambridge, MA), Syrian hamster anti-T1α (provided by Dr. Maria Ramirez, Boston University School of Medicine), rabbit anti-ProSP-C (Millipore), rabbit anti-SP-B (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-aquaporin 5 (Millipore, Temecula, CA), mouse anti-vimentin (Millipore), and mouse anti-cytokeratin 5, 8 (Millipore). MHV-1 infection was evaluated using a monoclonal antibody that recognizes the nucleocapsid protein of MHV-1 (provided by Dr. Julian Leibowitz, Texas A&M University). Goat antiserum NR-3418, which recognizes the hemagglutinin protein of PR8 (BEI Resources) was used to detect PR8 infection. After washing unbound primary antibodies, cells were incubated for 15 min in blocking buffer at RT followed by incubation with secondary antibody diluted in blocking buffer at 37°C for 1 hr. The secondary antibodies used include goat anti-mouse-488 or anti-rabbit-555 (Invitrogen), rabbit anti-hamster-FITC (Abcam), and donkey anti-goat 555 (Invitrogen). Cells were stained with DAPI (Vector Labs, Burlingame, CA) to visualize nuclei and were photographed on a Nikon Eclipse Epifluorescent Microscope with Hamamatsu digital camera and MetaMorph software (Molecular Devices).

2.3.4 Western blot analysis

Cells were lysed in RIPA buffer (25 mM Tris-HCl; pH 7.6, 1 M NaCl, 1% NP-40, 0.5% sodium deoxycholate and 10% SDS) with protease inhibitors. Equal amounts of cell protein, as determined by Bicinchoninic acid (BCA) protein assay (Thermo Scientific, Rockford, IL), were electrophoresed on a 4-20% SDS-PAGE gel (Thermo Scientific) with Bench Marker (Invitrogen) as a size standard. Proteins were transferred to PVDF membranes and blocked with 5% nonfat dry milk in TBS-Tween (20 mM Tris pH 8.4, 135 mM NaCl and 0.5% Tween-20). Membranes were incubated with primary antibody to T1α diluted in 0.5% nonfat dry milk/TBS-Tween 20 overnight at 4°C. After washing, membranes were incubated with horseradish peroxide (HRP)-conjugated anti-hamster secondary antibody (Abcam) for 1 hr at RT. Equal protein loading was evaluated using an antibody specific for β-actin conjugated to HRP (Abcam). HRP was detected on film with Pierce enhanced chemiluminescence (ECL) substrate (Thermo Scientific).

2.3.5 Cytokine gene expression analysis

Total cellular RNA was isolated using Trizol Reagent, according to the manufacturer's recommendations (Invitrogen). Mouse Inflammatory Cytokines and Receptors RT^2 Profiler PCR Arrays were used to quantify the expression of 84 inflammatory genes in PR8-, MHV-1-, and mock-inoculated ATI cells (SABiosciences/QIAGEN, Valencia, CA). Where indicated, lipopolysaccharide (LPS; Sigma Aldrich; 1 ug/ml) was used to induce cytokine expression. Expression of IL-1β was quantified in mock-inoculated and virus-infected ATI and ATII cells by qPCR using published primer sequences (Oh *et al.*, 2011) and SYBR green in a StepOnePlus instrument (Applied Biosystems, Carlsbad, CA).

Expression of β-actin was quantified to determine relative expression levels, using primers: forward, 5'-AAGTCCCCTCACCCTCCCAAAAG-3' and reverse, 5'-AAGCAATGCTGTCACCTTCCC-3'. Relative expression was determined by the reciprocal of the ΔCt (1/Ct [IL-1β] – Ct [β-actin]). Means and standard errors from at least three experiments were used to test for statistical significance compared to expression in mock-treated cells using an unpaired *t* test and $p<0.05$ was determined to be significant.

2.4. Results

2.4.1 Identification of antibodies to distinguish ATI and ATII phenotypes

In order to confirm that the cultured cells expressed proteins that are characteristic of ATI or ATII cells *in vivo*, identification of antibodies that distinguished ATI/ATII phenotypes was assessed by IFA on cell lines derived from mouse lung epithelium and fibroblasts cells. We observed that antibodies for aquaporin 5, SP-B and SP-C did not react with the expected cell lines (Figure 2.1). SP-B and SP-C antibodies, which should be specific for ATII cells bound to E10 (ATI) and 17C1-1 (fibroblast) cells, but not MLE-15 (ATII) cells. The antibody for aquaporin 5, which should be specific for ATI, bound non-specifically to the MLE-15 (ATII) cells but not to E10 (ATI) cells. This was further examined on the primary cells (Figure 2.2). On day 1, the primary cells have the cuboidal shape of ATII cells and by day 5 they attain the flat morphology that corresponds with trans-differentiation to an ATI phenotype. On day 1, SP-C antibody bound specifically to ATII phenotype but non-specifically to ATI phenotype on day 5. Similarly, the antibody for aquaporin 5, which should be specific for ATI phenotype on day 5, also bound non-specifically to ATII phenotype on day 1. These antibodies were not utilized in characterizing

the phenotypes of the alveolar epithelial cells. We then used other antibodies that have been found to react with cell markers for ATI and ATII phenotypes. Expression of LBP180 was evaluated to identify ATII phenotype (Mulugeta *et al.*, 2002). T1α is a marker of differentiated ATI cells that is not expressed by ATII cells, and was used to monitor expression of an ATI phenotype (Dobbs *et al.*, 1988; Ramirez *et al.*, 2003). In Figure 2.3, T1α antibody bound specifically to E10 cells (ATI) but not to MLE-15 (ATII) or 17C1-1 (fibroblast) cells. Likewise, LBP180 bound specifically to the respective cell line, MLE-15 (ATII) cells, but not to E10 (ATI) or 17C1-1 (fibroblast) cells. We further assessed the specificity of vimentin and cytokeratin 5, 8 on their respective cell lines. As shown in Figure 2.3, vimentin bound specifically to 17C1-1 (fibroblasts) cells, but not to E10 (ATI) or MLE-15 (ATII) cells. Cytokeratin 5, 8 antibodies also bound specifically to E10 (ATI) and MLE-15 (ATII) cells, but not to 17C1-1 (fibroblast) cells. Vimentin and cytokeratin 5, 8 antibodies were used to detect any contaminating fibroblasts in our cultures and to confirm the epithelial nature of the ATI and ATII cells, respectively.

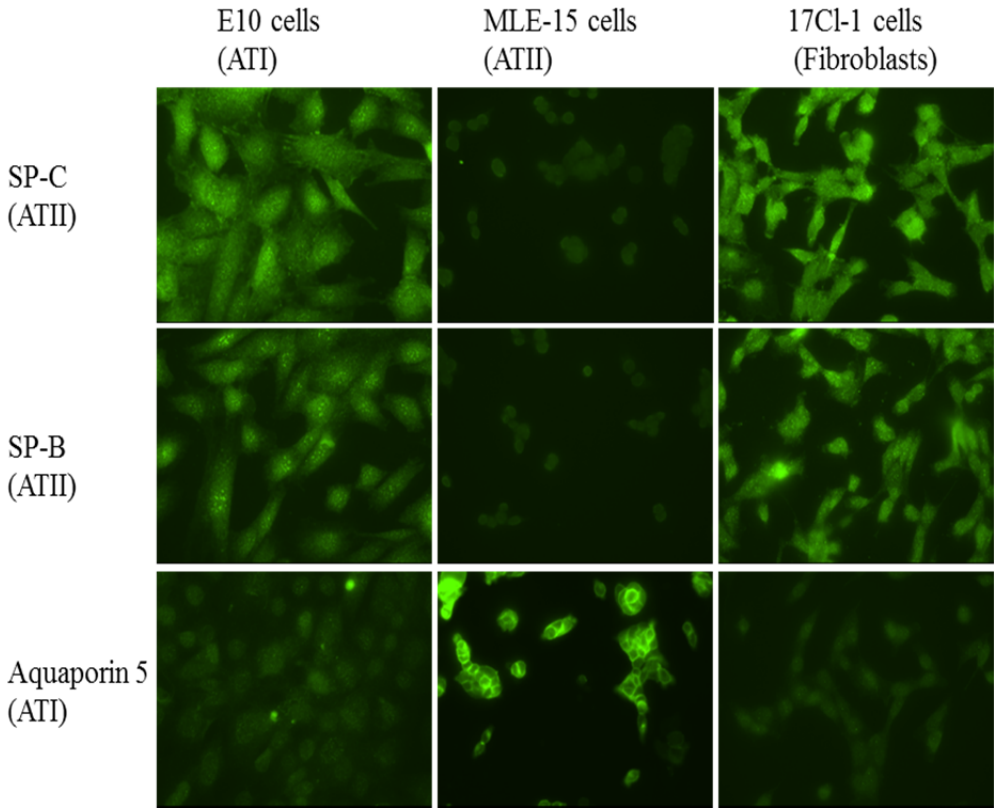

Figure 2.1 Non-specific binding of antibodies on murine alveolar epithelial and fibroblast cell lines. Immunofluorescence assay was utilized to detect ATII (SP-C and SP-B) and ATI (aquaporin 5) marker proteins on cell lines with ATI (E10), ATII (MLE-15) or fibroblast (17C1-1) phenotypes.

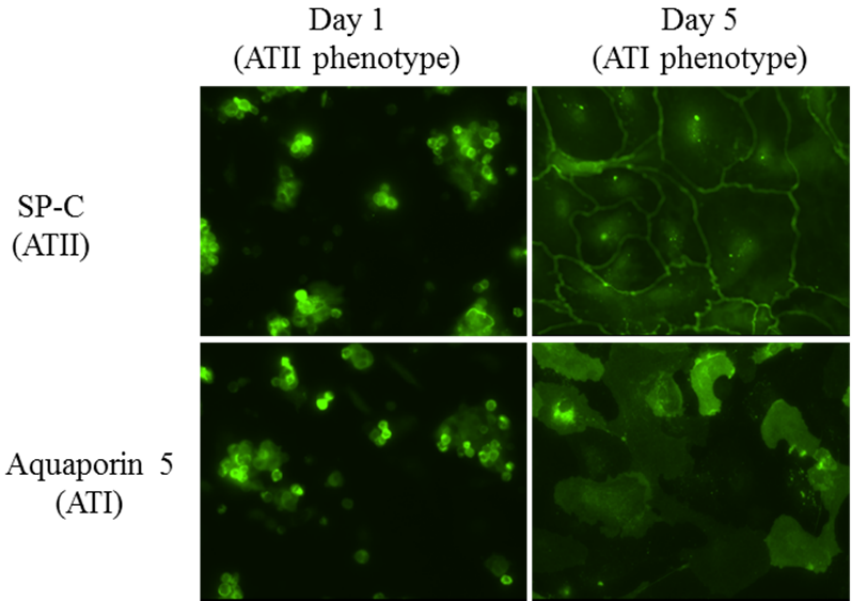

Figure 2.2 Non-specific binding of ATI and ATII marker antibodies on ATI and ATII primary cells with an ATI or ATII phenotype. Primary ATII cells were cultured on collagen-coated coverslips for the indicated times and expression of ATII (SP-C) and ATI (aquaporin 5) marker proteins were analyzed.

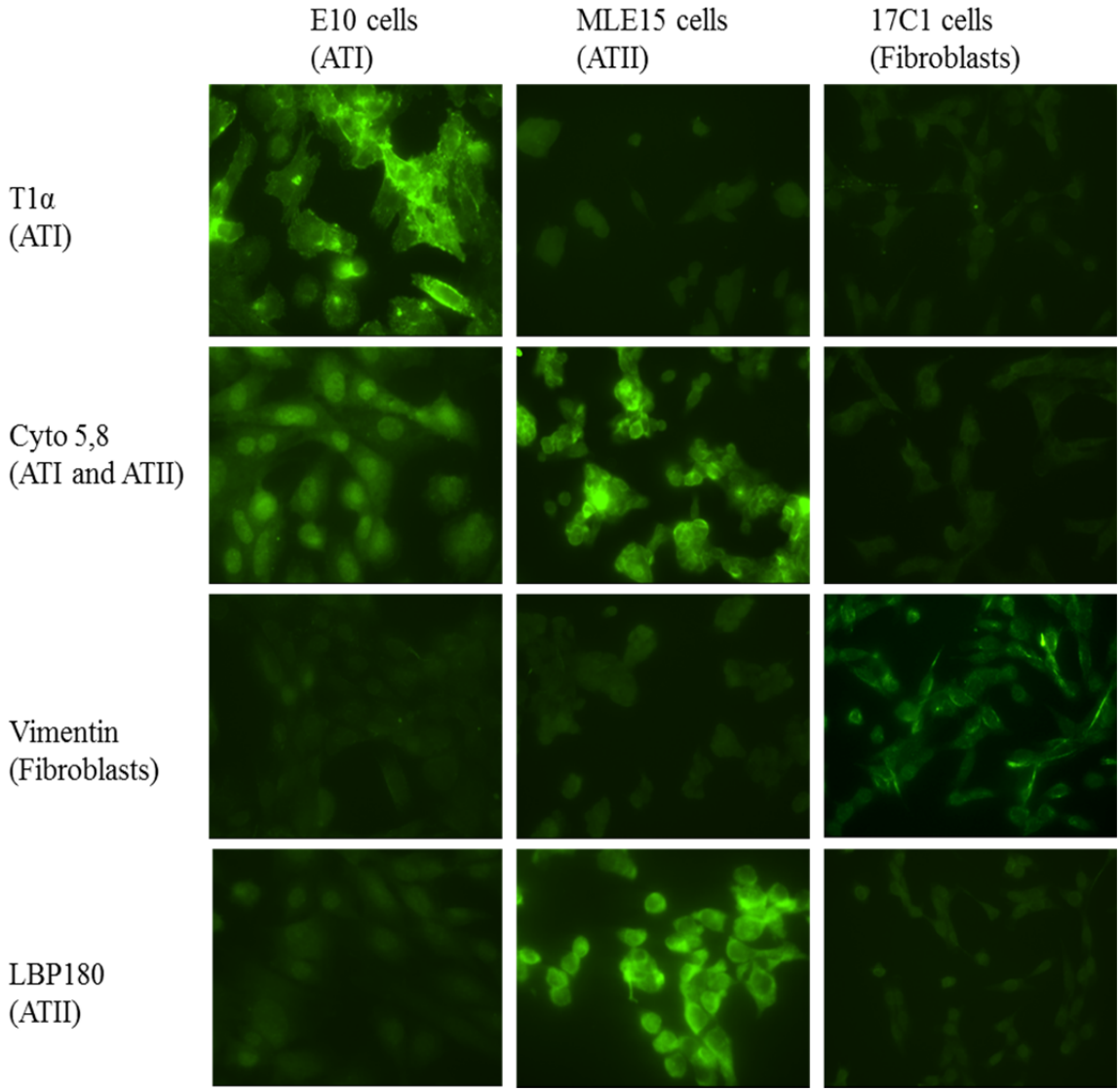

Figure 2.3 Specific binding of T1α, cytokeratin, vimentin and LBP180 antibodies to the respective cell lines. Immunofluorescence staining was used to detect ATI (T1α), epithelial (cytokeratin 5, 8), fibroblasts (vimentin) and ATII (LBP180) cell markers.

2.4.2 Optimization of ATII isolation

Initially there were challenges in the isolation of ATII cells due to contaminating fibroblast cells in the cultures. ATII cells cultured on collagen-coated coverslips showed expression of vimentin (fibroblasts-specific) on day 5 of culture (Figure 2.4A). We then used cis-OH proline to inhibit the growth of fibroblasts. Fibroblast proliferation relies on collagen, which is rich in proline. cis-OH proline is a proline analogue that gets incorporated into procollagen polypeptides to prevent the chains from folding into a triple helical conformation, thereby reducing its synthesis (Kao & Prockop, 1977). A previous study used cis-OH proline to remove fibroblasts from rat ATII cell cultures (Willis *et al.*, 2005), however this proved to be a challenge on the mouse ATII cell culturing in our study. ATII cells cultured in the presence of cis-OH proline had reduced expression of T1α when compared to cultures without cis-OH proline (Figure 2.5). As an alternative, we removed contaminating fibroblasts by plating freshly isolated ATII cells on three subsequent tissue treated dishes, called panning. This resulted in rapid adherence of the fibroblasts on tissue treated dishes (Figure 2.4B), after which the non-adherent ATII cells were plated on collagen/matrigel or fibronectin. After panning, the analysis of freshly isolated cells by IFA for expression of ATII cell marker protein LBP180 ranged from 87.7 to 90.7% positive (Figure 2.6).

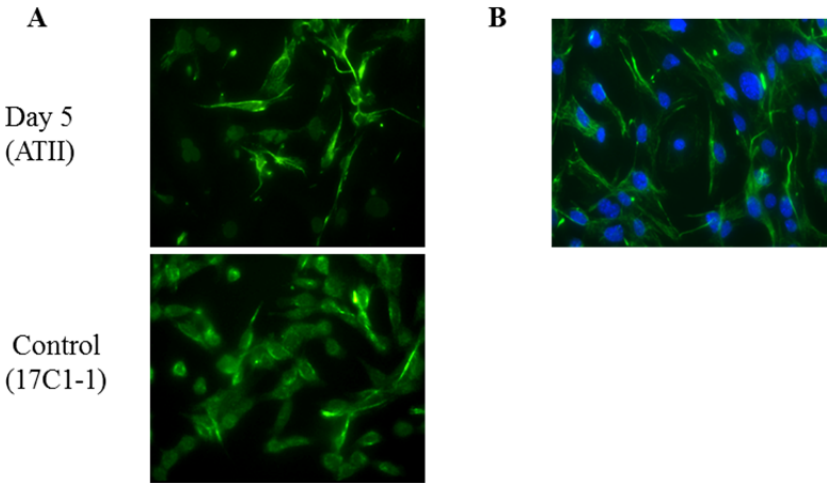

Figure 2.4 Fibroblast contamination of ATII cell cultures. (A) ATII cells were cultured on collagen-coated coverslips for 5 days and fibroblast contamination was detected by immunofluorescence using antibody to vimentin (green). 17Cl-1 cells were used as a positive control. (B) Fibroblasts removed during ATII cell isolation. Expression of vimentin was analyzed by immunofluorescence (green) and nuclei visualized by DAPI (blue).

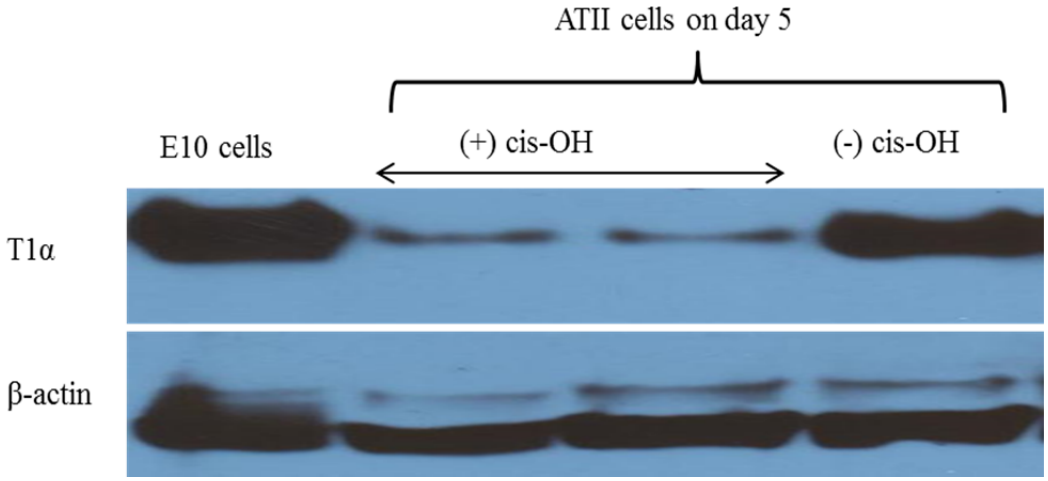

Figure 2.5 ATII cells cultured in the presence of cis-OH proline have reduced expression of T1α on day 5. ATII cells were cultured on collagen coated coverslips and given DMEM in the presence or absence of 100 ug/ml cis-OH proline and allowed to trans-differentiate for 5 days. Cell lysates were analyzed by western blot using antibody against T1α. E10 cells are a positive control. β-actin was used as a protein loading control.

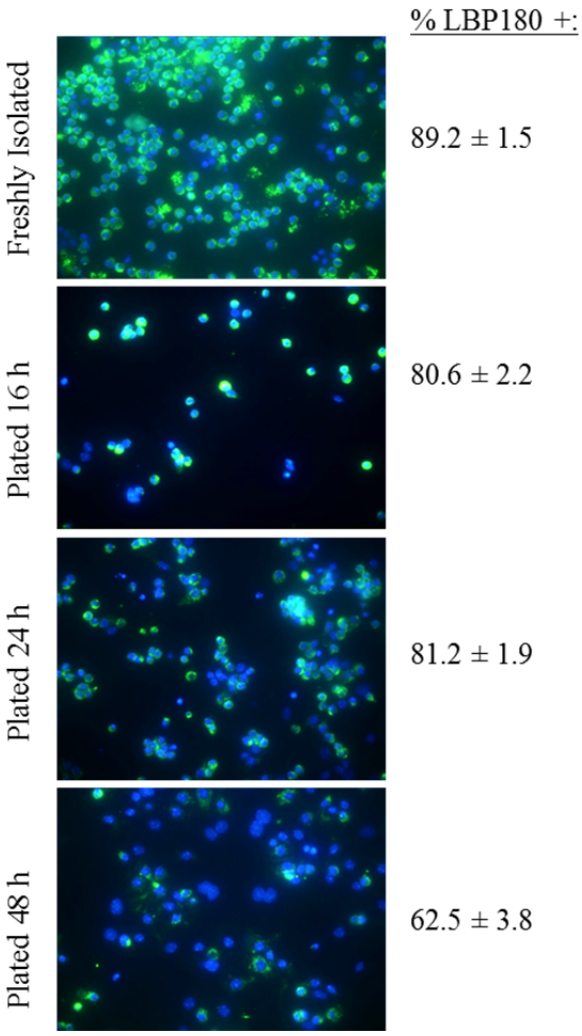

Figure 2.6 Expression of ATII cell marker protein, LBP180, is rapidly lost upon culture of ATII cells on fibronectin. ATII cells were cytospun onto glass slides (Freshly Isolated) or plated on fibronectin-coated coverslips for 16, 24, or 48 h. Expression of LBP180 was analyzed by immunofluorescence (green) and nuclei were visualized by DAPI staining (blue). Images were obtained from 7 random fields and the cells were counted to determine the percent positive for LBP180 antigen. The values to the right of each panel are the mean percentages and standard errors from these counts.

2.4.3 Phenotypes of ATI and ATII cultures

Antibodies against LBP180 and T1α were accurate at distinguishing between ATI and ATII continuous cell lines and did not recognize fibroblasts (Figure 2.3). Hence, LBP180 and T1α antibodies were further utilized to characterize the ATI and ATII phenotypes of primary cultures. Freshly isolated ATII cells were analyzed for LBP180 expression by IFA and random fields were counted to estimate the percentage of positive cells. Approximately 90% of freshly isolated cells expressed the ATII-specific protein, LBP180 (Figure 2.6). The percentage of cells expressing LBP180 rapidly decreased during culture on fibronectin-coated coverslips, to approximately 62% positive by day 2 after isolation (Figure 2.6 and Figure 2.7A). Further culture of murine ATII cells on fibronectin resulted in significantly reduced expression of LBP180 (ATII-specific) by day 4 after isolation and LBP180 was not detected on day 7 (Figure 2.7A, left panels). Under these same culture conditions, the expression of T1α (ATI-specific) was increased by day 4 after isolation, which increased further by day 7 (Figures. 2.7A and B). Thus, concomitant with the loss of LBP180 expression, these primary cultures acquired expression of T1α, suggesting a transition from a predominantly ATII to ATI phenotype.

In order to promote expansion of the ATII cells while maintaining their phenotype, mixtures of collagen/matrigel in different media were tested. The mixture containing 30% collagen and 70% matrigel caused ATII cells to form clumps that made the apical surface to face inward, making it inaccessible for IFA analysis (Table 2.1). The cells on 70% collagen and 30% matrigel and provided with KGF in the growth medium maintained expression of LBP180 through 7 days after isolation, with maximal expression on day 5 (Figure 2.8A, right panels). Exclusion of KGF from the medium resulted in significantly fewer LBP180-

expressing cells on days 5 and 7 after isolation (Figure 2.8A, left panels). Furthermore, ATII cells cultured on 70% collagen and 30% collagen with KGF did not express an ATI cell-specific protein, T1α, in comparison to trans-differentiated ATI cells cultured on fibronectin (Figure 2.8B). Thus, murine ATII cells maintained expression of LBP180 for short time periods (2 days) of culture on fibronectin alone and for longer time periods (5-7 days) when cultured on 70% rat tail collagen and 30% matrigel matrix with KGF. The optimized ATI and ATII culture conditions are summarized in Figure 2.9.

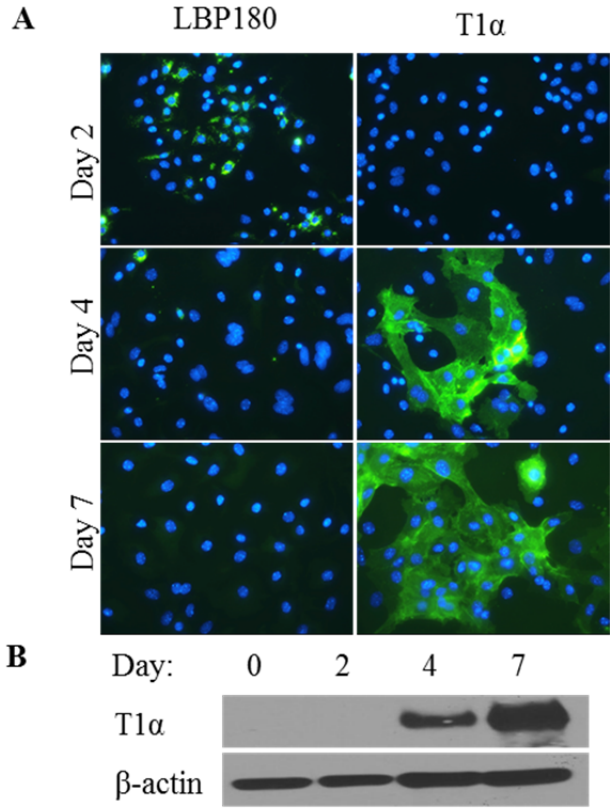

Figure 2.7 Trans-differentiation of murine ATII cells to an ATI cell phenotype. (A) ATII cells were cultured on fibronectin-coated coverslips for the indicated times and immunofluorescence assay was used to detect expression of phenotypic marker proteins of ATII cells, LBP180, or ATI cells, T1α. Nuclei were stained with DAPI, (blue). (B) ATII cells were lysed on the day of isolation (day 0) or cultured on fibronectin and lysed on the indicated days. Cell lysates were analyzed by western blot analysis using antibody against T1α or β-actin, as a protein loading control. The images shown are representative of three replicate experiments.

Table 2.1 Phenotypic characterization of ATII cells cultured under various collagen/matrigel ratios and media formulations.

% Collagen:% Matrigel ratio	Medium	Results
30:70	Complete DMEM+KGF	ATII cells formed aggregates and the apical surface was inaccessible for IFA analysis
30:70	Complete BEGM+KGF	
70:30	Complete DMEM +KGF	ATII phenotype (a higher proportion of cells expressed LBP180)
70:30	DMEM/cs-FBS+KGF	ATII phenotype

DMEM= Dulbecco's modified eagle medium; **BEGM**= Bronchial epithelial growth medium; **FBS**= Fetal bovine serum; **cs-FBS**= charcoal stripped FBS.
Complete DMEM= DMEM + 10% FBS+2% antibiotics; **KGF**= 10 ng/ml keratinocyte growth factor; **Complete BEGM**= BEGM + 2% antibiotics + 1% glutamine + 1% cs-FBS.

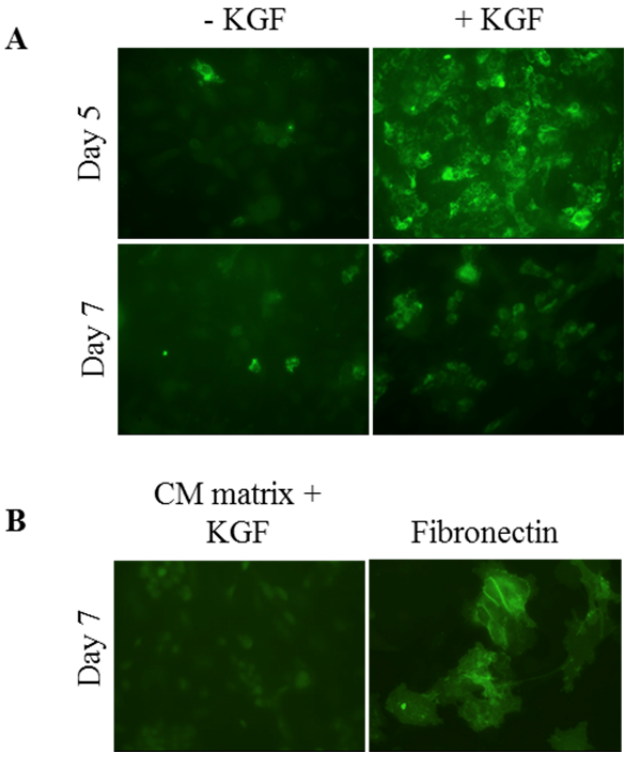

Figure 2.8 Maintenance of ATII cell phenotype in cultured murine cells. ATII cells were cultured on 70% collagen/30% matrigel (CM) matrix for 5 or 7 days with or without keratinocyte growth factor (KGF) in the medium. Immunofluorescence assay was used to detect the expression of (A) ATII marker protein, LBP180, or (B) ATI marker protein, T1α. Cells cultured on fibronectin were used as a positive control for T1α expression

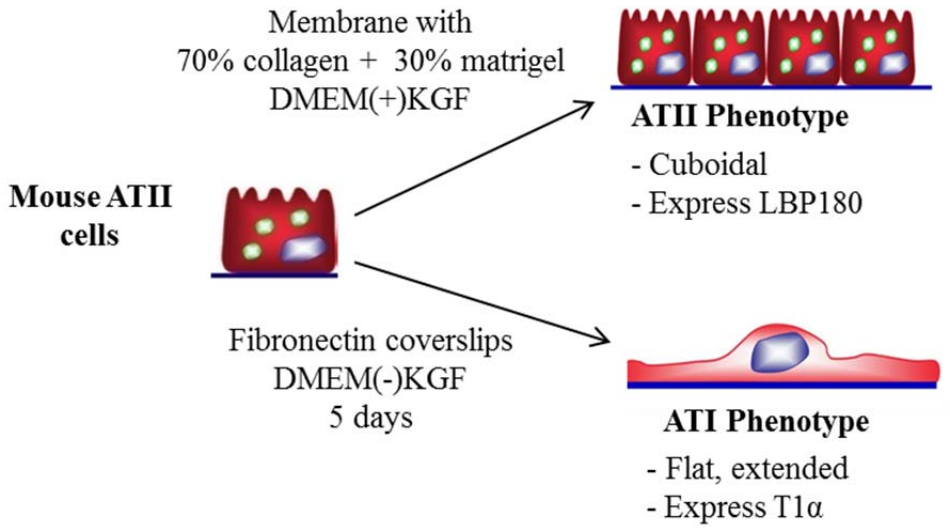

Figure 2.9 Schematic representation of culture conditions for primary differentiated alveolar epithelial cells. ATII cells are isolated from mouse lung cells and cultured to maintain an ATII phenotype or trans-differentiate into an ATI phenotype.

2.4.4 MHV-1 replicates in primary murine cultures with an ATI or ATII cell phenotype.

ATI cells were inoculated with MHV-1 and monitored for cytopathic effects (CPE), the presence of viral antigen, and production of infectious virus over time. MHV-1 infection of ATI cells resulted in the formation of syncytia and rounding up of cells in the monolayer by 24 hours post infection (h p.i.) (Figure 2.10A, top panels and 2.10B). Viral nucleocapsid protein antigen was detected in approximately 50% of the cells by 12 h p.i., and expanded to the majority of cells in the culture by 24 h p.i. (Figure 2.10A, top panels). The presence of viral antigen corresponded to increased release of infectious virus through 24 h p.i. (Figure 2.10C).

ATII cultures were also tested for susceptibility to infection by MHV-1. In agreement with infection of ATI cells, viral antigen was detected in ATII cells at 12 and 24 h p.i., and the titer of infectious virus in the medium increased over a similar time course (Figure 2.10A, bottom panel and 2.10C). In contrast to infection of ATI cells, MHV-1 did not induce syncytia formation or visible CPE in ATII cell cultures. Despite a lower proportion of viral antigen-expressing cells, cultures with an ATII phenotype produced higher titers of virus through 24 h p.i. (Figure 2.10C). Syncytia formation and cell death seen in the ATI cells may limit the production of MHV-1 compared to infection in ATII cultures. These results show that MHV-1 established a productive infection in cultures with either ATI or ATII cell phenotypes, which differed in CPE and virus production.

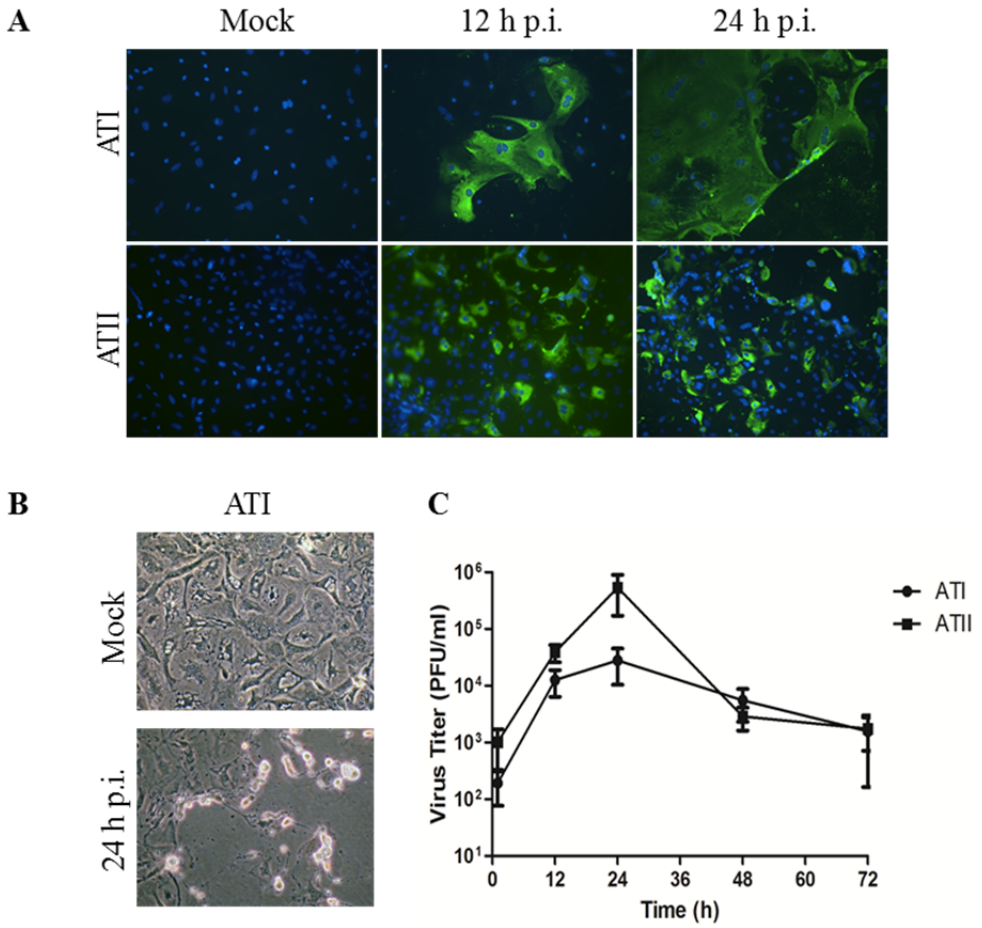

Figure 2.10 Susceptibility of primary murine alveolar epithelial cells to infection by murine coronavirus, MHV-1. (A) Murine cells were cultured as either an ATI or ATII cell phenotype for 5 days, then were inoculated with MHV-1 for 12 h or 24 h. Infection was analyzed by immunofluorescence assay of viral nucleocapsid protein (green) and nuclei were stained with DAPI (blue). (B) Cells were cultured on fibronectin (ATI phenotype) and inoculated with MHV-1 or mock-inoculated. Cells were photographed on phase contrast at 24 h p.i. (C) Infection of MHV-1-inoculated ATI and ATII cells was analyzed by plaque assay of supernatant medium at the indicated times. The mean virus titers and standard errors from four replicate experiments are shown.

2.4.5 PR8 replicates in primary murine cultures with an ATI or ATII cell phenotype

The PR8 strain of influenza A virus is frequently used as a model for viral pneumonia, and the spread of PR8 to the alveoli in infected mice corresponds with increased disease severity (Blazejewska *et al.*, 2011; Hrincius *et al.*, 2012). To determine whether primary murine cells with an ATI or ATII cell phenotype are susceptible to PR8 infection *in vitro*, primary cultures were inoculated with PR8 and infection was analyzed by IFA and plaque assay. Like MHV-1, PR8 infected primary cultures with both ATI and ATII cell phenotypes (Figure 2.11). However, the kinetics of infection were slower, with the peak of viral antigen expressing cells present at 48 h p.i. Although CPE was not apparent, reduced viral antigen expression late in infection (72 h p.i.) suggests a decline in the number of susceptible cells in the culture.

The viral growth curves for PR8 replication in ATI and ATII cells were indistinguishable (Figure 2.11C). In both ATI and ATII cultures, fewer cells were infected by PR8 compared to MHV-1. As our phenotypic analysis of these cultures demonstrated heterogeneity in expression of ATI and ATII marker proteins (Figs 2.7 and 2.8), we next determined whether PR8 infects differentiated cells or other cells within these cultures. Co-localization of viral antigen and T1α (ATI-specific protein) was evaluated in ATI cultures 24 h after inoculation with PR8 by dual IFA. A majority of cells that contained PR8 antigen were also positive for T1α expression, which demonstrated that PR8 infects cells with an ATI cell phenotype (Figure 2.11B, to panel). However, many T1α positive cells were not infected by PR8. Similarly, in cells cultured with an ATII phenotype, PR8 antigens were detected in cells expressing ATII phenotypic marker, LBP180 (Figure 2.11B, bottom panels). However, PR8 infection was not exclusively found in LBP180-positive

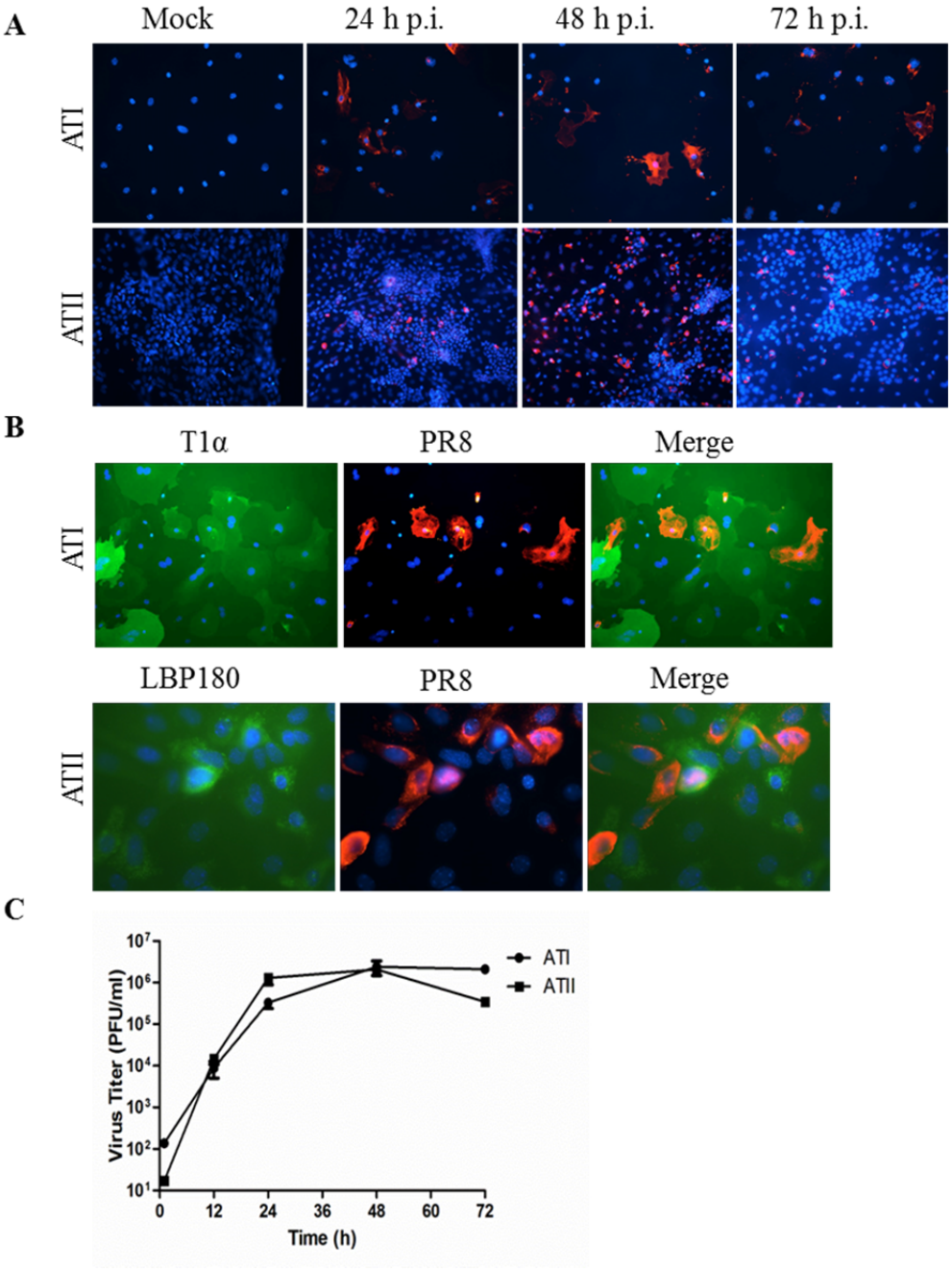

Figure 2.11 Susceptibility of primary murine alveolar epithelial cells to infection by influenza A virus, PR8. (A) Murine cells were cultured as either an ATI or ATII cell phenotype for 5 days, then were inoculated with PR8. Infection was analyzed by immunofluorescence assay of viral hemagglutinin protein (red) and nuclei were stained with DAPI (blue) at the indicated times post infection (p.i.). (B) Co-localization of ATI (T1α) or ATII (LBP180) phenotypic proteins (green) and PR8 antigen (red) was analyzed by dual IFA 24 p.i. Nuclei were stained with DAPI (blue). (C) Infection of PR8-inoculated ATI and ATII cells was analyzed by plaque assay of supernatant medium at the indicated times. The mean virus titers and standard errors from five replicates are shown.

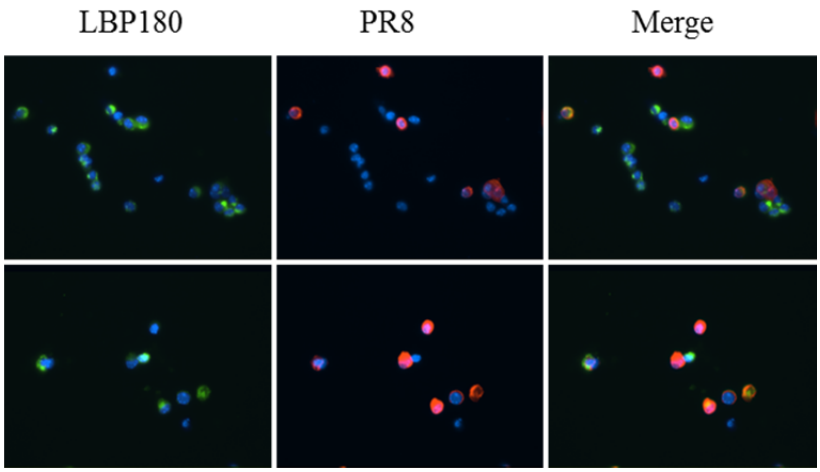

Figure 2.12 Co-localization of PR8 and LBP180 antigens in ATII cells infected on day 1 after isolation. Murine ATII cells were plated on fibronectin-coated coverslips overnight, then inoculated with PR8. Expression of viral antigen (red) and ATII cell phenotypic marker LBP180 (green), were analyzed by IFA 24 h p.i. The two rows of images are from two independent experiments.

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cells. A majority of ATII cells plated on fibronectin remained LBP180 positive by day 2 after isolation (Figure 2.6). Therefore, we inoculated ATII cells with PR8 one day after isolation and performed dual IFA for LBP180 and PR8 antigens 24 h p.i (Figure 2.12). As with our ATII cells plated on 70% collagen and 30% matrigel matrix, some but not all LBP180 positive cells were also positive for PR8 antigens. Taken together, these experiments demonstrate that PR8 does infect alveolar epithelial cells expressing ATI or ATII specific proteins.

2.4.6 Cytokine gene expression in virus-infected alveolar epithelial cells

Alveolar epithelial cells express cytokines and chemokines in response to viral infection, thereby contributing to the influx of inflammatory cells into the lungs. We evaluated the response of ATI cells to infection by MHV-1 and PR8 using RT-PCR arrays

that quantify RNA for 84 cytokines. Compared to mock-inoculated cells, PR8 and MHV-1 induced mRNA expression of a shared group of cytokines (IL-10, IL-16, IL-1β, IL-36α, TNF-α, LT-α, LT-β), chemokines (CCL2, 4, 7, 8, CXCL1, 10, 14), and receptors (CCR3, TNFRII) (Table 2.3). In addition to these shared cytokines, MHV-1 induced expression of several additional genes that were not induced by PR8 infection in ATI cells (Table 2.4). In contrast, only three chemokine genes (CCL5, CCL9 and CXCL11) were differentially expressed by PR8-infected cells.

As a canonical pro-inflammatory cytokine that contributes to the local recruitment and activation of inflammatory cells in addition to systemic symptoms of viral infection, the production of IL-1β has important consequences for both viral clearance and disease severity. Expression of IL-1β mRNA was quantified by qRT-PCR in ATI cells infected by PR8 or MHV-1 at 6 and 24 h p.i. (Figure 2.13A), and in ATII cells infected by PR8 or MHV-1 at 24 h p.i. (Figure 2.13B). LPS was used as a positive control to induce IL-1β expression in both cell types. Expression of IL-1β was induced significantly by PR8 and MHV-1 infection of ATI cells by 24 h p.i (Figure 2.13A). MHV-1 infection stimulated a similar increase in IL-1β expression in both ATI cells and ATII cells (Figures 2.13A and B); however PR8 infection in ATII cells stimulated a lower level of IL-1β expression, which did not reach statistical significance (Figure 2.13B). Interestingly, while both ATI and ATII cells expressed IL-1β in response to LPS, expression in ATI cells was induced to a higher level. These results show that primary murine alveolar epithelial cells respond differently to viral infection and LPS stimulation under culture conditions that generate an ATI or ATII phenotype.

Table 2.3 Cytokine gene expression in ATI cells infected by PR8 and MHV-1[a]

	Fold Change vs. Mock[b]	
Gene	**PR8**	**MHV-1**
BCL6	2.8	4.0
CCLl2	2.2	3.2
CCL20	3.1	11.1
CCL4	2.1	2.0
CCL7	2.2	3.0
CCL8	2.6	2.3
CCR3	3.2	3.8
CXCL1	5.0	11.5
CXCL10	8.0	5.2
CXCL13	5.6	2.1
IL-10	2.4	10.7
IL-16	2.8	5.8
IL-1β	2.1	10.3
IL-36α	3.0	3.7
LT-α	2.6	5.6
LT-β	2.3	25.2
TNF-α	9.1	6.0
TNFRII	2.7	5.2

[a]RNA was extracted from PR8 or MHV-1 infected ATI cells 24 h p.i. and mRNA for inflammatory cytokines and receptors was quantified by qPCR Array (SABiosciences). Genes included are those with at least a two fold increase compared to mock-inoculated cells in at least two replicate experiments.
[b]Fold change values are representative values from three (PR8) or two (MHV-1) replicate samples.

Table 2.4 Cytokine genes expressed in ATI cells infected by MHV-1 but not PR8[a]

Gene	Fold Change vs. Mock[b]
ABCF1	2.7
AIMP1	3.3
CCL17	4.7
CCL24	2.9
CCL25	2.6
CCL3	2.3
CCR9	6.2
CXCl5	13
IL-10Rβ	3.9
IL-11	30
IL-15	2.0
IL-1α	6.8
IL-1R1	2.9
IL-3	7.2
IL-6Rα	3.4
IL-6ST	3.8
TGFβ1	3.9
CXCL12	3.6
IL-2Rγ	4.7
TNFRSF1A	3.8
TOLLIP	2.7

[a]RNA was extracted from PR8 or MHV-1 infected ATI cells 24 h p.i. and mRNA for inflammatory cytokines and receptors was quantified by qPCR Array (SABiosciences). Genes included are those with at least a two fold increase in MHV-1-infected compared to mock-inoculated cells that have no increase in PR8-infected cells.
[b]Fold change values are representative values from two (MHV-1) replicate samples.

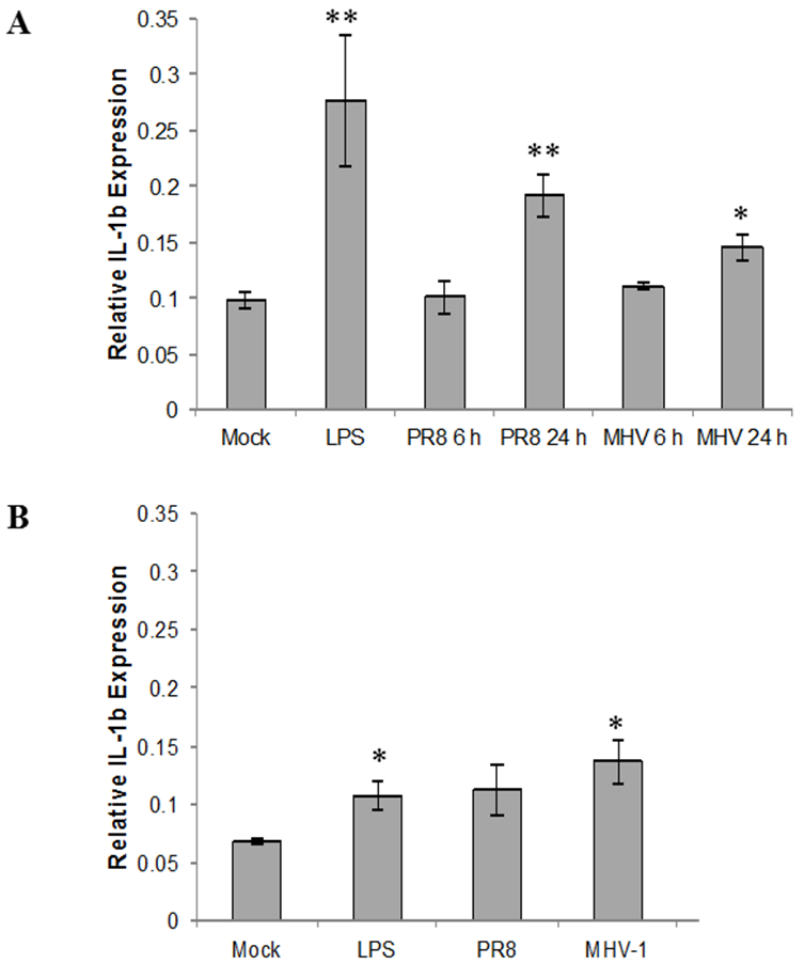

Figure 2.13 Expression of IL-1β by primary murine alveolar epithelial cells in response to respiratory viral infection. Murine cells were cultured as an ATI (A) or ATII (B) cell phenotype, infected by the indicated viruses, and IL-1β mRNA was quantified by RT-qPCR. Mock-inoculated and LPS-treated cells were used as negative and positive controls, respectively. Expression of IL-1β was normalized to that of β-actin and the means and standard errors from 3-5 replicate experiments are shown. Statistically significant differences in expression compared to mock samples were determined by unpaired t test. *= $p<0.05$, **= $p<0.005$.

2.5. Discussion

Due to its critical function in respiration, damage to the alveolar epithelium by viral infection results in severe, potentially fatal, disease. In order to design novel therapies that successfully limit this damage, it is imperative that we identify the mechanisms that contribute to lung pathology during infection of alveolar epithelial cells. The response of differentiated ATI and ATII cells to viral infection has been studied in a variety of *in vitro* models. Although mouse ATII cell isolation and culture has been performed and trans-differentiation into ATI characteristics was reported (Corti *et al.*, 1996; Herold *et al.*, 2006; Messier *et al.*, 2012; Rice *et al.*, 2002), full characterization of the ATI and ATII phenotypes in primary murine alveolar cells is still lacking. We modified other published methods to optimize isolation and culture of murine ATII cells. To characterize the primary alveolar epithelial cells, antibodies to distinguish ATI and ATII phenotypes were optimized on continuous cell lines. T1α and LBP180 bound specifically to the ATI and ATII cell lines, respectively. The incorporation of panning of ATII cell suspensions to remove fibroblasts increased the purity of our ATII cells. Culturing these cells in a mixture of 70% collagen and 30% matrigel with KGF maintained the cells in the ATII phenotype as shown by the cuboidal morphology, presence of lamellar bodies and expression of LBP180. As in the rat model of ATII cell culture, the addition of KGF promoted cell proliferation in mouse ATII cell cultures (Mason, 2002; Yano *et al.*, 2000). In validation of the concept of plasticity of the ATII cells, we were able to show that culture of mouse ATII cells on fibronectin coated coverslips allowed the cells to trans-differentiate into an ATI phenotype, characterized by expression of T1α. This *in vitro* system will be invaluable in future studies to identify the

mechanisms of pathogenesis during viral infection of the alveolar epithelium that can be directly compared to robust murine models of viral pathogenesis *in vivo.*

The susceptibility of the alveolar epithelial cells to infection by respiratory viruses was compared. We evaluated the susceptibility of murine ATI and ATII cells for infection by a murine coronavirus, MHV-1. MHV-1 infects the respiratory tract and causes disease with a wide range of severities, depending upon the genetic line of mice (De Albuquerque *et al.*, 2006; Khanolkar *et al.*, 2009b). Upon intranasal inoculation with MHV-1, BALB/cJ mice exhibit some of the pathological characteristics of SARS in humans, including pulmonary congestion, alveolar and interstitial inflammation, and hyaline membranes. However, intranasal infection of MHV-1 is not lethal in BALB/cJ mice, which show only mild clinical signs of disease and recover completely. In contrast, infection of A/J mice with MHV-1 results in severe lung pathology and death of infected mice. The lung pathology seen in MHV-1 infected A/J mice is similar to fatal SARS cases in humans, including severe edema, thickening of the alveolar epithelium, interstitial inflammation, fibrin deposits, and hyaline membranes. Although MHV-1 virions have been detected by electron microscopy in alveolar macrophages late during infection of A/J mice, infection of alveolar epithelial cells early during infection cannot be excluded. Furthermore, ATI cells express fibroleukin during MHV-1 infection of A/J mice, suggesting a role for these cells in fibrin deposition in the alveoli (De Albuquerque *et al.*, 2006). Primary murine ATI cells provide an attractive model for studying the signaling pathways involved in MHV-1-induced fibroleukin expression and their potential role in fibrin deposition during viral infection in the lung. The mechanisms responsible for the differential pathogenesis seen during respiratory infection of various mouse strains by MHV-1 have not been determined. Future studies using primary

alveolar epithelial cells from different genetic lines of mice will determine the potential contributions of these cell types in pathogenic outcomes of infection.

The pulmonary pathology in fatal cases of influenza viral pneumonia is characterized by diffuse alveolar damage, including the presence of hyaline membranes, inflammatory cells, and edema in the alveoli (Liem *et al.*, 2008; Nakajima *et al.*, 2012; Shieh *et al.*, 2010). The mouse-adapted PR8 strain of influenza A virus has been widely studied as a model for influenza pneumonia because it induces similar pathology in specific inbred strains of mice (Blazejewska *et al.*, 2011; Fukushi *et al.*, 2011). The virulence of different PR8 variants in various murine genetic lines is correlated with increased viral replication and spread to the alveolar regions of the lung (Blazejewska *et al.*, 2011). Infection of the alveoli by PR8 results in dramatic pathology associated with inflammation, leukocytic infiltration, and destruction of the epithelial surface (Blazejewska *et al.*, 2011; Fukushi *et al.*, 2011; Loosli *et al.*, 1975). Epithelial damage is likely due to a combination of direct viral cytopathic effects and the inflammatory response. Inhibition of inflammatory mediators during influenza virus infection in mice lessens morbidity and mortality, suggesting that the inflammatory response is a critical determinant of severe disease outcomes (Herold *et al.*, 2008; Le Goffic *et al.*, 2006b; Vlahos *et al.*, 2011). Herold et al. further demonstrated that macrophages recruited to the lungs during PR8 infection in mice have a direct role in alveolar damage by inducing apoptosis of ATI cells (Herold *et al.*, 2008). Murine ATII cells cultured to acquire an ATI cell phenotype *in vitro* have been used to study influenza virus infection in the alveolar epithelium and how infected ATI cells interact with monocytes to mediate disease pathology (Herold *et al.*, 2008; Herold *et al.*, 2006). Our study confirmed their results demonstrating susceptibility of and cytokine expression by murine ATI cells in response to PR8 infection

(Herold *et al.*, 2006; Tate *et al.*, 2011). We also established the susceptibility of primary murine cells with an ATII phenotype to PR8 infection and showed that cells with an ATI, but not ATII, phenotype express IL-1β in response to PR8 infection. While infection of ATI and ATII cultures by PR8 was not as robust as MHV-1, a significant amount of infectious PR8 was released from these cultures. We further demonstrated that PR8 infection co-localized with ATI and ATII phenotypic markers, suggesting infection of these cell types. However, in ATII cell cultures, PR8 antigens were also detected in LBP180-negative cells. While the exact nature of these cells is not known, many possibilities exist. Our fresh ATII cell isolations ranged from 87.7 to 90.7% LBP180-positive (Figure 2.6), which is similar to the purity reported by others (Corti *et al.*, 1996; Messier *et al.*, 2012). Thus, the LBP180-negative cells could be a contaminating subpopulation of cells from the isolation procedure, including fibroblasts, which were excluded by panning (Figure 2.4), endothelial cells, or white blood cells, which were selectively excluded by negative selection using the CD16/32 or CD45 antibodies. Furthermore, ATII cells can trans-differentiate into ATI cells or undergo epithelial to mesenchymal transition when cultured *in vitro* (Kim *et al.*, 2006). The LBP180-negative cells in our cultures may be ATII cells that are no longer fully differentiated. However, our dual antigen labeling demonstrated that alveolar cells with either ATI or ATII phenotype can be infected by PR8. As ATI and ATII cells are both targeted in severe influenza virus infections, it is important to identify the responses of these unique cell types to infection. In addition to inflammation-induced damage, electron microscopy has demonstrated budding of PR8 virions from ATI and ATII cells early during infection with extensive damage to the epithelial surface over time (Loosli *et al.*, 1975). Differentiated cultures of alveolar epithelial cells will be critical for identifying the

mechanisms responsible for direct viral damage to the alveolar epithelium during infection.

The pathogenesis of respiratory viral infections is often correlated with an excessive inflammatory response in the lungs. Alveolar epithelial cells express proinflammatory cytokines and chemokines in response to viral infection and may be important in the initiation of these damaging inflammatory responses (Herold *et al.*, 2006; Miura *et al.*, 2007; Rzepka *et al.*, 2012; Wang *et al.*, 2011). In this study, we compared cytokine expression by murine ATI cells infected by MHV-1 or PR8, and IL-1β expression by ATI and ATII cells in response to infection by MHV-1 or PR8. Infection of ATI cells by MHV-1 or PR8 induced similar cytokine profiles, which correspond to cytokine expression in the lungs of infected mice (Alberts *et al.*, 2010; Leibowitz *et al.*, 2010; Srivastava *et al.*, 2009). Interestingly, MHV-1 also stimulated expression of several cytokines and chemokines that were not expressed upon PR8 infection. It is not known whether this difference has biological relevance during *in vivo* infection. The inflammatory response to MHV-1 has been characterized in A/J mice, which have severe disease outcomes to infection (De Albuquerque *et al.*, 2006). Our primary cells were isolated from C57BL/6 mice, which have mild infection in the respiratory tract by MHV-1 (De Albuquerque *et al.*, 2006). The mechanisms that underlie the differences in pathogenesis of MHV-1 infection in different genetic lines of mice have not been clearly defined. In general, increased cytokine expression correlates with disease severity in both MHV-1 and PR8 infection models. Thus, it will be important to identify the mechanisms whereby alveolar epithelial cells detect viral infection and determine their role in the detrimental inflammatory response to viral infection in the lung. ATI and ATII cells are both targets for viral infection within the alveoli. In this study, we directly compared expression of IL-1β by murine ATI and ATII cells in response

to viral infection. IL-1β expression was induced by LPS to a high level in ATI cells, but only moderately in ATII cells. Raoust et al. also observed cytokine expression by primary murine cells cultured with an ATI cell phenotype upon treatment with LPS (Raoust *et al.*, 2009). Another study demonstrated that murine ATII cells cultured similarly to those in our study express chemokines CCL2 and CXCL2 in response to LPS treatment, suggesting that these cells are capable of LPS-induced responses through TLR4 signaling (Bello-Irizarry *et al.*, 2012). Infection by PR8 and MHV-1 induced IL-1β expression by 24 h p.i. in ATI cells. Viral infection induced expression of IL-1β in ATII cells, yet similar to LPS, expression was not as robust as in ATI cells. Thus, ATI and ATII cells responded differently to the same viral infections, and the level of IL-1β expression was dependent both on the cell phenotype and the specific virus. Other studies have reported differential expression of cytokines by primary alveolar epithelial cells cultured with an ATI or ATII cell phenotype (Mir-Kasimov *et al.*, 2012; Yu *et al.*, 2011). Human primary alveolar epithelial cells cultured with an ATI or ATII phenotype express very similar levels of cytokine mRNA upon infection by influenza viruses (Yu *et al.*, 2011). Despite similarities in mRNA levels, secretion of MCP-1 is dramatically increased upon influenza virus infection of ATI, but not ATII, cells (Yu *et al.*, 2011). A similar finding between our study and others is the observation that despite having a similar susceptibility to viral infection, cultures with ATI and ATII phenotypes can have different responses to these infections. Determining the mechanisms whereby ATI and ATII cells detect viral infections and regulate cytokine expression in the lungs is a critical next step of these studies. Primary murine alveolar epithelial cells will be crucial in the identification of these mechanisms.

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Chapter 3

Proteomics analysis of primary murine alveolar type II epithelial cells infected with influenza A virus

3.1 Abstract

The alveolar type II (ATII) epithelial cells are directly targeted by influenza A virus (IAV) in fatal human cases of avian H5N1 and 2009 pandemic H1N1 viruses. Infection of mice with IAV similarly results in fatal disease when ATII cells are infected, which correlates with diffuse alveolar damage and destruction of the epithelial surface. To understand the cellular mechanisms of IAV pathogenesis, ATII cells were isolated from murine lungs. Cells were cultured to maintain a differentiated ATII cell phenotype, infected with IAV and cell monolayers harvested for mass spectrometry analysis of infected and uninfected cells. Bioinformatics analyses were used to identify cellular proteins differentially regulated during infection. IAV infection up-regulated proteins including those associated with interferon signaling, antigen presentation, and cytoskeleton regulation. In addition, the expression of proteins including those involved in mitochondrial metabolism, chromatin formation and apoptosis was down-regulated. Moreover, proteins that are phenotypic markers of ATII cells *in vivo* were identified, confirming that these cultures maintained an ATII cell phenotype *in vitro*. Among the four surfactant proteins made by ATII cells, SP-B was down-regulated by IAV infection. Western blot analysis of selected proteins confirmed the proteomics data. In contrast to the finding that IAV infection down-regulated SP-B *in vitro*, IAV-infected mice had increased levels of SP-B in bronchoalveolar lavage fluid compared to uninfected mice. Investigation of changes in ATII cell proteome

profiles could be beneficial for elucidating cellular processes involved in IAV pathogenesis, which may provide insight into potential therapies to help reduce fatal disease.

3.2 Introduction

Influenza A virus (IAV) is a common cause of human respiratory tract infections with epidemics causing significant morbidity and mortality worldwide. The major concern with IAV is the high genetic variability of the virus that often results in the rapid emergence of variants that escape the immunity induced by available vaccines. The diverse animal species infected by IAV further increases the rate of genetic reassortments resulting in occasional influenza pandemics. Like other viruses, IAV depends on the host cellular machinery for replication and this is orchestrated by the complex interactions between the host and the viral proteins (Engelhardt & Fodor, 2006; Konig *et al.*, 2010; Taubenberger & Kash, 2010). These viral proteins can hijack the cellular machinery but the host is able to mount antiviral responses to counter-act the effects of the virus. Thus, this balance reflects the complex interaction of the influenza virus with the host cellular proteome that needs to be unraveled.

The epithelial cells of the upper and lower respiratory tracts are the primary sites of virus replication in IAV infections. However, infection of the lower respiratory tract is the most severe as it may result in damage of the alveolar epithelium thus compromising the gas exchange in the lungs. The alveolar type II (ATII) epithelial cells located in the lower respiratory tract are involved in regeneration to repair the injured alveolar epithelium and produce pulmonary surfactant phospholipids and proteins, which are critical to prevent alveolar collapse (Rooney *et al.*, 1994). ATII cells have been found to be directly targeted

by IAV in fatal human cases of avian, H5N1 and 2009 pandemic H1N1 virus infections (Mauad *et al.*, 2010; Shieh *et al.*, 2010; Uiprasertkul *et al.*, 2005; Weinheimer *et al.*, 2012). Influenza A/PR/8/34 H1N1 virus (PR8), a mouse adapted influenza strain, induces destruction of ATII cells in the alveoli of mice (Loosli *et al.*, 1975; Yetter *et al.*, 1980), making it a suitable model to study IAV pathogenesis in the lung. Primary murine ATII cells thus provide a powerful *in vitro* model to determine the effects of viral infection on these specialized pulmonary cell types, which corresponds to pathology in the mouse model. We have shown that murine ATII cells are susceptible to PR8 infection *in vitro,* resulting in secretion of various pro-and anti-inflammatory cytokines and several chemokine molecules (Kebaabetswe *et al.*, 2013) (Chapter 2). This approach, however, did not offer a comprehensive viral-host protein interaction that could be important to the life cycle of IAV during replication in the ATII cells. Hence, examining protein expression in IAV-infected ATII cells could offer a global picture of how these cells interact with IAV at the cellular level.

Proteomics studies of influenza virus infections in macaques (Baas *et al.*, 2006a; Brown *et al.*, 2010), continuous cell lines (Coombs *et al.*, 2010; Dove *et al.*, 2012; van Diepen *et al.*, 2010; Vester *et al.*, 2009), primary human bronchial epithelial cells (Kroeker *et al.*, 2012), primary human alveolar macrophages (Liu *et al.*, 2012), and primary human monocyte-derived macrophages (Lietzen *et al.*, 2011), have provided insight into the regulation of host gene expression and response to IAV infection. However, cellular responses to IAV infection are also, in part, specific to the cell type and localization in the lungs (Ibricevic *et al.*, 2006), and more studies using primary cells from the lungs are therefore needed to elucidate the host response in detail. Analysis of primary ATII cells

allows us to understand the host proteome interaction with IAV in biologically relevant cells. In this study, our aim was to utilize the *in vitro* model of primary ATII cells and examine the protein profiles following infection with IAV. Differentially expressed proteins were identified and the functions of some of these proteins in PR8 pathogenesis are discussed.

3.3 Materials and methods

3.3.1 Virus preparation

Human influenza A/PR/8/34/H1N1 virus (PR8) was obtained from BEI Resources. Madin-Darby canine kidney (MDCK) cells were purchased from American Type Culture Collection. PR8 was propagated and titrated by plaque assay in MDCK cells in media containing 0.1% bovine serum albumin (BSA) (EMD, Millipore Corp, Billerica, MA) and TPCK-trypsin (Invitrogen; 1 ug/ml).

3.3.2 Alveolar type II cell culture and virus infections

Animal protocols were approved by the University of Idaho Animal Care and Use Committee according to the National Research Council Guide for the Care and Use of Laboratory Animals. ATII cells were isolated from 6-8 week old C57BL/6 mice as previously described (Kebaabetswe *et al.*, 2013) (Chapter 2). Briefly, to maintain an ATII cell phenotype, ATII cells were cultured on millicell inserts (EMD) coated with 70% rat tail collagen and 30% BD Matrigel (BD Biosciences, San Jose, CA), in DMEM/10% FBS supplemented with keratinocyte growth factor (KGF; 10 ng/ml; ProSpec, Rehovot, Israel)

for 5 days. Quadruplicate cultures of cells were inoculated with PR8 in the presence of TPCK-trypsin or mock treated with media for 24 h.

3.3.3 Protein digestion, LC-MS and bioinformatics analysis

To harvest proteins for mass spectrometry, ATII cells were isolated from collagen/matrigel by dissolving the matrix in collagenase and dispase. The cells were washed in 150 mM ammonium bicarbonate buffer followed by lysis in 8 M urea. The samples were sent to the Pacific Northwest National Laboratory (Richland, WA) for liquid chromatography-mass spectrometry (LC-MS) analysis, protein identification and quantification and statistical analysis (Webb-Robertson *et al.*, 2010). Functional analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.7 (Dennis *et al.*, 2003; Huang *et al.*, 2009).

3.3.4 Western blot and densitometry analysis

Mock and PR8 infected ATII cells were lysed in NP40 lysis buffer (1% NP40, 0.1% SDS, 0.5% sodium-deoxycholate) and protease inhibitor cocktail (1 mg/ml aprotinin, 1 mg/ml leupeptin and 100 mM PMSF). Equal volumes of samples were electrophoresed on 4-20% SDS page gels (Thermo Scientific Rockford, IL) and transferred to polyvinylidene difluoride (Thermo Scientific, Rockford, IL) or nitrocellulose (Schleicher and Schuell, Keene, NH) membranes. After blocking with either 5% BSA or milk, membranes were incubated with antibodies against BST-2 (Santa Cruz Biotechnology), Ezrin (Abcam, Cambridge, MA), Histone H2B (Abcam), SP-B (Santa Cruz Biotechnology), IFITM3 (Santa Cruz Biotechnology), or TLR3 (eBioscience, San Diego, CA). With the exception of TLR3,

all blots were incubated with either rabbit or goat horseradish peroxidase (HRP) conjugated secondary antibodies (Thermo Scientific and Southern Biotech, Birmingham, AL, respectively). TLR3 was incubated with anti-rat IgG (eBioscience) as secondary followed by avidin-HRP (eBioscience) as tertiary. A β-actin antibody conjugated to HRP was used as a loading control (Abcam). HRP was detected on film with Supersignal West Pico chemiluminescence substrate (Thermo Scientific). Protein bands were quantified by densitometric scanning of exposed film and quantitation with Quantity One (Bio-Rad, Hercules, CA). For each protein, the specific densitometry was determined by subtracting the area containing the band from average of adjacent empty areas (above and below the band). Normalization ratio to β-actin was calculated by taking each experimental value divided by the corresponding β-actin value.

3.3.5 Immunofluorescence microscopy

After 24 h, PR8 and mock-infected cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Viral antigen was detected with goat antiserum NR-3148, which recognizes the hemagglutinin protein of PR8 (BEI Resources). SP-B was detected using the goat polyclonal SP-B antibody (Santa Cruz Biotechnology, Dallas, TX). Anti-goat-555 secondary antibody (Invitrogen, Carlsbad, CA) was used to detect both the PR8 infection and SP-B. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei and were photographed on a Nikon Eclipse Fluorescence Microscope with Hamatsu digital camera and Metamorph software (Molecular Devices).

3.3.6 Preparation of bronchoalveolar lavage fluid for SP-B analysis

C57BL/6 and BALB/c mice were infected by intranasal inoculation of PR8 ($1x10^4$ plaque forming units) or mock infected (controls) with sterile PBS. As mentioned above plaque assay was performed on MDCK cells to titrate the virus. The PR8-infected mice assayed in this study were sacrificed on days 6-9 before the 14-day experimental period due to loss of weight and severe disease. Lungs were lavaged with 1 ml aliquots of sterile PBS. Proteins were prepared by concentrating 250 ul of bronchoalveolar lavage fluid in nanosep centrifugal membrane filters with a cut off of 3 kDa (Pall Life Sciences, Ann Arbor, MI). Using membrane filters with this size meant all proteins with a molecular size larger than the membrane filter were retained on the surface of the membrane. All of the SP-B cleavage intermediates are larger than 3 kDa. The nanosep columns were centrifuged at 14,000 x g for 15 min. Fifteen microliters of resulting concentrate was used for SP-B western blot analysis, discussed above.

3.4 Results and discussion

Primary ATII cells were isolated from mice and cultured to maintain an ATII phenotype *in vitro*, as previously described (Kebaabetswe *et al.*, 2013) (Chapter 2). Five days after isolation, cultures were infected with PR8 or mock-inoculated. Cells were fixed 24 h after infection and analyzed for viral infection by immunofluorescence assay and proteins harvested for western blot. The PR8-infected cells expressed the viral surface antigen, hemagglutinin (HA) (Figure 3.1A). This was consistent with the western blot analysis that further detected the different forms of HA, uncleaved precursor, HA0 and cleaved forms consisting of two-disulfide-linked chains, HA1 and HA2 (Figure 3.1B) (Lamb

& Krug, 2001). The cleavage of the HA molecule is required for viral infectivity as it allows for virus uncoating and genome release into the cytoplasm. These data are in agreement with our previous study that demonstrated productive infection of murine ATII cells by PR8 (Chapter 2).

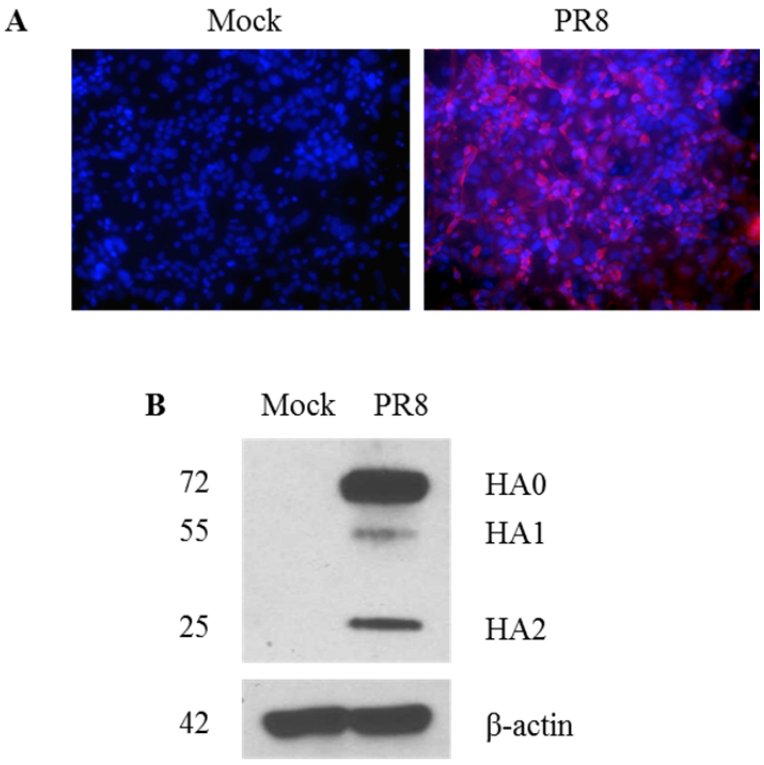

Figure 3.1 Influenza viral infection of ATII cells. Primary murine ATII cells were mock-treated or infected with PR8. At 24 h post-infection, cells were analyzed by (A) immunofluorescence assay of viral hemagglutinin protein (red) and nuclei were stained with DAPI (blue), and (B) western blot analysis of viral HA protein isoforms in whole cell lysates. β-actin was used as a loading control. Molecular weights in kDa are provided to the left. The images shown are representative of three replicate experiments.

3.4.1 Influenza proteins identified by LC-MS

PR8-infected and mock-infected ATII cells were lysed and proteins were analyzed by LC-MS. We detected 5 of the 11 IAV proteins (HA, NA, NP, M1 and NS1) (Table 3.1). Influenza viral proteins have been detected before by LC-MS/MS from PR8-infected primary human alveolar macrophages and embryonic kidney 293T cells (Emmott *et al.*, 2010; Liu *et al.*, 2012). These studies detected similar proteins as in our study with the exception of NA. There were high numbers of peptides for HA and NP in the infected cells (Table 3.1). We also detected HA and NP in mock-infected cells and this could be due to background noise during detection. The NP is required for the encapsidation of the newly synthesized viral RNA once replication is complete; it wraps around the viral RNA and packages it into viral ribonucleoproteins together with the newly synthesized polymerase proteins. The M1 and NS1, through their nuclear export signals facilitate transport of the newly synthesized viral nucleoproteins for assembly at the plasma membrane. NA is required for the cleavage of the sialic acids on the cell surface and newly produced viral particles to be released from infected cells whereas HA is required for the subsequent viral receptor binding for new infections. We did not detect the rest of the viral proteins, NS2, M2, PB1, PB2, PB1-F2 and PA. This has also been observed in other studies (Emmott *et al.*, 2010; Liu *et al.*, 2012). This is likely due to the lower levels of expression of these proteins during viral replication that could not be detected by LC-MS. Furthermore, NS2 and M2 are encoded by the spliced messenger RNA from NS1 and M1 respectively, and the level of spliced viral transcripts is only 10% that of unspliced in infected cells (Lamb *et al.*, 1980), hence they might have been too low to be detected.

Table 3.1 Influenza A virus proteins identified by LC-MS

Uniprot Accession ID	Gene Name	Number of Peptides identified	Fold Change[1] (P-value)
P03466	Nucleoprotein (NP)	61	18.2 (0.049)
P03452	Hemagglutinin (HA)	39	15.3 (0.075)
P03468	Neuraminidase (NA)	8	*(0.001)
P03485	Matrix protein 1 (M1)	8	*(0.014)
P03496	Non-structural protein 1 (NS1)	4	*(0.001)

[1]Fold change expressed as ratio of signal intensity in PR8-infected/mock, P-value determined by student's t-test, or g-test in the cases*

* Not detected in mock.

LC-MS analysis was used to compare the ATII protein profiles in PR8-infected and mock-infected cells using four samples for each treatment. We identified 156 differentially regulated proteins, 50 up-regulated (Table 3.2 and Table 3.3) and 107 down-regulated (Table 3.4) by PR8 infection, the tables are discussed under their respective sections. Functional analysis of the differentially regulated proteins using the DAVID database showed that a significant proportion of proteins that were up-regulated by PR8 infection belong to the interferon-induced proteins, cytoskeleton regulation, and class I antigen presentation pathway. Proteins that were decreased by infection included those involved in mitochondrial metabolism, apoptosis and chromatin formation. The significance of the functional groups of proteins is discussed below.

3.4.2 Proteins increased by PR8 infection

It is well documented that influenza virus infection triggers activation of interferon (IFN) inducible proteins by various mechanisms. In our study, several IFN-induced proteins were up-regulated, including BST-2, IFITM3, IFP35, TLR3, β2M, OAS1A p42 and the OAS2L p54 (Table 3.2). Influenza uses the NS1 protein to attenuate the IFN response to infection but our results suggest that PR8 does not completely impair IFN signaling in the ATII cells. Since we used a later time point when viral titers were increased, the viral products could have become too high for the triggering of IFN response to be fully inhibited by NS1. On the other hand, even though the NS1 viral protein can antagonize interferon response by blocking translation and sequestering viral RNA, this is strain-dependent (Mibayashi *et al.*, 2007). The NS1 protein of the influenza strain PR8 has been found to be less inhibitory to the IFN response than other strains of IAV (Haye *et al.*, 2009), agreeing with our study. Our analysis did not identify the Mx1 protein, which is an IFN-induced protein that confers immunity to influenza disease and has been identified in other quantitative proteomic studies (Coombs *et al.*, 2010; Vester *et al.*, 2009). This is in agreement with the findings of Wang et al. who show that primary human ATII cells do not have increased Mx1 protein upon infection by IAV (Wang *et al.*, 2009).

Pathogen recognition receptors such as toll-like receptors (TLRs) detect virus-associated molecular patterns, triggering a signaling cascade that leads to synthesis and secretion of type I IFNs. TLR3 is constitutively expressed by the alveolar epithelial cells in the lung and plays a key role in immune responses to IAV by detecting the dsRNA intermediates during virus replication (Guillot *et al.*, 2005). We observed an increase of TLR3 in PR8-infected ATII cells and did not detect it in the mock-infected cells (P-value

0.014) (Table 3.2). The following *in vitro* studies have established a role for TLR3 in the response of lung epithelial cells to IAV infections. Recognition of dsRNA in lung epithelial cells by TLR3 induces secretion of cytokines IL-8, IL-6, RANTES, IFN-β and up-regulation of major adhesion molecules (Guillot *et al.*, 2005). TLR3 activation also provides a link between the innate immune system and the adaptive immune system by enhancing the cross-presentation of peptides by dendritic cells to $CD8^+$ T cells to kill virus-infected cells (Enelow *et al.*, 1998). Despite this, *in vivo* studies using mouse models have reported that TLR3 activation and eventual release of inflammatory mediators contributes to debilitating effects, resulting in severe lung damage. Mice deficient in TLR3 have significantly decreased inflammatory cytokines, lower numbers of $CD8^+$ T cells, and longer survival compared to controls (Le Goffic *et al.*, 2006a). We are not sure if ATII cells use TLR3 to counteract PR8 infection, but the fact that it was increased in our study allows us to speculate that it might be essential during response to PR8 infection in the lung. Due to its functions in inducing both protective type I IFN responses and detrimental inflammatory responses, TLR3 might contribute to both viral clearance and the severe lung damage that is seen in PR8 mouse models (Loosli *et al.*, 1975; Yetter *et al.*, 1980).

The interferon-inducible transmembrane (IFITM) proteins and in particular, IFITM3, has been found to inhibit viral entry and replication of viruses including IAV, SARS coronavirus, dengue virus, and West Nile virus (Brass *et al.*, 2009; Huang *et al.*, 2011a). There was significant increased levels of IFIMT3 in infected ATII cells by LC-MS (P-value 0.001) and did not detect it in mock-infected cells (Table 3.2). The role of IFITM3 in influenza virus infections has been well-documented. It inhibits viral fusion, nuclear translocation of viral nucleoprotein and increases the size of autolysosomes for virus

trapping and eventual degradation (Brass *et al.*, 2009; Feeley *et al.*, 2011). Studies in mice and humans have also shown that IFITM3 protects against influenza disease. A mutant human *IFITM3* allele associated with reduced restriction activity was highly prevalent in patients hospitalized for influenza (Everitt *et al.*, 2011). IFITM3 knockout mice infected with IAV have significant weight loss, which corresponds with pulmonary expression of proinflammatory cytokines and chemokines in the lungs (Everitt *et al.*, 2011). Moreover, IFITM3 knockout mice infected with PR8 have greater mortality and higher viral load compared to wild-type mice (Bailey *et al.*, 2012). Bailey et al. further shows that IFITM3 localizes to the ATII cells in absence of infection (Bailey *et al.*, 2012). Hence, the basal levels of IFITM3 may provide protection to the host against IAV before the IFN response to viral infection is induced.

BST-2 is a transmembrane protein that has been shown to restrict the release of enveloped viral particles including HIV, dengue virus, and vesicular stomatitis virus from the cell surface (Fitzpatrick *et al.*, 2010; Pan *et al.*, 2012; Weidner *et al.*, 2010). BST-2 works by tethering mature virions to the cell surface, causing their subsequent endocytosis and proteosomal degradation in the lysosome (Neil *et al.*, 2008). We observed significant up-regulation of BST-2 expression in PR8-infected ATII cells (Fold change 25.4, P-value <0.001) (Table 3.2). Up-regulation of BST-2 during infection has also been observed by LC-MS/MS in Japanese encephalitis virus infected cells (Zhang *et al.*, 2013). BST-2 is a lipid raft associated membrane protein (Kupzig *et al.*, 2003) and IAV utilizes lipid rafts for budding (Takeda *et al.*, 2003). However, there have been contradictory findings regarding the role of BST-2 in IAV pathogenesis. Studies report that BST-2 inhibits the release of influenza virus-like particles but does not restrict the wild-type virus ((Watanabe *et al.*,

2011; Yondola *et al.*, 2011). In contrast, Mangeat et al. shows that BST-2 partially inhibits the release of wild-type viral particles from infected cells (Mangeat *et al.*, 2012), making it important in restricting IAV production. Although additional studies are needed to determine whether BST-2 inhibits release of PR8 from ATII cells, our results demonstrate that BST-2 is part of the host cellular defense against viral infection in these cells.

Apart from activation by IFN, the RNase L pathway is also activated by the viral dsRNA, which is a replicative intermediate of IAV. The binding of dsRNA to 2'-5' oligoadenylate (OAS) results in the activation of RNase L to degrade viral RNA and also block translation of host proteins (Chakrabarti *et al.*, 2011). We detected two isoforms of OAS: OAS1A p42 and OAS2L p54 in PR8-infected cells (Table 3.2). We are not sure if the RNase L pathway was activated in ATII cells but the increased levels of these isoforms in PR8-infected cells suggest that the cells may use this pathway to inhibit viral replication. The 2'-5' oligoadenylate synthetase is also induced in IAV-infected A549 cells, an ATII epithelial cell line (Ronni *et al.*, 1997).

In addition to cellular restriction factors, type I IFNs also activate the MHC class I antigen processing and presentation pathway, which is critical for recognition of virus-infected cells by cytotoxic T cells. Several components of MHC I proteins such as β-2-microglobulin and various alpha chains were also up-regulated by PR8 infection of ATII cells (Table 3.2). In addition, we observed up-regulation of proteins involved in the class I antigen processing pathway such as TAP binding protein and proteasome subunits beta 8 and 10 (Table 3.3). This may suggest that, under the influence of IFN induction enhanced MHC I antigen processing and presentation by the ATII cells may render these cells to be targeted by cytotoxic, $CD8^+$ T cells during PR8 infection in the lungs.

Table 3.2 Interferon-induced proteins increased by PR8 infection of ATII cells

Uniprot Accession ID	Gene Name	Number of Peptides identified	Fold Change[1] (P-value)
Q9CQW9	Interferon induced transmembrane protein 3 (IFITM3)	2	*(0.001)
Q9Z2F2	2'5'-Oligoadenylate synthetase-like 2 (OAS2L p54)	1	*(0.001)
P11928	2'5'-Oligoadenylate synthetase 1A (OAS1A p42)	1	*(0.001)
Q8R2Q8	Bone marrow stromal antigen 2 (BST-2)	4	25.4 (<0.001)
Q99MB1	Toll-like receptor 3 (TLR3)	2	*(0.014)
Q9D8C4	Interferon-induced protein 35 (IFP35)	1	*(0.014)
O35309	N-myc and STAT interactor	1	*(0.014)
P01896	MHC Class I alpha chains (H2D)	1	*(0.014)
P04223	MHC Class I alpha chain (H2K)	2	*(0.014)
P01897	MHC Class I alpha chain (H2L)	3	*(0.014)
P01902	MHC Class I alpha chain (K-D)	1	*(0.014)
P01898	MHC Class I alpha chain (Q10)	2	*(0.014)
P01887	β-2 microglobulin (B2M)	2	*(0.001)

[1]Fold Change expressed as ratio of PR8/mock, P-value determined by student's t-test or g-test.
*Present in PR8, absent in mock.

Cytoskeletal components undergo alterations during viral infections and are especially important during infection in enveloped viruses such as IAV (Arcangeletti *et al.*, 1997; Avalos *et al.*, 1997). The viral NP and M1 proteins detected in this study interact with cytoskeletal proteins to regulate viral replication, assembly, and budding (Avalos *et al.*, 1997). We observed up-regulation of various cytoskeleton proteins including keratins and ezrin (Table 3.3). Keratins are structural proteins important in the formation of intermediate filaments and are expressed in cell type restricted patterns in the respiratory tract. Forbus et al. indicates that keratins may be linked to TNF and NF-κB signaling pathways (Forbus *et al.*, 2006), and thus might be important in mediating inflammatory responses to viral

infections in the alveolus. The interaction and induction of cytoskeletal proteins by IAV has been studied by proteomics. Cytoskeletal proteins are significantly up-regulated in the lungs of mice infected with avian H5N1, correlating with severe tissue damage observed in the lungs (Zhao *et al.*, 2012). Cytokeratins are also up-regulated in H9N2-infected gastric epithelial cells and in 2009 (H1N1) and classical swine H1N1 infected porcine alveolar macrophages (Liu *et al.*, 2008; Zhu *et al.*, 2012). Ezrin is another cytoskeleton protein that is localized in apical microvilli in polarized epithelial cells (Bretscher *et al.*, 2002). It is involved in controlling cell shape, movement and directional migration (Prag *et al.*, 2007). These movements could be important for the spread of virus to the neighboring uninfected cells. Furthermore, ezrin has also been linked to BST-2. Ezrin is mislocalized in BST-2 knockdown cells and the re-expression of BST-2 restores ezrin to the apical membrane (Rollason *et al.*, 2009), thus it is plausible to speculate that these two proteins could be used by the ATII cells to prevent spread of PR8 infection in the lungs. Myosin 1b is involved in the traffic of cargo along the endocytic pathway (Almeida *et al.*, 2011). Hence, myosin 1b may be up-regulated in PR8-infected cells to act as a transport carrier of viral particles to the endosome and also facilitates movement between endosome and the nucleus. These results implicate the potential roles of the cytoskeleton proteins in virus entry, regulation of gene expression and budding during IAV infection in the ATII cells.

Table 3.3: List of proteins significantly up-regulated in PR8-infected ATII cells identified by LC-MS

Uniprot Accession ID	Gene Name	Number of Peptides identified	Fold Change[1] (P-value)
Immune response and Proteasome Proteins			
P35700	Peroxiredoxin 1	1	*(0.014)
Q9R233	TAP binding protein (Tapasin)	4	*(0.001)
Q6ZQA6	Immunoglobulin superfamily, member 3	1	*(0.014)
O35955	Proteasome, subunit, beta type 10	1	*(0.001)
P28063	Proteasome, subunit, beta type 8	1	*(0.014)
RNA processing and Translational Machinery			
P62631	Eukaryotic translation elongation factor 1 alpha 2	2	1.33 (0.004)
Q8VEG4	Exonuclease 3'-5' domain containing 2	1	*(0.014)
P47963	60S ribosomal protein L13	1	*(0.014)
P14115	Ribosomal protein L27a	1	1.50 (0.032)
Q9Z2X1	Heterogeneous nuclear ribonucleoprotein F	1	2.10 (0.043)
Mitochondrial and Cellular Metabolism			
O08553	Dihydropyrimidinase-like 2	1	1.78 (0.039)
Q64467	Glyceraldehyde-3-phosphate dehydrogenase	2	1.53 (0.044)
Q91VC9	Growth hormone inducible transmembrane protein	1	*(0.014)
O35143	ATPase inhibitory factor 1	1	1.46 (0.025)
P35486	Pyruvate dehydrogenase E1 alpha 1	1	*(0.014)
P17182	Enolase 1, alpha non-neuron	1	*(0.014)
Q05769	Prostaglandin-endoperoxide synthase 2	3	2.68 (0.006)
Cytoskeleton Regulation			
P08730	Keratin 13	2	2.23 (0.006)
Q61414	Keratin 15	2	2.23 (0.006)
Q9Z2K1	Keratin 16	2	2.89 (0.013)
Q9D312	Keratin 20	1	2.49 (0.042)
Q8CCX5	Keratin 222	1	2.49 (0.042)
Q6IFZ6	Keratin 77	4	2.28 (0.049)
Q8BYA0	Tubulin-specific chaperone d	1	1.84 (0.017)
P26040	Ezrin	4	1.40 (0.045)
P46735	Myosin 1B	1	2.26 (0.021)

Intracellular Transport and Trafficking Proteins			
Q8K273	Membrane magnesium transporter 1	1	*(0.001)
Q8R143	Pituitary tumor-transforming 1 interacting protein	1	2.45 (0.026)
Q6PD26	Phosphatidylinositol glycan anchor biosynthesis	1	2.33 (0.039)
Q9Z0J0	Niemann Pick type C2	1	2.28 (0.016)
P63094	Guanine nucleotide binding protein G subunit	1	*(0.014)
Q9CQW2	ADP-ribosylation factor-like 8B	1	3.58 (0.038)
Cell Signalling and Adhesion			
Q07797	Lectin, galactoside-binding protein	3	3.51(<0.001)
P05533	Lymphocyte antigen 6 complex, locus A	4	4.23 (0.018)
P27046	Mannosidase 2, alpha 1	1	*(0.014)
P97427	Collapsin response mediator protein 1	1	1.78 (0.039)
P11835	Integrin beta 2	1	1.80 (0.037)

[1]Fold change expressed as ratio of PR8/mock, P-value determined by student's t-test or g-test

*Present in PR8, absent in mock.

3.4.3 Proteins decreased by PR8 infection

The majority of proteins that were down-regulated by PR8 in our study were associated with either the outer or inner mitochondrial membranes (Table 3.4). Mitochondrial proteins that were down-regulated in our study are involved in cellular metabolism, transition pore complexes, and generation of metabolites and energy. The mitochondria functions to provide energy, cellular respiration and modulating cell death pathways (Arnoult *et al.*, 2009; Ohta & Nishiyama, 2011). Alterations in mitochondrial proteins and integrity have been observed in A549 cells infected with human respiratory syncytial virus (Munday *et al.*, 2010). In gastric epithelial cells infected with H9N2, there are decreased levels of mitochondrial prohibitin proteins, which are required for apoptosis (Liu *et al.*, 2008). The IAV protein, PA has been found to associate with the mitochondrial proteins during infection. PA binds to the mitochondrial proteins that mediate outer voltage-dependent anion channel proteins, (VDACs) or inner membrane permeabilization

(transmembrane proteins) (Bradel-Tretheway *et al.*, 2011). Proteins belonging to these two mitochondrial compartments were down-regulated in our study (Table 3.4). Reduced expression of these proteins may render them incapable of fulfilling their functions thereby altering the permeability and the membrane potential of mitochondria. This may reduce the capacity for energy transduction in the mitochondria leading to deregulation of critical cellular processes. Other viruses also have proteins that alter the mitochondria; the cytomegalovirus β2.7 RNA interacts with complex 1 of the respiratory chain stabilizing mitochondrial transmembrane potential for a continued ATP production, leading to increase in the viability of infected cells and allowing for continued viral replication (Reeves *et al.*, 2007). Because of the importance of the mitochondria in cell death, it is not surprising that IAV targets mitochondrial function to subvert the cell death pathway.

Prevention of apoptosis is important to allow for the replication of the viral genome before the host cell dies. Viruses can prevent pro-apoptotic molecules such as cytochrome c (down-regulated in our study, Table 3.4) from initiating apoptosis by selectively binding or sequestering the Bax protein, which is required to initiate apoptosis in the cell (Arnoult *et al.*, 2009). The NS1 protein of IAV down-regulates apoptosis by inhibiting the activation of PKR and IRF3, which are the cellular promoters of apoptosis, thus delaying apoptosis in infected cells (Zhirnov *et al.*, 2002). Further, NS1 and M1 can cooperate to down-regulate apoptosis during viral replication by reducing the sensitivity of cells to IFNs and interfering with host caspase proteins, respectively (Balachandran *et al.*, 2000). Hence, PR8 may utilize the strategy of inhibiting apoptosis in ATII cells for its own benefits. The ubiquitin-proteasome is the major intracellular protein degradation pathway and also an important regulator of many cellular processes. Various components of the proteasome pathway

including proteasome 26S, proteasome beta subunit 5, and proteasome alpha subunit 2 were down-regulated (Table 3.4). The down-regulation of these proteins may suggest that IAV is able to avoid degradation of proteins that might otherwise be essential for viral replication thus allowing for efficient replication in the ATII cells.

Proteins involved in chromatin formation were down-regulated by PR8 infection including histone family proteins such as histone H4, histone H2b and core histone H2A.1 (Table 3.4). Histones play role in gene regulation (Marino-Ramirez *et al.*, 2005). Although IAV as an RNA virus does not form nucleosome structures, it can associate with nuclear matrix during replication and transcription. Histone proteins were down-regulated in proteomic analysis of primary human alveolar macrophages infected with IAV (Liu *et al.*, 2008). The H3N2 virus NS1 protein mimics the histones by possessing the histone-like sequence that targets the human transcription elongation complex, resulting in suppression of the antiviral response (Marazzi *et al.*, 2012). The human transcription elongation complex has a role in antiviral response by activating promoters of IL-6 responsive genes (Youn *et al.*, 2007). Hence, reduced expression of histones could allow the virus to replicate without having to contend with cellular chromatin and also prevent antiviral response.

Table 3.4 List of proteins significantly down-regulated in PR8-infected ATII cells identified by LC-MS

Uniprot Accession ID	Gene Name	Number of Peptides identified	Fold Change[1] (P-value)
Apoptosis Associated Proteins			
O35593	Proteasome, 26S subunit, non-ATPase, 14	1	*(0.014)
P49722	Proteasome, subunit, alpha type 2	17	0.76 (0.032)
P17047	Lysosomal-associated membrane protein 2	2	0.25 (0.014)
O55234	Proteasome, subunit, beta type 5	13	0.66 (0.017)
P32261	Serine (or cysteine) peptidase inhibitor, clade C	1	*(0.014)
P01029	Complement component 4A/4B	1	0.07 (0.027)
P04186	Complement factor B	1	0.07 (0.018)
Chromatin formation and DNA binding			
Q9QZQ8	Core histone H2A.1	5	0.62 (0.001)
O54962	Barrier to autointegration factor 1	2	0.72 (0.008)
Q3U1J4	Damage specific DNA binding protein 1	1	0.51 (0.015)
P62806	Histone H4	55	0.68 (0.026)
Q64524	Histone cluster 2, H2be	29	0.89 (0.05)
Q9D2U9	Histone H2ba, H2bb	34	0.87 (0.025)
Q8VEK0	Cell cyle control 50A	1	0.52 (0.047)
Mitochondria and Cellular Metabolism			
O35683	NADH dehydrogenase 1 alpha subcomplex, 1	1	0.51 (0.027)
Q9DCJ5	NADH dehydrogenase 1 alpha subcomplex, 8	2	0.52 (0.018)
Q9DC69	NADH dehydrogenase 1 alpha subcomplex, 9	1	0.48 (0.027)
Q9CQZ6	NADH dehydrogenase 1 beta subcomplex 3	1	0.39 (0.042)
Q9DCS9	NADH dehydrogenase 1 beta subcomplex, 10	3	0.6 (0.028)
Q9CPU2	NADH dehydrogenase 1 beta subcomplex, 2	1	*(0.014)
Q9CQH3	NADH dehydrogenase 1 beta subcomplex, 5	1	0.64 (0.036)
Q3UIU2	NADH dehydrogenase 1 beta subcomplex, 6	1	0.6 (0.003)
Q91YT0	NADH dehydrogenase flavoprotein 1	1	0.73 (0.017)
Q9DBX6	Cytochrome P450, family 2, subfamily s, polypeptide 1	1	0.61 (0.04)
Q9D0M3	Cytochrome c-1	2	0.73 (0.002)
P43024	Cytochrome c oxidase, subunit VI	6	0.5 (0.023)
Q921G7	Electron transferring flavoprotein, dehydrogenase	2	*(0.014)
Q8BW75	Monoamine oxidase B	6	0.64 (0.016)
Q9CZ13	Ubiquinol-cytochrome c reductase core protein 1	5	0.69 (0.005)
Q99JR1	Sideroflexin 1	4	0.61 (0.037)
Q91V61	Sideroflexin 3	1	0.56 (0.027)
Q9D855	Ubiquinol-cytochrome c reductase binding protein	2	0.71 (0.005)
Q9CQA3	Succinate dehydrogenase Ip subunit	1	0.66 (0.028)
Q8K2I3	flavin containing monooxygenase 2	1	0.58 (0.026)
P16125	lactate dehydrogenase B	1	*(0.014)

Q8R127	Saccharopine dehydrogenase (putative)	2	0.56 (0.002)
P22437	Prostaglandin-endoperoxide synthase 1	2	0.44 (0.007)
P47740	Aldehyde dehydrogenase family 3, subfamily A2	1	0.81 (0.003)
Q8C165	Peptidase M20 domain containing 1	1	*(0.014)
Q9R112	Sulfide quinone reductase-like (yeast)	3	0.52 (0.01)
Q91X52	Dicarbonyl L-xylulose reductase	1	*(0.014)
Q922D8	Methylenetetrahydrofolate dehydrogenase	1	*(0.014)
Q8C7K6	Prenylcysteine oxidase 1 like	1	*(0.014)
Q60716	Procollagen-proline, alpha II polypeptide	1	*(0.014)
O55126	Glioblastoma amplified sequence	3	0.66 (0.037)
Q791T5	mitochondrial carrier homolog 1 (C. elegans)	1	0.48 (0.043)
Q99JB2	Stomatin (Epb7.2)-like 2	4	0.72 (0.002)
P09925	Surfeit gene 1	1	*(0.014)
Q78IK4	Apolipoprotein O-like	1	*(0.014)
O55125	Protein NipSnap homolog 1	1	0.67 (0.04)
RNA Processing and Translational Machinery			
Q6ZWM4	LSM8 homolog, U6 small nuclear RNA associated	1	*(0.014)
O35381	Acidic, nuclear phosphoprotein 32 family A	1	0.82 (0.005)
Q9EST5	Acidic, nuclear phosphoprotein 32 family B	1	0.82 (0.005)
P12023	Amyloid beta (A4) precursor protein	1	*(0.014)
P63325	40S Ribosomal protein S10	1	0.74 (0.02)
Q8N7N5	WD repeat domain 42A	1	*(0.014)
Q80ZM7	TFIIA small subunit; transcription factor II A,	1	*(0.014)
P57784	Small nuclear ribonucleoprotein polypeptide A'	1	0.51 (0.045)
Intracellular Protein Transport			
Q9DB05	N-ethylmaleimide sensitive fusion protein, protein α	1	*(0.014)
Q99PT1	Rho GDP dissociation inhibitor (GDI) alpha	1	0.85 (0.043)
Q01405	SEC23A (S. cerevisiae)	1	*(0.014)
P17426	Adaptor protein complex AP-2, alpha 1 subunit	1	0.59 (0.01)
Q8BU14	SEC62 homolog (S. cerevisiae)	1	*(0.014)
Q9DBG7	Signal recognition particle receptor ('docking protein')	1	0.73 (0.015)
O88441	Metaxin 2	1	*(0.014)
Q6P069	Sorcin	2	*(0.014)
Q3TDQ1	STT3, subunit of the oligosaccharyltransferase complex	1	0.3 (0.01)
P57759	Endoplasmic reticulum protein 29	1	*(0.014)
Q8BGH2	Sorting and assembly machinery component 50	3	0.68 (0.017)
Cell Signalling and Adhesion			
Q07113	Insulin-like growth factor 2 receptor	3	*(0.014)
Q3V3R4	Integrin alpha 1	1	*(0.014)
P11688	Integrin alpha 5 (fibronectin receptor alpha)	4	0.60 (0.002)
O55022	Progesterone receptor membrane component 1	1	0.57 (0.048)

Q61555	Fibrillin 2	1	0.56 (0.001)
Q9Z1Y4	Thyroid hormone receptor interactor 6	1	*(0.014)
Q61490	Activated leukocyte cell adhesion molecule	6	0.64 (0.002)
Q91X78	ER lipid raft associated 1	1	*(0.014)
Q8R2Y2	Melanoma cell adhesion molecule	6	0.5 (0.006)
Q91XD7	Cysteine-rich with EGF-like domains 1	1	0.51 (0.038)
Lipids and Polysaccharides Metabolism			
Q64435	UDP glycosyltransferase 1 family polypeptide A13	6	0.78 (0.05)
Q80UM7	Mannosyl-oligosaccharide glucosidase	2	*(0.014)
P70419	Polypeptide N-acetylgalactosaminyltransferase 3	1	0.47 (0.028)
Transmembrane Transporters, Transducers			
Q8R1V4	Transmembrane emp24 protein transport domain 4	2	0.64 (0.049)
Q9DBS1	Transmembrane protein 43	11	0.77 (0.037)
Q01853	Transitional endoplasmic reticulum ATPase	5	0.51 (0.044)
Q9CPQ8	ATP synthase, H+ transporting, mitochondrial F0 complex	4	0.55 (0.02)
Q9Z2Z6	Mitochondrial carnitine/acylcarnitine translocase, 20	3	0.52 (0.018)
Q9QZD8	Mitochondrial carrier, dicarboxylate transporter, member 10	1	0.58 (0.001)
Q5IRJ6	Solute carrier family 30 (zinc transporter), member 9	1	*(0.014)
O88343	Solute carrier family 4 (anion exchanger), member 4	1	*(0.014)
P57716	Nicastrin	3	0.57 (0.002)
Q69ZN7	Myoferlin	5	0.63 (0.008)
Q8C166	Copine I	11	0.67 (0.0290
Q60932	Voltage-dependent anion channel 1	6	0.71 (0.002)
Q60930	Voltage-dependent anion-selective channel protein 2	21	0.80 (0.042)
P21279	Guanine nucleotide binding protein, alpha q polypeptide	1	0.62 (0.014)
P63030	Brain protein 44-like protein	1	*(0.014)
Plasma Membrane			
Q9DCV5	DNA segment, Chr 14, ERATO Doi 449, expressed	1	*(0.014)
P15532	Nucleoside diphosphate kinase A (NDK A)	1	0.59 (0.016)
Q78XF5	Oligosaccharyltransferase complex subunit	1	0.65 (0.042)
Q8VCM8	Nicalin homolog (zebrafish)	2	0.47 (0.002)
Q8BJS4	Unc-84 homolog B (C. elegans)	1	0.48 (0.001)
Q8R0I4	TM2 domain containing protein 2	1	*(0.014)
P57096	Prostate stem cell antigen	1	*(0.014)
Q8R502	Leucine rich repeat containing protein 8C	1	0.67 (0.011)
Q3UM45	Protein phosphatase 1, regulatory (inhibitor) subunit 7	1	0.68 (0.04)

[1]Fold Change expressed as ratio of PR8/mock, P-value determined by student's t-test or g-test.
*Absent in PR8, but present in mock

3.4.4 Validation of proteomics data

To validate the mass spectrometry based quantification, BST-2, IFITM3, ezrin, TLR3, and histone H2B were selected for western blot and densitometry analysis (Figure 3.2). These proteins were chosen based on their importance to IAV pathogenesis. The proteomics data showed that BST-2 was significantly up-regulated in PR8-infected cells compared to mock (P-value <0.001) (Table 3.2). In agreement with the proteomics analysis, the western blot shows that expression of BST-2 was enhanced in PR8-infected cells, showing multiple glycosylated forms of BST-2 with multiple bands between 25-55 kDa (Figure 3.2A), which has also been observed in other studies (Mangeat *et al.*, 2012; Watanabe *et al.*, 2011). The multiple glycosylated forms have been stipulated to directly correlate with the antiviral activity of BST-2 (Mangeat *et al.*, 2012; Watanabe *et al.*, 2011). There were increased levels of TLR3 and minimal increase of IFITM3 in PR8-infected cells as shown by western blot analysis (Figure 3.2A). This was in agreement with the proteomics data. Meanwhile, IFITM3 and TLR3 were not detected in the mock samples by proteomics analysis (P-values, 0.001 and 0.014, respectively) (Table 3.2). The failure to detect them in LC-MS could mean these proteins might have been too low for detection. In addition, western blot detects whole protein compared to LC-MS that detects only peptides. Ezrin was also increased in PR8-infected cells in western blot analysis (Figure 3.2A). A higher number of peptides were detected for ezrin compared to other proteins involved in cytoskeleton regulation (Table 3.3). Histone 2b proteins were down-regulated in proteomics and a high number of peptides belonging to these proteins were detected (P-values, 0.05 and 0.03, respectively). The histone 2b Western blot was similar for mock- and PR8-infected cells, though they were distinguishable especially by densitometry analysis (Figure 3.2B). However, more blots are

needed for densitometry analysis. The functions of these proteins and their involvement in IAV pathogenesis has been discussed above.

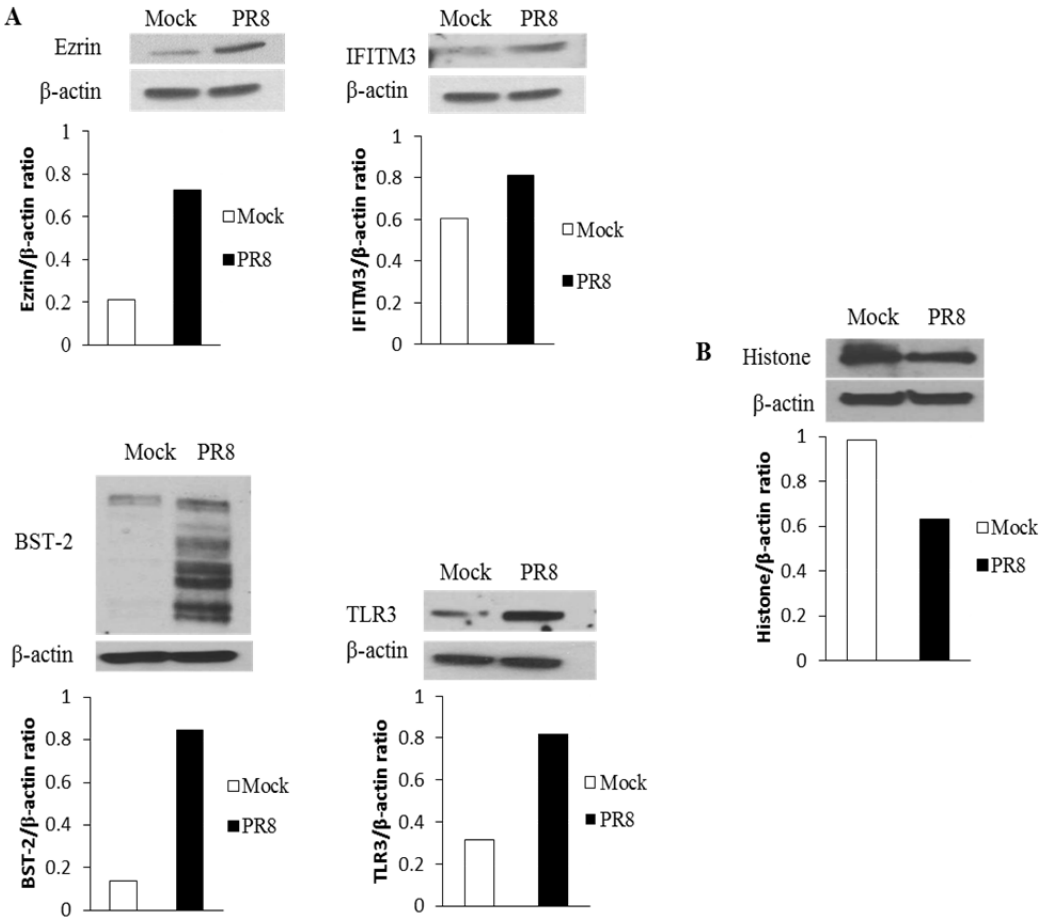

Figure 3.2 Western blot validations of LC-MS data. At 24 h post-infection, equal amounts of whole cell lysates of PR8-infected or mock-treated ATII cells were subjected to western blot analysis. Protein bands intensities (shown in graphs) were quantified by densitometry. The protein values were background corrected, then expressed as the ratio of candidate protein to β-actin (A) Proteins that were up-regulated by PR8 in proteomics (Ezrin, IFITM3, BST-2 and TLR3), (B) Protein (histone 2B) that was down-regulated by PR8 in proteomics analysis. Western blot images are representative of three replicate experiments, while densitometry quantification was performed once.

3.4.5 ATII-specific proteins identified by LC-MS

To confirm that our primary ATII cultures represent the phenotype of ATII cells in the lung, the presence of phenotypic markers was evaluated by proteomics. Through mass spectrometry analysis, we identified ATII cell-specific proteins in both PR8-infected and mock-infected samples, further confirming that these cultures maintained an ATII phenotype *in vitro*. As shown in Table 3.5, we identified multiple ATII-specific proteins in addition to the four pulmonary surfactant proteins that are the signature of the ATII cells: surfactant proteins (SP)-A, B, C and D (Weaver & Whitsett, 1991). These phenotypic markers have been identified by a thorough analysis of the molecular phenotypes of rat pneumocytes, which defined markers of ATII phenotypes by microarray analysis (Gonzalez *et al.*, 2005). In addition to markers of the ATII cell phenotype, of the 26 proteins that Gonzalez et al. found to be ATI-specific we detected only three in our ATII cultures: sparc, caveolin and agrin (Gonzalez *et al.*, 2005). This might occur as ATII cells have the ability to trans-differentiate into cells with an ATI phenotype where they gain expression of ATI-specific proteins. The well recognized ATI phenotypic markers, T1α and aquaporin 5 were not detected in our study. Thus, our ATII cultures express a majority of proteins that correspond to a differentiated ATII cell phenotype, and only a minority of ATI-specific proteins. PR8 infection inhibited expression of SP-B, but did not alter the expression of SP-A, C, D, or other ATII-specific proteins (Table 3.5).

Table 3.5 ATII phenotypic proteins identified by LC-MS

Uniprot Accession ID	Gene Name	Number of Peptides identified	Fold Change[1] (P-value)
Q02496	Mucin 1	3	1.009 (0.974)
Q8VCM7	Fibrinogen gamma chain	5	0.955 (0.865)
P35242	Surfactant-associated protein A	3	0.891 (0.614)
P50405	Surfactant-associated protein B	8	*0.37 (<0.001)
P21841	Surfactant-associated protein C	2	0.905 (0.771)
P50404	Surfactant-associated protein D	5	0.893 (0.774)
P97370	Sodium/potassium-transporting ATPase subunit beta-3	2	0.670 (0.323)
Q91VS7	Microsomal glutathione S-transferase 1	10	0.494 (0.068)
P11672	Neutrophil gelatinase-associated lipocalin	1	1.277 (0.397)

[1]Fold change expressed as ratio of PR8/mock, P-value determined by student's t-test

* Significantly regulated by PR8 infection.

3.4.6 Analysis of SP-B *in vitro* and *in vivo*

SP-B was significantly down-regulated by PR8 infection of ATII cells (P-value <0.001) (Table 3.5). Hence, we further validated the LC-MS data by utilizing immunofluorescence and Western blot analysis of PR8-infected ATII cells *in vitro*. We also performed western blot analysis of bronchoalveolar lavage fluid from mice infected with PR8. Cytoplasmic immunofluorescence and Western blot showed that SP-B was expressed in mock-infected ATII cells, whereas it was diminished in PR8-infected cells (Figure 3.3A and 3.3B). These changes in SP-B expression correlate with decreased levels of SP-B peptides as observed by proteomic analysis. A previous study using ELISA to detect surfactant proteins showed that PR8 does not alter SP-B secretion levels, but reduces secretion of SP-A and SP-D from human ATII cells cultured *in vitro* (Wang *et al.*, 2011). However, the study evaluated the secretion of SP-B rather than the protein from cellular lysates as was done in our study. In the context of our study, the preservation of SP-A and SP-D levels may be important for the augmentation of virus clearance, as these proteins are involved in innate immune responses in the alveolus. The decrease in SP-B may be due to the production of inflammatory molecules secreted by the cells during infection. Cytokine expression was reported to have effects on surfactant homeostasis and consequently inhibition of SP-B during *Pneumocystis carinii* pneumonia in mice (Beers *et al.*, 1999). Secretion of inflammatory cytokines such as tumor necrosis factor (TNF) has been shown to decrease the expression of SP-B in cultured alveolar epithelial cells (Wispe *et al.*, 1990) and other cytokines such as IL-1β may also down-regulate the expression of SP-B in ATII cells (Bry *et al.*, 1996; Ingenito *et al.*, 2001). Down-regulation of SP-B in the lungs could cause disruption of the intracellular routing of surfactant phospholipids and proteins to the lung surface, which may lead to respiratory

distress. The lack of SP-B through genetic mutations in mice and SP-B deficiencies in humans has been associated with alterations in surfactant biophysics and lethal respiratory failure at birth and death (Beers *et al.*, 2000; Clark *et al.*, 1995). Furthermore, studies have shown that single nucleotide polymorphisms in *SP-B* are associated with severe influenza disease in humans (Currier *et al.*, 2008; To *et al.*, 2013).

The goal of our *in vivo* analysis of SP-B was to examine whether PR8 infection would cause decreased SP-B expression in mice. However, in contrast to the down-regulation of SP-B in PR8-infected cultured ATII cells, we observed increased levels of SP-B in bronchoalveolar lavage fluid from mice infected with PR8 (Figure 3.4A and 3.4B). The increased levels of SP-B may be due to the destruction of the alveolar barrier, which is formed by the ATI cells (Fukushi *et al.*, 2012). This damage might allow for the release of surfactant from the inside of the cell to the extracellular space, hence we observed an increase of SP-B in PR8-infected mice compared to controls, where we did not detect any SP-B in bronchoalveolar lavage fluid. We observed a pro-SP-B intermediate of 24 kDa. In ATII cells, 24 kDa intermediate is further cleaved into active mature protein of 8 kDa, which gets released into the alveolar space together with the surfactant phospholipids (Rooney *et al.*, 1994). We speculate that the pro-SP-B intermediate was increased to replenish the pulmonary surfactant that is being released out of the alveoli to the extracellular environment. Additionally, the increase in SP-B could be due to ATII hyperproliferation, which would be required for trans-differentiation into ATI cells to repair the injured lung. Other studies have observed increases in serum levels of SP-A and SP-D due to pulmonary surfactant leakage from the alveolar lumen to capillaries in PR8-infected mice (Fukushi *et al.*, 2011). Hyperoxic lung injury in mice was shown to promote an increase of SP-B into the

alveolar space (Tokieda *et al.*, 1999). Our study did not look at the quantitative or qualitative analysis of pulmonary surfactant in these mice and whether other surfactant proteins were also changed. Nevertheless, our results show that PR8 alters the levels or location of SP-B in the lungs, which might disrupt surfactant homeostasis and compromise the ability to breathe.

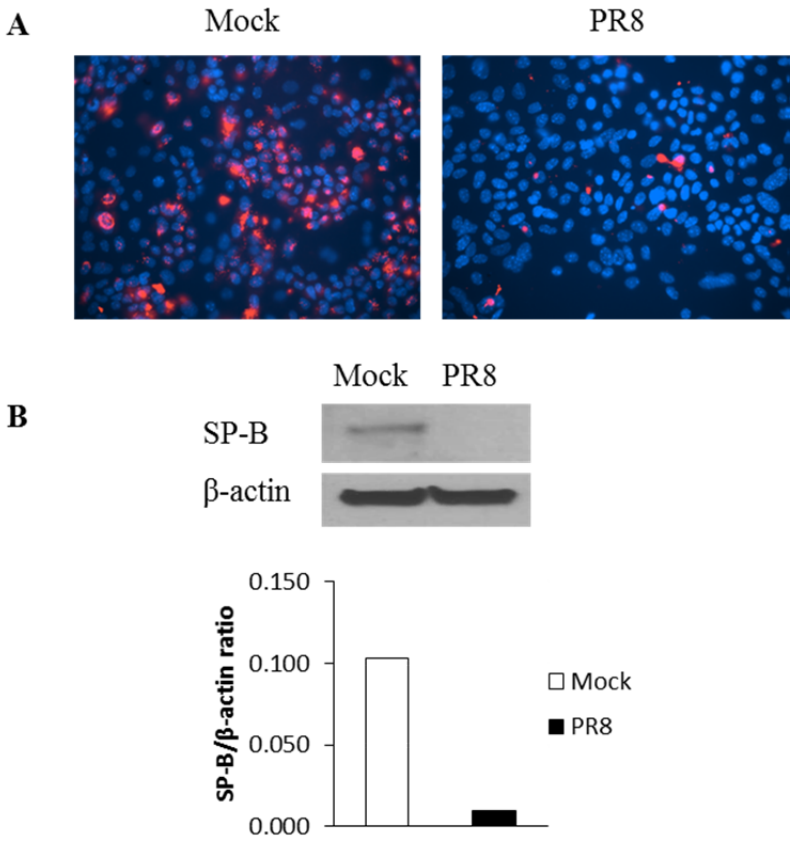

Figure 3.3 PR8 infection inhibits expression of SP-B in ATII cells. ATII cells were mock-treated or infected with PR8. At 24 h post-infection, cells were analyzed by (A) immunofluorescence assay of SP-B protein, (red) and nuclei were stained with DAPI (blue), and (B) western blot analysis using an antibody against SP-B. β-actin was used as a loading control. The graph shows quantitative densitometric analysis of the western blot image. Protein ratio is normalized to β-actin. Images for IFA and western blot are representative of two experiments while densitometry was performed once.

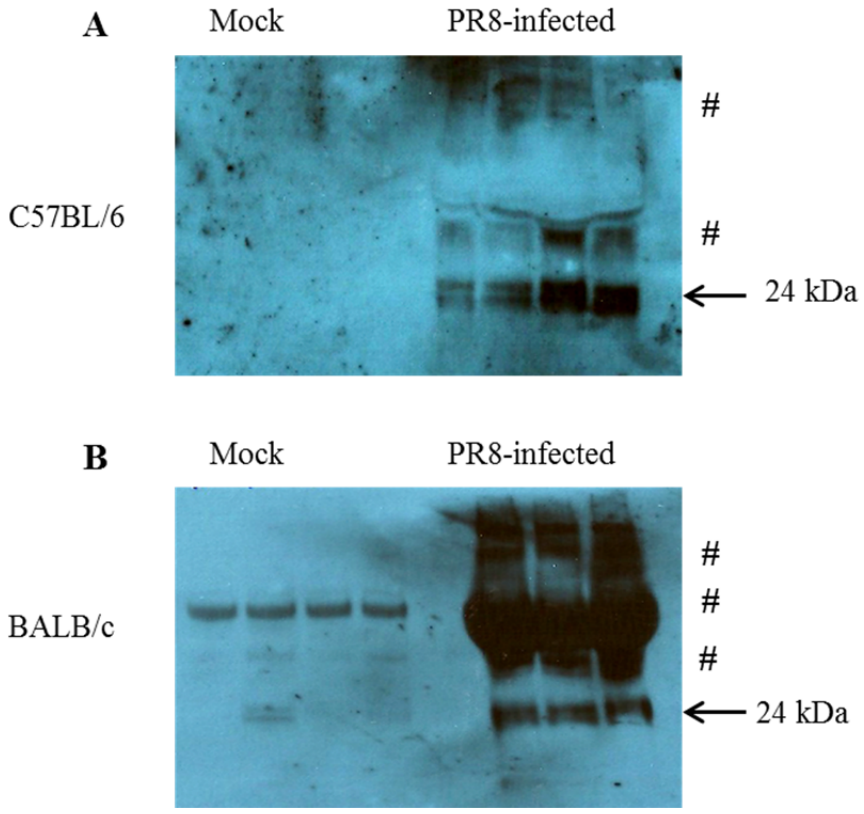

Figure 3.4 Levels of SP-B in bronchoalveolar lavage fluid from mice infected with PR8. Bronchoalveolar lavage fluid was prepared from (A) C57BL/6, and (B) BALB/c mice infected with PR8 or mock treated with cell culture medium. The arrows denote the position of the 24 kDa SP-B band. All the other bands were non-specific (#). Lanes were loaded with equal volumes of concentrated bronchoalveolar lavage fluid. Western blot image is representative of two replicate experiments.

3.5 Conclusion

The PR8 murine model is useful in the study of IAV pathogenesis as the immune response parameters in the lung can be monitored and evaluated directly. Several studies have used transformed cell lines that do not quite represent the real picture of what is going on in the lungs. Hence, rather than study transformed cell lines, we established cultures of primary murine ATII cells to compare the effects of virus-cellular interactions in a physiologically relevant cell type. This allows us to make direct correlations with the wealth of information that has been published on PR8 pathogenesis in live mice. The changes we observed in the cellular proteins of PR8-infected ATII cells confirmed aspects of what is already known about changes in the host proteome, validating our LC-MS data. The up-regulated proteins may play a role in the antiviral defense against PR8 whereas the down-regulated proteins may favor virus replication in the lung. Using primary murine ATII cell cultures has helped us identify that influenza virus down-regulates SP-B, which is required for the secretion of pulmonary phospholipids to prevent collapse of the lung. Although additional studies are needed to explore the detailed mechanisms of the differentially expressed proteins, our results demonstrate the importance of these proteins to ATII antiviral defense against PR8.

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Chapter 4

General discussion and future directions

Respiratory tract infections are the leading cause of infectious diseases globally. The emergence of SARS-CoV and the occurrence of the 2009 pandemic influenza H1N1 from animal reservoirs posed a threat to human health. Although the outbreaks of these viruses were contained, SARS-like viruses that are circulating in bats or avian influenza virus strains in birds may provide a pool of viruses with a potential for re-emergence into human populations. This is evidenced by the recent emergence of the MERS-CoV and H7N9 outbreak (Nicoll & Danielsson, 2013), which further necessitates the need to understand the mechanisms of increased pathogenicity of these viruses. Due to the lethal potential of these viruses, it is important that therapies are developed that can be universally applied. Therapeutic strategies that target the host, rather than the individual virus strains, would be more effective against a diversity of respiratory viruses.

There is a need to define the host-pathogen interaction required for initiating and regulating the innate immune response to viral infection in the lungs so we can target excessive responses. The pulmonary lesions observed in SARS patients were thought to be mediated by a robust increase in proinflammatory cytokines in response to viral replication in the lungs (Chien *et al.*, 2006; Gu *et al.*, 2005). Infection with H5N1 and 2009 pandemic H1N1 viruses also demonstrates that pulmonary damage and acute respiratory syndrome correlates with the excessive inflammatory responses in the host (Itoh *et al.*, 2009; Taubenberger & Morens, 2009). The occurrence of this “cytokine storm” contributes to increased disease severity. The importance of the alveolar epithelial cells as the primary

targets of respiratory viruses in the lung and their involvement in mediating immune responses suggests that they are the critical determinants of whether an effective or detrimental inflammatory response to respiratory viral infection occurs.

In order to decipher the role of the alveolar epithelial cells in the inflammatory response to respiratory viral infection, in Chapter 2 of this work murine alveolar epithelial type I (ATI) and II (ATII) cells were maintained with differentiated phenotypes under optimal culturing conditions. Several cytokines and chemokines have been implicated in the development of disease in SARS and influenza in the lung. Hence, in Chapter 2 of this work the innate immune response of the alveolar epithelial cells to influenza A virus (IAV) and mouse hepatitis virus-1 (MHV-1) infection was examined. MHV-1 is a respiratory model of SARS disease. Both viruses induced mRNA expression of IL-1β and TNF-α, these cytokines are the hallmark of the pro-inflammatory cytokines that are readily induced in response to IAV or SARS-CoV infections and act in synergistic fashion to contribute to the induction and development of disease symptoms (Baas *et al.*, 2006b; Julkunen *et al.*, 2000; Kong *et al.*, 2009). TNF-α is reported to have deleterious effects on the disease progression (Hussell *et al.*, 2001). TNF depletion reduces inflammatory cells, cytokine production in the lung and disease severity in mice challenged with IAV or respiratory syncytial virus. In mice deficient in TNF receptor-R1, there is significant reduction in morbidity and mortality compared to wild-type mice following IAV infection (Belisle *et al.*, 2010). Induction of TNF-α expression was also induced during infection of the rat ATI cells with rat coronavirus (Miura *et al.*, 2007).

Production of IL-1β has been found to occur through activation of the inflammasome during infection (Barlan *et al.*, 2011; He *et al.*, 2010; Ichinohe *et al.*, 2009). Inflammasomes

are multiprotein complexes that allow for activation of caspase-1, a regulator of inflammatory responses through its capacity to activate proIL-1β and cleave it into a mature protein (Kuida *et al.*, 1995; Meylan *et al.*, 2006). The nucleotide-binding oligomerization domain-like receptor (NLR) with adaptor proteins has been implicated as the caspase-1 activating-inflammasome (Ogura *et al.*, 2006; Sutterwala *et al.*, 2006). The induction of IL-1β mRNA in this study suggests proIL-1β protein may be made by the cells and hence, there may be regulation of the inflammasome in alveolar epithelial cells. In IAV-infected macrophages, IL-1β secretion is dependent on caspase-1 and NLRP3 pathways (Allen *et al.*, 2009). Attempts to determine the pathway of activation of IL-1β in the ATI cells and whether it is cleaved into a mature protein proved inconclusive in our study. However, a study by Pothlichet et al. show that RIG-I directly activates the inflammasome in lung epithelial cell line and modulates IL-1β secretion during infection with IAV (Pothlichet *et al.*, 2013). Whether alveolar epithelial cells use this pathway is not yet known. IL-1β signaling is through the IL-1 receptor and in this study MHV-1-infected ATI cells had induction of mRNA of two IL-1 receptors, IL-1α (IL-1 alpha) and IL-1R (IL-1 receptor). Gene knockout of IL-1R shows signaling through IL-1 receptor to be protective in IAV infection (Belisle *et al.*, 2010). Molecules produced from IL-1 signaling such as IL-1β could be central in communication between alveolar epithelial cells and monocytes during initiation of inflammation and development of adaptive responses. It would be interesting to know if the expression of these molecules was from infected or noninfected cells. A previous study on rat ATI cells infected with rat coronavirus reports that the virus stimulated noninfected cells to secrete inflammatory molecules in an IL-1 dependent mechanism (Miura *et al.*, 2007); hence paracrine signaling by IL-1 receptors would protect the alveolar

epithelium from infection by providing robust local immunity to curb viral load, but may also contribute to damaging inflammation at the site of infection. This paracrine stimulation could also affect the alveolar macrophages to further magnify the inflammatory immune responses.

Receptors for IL-6, an important cytokine in immune response to SARS-CoV were induced in MHV-1-infected ATI cells. These are IL-6Rα (IL-6 receptor alpha) and IL-6ST (IL-6 signal transducer). IL-6 is a multifunctional cytokine, involved in growth and differentiation, and may contribute to T cell mediated inflammatory reactions in the lungs (Van Snick, 1990). L-6 has been shown to be released by epithelial cells during lung injury (Hierholzer *et al.*, 1998). Reports suggest that IL-6 may contribute to the severe immunopathology that is observed in lung injury during coronavirus infection. Expression of IL-6 and other cytokines are elevated during early phase of SARS and was correlated with pulmonary damage (Jiang *et al.*, 2005). In MHV-1 infected mice, IL-6 mRNA levels also correlates with lung pathology (Leibowitz *et al.*, 2010). The effects of IL-6 have been found to be synergistic with those of IL-1 and TNF-α in causing severe disease. Meanwhile, however, IL-6 receptor knock-out mice infected with IAV show that IL-6 could also be important in protecting neutrophils from virus induced cell death in the lung and promotes neutrophil-mediated viral clearance (Dienz *et al.*, 2012), and thus it could also contribute to limiting viral infection in the alveolar epithelium.

An array of chemotactic cytokines including CCL2/MCP-1 and CXCL10/IP-10 are expressed by the respiratory epithelial cells during response to both coronavirus and IAV infections. MCP-1 and IP-10 are macrophage chemoattractants and mediate inflammatory response by further recruiting neutrophils to the infected site (Baggiolini, 1998). In the

present study, there was increased expression of MCP-1 and IP-10 in both IAV and MHV-1 infected ATI cells. In mice infected with mouse-adapted SARS-CoV, Urbani strain, the expression of MCP-1 and IP-10 is biphasic (Chen *et al.*, 2010). Early on day 2 activation of these chemokines plays role in controlling the virus, resulting in recruitment of macrophages and NK cells to the lung. MCP-1 and IP-10 are later expressed at day 7, correlating with infiltration of T cells, neutrophils and coinciding with onset of pneumonitis and later viral clearance. Furthermore, blocking MCP-1 using anti-MCP-1 monoclonal antibody results in enhanced alveolar epithelial damage during IAV pneumonitis in mice (Narasaraju *et al.*, 2010). These correlate with decreased lung expression patterns of surfactant protein C and T1α and pronounced apoptosis in anti-MCP-1 treated animals. In addition, mice lacking MCP-1 have increased viral load and higher levels of TNF-α, IL-6 and MIP-2 (Dessing *et al.*, 2007). This shows that MCP-1 and IP-10 may contribute to protective immune response during influenza infection and SARS-CoV infections.

We measured the expression of cytokines and chemokines in this study late during infection at 24 h when viral titers were at a peak. Hence, this suggests that these inflammatory molecules may be involved in causing the lung pathology that is often seen late during infection of mice. In addition, rat coronavirus infection of ATI cultures in our lab show that CXC chemokines that signal through CXCR2, including CXCL1, 5 (detected in our study), inhibit polymorphonuclear leukocytes apoptosis and activation of caspase 8 and 9 (Rzepka et al., 2012). However, taken together the functions of cytokines and chemokines with increased mRNA expression in this study shows that there are involved in various degrees in the alveolar epithelium in limiting virus infection or contributing to pneumonitis. It is also important to note that not all infected cells might be source the expression of these

inflammatory mediators as some bystander cells, that are not infected could be activated to express cytokines and chemokines. We did not measure these inflammatory mediators at earlier time points to determine whether the same will be secreted by the cells in late time points.

The changes in proteome profiles could help in elucidating cellular processes involved in viral infections. Hence, in Chapter 3 of this work ATII cells were infected with IAV to assess the protein profiles that are differentially regulated during IAV interaction with these cells. There was significant increase of IFN α/β response genes (TLR3, IFITM3, BST-2) in ATII cells infected with IAV. IFN response has a well-characterized antiviral activity and many other functions involved in the adaptive immunity including augmentation of MHC expression (Van Reeth, 2000). Mice lacking functional receptors for IFN-α/β are hypersensitive and fail to restrict even usually non-pathogenic IAV and have high viral replication and increased disease severity (Mordstein *et al.*, 2008). The data in this study further add to what is already known about the involvement of the IFN-response in IAV infections, but more specifically to how the primary ATII cells interact with IAV at the proteome level during infection. Meanwhile, however, more studies are needed to examine functional roles of these genes in the ATII-IAV interaction. Gene silencing studies using RNA interference could be done in the ATII cells to examine the roles of these proteins. In addition, ATII cells could be isolated from knock-out mice, lacking each of these genes and then infected with IAV to further study how the cells will respond to viral infection in their absence. With respect to BST-2, electron microscopy analysis to visualize if the protein does tether the viral particles to the plasma membrane of the ATII could be performed. Lastly, it will be interesting if this study could further examine the proteome of IAV infected ATI

cells to determine if there are differences in the way IAV interact with each of the cell types of the alveolar epithelium.

In using proteomics, we further validated that our ATII cultures maintained their differentiated phenotype *in vitro* with the expression of signature pulmonary surfactant proteins that are important to the phenotype and functions of ATII cells in the lung. While the rest of the ATII phenotype proteins detected in our study remained unchanged during IAV infection, the pulmonary surfactant protein B (SP-B) was significantly decreased by IAV infection. A comparison was done to correlate whether what is observed *in vitro* would also be true in mice infected with IAV. There was no correlation, but rather increased levels of SP-B in bronchoalveolar lavage fluid from IAV-infected mice compared to the controls. Surfactant proteins were shown to increase in serum of PR8-infected mice in association with aggravation of pneumonia up to 6 days post infection (Fukushi *et al.*, 2011). The mice in our study were sacrificed between days 6-9 post-infection when they showed clinical symptoms associated with severe disease including significant weight loss. These may compromise the ability of the host to breathe.

In conclusion, we show that alveolar epithelial cells could be important to the host response to respiratory viral infections, which will either promote or reduce pathogenesis in the lungs. Other studies have shown that PR8 and MHV-1 cause pathologies in the lung, but we further show that ATI and ATII could be the source of some of the inflammatory mediators that is seen during pulmonary infection by these viruses. Several cytokines and chemokines that were detected in ATI and ATII cultures in our study are also expressed in lungs of mice infected with MHV-1 and PR8. Hence our data correlates with studies using *in vivo* models. For instance histology sections of lungs infected with PR8 show large areas

of virus-infected cells in the alveolar regions more than in other areas of the lungs and the alveoli filled with inflammatory cells (Blazejewska *et al.*, 2011). Furthermore, the presence of virus in the alveoli and alveolar ducts causes severe alveolar collapse (Fukushi *et al.*, 2012). These studies report the occurrence of these features early during infection on days 1-2 (Blazejewska *et al.*, 2011; Fukushi *et al.*, 2012), thus this may implicate the alveolar epithelial cells in the first wave of cytokines and chemokines in response to viral infection. Overall, this work has shown that murine alveolar epithelial cells can be maintained in culture and used as an *in vitro* model to study virus-host interactions in a specific cell-type that otherwise would be challenging *in vivo*. This study also offers a way in which cytokines and chemokines that were induced during MHV-1 and PR8 infection and the pathways involved in the induction of the protein profiles observed in ATII cells could be manipulated in the mouse model and lead to the design of therapeutic strategies and drug interventions.

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Appendix A

Protocol Approval from Animal Care Unit (University of Idaho)

University of Idaho

Office of Research Assurances
Institutional Biosafety Committee
PO Box 443010
Moscow ID 83844-3010

Phone: 208-885-6162
ibc@uidaho.edu

TO: Tanya Miura

RE: IBC# **B-012-12,** Approved May 24, 2012 and Expires May 23, 2015

Pathogenesis of respiratory viral infections

This notification is to inform you that the above named biosafety application has been approved by the Institutional Biosafety Committee (aka the Biohazards Committee). This approval is good for three years.

Should you make changes to this protocol please verify with the Biosafety Officer what modifications, if any, are needed to keep your approval current.

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Appendix B

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Lemme