Pneumocystis Colonization and Chronic Obstructive Pulmonary Disease in a Simian Model of HIV Infection

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Potent anti-retroviral therapy has transformed HIV infection from an acute to a chronic disease. Consequently, diseases previously not prevalent in HIV+ persons have emerged. For example, HIV-infected persons are at increased risk for developing COPD. *Pneumocystis* (Pc), a fungal opportunistic pathogen, has been associated with HIV and COPD. Pc colonization- the presence of Pc in subjects without clinical symptoms of *Pneumocystis* pneumonia- is increased in COPD patients. Furthermore, HIV+ individuals are at elevated risk for both Pc colonization and emphysema. Together, these observations suggest that COPD in HIV+ individuals involves Pc colonization. We used a simian/human immunodeficiency virus (SHIV) model of HIV infection to study pulmonary effects of Pc colonization.

SHIV-infected/Pc-colonized monkeys developed obstructive pulmonary disease characterized by increased emphysematous tissue and bronchial-associated lymphoid tissue. Elevated Th2 cytokines and pro-inflammatory mediators in bronchoalveolar lavage fluid coincided with Pc colonization and pulmonary function decline. These results indicate that Pc colonization may be a risk factor for development of HIV-associated COPD.

Gene expression profiles in the lung tissue of these animals evaluated by microarray analysis revealed differential expression of 243 genes in the obstructed SHIV/Pc monkeys compared to SHIV-only monkeys with normal lung function. Potentially relevant differentially expressed genes included genes involved in inflammation, protease/antiprotease balance, redox

balance and tissue homeostasis, thus indentifying factors and pathways involved in early development of SHIV-associated COPD and revealing several novel, possible therapeutic targets.

In a second cohort of animals, airway obstruction development associated with Pc colonization was recapitulated. To directly correlate pulmonary function decline with presence of Pc, a subset of the Pc-colonized monkeys was treated with the anti-Pc drug, TMP-SMX, after significant airway obstruction had occurred. No further pulmonary function decline was observed in either the treated or untreated animals up to a year after initiating TMP-SMX treatment. These results indicate that Pc-associated induction of airway obstruction takes place early after onset of colonization followed by an extended period of containment of the effects of Pc.

These results demonstrate a key role for Pc in the early development of SHIV-associated COPD. Furthermore, they reveal multiple potential mediators of Pc-induced airway obstruction.

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In closing, I believe that Spongebob Squarepants said it best when he said, "Remember, licking doorknobs is illegal on other planets!"

1.0 INTRODUCTION

With the outbreak of the AIDS epidemic in the 1980s, the opportunistic fungal pathogen, *Pneumocystis jirovecii*, gained significant prominence as the causative agent of *Pneumocystis* pneumonia (PcP), the most prevalent opportunistic infection in patients who have acquired immune deficiency syndrome (AIDS) (1). Even with the widespread implementation of potent antiretroviral drug cocktail therapies and prophylactic drug use against *Pneumocystis* (Pc), PcP continues to be a serious but common affliction in AIDS patients (174, 243). The mortality associated with developing the disease is as high as 63% in individuals not on antiretroviral therapy but can still be as high as 33% in patients who are on antiretroviral therapy (159, 247).

Other immunosuppressed groups are also at risk of contracting PcP. These include individuals on immunosuppressive medications, patients with malignancies that are either hematological or solid, transplant recipients and individuals with genetic immunodeficiencies (314, 359).

These data clearly indicate that a fulminant infection with Pc can be extremely serious. What is not as well understood are the consequences of a subclinical infection with Pc wherein no clinical symptoms of PcP are apparent and the organism cannot be detected microscopically in respiratory samples but, rather, by the highly sensitive method of polymerase chain reaction (PCR) (colonization). In the population infected with the human immunodeficiency virus (HIV), rates of colonization as high as 69% have been observed (147). This is significant because it has

been shown in the HIV+ population, which is predisposed to high rates of chronic obstructive pulmonary disease (COPD) (66) and accelerated emphysema (80), that PcP can result in COPD-like changes in pulmonary function after resolution of the pneumonia (248). The association between Pc colonization and COPD warrants further investigation.

In short, more studies are required to enhance our current level of understanding of Pc in order to stem morbidity and mortality rates associated with acquiring this organism.

1.1 A HISTORICAL LOOK AT *PNEUMOCYSTIS*

Pc was first described by Carlos Chagas in 1909 in a guinea pig model of *Trypanosome cruzi* infection (46). At the time, Chagas thought he had identified a new form of trypanosome. Shortly thereafter, Antonio Carinii arrived at the same conclusion when he discovered similar organisms in infected rat lung tissue (44). It was not until two years later that the organism identified by the two researchers was recognized as a separate species (74). At this time, the organism was called *Pneumocystis carinii* after Antonio Carinii and highlighting its tropism for the lungs.

Histological evidence demonstrating the Pc lifecycle to consist of a small trophic form and a larger cyst form, similar to protozoans, led to its classification as a trypanosome. However, in 1988, Pc was placed in the fungal kingdom because sequencing of its small ribosomal RNA subunit indicated a greater familial link to fungi (85).

In addition to its change in taxonomic status, the nomenclature for the organism has also evolved significantly. The reason for this stems from observations of Pc organisms in nearly every mammalian species. Analysis of the major surface glycoprotein gene, which is expressed

on the surface of Pc, has led to the conclusion that a genetically distinct variety of the organism with stringent host specificity is harbored by each mammalian species (342). This has led to the naming convention in which each form of *P. carinii* received a forma specialis (f.sp.) designation indicating the host species that it infects. For example, *Pneumocystis carinii* f.sp. *hominis* and *Pneumocystis carinii* f.sp. *murina* were the names given to the forms that infect humans and mice, respectively. Recently, though, the form that infects humans was renamed *Pneumocystis jirovecii* after Jirovec who first described Pc in humans (341).

1.2 BIOLOGY OF *PNEUMOCYSTIS*

1.2.1 Lifecycle of Pc

Descriptions of the lifecycle of Pc come solely from microscopic examination of infected lung tissue because it cannot be purely cultured *in vitro*. In the lungs Pc principally occurs in two forms: the trophic form and the cyst. The smaller trophic form is 1-4 μm in diameter and outnumbers the larger mature cyst form, which is 8-10 μm, by about 10 to one (358). Pc infection is established by tight adherence of these trophic forms with type I alveolar epithelial cells by interdigitating its membranes with that of the host cell (371). However, structure and barrier functions of the alveolar cells do not appear to be disrupted by this interaction (21).

There are three intermediate cyst stages (early, intermediate and late) called “precysts” containing two, four and eight nuclei, respectively (229). It is believed that the mature cyst form, which also has eight nuclei, gives rise to the trophic form which can reproduce either sexually by vegetative growth or conjugation to reform the cyst, or asexually by binary fission (358).

1.2.2 Surface molecules of Pc

The binding of Pc to host cells is largely mediated by conjugation of glycoprotein A (gpA or major surface glycoprotein (MSG)), the major surface protein on Pc, with the host proteins, fibronectin and vitronectin (208). This 120 kDa protein complex is heavily glycosylated containing multiple carbohydrates including galactose, mannose and glucose (69, 113). Alveolar macrophages (AM), which are ultimately responsible for clearing Pc from the lungs, have mannose receptors that recognize gpA to take up the organism (262). However, Pc has developed mechanisms to evade this arm of host innate immunity. Pc can shed or secrete gpA molecules in order to block phagocytosis by AM (196). Moreover, Pc can induce AM to secrete mannose receptors which consequently blocks gpA-mediated uptake by AM (97). Glycoprotein A also plays a role in escaping recognition by the adaptive arm of the immune system. Even though only a single form of gpA is expressed on the Pc surface at one time, gpA is encoded by approximately 100 genes resulting in extensive surface variability which may be instrumental in evading adaptive immune responses (342, 359).

One study in mice deficient for the mannose receptor showed that clearance of Pc can still take place in the absence of this important Pc recognition receptor despite being CD4+ T cell depleted (348). The reason for this is that AM can also recognize Pc via the beta-1,3-glucan molecule, another major component of the Pc cell wall (228). Although a number of potential receptors for beta glucans exist, the main receptor on AM is the Dectin-1 receptor and has been shown to mediate killing of Pc (335).

1.3 EPIDEMIOLOGY OF *PNEUMOCYSTIS* INFECTION

Due to the fact that Pc cannot be continuously cultured *in vitro,* the precise epidemiology surrounding transmission of the organism remains in question. However, there are three prevailing theories to explain how Pc infection takes place.

1.3.1 Reactivation of a latent infection

It has been suggested that children act as the reservoir for Pc as reports have shown that the majority of children have antibodies to the organism. In one such study, two thirds of normal, immunocompetent children were positive for serum antibodies against Pc by four years of age (274). However, in a second more recent study examining both Pc colonization and seropositivity to Pc in normal subjects up to two years of age, a colonization rate of 32% and a seroconversion rate of 85% was observed (367). Data such as these form the foundation for the hypothesis wherein individuals who acquire Pc at a very young age develop pneumonia if their immune system fails in subsequent years. Nevertheless, there are a number of reports that refute this hypothesis. For example, Chen, *et al* showed that severe combined immunodeficiency (SCID) mice, which lack an adaptive immune system, that have recovered from a Pc infection through spleen cell reconstitution failed to reactivate the infection after depletion of CD4+ T cells. Furthermore, neither Pc organisms or Pc DNA was detected in lungs of these mice three weeks after spleen cell reconstitution (50). Another study evaluating the genotypes of Pc in patients with repeated PcP identified a different variant of Pc from the one in the original infection in 50% of the cases indicating that reactivation of a previously encountered strain of Pc is not the sole source of Pc infections that develop later in life (179).

1.3.2 Acquisition via environmental exposure

Another reservoir that has been proposed is the environment. The discovery of Pc DNA from both rat and human in air filters supports this hypothesis (370). Environmental transmission of the organism is further upheld by the finding of Pc in pond water (45). Finally, despite a lack of direct evidence, it has been inferred that Pc also exists in soil (253). In this study, HIV+ individuals who gardened and/or hiked were found to be at higher risk for contracting PcP.

1.3.3 Person-to-person transmission

Currently, the method of Pc transmission that is most widely accepted is that of person-to person transmission. There is an abundance of evidence to support this theory such as the report by Singer, *et al* showing clustering of PcP cases in patients residing in an oncology ward at a single hospital (329). In another clustering event, five kidney transplant patients who attended a clinic shared by AIDS patients all developed PcP within a 22 month period where no cases had been reported in over five years for 114 other transplant patients who had been treated with the same immunosuppressive protocol (48). There are also a number of animal studies that support lateral transmission of Pc. In mice immunosuppressed with dexamethasone, Pc transmission in multiple strains of mice was accomplished simply by cohousing healthy immunosuppressed mice with mice that have PcP(277). A high incidence rate of PcP in a regional primate center colony of simian immunodeficiency virus (SIV)-infected rhesus macaques provided further evidence of horizontal transmission (369). In this report, 51% of terminally ill monkeys that were housed together developed PcP. In contrast, none of the SIV-infected monkeys that were isolated contracted the disease. Lastly, Gigliotti and colleagues showed that immunosuppressed mice

with PcP can transmit Pc to healthy, immunocompetent mice via brief periods of cohousing that in turn were able to transmit the organism to other normal mice (117). These data strongly suggest a means whereby Pc can be maintained in the environment.

1.4 AT-RISK POPULATIONS FOR *PNEUMOCYSTIS* INFECTION

1.4.1 Pc infections in non-HIV infected individuals

The first reports of clinical disease caused by Pc were in the 1940s in orphanages right after World War II (102). In the reports, malnourishment of many of the children in the orphanages was associated with pneumonia accompanied by infection with Pc. Since that time, Pc infections have been noticed with increasing frequency in patients who are immunocompromised for a variety of reasons. For example, transplant patients are generally susceptible because of the need for lifelong immunosuppresive therapies to prevent rejection. Still other individuals have genetic disorders, such as chronic granulomatous disease (CGD) or Wiscott-Aldrich Syndrome (WAS), which adversely affect their immune systems resulting in predisposition to Pc infections. Patients with cancer are also prone to Pc infections. In particular, those with hematologic malignancies such as non-Hodgkins lymphoma, acute lymphoblastic and myeloid leukemias and chronic lymphocytic leukemia suffer from a 33% mortality rate due to PcP (291). The requirement for effective management of Pc infections in these patients is vital because, collectively, the mortality rate for PcP in non-AIDS patients is 30-60% (359).

1.4.2 Pc infections in HIV+ individuals in the era of antiretroviral therapy (ART) and anti-Pc prophylaxis

It was the AIDS epidemic that led to Pc becoming a widely recognized serious threat as a lethal, opportunistic fungal infection. The primary reason for this is that PcP became the leading AIDS-defining illness in the HIV+ population due to severe immunosuppression arising from extensive destruction of host CD4+ T cells which results in extreme susceptibility to microbial infections and illnesses that are not typically seen in healthy individuals. However, the combination of ART, which directs a potent cocktail of drugs against HIV, and trimethoprim-sulfamethoxazole (TMP-SMX, aka bactrim), the drug of choice for treatment and prophylaxis of Pc, has driven PcP incident rates in the HIV+ population down from approximately 75% to 3-4% (243). Yet, increased usage of PCR as a highly sensitive detection technique has revealed that Pc still occurs in asymptomatic HIV+ subjects at rates as high as 46% (246). Even higher rates (69%) have been observed in patients presenting with respiratory symptoms but who were not diagnosed as having PcP (147). These studies showing carriage of Pc in the absence of PcP development call in to question the long-term effects of colonization in host lungs by the organism.

1.5 INFLAMMATORY RESPONSES TO *PNEUMOCYSTIS* INFECTION

1.5.1 Innate immune responses in Pc infection

Structural epithelial cells may play a vital role in Pc infection. Indeed, as the substrate to which Pc organisms attach, epithelial cells are likely to initiate host immune responses that are to

follow. *In vitro* studies in which either Pc or its surface molecules, gpA or beta-glucans, are incubated with epithelial cells supports this reasoning as these cells were observed to secrete chemokines that attract or stimulate neutrophils (interleukin (IL)-8 and CXCL2 (23, 131) and monocytes or macrophages (CCL2 and IL-6) (276, 374).

Natural killer (NK) cells have not been extensively studied in the context of Pc infection. One study indicating NK cell involvement was performed in CD4+ T cell-depleted mice that received interferon (IFN)-γ-expressing adenoviral vector just before challenge with Pc (186). Mice that upregulated IFN-γ by gene transfer were able to resolve the Pc infection which correlated with significant increases of CD8+ T cells and NK cells in the lungs.

Like NK cells, studies on dendritic cell (DC) involvement in immunity against Pc are limited. One study showed that neonatal mice challenged with Pc exhibit delayed clearance of the organism compared to adults due, in part, to reduced recruitment of CD11c+ immature DC to the lungs (105). In another study, DC genetically modified to express CD40 ligand (CD40L) and pulsed with Pc antigen were administered to CD4+ T cell-depleted mice as a CD4+ T cell-independent vaccine (386). Significant protection conferred on these mice from subsequent challenge with Pc was associated with Pc-specific IgG antibody response.

It is widely believed that lung damage arising from Pc infections is mediated by host responses to the organism rather than a result of harm induced by Pc itself. In support of this theory, studies of neutrophils in Pc infections almost universally point to these cells as playing a negative role. Evaluation of neutrophil numbers and levels of the chemokine largely responsible for neutrophil attraction, IL-8, in bronchoalveolar lavage (BAL) fluid of patients with PcP reveals a strong correlation between these two elements of innate immunity and impairment of pulmonary function and/or clinical severity of disease (24, 300, 330). These observations in

humans have been recapitulated in rodent and monkey models of Pc infection (22, 29, 54, 65). Due to conflicting reports, the effector function responsible for the increased morbidity associated with neutrophils has yet to be elucidated. It has been suggested that increased superoxide production may be responsible since neutrophils cultured with Pc upregulate production of this potentially damaging reactive oxygen species (ROS) (200). However, neutrophils from HIV+ patients show decreased production of superoxide when cultured with Pc making this a less likely scenario (201). Hence, a mechanism for neutrophil-mediated pulmonary damage associated with Pc infection requires further investigation. Even if neutrophil-associated ROS are found not to mediate tissue damage, neutrophils can express a variety of other products such as proteinases and cationic peptides that can potentially cause lung damage (240). Despite frequent correlations made between neutrophils and Pc-associated lung damage, there are schools of thought that suggest that this link is more associative rather than causative (225). In fact, using a series of knockout (KO) mice and mice depleted of neutrophils, one study showed that neither neutrophils nor reactive oxygen species contribute to lung damage in PcP (349). Thus, other immune mediators and cell types must be considered in Pc-related tissue damage scenarios.

Of all of the immune system components discussed thus far, AM have been the most extensively studied in conjunction with Pc infection. As stated above, the multiple receptors available to AM for uptake of the organism make them a likely key player in the clearance of Pc from host lungs. Their importance seems to be underscored by observations that they increase in numbers and level of activation in response to Pc infection (206). In conflicting reports, macrophage numbers were observed to be decreased, rather than increased, in both human and rat PcP (94, 194, 383). Further investigation indicated that these decreases in AM were due to

increased apoptosis (197), and that blockage of the apoptosis with caspase inhibitors could enhance AM activity against Pc in rats and mice with PcP, thus prolonging their survival (195). Either way, the critical role of AM was convincingly shown by Limper *et al*. in a study in which rats were treated with liposomal dichlormethylene diphosphonate to selectively deplete AM (207). In the rats that received this compound, AM were depleted by more than 85%. Twenty four hours after a subsequent Pc challenge, the AM-depleted rats had significantly higher numbers of Pc organisms in their lungs than control rats. The mechanism by which AM kill Pc after phagocytosis appears to be mediated by ROS as studies have shown both superoxide and hydrogen peroxide production to be increased in AM cultured with Pc (139, 199). In a follow-up study, Steele *et al*. showed that *in vitro* incubation of Pc with physiologic concentrations of hydrogen peroxide can kill the organism (335).

1.5.2 Adaptive immune responses to Pc infection

The involvement of γδ T cells in Pc infection has not been studied extensively, although it has been shown that AIDS patients with PcP have elevated numbers of the cells in both blood and BAL fluid (167). Moreover, increased γδ T cell numbers were observed in the lungs of normal mice that had received inocula of Pc (336). It was further observed in this study that mice lacking γδ T cells had accelerated clearance of Pc compared to wild type controls, which correlated with increases in CD8+ T cell numbers and IFN-γ levels in the lungs, suggesting that γδ T cells impose some sort of regulation of CD8+ T cell recruitment into the lungs in the context of a Pc infection.

Immune response orchestration largely depends on the activity of CD4+ T helper cells which modify both innate and adaptive immunity. In innate immunity, they maximize the

activity of phagocytes such as macrophages. In adaptive immunity, their activity influences B cell antibody class switching and leads to activation of cytotoxic T cells. It, therefore, comes as no surprise that these cells are critical in the host defense against Pc infection. Furthermore, since CD4+ T cell destruction is the hallmark of HIV infection, it follows that Pc infections are often associated with individuals who are infected with this virus. This is also true of animal models of Pc infection which are typically designed around exploiting low or non-existent CD4+ T cell levels such as in nude mice, which do not have any T cells due to the absence of a thymus (373), and SCID mice, which lack the ability to make B or T cells (372), or by inducing susceptibility Pc infection by driving down CD4+ T cell levels through the use of monoclonal antibodies (324), corticosteroids (47) or a lentivirus (84). Underscoring the importance of CD4+ T cells, animals in most of these models can develop severe Pc infections despite having functional neutrophils and macrophages. In trying to gain a clearer understanding of the role CD4+ T cells play in immune responses against Pc, Shellito *et al*. attempted to determine whether T-helper type 1 (Th1) responses, which involve cytokines such as IFN-γ and lead to induction of cell-mediated immunity and phagocyte activation, or T-helper type 2 (Th2) responses, which involve cytokines such as IL-4 and IL-13 and promote humoral responses, are important (325). In their analysis of CD4+ T cell responses to Pc infection in lymph node and lung tissue, they found that the overall CD4+ T cell response involved both subsets of CD4+ T cells but was predominately Th2-skewed. In support of the importance of a Th2-skewed response, Zheng and colleagues introduced Pc-pulsed DCs that had been transduced with CD40L, which is normally found on CD4+ T cells and is key in inducing B cells to generate IgG antibodies, into CD4+ T cell-depleted mice (386). After Pc challenge, the DC-vaccinated mice mounted high titers to Pc and were protected from infection.

Although it has not been shown that CD8+ T cells specifically interact with and kill Pc, there is evidence that these cells do play a significant role in Pc infections. For example, in a study using mice depleted of CD4+ T cells and mice depleted of both CD4+ and CD8+ T cells, it was observed that the mice depleted of both T cell subsets developed a more severe Pc infection than those depleted of only CD4+ T cells suggesting that the CD8+ T cells provide some form of support in immune responses against Pc (20). Additionally, as mentioned previously, mice deficient in γδ T cells clear Pc infection more rapidly than wild type mice, a finding that correlated with increases in levels of CD8+ T cells and IFN-γ into the lungs (336). In another study, an IFN-γ expressing adenovirus introduced into the lungs of mice led to recruitment of CD8+ T cells to the lungs accompanied by induction of the chemokine CXCL10. When these CD8+ T cells were purified from the lungs and incubated *in vitro* with macrophages, enhanced killing of Pc by the macrophages was observed (231). The *in vivo* activity of the activated CD8+ T cells was then assessed by adoptively transferring them into Pc-infected SCID mice which were found to have significantly lower Pc burdens compared to SCID mice that received a mock adoptive transfer of cells (231). Although these studies indicate that CD8+ T cells augment immune responses against Pc infections, there is evidence indicating that they also contribute to tissue damage associated with Pc infection. In a study designed to identify specific cellular involvement in lung damage, researchers depleted mice of both CD4+ and CD8+ T cells which went on to develop PcP upon Pc challenge but maintained normal lung function and demonstrated no evidence of lung injury. On the other hand, mice depleted of CD4+ T cells only followed by challenge with Pc developed severe lung inflammation and exhibited substantial lung damage suggesting that CD8+ T cells, at least in part, mediate Pc infection-associated lung damage (379).

B cells and antibodies play a significant role in host defenses against Pc infection. Multiple studies have shown that passive transfer of IgM antibodies specific for Pc is, at least, partially protective against Pc infection (114, 118). It has also been shown by Harmsen and colleagues that Pc-specific IgG antibodies generated in immunocompetent mice through repeated exposure to Pc are protective against Pc challenge in the context of CD4+ T depletion (133). Furthermore, Garvy *et al*. showed that immunization of mice deficient in either IFN-γ or IL-4 could both mount protective Pc-specific antibody responses after CD4+ T cell depletion even though different subclasses of IgG were generated by each group demonstrating that induction of a specific antibody subclass is not critical for protection against Pc (106). Reports of decreased Pc-specific antibody titers in HIV+ subjects also suggest that anti-Pc antibodies may be important in protection against Pc infections (143, 299). The importance of having existing high antibody titers against Pc prior to a period of susceptibility was upheld by nonhuman primate studies in our lab showing correlation between high anti-Pc antibodies and protection against Pc colonization after immunosuppression by lentivirus (Kling, Shipley *et al*. (submitted)).

These studies clearly show the importance of antibodies in host responses against Pc but fail to demonstrate whether protection was a product of only the antibody activity or if B cells play a role beyond secretion of antibodies. One study investigating CD4+ T cell-B cell interactions blocked CD40L with monoclonal antibodies in mice which resulted in reduced clearance of Pc that correlated with decreased Pc-specific IgG and decreased CD4+ T cell activation (377). Though it was clear that the CD40-CD40L interaction was important in Pc clearance, the question of whether B cell-mediated antibody production or activation of CD4+ T cells or both were impaired was not answered. In an attempt to answer this question, SCID mice received splenocytes depleted of immunoglobulin secreting cells, and it was found that the mice

could not clear their Pc infection (134). Another study probing the same question found that mice deficient of B cells are extremely susceptible to PcP (223). However, neither of these studies showed whether host susceptibility to Pc in these studies was a consequence of decreased B cell-mediated antibody production or decreased B cell-mediated CD4+ T cell activation. Through performance of a series of experiments, one study showed that: 1) Pc-specific IgM produced by CD40- (which is normally expressed by B cells) deficient mice is not sufficient to clear a Pc infection; 2) mice lacking CD40 only on B cells were not able to produce Pc-specific IgG but were able to resolve Pc infections albeit at a greatly reduced rate compared to wild type mice and 3) mice deficient for both B cells and CD40 had reduced numbers of activated CD4+ T cells in the lungs (214). These results strongly suggest a role for B cells (that of activating CD4+ T cells) in host defenses against Pc infections.

Pc presence in the lungs of a host elicits secretion of a cascade of cytokines and chemokines, several of which have already been discussed. Generally, the types of cytokines induced in response to Pc presence are proinflammatory. Heightened expression of IFN-γ, tumor necrosis factor (TNF)-α, IL-1, IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) have all been observed to be upregulated either in *in vitro* or *in vivo* studies upon introduction of Pc into the various models (51, 53, 153, 265, 355). The proinflammatory environment created by expression of these cytokines seems to serve a useful purpose as rodent studies have shown that upon depletion of some of these (TNF-α, IL-1 and GM-CSF), impaired clearance of Pc is the result (52, 53, 187, 265). In fact, in some cases augmenting the host's expression of some of these cytokines resulted in enhanced protection from or clearance of Pc without exacerbating inflammatory responses. Kolls et al. demonstrated that gene transfer by an IFN-γ-expressing adenovirus could protect CD4+ T cell-depleted mice from Pc infection (186).

Similarly, Mandujano and colleagues showed that subcutaneous administration of GM-CSF to CD4+ T cell-depleted/Pc-infected mice resulted in decreased Pc burden (221). Interestingly, these same two cytokines were also observed to dampen overly exuberant inflammatory responses. Neutralization of IFN-γ in the context of a Pc infection led to exacerbation of inflammatory responses wherein infiltrates consisting of multinucleated giant cell, neutrophils and eosinophils were observed in the lungs of IFN-γ-depleted mice compared to controls (104). GM-CSF neutralization led to a similar outcome in Pc-infected GM-CSF KO mice that exhibited increased lung infiltration of macrophages, neutrophils and lymphocytes (265). In light of the proinflammatory response induced by Pc, investigators have examined the effects of an anti-inflammatory cytokine, IL-10, in the context of a Pc infection. For instance, pre-treating CD4+ T cell-depleted mice with an IL-10-expressing adenovirus vector did not increase Pc clearance rates but did reduce associated inflammation (295). In another study involving IL-10-deficient mice, it was observed these mice were able to clear Pc infection more rapidly than wild type mice, which correlated with increased CD4+ and CD8+ T cell responses and earlier influx of neutrophils into the lungs (284). However, when CD4+ T cells were depleted in these mice, they no longer exhibited enhanced Pc clearance. In humans, when a limited panel of proinflammatory cytokine levels were assessed in BAL fluid from HIV+ patients with acute PcP, only IL-1 showed up as significantly increased in these subjects compared to HIV+ subjects who were asymptomatic (271). This same group of investigators also looked at proinflammatory cytokines in the BAL fluid of patients with acute PcP but who were HIV-. In these subjects, TNF-α was significantly elevated compared to healthy control subjects (272). With respect to studies in humans, it has been observed that HIV infection of macrophages results in the inability to secrete

either TNF-α or IL-1 suggesting a possible mechanism that could explain the increased susceptibility of the HIV+ population to Pc infections (172).

1.6 RELATIONSHIP BETWEEN *PNEUMOCYSTIS* AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE

1.6.1 COPD

The GOLD (Global Initiative for Chronic Obstructive Lung Disease) Global Strategy has formally defined chronic obstructive pulmonary disease (COPD) as a typically progressive disease state characterized by poorly reversible airflow that is accompanied by an abnormal inflammatory response in the lungs (www.goldcopd.com/workshop/index/htmt). COPD includes the two main sub-phenotypes of chronic bronchitis and emphysema and the lesser recognized sub-phenotype, obstructive bronchiolitis. Chronic bronchitis is characterized by clinical symptoms that include persistent cough and phlegm production over a three month period recurring in at least two consecutive years and cannot be attributed to another condition (3). Emphysema is defined anatomically as permanent enlargement of airspaces distal to the terminal bronchioles and is associated with destruction of alveolar walls (3). Other pathological hallmarks of emphysema are: increased lung compliance, and loss of alveolar-capillary units due to destruction of parenchymal tissue. Obstructive bronchiolitis is typically recognized clinically as chronic bronchitis; however, examination of surgically resected tissue reveals involvement of small and peripheral pulmonary airways (< 2 cm in diameter) that are collapsed and often contain mucosal and inflammatory exudates (350). COPD has become a global concern as it is

predicted to become the third leading cause of death worldwide by the year 2020 (251). The morbidity and mortality associated with COPD are tremendous. Approximately 2.7 million people died of the disease worldwide in the year 2000 (268). Furthermore, according to statistics from the year 2000 in the United States alone, an estimated 24 million adults have evidence of impaired lung function and COPD was responsible for 8 million physician office and hospital outpatient visits, 1.5 million emergency room visits, 726,000 hospitalizations and 119,000 deaths (222). Despite the relative ease of diagnosis, little progress has been made toward stemming this enormous health burden as current knowledge of the disease does not provide for a curative treatment. Moreover, although smoking is known to be the primary risk factor for development of COPD, only about 15% of smokers develop the disease prompting questions as to the actual cause of the disease (93). Therefore, roles played by genetic and environmental factors, such as infectious agents, must be considered in disease pathogenesis.

1.6.2 HIV and COPD

HIV infection has historically been associated with pulmonary complications. Although widespread use of ART and PcP prophylaxis in HIV+ patients has resulted in greatly decreased rates of infectious pathogen-associated pulmonary problems, it has been reported that respiratory symptoms such as cough, phlegm production, dyspnea, and wheezing remain prominent in these individuals compared to control subjects (83). In addition, evidence shows that HIV+ individuals are at increased risk of developing COPD earlier and at a greater frequency than the HIV- population (66, 79, 81). Diaz *et al*. reported on a small group of HIV+ patients aged 32-55 years that exhibited radiographic evidence of emphysema and pulmonary function test data revealing prominent air trapping, hyperinflation and decreased carbon monoxide diffusing

capacities (Dlco) (79). Since the abnormal pulmonary function results were not proportional to the amount of reported tobacco use, it was concluded that HIV infection led to heightened susceptibility to cigarette smoke damage. The observation of reduced Dlco in this study is supported by other reports of HIV+ individuals being at increased risk of having impaired Dlco, even in the absence of overt pulmonary disease (82, 99, 254). This may be due to reduction in overall capillary volume in the lungs which, in turn, can lead to parenchymal lung destruction (82, 309). This phenomenon may provide some explanation for studies that have drawn correlations between HIV infection and increased risk for COPD development even when controlling for tobacco use and adjusting for age, pack years of smoking, IV drug use and alcohol abuse (66, 81).

1.6.3 Infections and COPD

Multiple studies have reported on the potential involvement of infectious agents in COPD development (33, 144, 224, 318). In support of pathogenic involvement in COPD pathogenesis are observations of bronchus-associated lymphoid tissue (BALT), small concentrations of lymphoid tissue typically associated with regional infections, in the lungs of smokers (32, 145, 289). These studies suggest the existence of a continuum wherein BALT is rarely observed in healthy non-smokers (145), is found in about 5% of smokers who have normal lung function (GOLD-0) and those with mild to moderate airway obstruction (GOLD-1 and GOLD-2, respectively) (32, 145, 289), and spikes in frequency (~27-33%) in severe and very severe airway obstruction (GOLD-3 and GOLD-4, respectively) (145).

In contrast to healthy airways which are sterile, a variety of pathogens including bacteria, atypical bacteria, viruses and fungi are often found in the airways of COPD patients (319).

Infectious agents can contribute to COPD pathogenesis in two different ways. In one hypothesis, they play a role in the development of COPD by upregulating inflammatory mediators in the lungs that act in concert with other insults, such as cigarette smoking, to promote lung pathology. A second hypothesis states that microbial pathogens cause acute symptoms associated with infections in the context of COPD known as exacerbations. These exacerbations are associated with vigorous host inflammatory responses that may be chronic and dysregulated resulting in significant structural lung damage and progression of airway obstruction. It is believed that the damage caused by these infections impairs the lungs' ability to clear infections making further infections and subsequent damage likely. This is known as the Vicious Circle Hypothesis of infection and inflammation in COPD (317).

1.6.3.1 Viruses and COPD

Multiple studies have shown associations between viral infections and COPD development or exacerbation. For example, adenovirus has been implicated in COPD pathogenesis in both an animal model (233) and in humans (288). In the former study, guinea pigs with a latent adenoviral infection that were exposed to cigarette smoke developed a more severe form of emphysema than those exposed to cigarette smoke alone, which correlated with a greater influx of inflammatory cells (233). In the human study, resected lung tissue from subjects was evaluated for inflammatory cell infiltrates and computed tomography (CT) scans were performed to assess extent of emphysematous damage. Absolute numbers of inflammatory cells in lung tissues correlated with number of alveolar epithelial cell expressing adenovirus E1A protein which, in turn, correlated with severity of emphysema suggesting that latent adenovirus infection amplifies inflammation associated with cigarette smoking which then accelerates emphysema pathogenesis (288).

Other viruses have been linked to exacerbations of COPD which is significant because exacerbations are associated with increased progression of COPD. These include rhinovirus, respiratory syncytial virus (RSV) influenza viruses A and B (311) and human metapneumovirus (226). Studies show that the exacerbations are typically associated with increased inflammation (227, 385).

1.6.3.2 Bacteria and COPD

Bacteria have also been implicated in COPD pathogenesis. Bacteria that have been identified in COPD patients include *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* (315). *Chlamydia spp.* has also been associated with development of airway obstruction in humans as well as calves (158, 320). Although bacteria were often detected by standard culturing techniques and during exacerbations in the human subjects, in many cases for both humans and the animal model, bacteria could only be detected by PCR on respiratory samples, thus emphasizing how subclinical lung infections can result in destructive inflammatory processes (158, 315). Bacterial colonization is typically associated with inflammation involving cellular infiltrates, cytokines, chemokines and various proteinases (158, 266, 339).

1.6.3.3 Parasites and COPD

Although much more limited in scope, parasites have also been linked to COPD development. One report showed that experimental infection of rodents with the hookworm *Nippostrongylus brasiliensis* led to emphysema that was accompanied by an increase in the lungs of the numbers of alternatively activated macrophages producing MMP-12 (224).

1.6.3.4 Pc and COPD

One of the earliest studies linking Pc to COPD is attributed to Calderon and colleagues who reported an infection frequency of about 10% in COPD patients (40). Since that time, as detection techniques have become more sensitive, Pc has been identified in a disproportionate number of COPD patients compared to patients with other types of lung disorders. For example, in both cystic fibrosis and lung cancer, a Pc colonization rate of 7% has been reported (279, 327). In contrast, 41% of COPD patients were reported to be colonized with Pc (279). Another study evaluating HIV-negative subjects admitted to the hospital for suspected bacterial pneumonia found that 4.4% of the patients were colonized with Pc (137). Of these Pc-colonized patients, 63% were determined to have COPD compared to 20% of non-colonized patients. In support of a higher Pc colonization frequency in COPD patients versus patients who have other types of severe lung diseases, Morris *et al*. compared subjects with severe COPD (GOLD stage IV) to other patients with severe lung diseases (control group), all of whom underwent lung transplants (245). Pc colonization rates of 37% and 9% were observed in the COPD and control groups, respectively. Moreover, they discovered that Pc occurrence was linked to COPD progression as severity of COPD was correlated with Pc colonization, with 37% of subjects with severe airway obstruction (GOLD stage IV) colonized with Pc compared to only 5.3% of subjects with less severe obstruction (GOLD stages 0-III). Statistical analyses ruled out a number of clinical variables such as age, immunosuppressive therapy, use of TMP-SMX, other comorbid conditions and even smoking history as Pc colonization risk factors.

As previously discussed, the HIV+ population is at increased risk for development of COPD (66, 80). It is possible that latent infections might be involved in the pathogenesis of COPD given the immunocompromised state of many of these individuals. Morris and colleagues

investigated whether Pc might play this role and found that in otherwise healthy HIV+ individuals with no acute respiratory symptoms, subjects who were colonized with Pc were significantly more likely to have clinical airway obstruction, independent of smoking history (241).

Other evidence in support of an association between Pc and COPD is found in a report by Morris *et al*. wherein an undetectable or low antibody titer against Pc was found to be an independent predictor of more severe airway obstruction (244). This finding indicates a potential role for Pc colonization in COPD progression and suggests that anti-Pc antibodies may be important for protection against colonization.

Finally, striking similarities in host responses to COPD and Pc infection have been reported. In particular, lung inflammatory responses characterized by marked elevations in CD8+ T cells, macrophages and neutrophils that are commonly observed in COPD (77, 178, 303, 346) have also been observed during acute PcP in humans (300, 330), as well as, rodent models (22, 206, 349). While these observations apply to acute PcP infections, similar findings have been reported in nonhuman primate models of AIDS in which animals are inoculated with Pc (29, 267).

In addition to the similarities between the inflammatory responses, development of PcP in humans has also been shown to result in COPD-like changes in pulmonary function. For example, one small study of 10 HIV+ patients with acute PcP found a high incidence of small airways dysfunction characterized by significant decreases in maximum midexpiratory flow (MMEF), a commonly used measurement of COPD (92). Another study examining 169 HIV+ patients with acute PcP in addition to a variety of other AIDS-related diseases including AIDS patients who had resolved their PcP infections found that Dlco, forced expiratory volume in one

second (FEV_1) and peak expiratory flow (PEF), which are all measurements of COPD, were significantly reduced in patients with acute PCP and those who had resolved the disease (323). Moreover, these declines in pulmonary function appear to be permanent as revealed in a study by Morris *et al*. that monitored pulmonary function in 1149 HIV+ subjects for a median of four years (248). They found that declines in pulmonary function persisted for years after resolution of acute PcP and that the declines were indistinguishable from those associated with COPD.

Proposed mechanisms of lung damage for the two diseases also show similarities. The prevailing theory of COPD pathogenesis is that damage to lung parenchyma is the result of excessive proteolytic activity due to an imbalance of proteases and anti-proteases in the lungs (357). Evidence of excessive proteolytic activity has also been observed in PcP. In one report, detection of cysteine protease (cathepsin) enzymatic activity was evaluated in lung homegenates from rats that developed PcP resulting from steroid-mediated immunosuppression (136). In this study, it was found that Pc infection resulted in increased cathepsin B, H and L activity. Others have found that levels of matrix metalloproteinase- (MMP) 2 and/or 9 activity, both of which are type IV collagenases that have elastinolytic activity, were increased in response to Pc infections in immunosuppressed rats and correlated with lung injury and/or pulmonary inflammation (282, 345). In addition to these host-derived sources of proteases, Pc itself can express proteases capable of degrading components of host extracellular matrix (ECM) (12). Furthermore, Pc also expresses kexin, a serine protease with undefined function in the organism, which may also play a role in lung tissue destruction (191, 213).

Although these studies do not prove the existence of a causal relationship between Pc colonization and COPD development, the strong association warrants further investigation.

1.7 COPD PATHOGENESIS

It is likely that multiple pathogenic mechanisms contribute to the development of COPD. This section outlines the current prevailing schools of thought on the basis for disease development.

1.7.1 Inflammation

Since it is generally accepted that smoking is the main risk factor for COPD development and it is known that all smokers have some evidence of lung inflammation (255), it is believed that an amplified inflammatory response is largely responsible for inducing disease in the minority of smokers who advance to serious COPD.

Inflammation in COPD is characterized by an influx of several cell types that progresses as airway obstruction worsens. For example, macrophages, which are the most abundant cells found in the lungs of COPD patients as well as healthy individuals, are increased in the bronchioles and the alveoli of COPD patients (89, 263). Numbers of macrophages have been correlated with severity of airway obstruction (77). Additionally, a positive association has been observed between the numbers of macrophages in the alveolar walls and the presence of emphysema (89). The primary mechanism by which macrophages are believed to cause lung damage is through expression of proteases that can break down collagen and elastin, major components of the ECM. Studies using macrophages from COPD patients have shown increased production of these enzymes compared to macrophages from control subjects (90, 264), and animal studies support their role in COPD development (135, 313). Macrophages can also express several other mediators such as cytokines, chemokines and ROS which all have the potential to contribute to COPD pathogenesis.

Neutrophils are also thought to play a significant role in COPD development. The reason for this is that studies have shown correlations between extent of airway obstruction and numbers of neutrophils in bronchial tissue (77), sputum (270, 333) and airways (61, 360). Furthermore, studies have demonstrated that many of the enzymes secreted by neutrophils can produce the type of tissue damage encountered in smoking-related COPD (173, 204, 211).

Evidence of mast cell involvement in COPD pathogenesis has also been suggested in studies showing increased mast cell numbers in the airway walls of COPD patients (123). In addition, mast cells have also been implicated in a less direct manner in COPD development through the finding of elevated mast cell mediators in the airways of smokers (168). The precise manner in which they contribute to COPD is unknown. However, they can express many potential mediators including a number of proteases that can degrade lung tissue directly, several different cytokines, and chemokines which could possibly recruit other inflammatory cells such as neutrophils (18).

A role for eosinophils in COPD pathogenesis has also been suggested as multiple reports have identified these cells in various respiratory specimens of COPD patients (192, 298). While eosinophils are not known for eliciting tissue damage, their secondary granules store proteins that are toxic to bronchial epithelial cells such as eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophilic-derived neurotoxin and they are able to secrete a diverse range of cytokines (304).

In healthy individuals, very few lymphocytes are typically recovered from the airways (7). However, in COPD, lymphocytes, especially CD8+ T cells, accumulate in the airways (263), alveolar structures (301), pulmonary vessels (301) and the lymph nodes (302). Production of mediators such as perforin, granzyme B or cytokines by these cells could provide potential

mechanisms for disease development, but it is not known if any or all of these play a role. Although the mechanism through which they cause tissue damage is not understood, their numbers in the lungs have been directly correlated to degree of airflow limitation (89, 263, 288, 303).

CD4+ T cells typically infiltrate the lungs less extensively than CD8+ T cells in COPD. Nevertheless, increased numbers have been observed in both the air spaces and the parenchymal walls of the lungs of COPD patients (219, 288). CD4+ T cells are largely responsible, through secretion of an array of cytokines, for orchestrating and amplifying inflammatory responses of both adaptive and innate effector cells. For example, they are critical in priming CD8+ T cells for cytotoxic activity as well as maintaining memory CD8+ T cells. They also play a significant role in activating macrophages. Hence, their activity can be indirectly linked to tissue damage via their effects on other effector cells, and directly by the release of damaging cytokines.

There are dozens of soluble inflammatory mediators that have been implicated in COPD pathogenesis. Chemokines act to recruit inflammatory cells from the circulation into the lungs, proinflammatory cytokines amplify and perpetuate inflammation, T cell-secreted lymphokines are responsible for determining the pattern of inflammation, and growth factors maintain inflammation that leads to remodeling of lung tissue.

Though many chemokines have been identified as being elevated in various respiratory specimens from COPD patients, one that is consistently observed is CCL2 which has been found in sputum and BAL fluid (70, 361). This chemokine is a potent chemoattractant of monocytes which may, in part, explain the accumulation of macrophages in the lungs of COPD patients. Other chemokines that can also recruit macrophages, as well as T cells and eosinophils, and are frequently observed in COPD patients, are CCL3, CCL4 and CCL5 (43, 63, 98). These

chemokines have been particularly associated with exacerbations in COPD patients (389). Elevated IL-8, which is highly chemotactic for neutrophils, has also been commonly observed in COPD patients and seems to be especially important as it can serve as a marker of disease severity (96, 178, 380). Another group of chemokines receiving increased attention recently include CXCL9, CXCL10 and CXCL11, all of which are induced by IFN-γ and act to recruit T helper type 1 (Th1) cells. All of these chemokines appear to play important roles in COPD as they have been found to be elevated in sputum samples of COPD patients and correlated with disease severity (63).

Increased proinflammatory cytokine levels are also frequently observed in COPD and appear to play important roles in disease development. These cytokines act through activation of the transcription factor, nuclear factor (NF)-κB, leading to increased expression of inflammatory genes and subsequent amplification of inflammation. TNF-α, which has been reported to be elevated in sputum from COPD patients, especially during exacerbations (5, 178), is one example. When overexpressed in mice, TNF-α, which induces expression of IL-8 and MMPs, leads to classic pathologic features of emphysema (100). Additionally, another study demonstrated that TNF-α receptor knockout mice exposed to cigarette smoke developed a less severe form of emphysema than control mice (59). IL-1β, which is functionally similar to TNF-α, elicits production of multiple proinflammatory mediators including IL-2, IL-6, IL-8, CCL5, GM-CSF, IFN-γ and TNF-α (56). This cytokine can also stimulate elastolytic activity, especially that of MMP-9 (162, 203), which may be important in emphysema development. Similar to TNF-α, increased levels of it can be found in sputum from COPD patients and has been correlated with disease severity (307). IL-6, which has been found to be increased in sputum and BAL fluid from COPD patients (28, 331), is another cytokine with a wide array of

proinflammatory effects and likely plays a role in COPD development which, to date, is still unclear. Two other lesser studied cytokines have also been associated with COPD pathogenesis. Recently described, IL-32, was shown to be increased in expression by epithelial cells, macrophages and CD8+ T cells of COPD patients which was correlated with disease severity (37). Thymic stromal lymphopoietin (TSLP) is a member of the IL-7 family of cytokines and has also been shown to be elevated in the airway epithelium of COPD patients (381). This cytokine plays a dual role of mediating both Th1 and Th2 cells, albeit by different mechanisms (209).

Most literature describing cytokine secretion patterns in COPD indicate a predominantly Th1-skewed response (141, 220). However, Th2-biased responses have also been described in COPD (16, 17). The disparities among these studies may be due to heterogeneity in clinical samples arising from differences in disease severity. Given the complexity of COPD, it seems unlikely that a single pattern of cytokine secretion is involved but, rather, an overlap between Th1 and Th2 cytokines.

IFN-γ, a key Th1 cytokine that is a potent stimulator of macrophages, has been frequently implicated in COPD development. It is believed to play a major role in COPD development due to reports of increased expression of IFN-γ in T cells isolated from emphysematous lung tissue (127), BAL fluid (141) and peripheral blood (220), as well as, higher numbers of IFN-γ-secreting CD8+ T cells in the sputum of COPD patients (365). The importance of IFN-γ in COPD indicated by these clinical reports is supported by animal studies. For example, overexpression of IFN-γ in the lungs of mice was shown to lead to emphysema (375).

Th2 cytokines are receiving increased attention by investigators because of reports showing elevated levels of Th2 cytokines in COPD. IL-4, the archetypal Th2 cytokine, has been

shown to be increased in cytotoxic T (Tc) type 2 (Tc2) cells recovered from BAL fluid of patients with COPD (17). Similarly, Barceló *et al*. also observed a higher frequency of IL-4-secreting Tc2 cells in BAL fluid of COPD patients in addition to Tc2 cells secreting IL-13, another Th2 cytokine, both of which inversely correlated to degree of airflow obstruction (16). Plasma levels of IL-13 have also been shown to be inversely related to airway obstruction (202). Recent evidence gleaned from animal models also supports a role for IL-13 in COPD development. Zheng and colleagues demonstrated development of MMP- and cathepsin-dependent emphysema in the lungs of mice where IL-13 was overexpressed (387). IL-13 was also associated with development of emphysema in a parasite infection mouse model (*Nippostrongylus brasiliensis*) in which alternatively activated macrophages (AAM) secreting MMP-12 appeared to play a role in disease pathogenesis (224), and in a virus infection mouse model (Sendai virus) in which invariant natural killer T (NKT) cells induce macrophages to secrete IL-13 consequently driving disease development.

Another proinflammatory cytokine that has been associated with COPD is IL-18. Increases in IL-18 that correlate with disease severity have been observed in alveolar macrophages and CD8+ T cells from the airways(151), as well as, sputum (294) from COPD patients. In an animal model supporting these data, overproduction of IL-18 in the lungs of a mice mediated increased production of IL-13 and inflammatory cell influx into the lungs ultimately leading to development of emphysema (146).

A subset of CD4+ T cells that has been receiving increased attention in recent COPD research are the Th17 cells which play an important role in inflammatory processes. Their role and regulation in COPD is not well understood, but IL-17A, the predominant product of these cells, has been found to be elevated in sputum from COPD patients (362). Furthermore, IL-17-

expressing cells, as well as cells expressing IL-22 and IL-23 (other members of the IL-17 family of cytokines) have been identified in bronchial biopsies from COPD patients (78).

Many of the cytokines implicated in COPD pathogenesis act as growth factors that can support differentiation and survival of inflammatory cells, or promote airway remodeling through activation and proliferation of structural cells. One such cytokine is GM-CSF which governs these functions for neutrophils, macrophages and eosinophils. In COPD patients, elevated levels of GM-CSF that coincided with increased numbers of neutrophils have been observed in BAL fluid, particularly during exacerbations (14) and sputum (305).

Transforming growth factor (TGF)-β is a fibrogenic growth factor (188) that has been linked to COPD. TGF-β can induce proliferation of airway smooth muscle cells and fibroblasts in addition to stimulation of ECM deposition and epithelial repair. Its immunological role is usually that of immune regulation via the activity of regulatory T cells which results in the suppression of Th1, Th2 and Th17 cells. Increases observed in this growth factor in airway epithelial cells and macrophages of COPD patients (71, 354) may reflect a compensatory mechanism to inflammation associated with the disease. Perhaps even more relevant to disease development are the fibrotic effects caused by TGF-β which are mediated by increases in connective tissue growth factor (CTGF) which has been shown to be upregulated in microarray analyses of COPD patient lungs (256). Animal models showing this type of COPD development do not currently exist; however, Morris *et al*. demonstrated development of MMP-12-mediated emphysema in mice lacking the αvβ6 integrin which activates latent TGF-β (249).

Epidermal growth factor (EGF) is another fibrogenic growth factor (190), perhaps to a lesser extent than TGF-β, that has been found to be increased in the airway epithelium of COPD patients (368). It has been speculated to play a role in the pathophysiological mucous plugging

of small airways aspect of COPD development by its ability to induce mucin expression (353). The importance of this is supported by findings of Hogg *et al*. who showed that degree of airway obstruction was associated with the extent of small airways plugging by mucous (145). It has also been hypothesized that COPD pathogenesis might also occur through the activity of neutrophils recruited in response to increased IL-8 expression induced by EGF (343).

Another growth factor implicated in COPD development is vascular endothelial growth factor (VEGF). This growth factor is necessary for growth of new vessels and regulation of vascular leakage. Containing the highest concentration of all body tissues (25, 239), the lungs require VEGF to maintain homeostasis through enhanced cell proliferation (34). Therefore, it is significant that reduced levels of VEGF have been observed in the airways (347) and sputum (170), where levels were inversely correlated to extent of airway obstruction, of emphysema patients. In support of these clinical data, it has been shown that blockage of VEGF uptake in a rat model led to enlargement of airspaces indicative of emphysema (177). Interestingly, in contrast to emphysema patients, increased levels of VEGF, that inversely correlated to severity of airway obstruction, have been observed in sputum from chronic bronchitis patients (170).

1.7.2 Protease-Antiprotease Imbalance

The prevailing theory for emphysema development since the early 1960s is the protease-antiprotease imbalance theory. This hypothesis states that the proteolytic balance maintained in healthy lungs shifts towards a proteolytic phenotype when an excess of protease activity prevails due to an imbalance between local proteases and their respective antiproteases resulting in the breakdown of connective tissue components in the lung parenchyma. This theory first took hold when two discoveries about a specific enzyme inhibitor were made. The first is description of

the finding that the band for the protein, α1-antitrypsin, an inhibitor of the elastin degrading serine proteases secreted by neutrophils (neutrophil elastase (NE), cathepsin G and Proteinase 3 (Pr3)) was missing from a protein electrophoresis of serum from a patient in a respiratory hospital (198). The second discovery is the observation that patients deficient for this protein developed early onset emphysema (87). The still young protease-antiprotease hypothesis was subsequently reinforced by the use of experimental animal models wherein proteases such as the cysteine protease, papain (126), porcine pancreatic elastase (161) and NE (175) were instilled into the lungs resulting in emphysema-like disease. Since then, focus has shifted from NE being the sole mediator of emphysema when Janoff and colleagues pointed out that approximately 50% of the elastase activity in the lungs of smokers was actually from MMPs (160). This was supported by the finding that numbers of macrophages, which can secrete many MMPs, correlated with severity of emphysema (86). Further work in this arena has introduced the concept that enzymes other than elastases can participate in the connective tissue destruction of the lung parenchyma. Indeed, mounting evidence indicates that it is unlikely that a single protease, or even a single type of protease, is responsible for the matrix destruction in emphysema.

As mentioned, the original formulation of the protease-antiprotease hypothesis involved only the neutrophil-secreted serine proteases, particularly NE, as mediators of emphysema. This is because of correlations between extent of airway obstruction and neutrophil numbers in airways (360), sputum (333) and bronchial tissue (77), as well as studies demonstrating emphysema development in the lungs of animals instilled with human NE (211), cathepsin G (211) or Pr3 (173). However, because α1-antitrypsin therapy clinical trials have failed to provide protection against development of emphysema in humans deficient for this protein (338), and

because some investigators have reported the absence of a correlation between neutrophil numbers and severity of lung destruction (86, 89), the role for neutrophils and the serine proteases is currently in question.

Lysosomal cysteine proteases (cathepsins) are another type of protease that has been implicated in COPD pathogenesis. Although they are normally restricted to intracellular activity, they have the ability to degrade several ECM components including elastin and collagen. Macrophages (281), mast cells (378), smooth muscle cells (344) and fibroblasts (363) have all been observed to release cathepsins. Since the report showing that intratracheal instillation of cathepsin B in hamsters led to emphysema-like disease (204), other studies have been performed to elucidate the role of these enzymes in COPD development. For example, studies have shown that cathepsin L is significantly increased in alveolar macrophages and BAL fluid of smokers (351, 352). Furthermore, macrophages incubated with BAL fluid from COPD patients showed increased expression of cathepsin S (108). While these clinical studies hint at the importance of cathepsins in COPD development, seminal studies that clearly demonstrate their importance in disease development were performed in transgenic mice that employed inducible lung expression of either IFN-γ (375) or IL-13 (387) expression. In both of these studies, overexpression of the cytokines in the lungs led to emphysema-like disease that was mediated in part by multiple cathepsins which was demonstrated by administration of a cysteine protease inhibitor (E64) that significantly attenuated emphysematous changes (387).

A great deal of research investigating a proteolytic cause of emphysema has focused on the abnormal expression of MMPs, a family of 24 matrix-degrading enzymes required for development, tissue remodeling and repair. The MMPs of particular interest in COPD pathogenesis can be simplistically grouped, by substrate specificity, into the collagenases (MMP-

1 and -8 which cleave collagen types I, II and III), the stromelysins (MMP-3, -10 and -11 which are specific for laminin), the gelatinases (MMP-2 and -9 which most efficiently cleave type IV collagen but can also cleave elastin fibers and gelatin) and the elastases (MMP-7 and -12 which are specific for elastin). It should be noted that all of these MMPs can cleave substrates other than those listed but those that are listed are the ones for which they appear to be most specific.

Evidence pointing to a role for MMPs in COPD development includes observations of increased levels of collagenases in the BAL fluid of emphysema patients (91). Although this study did not identify the precise source of the collagenase activity in the BAL fluid, they speculated that it was from MMP-8 based on the rate of activity they observed. Using lung tissue specimens to quantify RNA expression and detect gene expression via *in situ* hybridization (ISH), Imai *el al*. also observed an increase of collagenase that was localized to type II pneumocytes in emphysema patients (149). In this case, the collagenase was identified as MMP-1. Other groups have also examined RNA expression in lung tissue of COPD patients to identify increased expression of gelatinases (MMP-2 and -9), as well as, collagenases (MMP-1 and -8) (264, 312). Both of these studies confirmed their RNA findings by evaluating enzymatic activity of respiratory specimens in zymographic assays. Due to their tremendous proteolytic potential over a broad range of substrates, considerable attention has centered on the role of macrophages in COPD development. Multiple studies have shown that macrophages from COPD patients upregulate MMP-1 and/or MMP-9 (90, 296), thus, enhancing elastolytic activity in the lungs of these patients (90, 297). Because of the vital nature of elastin in the lungs (125, 334), of particular interest among the MMPs is MMP-12 due to its potent elastolytic properties. Increased levels of MMP-12 have been found in sputum (75) as well as BAL fluid, BAL cells and tissue biopsies (238) of COPD patients. In a two-pronged study, Qu and colleagues showed

that MMP-12 mRNA expression was upregulated in biopsy tissue obtained from human cancerous and emphysematous lungs corroborating data generated in a murine study wherein overexpression of MMP-12 in mouse lungs resulted in development of cancer and emphysema (283). Underscoring a critical role for MMP-12 is the finding that an allele of the human MMP-12 gene containing single nucleotide polymorphism in the promoter was associated with reduced risk of COPD development in smokers (148).

Animal studies have given weight to these findings in humans. For example, mouse models have shed light on the possible mediators of MMP expression. One study found that overexpression of IL-1β in the lungs of mice led to emphysema mediated by inflammatory cell infiltrates and increased expression of MMP-9 and 12 (193). Prause and colleagues showed that intranasal instillation of IL-17 resulted in elevated MMP-9 that coincided with increased numbers of neutrophils in the BAL fluid of mice (278). Highlighting a potentially pivotal role of IL-13, Zheng *et al*. showed that overexpression of this cytokine in the lungs of mice led to enhanced inflammation and induction of MMP-2, -9, 12, 13 and -14 resulting in emphysema development (387). Inhibition of these MMPs resulted in reduced emphysema demonstrating that these enzymes, at least in part, mediate disease development. In a similar fashion, Wang *et al*. demonstrated that overexpression of IFN-γ in mouse lungs resulted in emphysema mediated in part by MMP-9 and -12 (375). In yet another transgenic mouse model in which lung macrophages expressed human MMP-9, adult mice developed emphysema characterized by significant airspace enlargement and decreased alveolar wall elastin (95). The importance of MMP-12 was clearly demonstrated in MMP-12 knockout mice that were exposed to cigarette smoke (135). While their wild type littermates developed emphysema that coincided with macrophage influx into the lungs, the knockout mice did not develop any signs of the disease nor

did they have increased numbers of macrophages in their lungs. Furthermore, instillation of CCL2 into the lungs of the knockout mice led to heightened levels of macrophages in their lungs, but they still did not develop disease demonstrating that MMP-12 is sufficient to induce smoking-induced emphysema.

As the overwhelming majority of studies on protease-antiprotease imbalance in COPD pathogenesis have focused on the protease side of the balance, there is very little literature on antiprotease levels in the disease. What is known is that all of these proteases are typically counteracted by an excess of antiproteases. The serine proteases are kept in check primarily by α1-antitrypsin in the lung parenchyma and secretory leukocyte protease inhibitor (SLPI) in the airways, both of which have been shown to ameliorate neutrophil-induced emphysema in an animal model (212, 340). Furthermore, in their mouse model of emphysema wherein induction of disease was effected by overexpression of IFN-γ in the lungs, Wang and colleagues observed that SLPI was downregulated (375). In addition, α1-antitrypsin was found to be downregulated in an IL-13 induction of emphysema mouse model (387). Four tissue inhibitors of MMPs (TIMP-1, -2, -3 and -4) along with α-macroglobulins counteract the activities of the MMPs. Levels of TIMP-1, which is known to inhibit MMP-9 activity, have been observed to be decreased in the plasma (321) and sputum (232) of COPD patients. Furthermore, TIMP-1 expression by alveolar macrophages obtained from BAL of COPD patients has been reported to be reduced compared to controls (275). Sputum levels of TIMP-2, the inhibitor of MMP-2, from COPD patients has been negatively correlated with airway obstruction (390). Additionally, Hirano *et al*. observed an association between COPD development and polymorphisms in the TIMP-2 sequence (140). In smoke exposed mice that developed emphysema,

immunohistochemical staining of lung tissue demonstrated that TIMP-2 levels were significantly lower than in control mice (366).

1.7.3 Oxidative stress

Smoking is the leading risk factor for development of COPD which largely explains why oxidative stress is thought to play an important role in disease pathogenesis as the chemical make-up of cigarette smoke contains more than 4700 compounds, including high concentrations of free radicals and oxidants (58). For this reason, an oxidant-antioxidant hypothesis, analogous to the protease-antiprotease hypothesis, for COPD development has been proposed in which oxidative stress caused by an imbalance of oxidants and antioxidants in favor of oxidants results in lung injury (217). The importance of this theory to COPD development lies in the potential of the oxidants to oxidize proteins, DNA or lipids which can directly result in lung injury or induce a variety of signaling cascades that may initiate the cell death process (130). In addition to cigarette smoke, there are also multiple cellular sources of oxidants. Therefore, since the work being expounded in this document centers on pathogenically-induced development of COPD, this section will focus on cell-derived oxidants.

As previously discussed, inflammation is a common feature of COPD characterized by recruitment and activation of a variety of different immune cell types. Immune cell activation can lead to secretion of proinflammatory cytokines that, in turn, can induce both phagocytic and nonphagocytic cells to generate reactive nitrogen species (RNS) and reactive oxygen species (ROS). For example, IFN-γ, TNF-α, GM-CSF, IL-1 and IL-6 can all induce macrophage expression of the RNS, nitric oxide (NO), by either the nitric oxide synthase 2 (NOS2) gene or the inducible nitric oxide synthase (iNOS) gene (76, 216). Additionally, IL-17 has also been

shown to stimulate NO production via the iNOS pathway by a number of structural cells, but not by macrophages. Interestingly, in addition to the proinflammatory IFN-γ, IL-4 has been reported to stimulate production of NO via the iNOS gene in airway epithelial cells (129). These data support observations of increased NO in the exhaled breath condensate of COPD patients (62, 230). Proinflammatory cytokines, especially IFN-γ and TNF-α (122), also mediate expression of ROS such as the superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2) in processes that include mitochondrial respiration, the xanthine/xanthine oxidase (X/XO) reaction and the respiratory or oxidative burst, which is the major ROS generating system. The respiratory burst is most commonly associated with immune cells such as neutrophils, monocytes and macrophages, but airway epithelial cells are also known to generate ROS via the respiratory burst (292). In COPD patients, particularly during exacerbations, increased ROS release by neutrophils in peripheral blood has been reported (250). In addition, H_2O_2 levels in breath condensate of COPD patients was found to be higher than in normal subjects which, again, was especially true during exacerbation episodes (260, 261).

While inflammatory processes can induce expression of oxidants, these resulting oxidants can, in turn, elicit further inflammation resulting in a vicious circle type of amplification that can result in tissue destruction. For example, oxidants can initiate signal transduction pathways via the transcription factor NF-κB (310). Once activated, this molecule is known to induce expression of several proinflammatory cytokines including IL-2 (138), IL-6 (13, 15), IL-8 (337), GM-CSF (13, 15), IFN-γ (185) and TNF-α (322). Oxidants can also amplify inflammation by inactivation of histone deacetylase 2 (HDAC2) which suppresses gene transcription of inflammatory genes by removing acetyl groups from histones, thus ensuring that chromatin remains tightly wound around histones (154-156).

1.7.4 Apoptosis

One other mechanism that may be at play in COPD pathogenesis is apoptosis. Similar to the previous "imbalance" models, it is believed by some that the balance between apoptosis taking place in the lungs and a reciprocal increase in proliferation of cells to replace the dying cells is shifted toward an excessive apoptosis phenotype. Reports of elevated apoptosis in the alveolar walls of the lungs of patients with emphysema (38, 142, 176) lend credibility to this theory. These findings led researchers to revisit a theory posited by Liebow stemming from his observations that alveolar septa in lung tissue from emphysema patients were extremely thin and almost avascular. Consequently, he hypothesized that alveolar septa disappearance characteristic of the disease may be the result of decreased blood supply to the small precapillary blood vessels (205). This, in turn, prompted researchers to speculate that VEGF, a potent angiogenic factor that promotes endothelial cell proliferation and vessel formation (88), might play a role in emphysema development. One reason is that the lungs contain the highest concentrations of VEGF of all of the body tissues (25, 239). Furthermore, both *in vitro* (109, 110) and *in vivo* (8) studies have shown that withdrawal of VEGF results in endothelial cell apoptosis. Assessments of VEGF levels in COPD patients have revealed decreased expression of VEGF and its receptors in lung tissue (176) and airway tissue (347). In a more comprehensive study evaluating the pathobiological link between oxidative stress and VEGF levels, severity of COPD correlated with NO levels and inversely correlated with VEGF levels in the sputum of COPD patients (171).

Animal studies largely support the findings in human disease. For example, Aoshiba and colleagues demonstrated that introduction of active caspase-3, an important mediator of cellular apoptosis, into the lungs of mice resulted in emphysema without excessive inflammation (10),

thus providing direct evidence that apoptosis of alveolar walls leads to emphysematous changes. Moreover, Suzuki *et al*. reported that VEGF levels were decreased in the airways of mice with smoke-induced emphysema (347), and Kasahara *et al*. showed in rats that blocking the effects of VEGF with an inhibitor of the VEGF receptor kinase leads to emphysema that developed in the absence of an inflammatory response (177). Similar development of emphysema without inflammation that was accompanied by upregulation of caspase-3 was reported by Tang and colleagues in a mouse model of lung-targeted ablation of the VEGF gene (356). In another study investigating the connection between oxidative stress and apoptosis-mediated emphysema, researchers discovered that the emphysematous effects of VEGF receptor blockade could be prevented by administration of a superoxide dismutase (SOD) mimetic, an antioxidant that removes superoxide anions (364). Treatment with the SOD mimetic was characterized by increased septal cell proliferation and enhanced activation of the anti-apoptotic protein, Akt. They went on to further show that apoptotic areas in the lungs, where activated caspase-3 was detected, were co-localized to the areas of antioxidant stress.

1.7.5 Use of microarray for the study of COPD pathogenesis

In aggregate, the many mechanisms and mediators discussed highlight the fact that COPD pathogenesis is a highly complex process. Furthermore, it likely involves multiple mediators and mechanisms that work in tandem. Given this complexity, the variable susceptibility of the general population to disease development and the fact that patients typically are not diagnosed until the late stages of the disease when therapies may be most effective, it is important to be able to identify biomarkers and/or susceptibility and progression factors.

DNA microarray is one such technology that can be used for this purpose. For example, Spira *et al*. used microarrays in order to evaluate gene expression patterns associated with emphysema in the lung tissue of patients who had undergone lung reduction surgery (332). In a study utilizing a similar approach, Golpon and colleagues also employed microarrays to analyze severely emphysematous lung tissue samples from patients with "usual" emphysema and patients with α1-antitrypsin-related emphysema (121). Ning and coworkers used microarrays in combination with serial analysis of gene expression (SAGE) to compare gene expression patterns of at-risk control smokers and COPD patients with moderate airway obstruction (GOLD-2) and discovered previously unreported candidate genes that could serve as molecular targets of the disease (256). In another study, microarray technology was used to assess gene expression of "normal" lung tissue (grossly uninvolved with simultaneously resected nodular tissue suspected of being cancerous) removed from patients with varying degrees of airflow obstruction (27). Gene expression biomarkers unique to COPD were distinguished from which a subset was identified that the investigators then used to reliably predict (97% accuracy) occurrence of disease in a separate and distinct data set independently obtained from a different population of COPD patients. Hence, microarray technology represents a powerful tool that can be used in the study of COPD pathogenesis.

1.8 STUDY OF *PNEUMOCYSTIS*-RELATED COPD DEVELOPMENT

Because Pc cannot be cultured continuously *in vitro*, animal models are required for molecular characterizations of the organism, as well as, the study of host immune responses to it.

1.8.1 Rodent models for the study of Pc-host interactions

The principal animal models that have been most widely used, and continue to be used, in the study of Pc are rodent models in which sustained Pc infection is achieved in mice that have immune deficiencies such as SCID, nude or genetically altered mice, or it is facilitated by immunosuppression mediated by the use of corticosteroids or selective depletion of specific immune cells (73, 132).

While these models have provided a wealth of information about Pc-host interactions, there are limitations inherent in their use. For example, broad suppression of immune responses with corticosteroids may compromise study of the various host immune mechanisms that may be of interest in Pc infections. Use of models wherein antibodies are employed to deplete CD4+ T cells goes to the opposite extreme of corticosteroid use. In targeting only the CD4+ T cells to mimic the immune deficiency of AIDS, the constellation of immune dysfunctions that accompanies the disease is circumvented. These dysfunctions may be relevant to the study of host responses to Pc in the context of AIDS-associated immunosuppression. In addition, some experimental procedures such as BAL washes cannot be repeatedly performed in mice which limits serial analyses of cell populations, biochemical composition and organism burden in the lungs during the course of infection. Moreover, since Pc exhibits exquisite host specificity (112, 115, 116), translation of data gleaned from rodent models of Pc infection to clinical Pc infections may not be optimal.

1.8.2 Nonhuman primate models for the study of Pc-host interactions

As an alternative to rodent models for the study of Pc-host interactions, the Norris lab has developed two nonhuman primate models of Pc infection in the context of AIDS-like immunosuppression. Infection of monkeys with simian immunodeficiency virus (SIV) results in AIDS-like disease with development of opportunistic infections, including PcP (19). Studies in SIV-infected nonhuman primates that are co-infected with Pc revealed extensive CD8+ T cell and neutrophil infiltration (29, 65, 267), as well as, rises in IL-8 and TNF-α in the lungs of animals with Pc (267) mirroring clinical reports of Pc infection, thus making this a useful model in the study of AIDS-related Pc infections. Another finding was that many of these animals experience a protracted, asymptomatic colonization period before developing PcP (29, 267).

The second model utilizes simian/human immunodeficiency virus (SHIV) instead of SIV to induce immunosuppression. SHIV is a chimeric SIV that expresses HIV envelope and other accessory proteins from HIV such as *tat*, *rev* and *vpu* and, like SIV, CD4+ T cell lymphopenia accompanied by wasting and susceptibility to opportunistic infections develop in monkeys infected with this virus paralleling the virulence of an acute HIV infection (84, 286). The main advantage to using SHIV is that immunosuppression after infection occurs much more quickly (2-3 weeks) compared to SIV infection (6-12 months), thus inducing earlier susceptibility to Pc colonization or infection.

These studies support the use of nonhuman primate AIDS models as an alternative to rodent models for studying host immune responses to Pc. They have greater relevance to humans in that the immune deficits experienced by animals infected with SIV or SHIV that become immunosuppressed are similar to those that develop in HIV+ individuals who develop AIDS. In addition, Pc derived from monkey is evolutionarily closer to Pc derived from humans

compared to rodent-derived Pc making it more relevant for understanding Pc infections in humans (259). Another benefit is the opportunity provided by these models to study the effects of inflammatory cells and mediators during the colonization phase. Finally, these nonhuman primate models are particularly well suited to longitudinal studies because serial samples that are difficult to obtain from humans or rodents are easily harvested.

1.9 SUMMARY

Despite the availability of antiretroviral therapy and prophylactic drugs for Pc infections, as the cause of PcP, this fungal pathogen remains one of the most serious opportunistic infection in the HIV+ population resulting in extensive morbidity and mortality. Moreover, Pc infections and the associated health problems are common in non-HIV-infected populations such as transplant and cancer patients.

However, the extent of the health burden attributed to this pathogen may be grossly underestimated as colonization with Pc without any overt clinical symptoms, which appears to be highly prevalent in both HIV+ and HIV- populations, may act as a contributing factor in the pathogenesis of COPD, a far more pervasive disease that is a leading cause of death worldwide. Mounting evidence implicating Pc in COPD development is found in reports documenting high rates of emphysema and Pc colonization in HIV+ smokers, as well as, increased rates of Pc colonization in COPD patients that corresponds with severity of the disease. Furthermore, COPD-like changes that have been observed after resolution of PcP and the similarities in the host immune responses in COPD and Pc infection add further support to the existence of an

association between Pc colonization and COPD. Despite these many lines of evidence, a causal link has still not been demonstrated.

How COPD development occurs is poorly understood. Although there are multiple hypotheses for mechanisms of COPD pathogenesis, efforts to pinpoint which, if any, are correct have been hindered by the wide variability in severity and expression patterns of the disease. To this end, the simian model of Pc infection is an excellent tool in the study of Pc-host interactions. It is highly relevant because the species of Pc that infects nonhuman primates is closely related to the species that infects humans, and study of the effects of Pc colonization can be evaluated longitudinally in the context of AIDS-like immunosuppression. Furthermore, samples used for assessment of disease severity and progression are easily obtained throughout the duration of the disease.

In sum, use of the nonhuman primate model of Pc infection offers a unique opportunity to study acute and chronic effects of Pc colonization and elucidate possible mechanisms involved in HIV-related emphysema development. Information gained may be valuable in prevention and treatment of obstructive airway disease.

1.10 CENTRAL HYPOTHESIS AND SPECIFIC AIMS

1.10.1 Central Hypothesis

Chronic subclinical infection (colonization) with Pc in the context of HIV infection results in a persistent inflammatory response that contributes to the acceleration and progression of COPD.

1.10.2 Specific Aim 1. To test the hypothesis that Pc colonization in a primate model of AIDS leads to progressive airway obstruction and development of COPD.

We anticipated that animals will break out into SHIV-infected and SHIV-infected/Pc-colonized groups based on differences in baseline anti-Pc titers. We monitored obstruction by performing pulmonary function tests (PFTs) on SHIV-infected monkeys by whole body plethysmography to collect spirometric, lung volume and lung compliance measurements. The nature of the obstruction was characterized by correlating Pc colonization and decreased lung function to COPD-associated changes in lung tissue by performance of computed tomography (CT) scans and histologic morphometry.

1.10.3 Specific Aim 2. To identify key immune mediators of SHIV/Pc-associated obstructive lung disease.

RNA was isolated from necropsied lung tissue samples. Using microarray technology, gene expression profiles were developed that distinguish responses associated with SHIV infection/Pc-colonization and responses associated with SHIV infection only. These studies generated a comprehensive molecular network of the inflammatory pathways associated with the progression of COPD in AIDS and identify key candidates for therapeutic intervention.

1.10.4 Specific Aim 3. To test the effect of treatment with trimethoprim-sulfamethoxazole on progression of pulmonary function decline in SHIV-infected monkeys colonized with Pc.

As in specific aim 1, animal groups included SHIV-infected and SHIV-infected/Pc-colonized monkeys. After significant obstruction became apparent in Pc-colonized animals, this group was be divided into subgroups in which one received TMP-SMX to eradicate Pc colonization, and the other group did not receive any treatment and was allowed to continue the normal course of disease induced by Pc colonization. PFTs, CT scans and histologic morphometry were performed to determine if pulmonary function had been stabilized and that further obstruction had been arrested.

2.0 PERSISTENT *PNEUMOCYSTIS* COLONIZATION LEADS TO THE DEVELOPMENT OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) IN A NON-HUMAN PRIMATE MODEL OF AIDS

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2.1 ABSTRACT

HIV-infected patients are at increased risk for development of pulmonary complications, including chronic obstructive pulmonary disease (COPD). Inflammation associated with sub-clinical infection has been postulated to promote COPD. Persistence of *Pneumocystis* (Pc) is associated with HIV and COPD, although a causal relationship has not been established. We used a simian/human immunodeficiency virus (SHIV) model of HIV infection to study pulmonary effects of Pc colonization. SHIV-infected/Pc-colonized monkeys developed progressive obstructive pulmonary disease characterized by increased emphysematous tissue and bronchial-associated lymphoid tissue. Elevated Th2 cytokines and pro-inflammatory mediators in bronchoalveolar lavage fluid coincided with Pc colonization and pulmonary function decline. These results support the concept that an infectious agent contributes to development of HIV-associated lung disease and suggests that Pc colonization may be a risk factor for the development of HIV-associated COPD. Furthermore, this model allows examination of early host responses important to disease progression thus identifying potential therapeutic targets for COPD.

2.2 INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is predicted to become the third leading cause of death worldwide by 2020 (251). COPD is characterized by development of irreversible airflow limitation and destruction of alveolar septa resulting in alveolar enlargement and airway obstruction. Although smoking is the primary risk factor for COPD, only 15-20% of smokers develop the disease suggesting other factors contribute to disease susceptibility.

COPD occurs earlier and more frequently in HIV-infected subjects compared to HIV-negative subjects (66, 81). How these complications develop is not understood, but sub-clinical or latent infections might be involved (144, 246). Evidence exists linking *Pneumocystis jirovecii*, a fungal opportunistic pathogen, to COPD development in HIV-negative smokers. Subjects with COPD tend to be colonized with *Pneumocystis* (Pc) more frequently than those with other chronic lung diseases, and Pc colonization is associated with severity of airflow obstruction (41, 245). HIV-infected persons are also at risk for Pc colonization, with colonization prevalence up to 69% (147, 242). Although these studies demonstrate association between Pc and COPD, a causal relationship has not been shown.

To examine whether persistent Pc colonization is a co-factor in HIV-related COPD pathogenesis, we developed a Pc colonization model using chimeric simian-human immunodeficiency virus (SHIV) in macaques. Excellent rationale exists for use of this model as studies have shown that Pc derived from humans and non-human primates to be phylogenetically most closely related (128, 259). We performed longitudinal studies to determine association between Pc colonization and progression of airway obstruction and emphysema in the context of an AIDS model.

2.3 METHODS

2.3.1 Animals

Twelve adult, Chinese-origin, cynomolgus macaques (*Macaca fasicularis*) obtained from National Primate Centers or vendors approved by the Department of Laboratory Animal Research, University of Pittsburgh were individually housed and maintained in a BSL2+ primate facility at the University of Pittsburgh. Before purchase, all animals were screened and found negative for simian retroviral infections. Animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Clinical evaluations were conducted monthly or as needed (29).

2.3.2 Virus Infection

Monkeys were infected as described (269) with $SHIV_{89.6P}$ (gift of Dr. Opendra Narayan, University of Kansas), which induces CD4+ T cell lymphopenia and AIDS-like disease with wasting and opportunistic infections(269, 286). Inoculations were repeated one month later to ensure infection in all animals. Viral loads were determined as described for blood and bronchoalveolar lavage samples (269).

2.3.3 Bronchoalveolar lavage (BAL)

Monkeys underwent BAL at baseline and at monthly intervals post-SHIV infection (29). Unfractionated BAL fluid (BALF) aliquots were used for bacterial, fungal and viral culture

(Antech Diagnostics, Pittsburgh, PA) and nested-PCR detection of Pc DNA(29). The remainder was filtered through a 40-micron cell strainer after which cell counts were performed and supernatants were used for cytokine analysis and quantitation of SHIV (269) . 1 x 10^5 cells were removed and stained with modified Giemsa stain (Dade Behring, Newark, DE) and differential counts performed manually (65). Recovered cells were prepared for flow cytometry as described (29).

2.3.4 Pc colonization of SHIV-infected macaques

To promote natural transmission of Pc, SHIV-infected macaques were continuously exposed by co-housing in the same room with 10-20 SIV- or SHIV-immunosuppressed macaques which served as a Pc source. None of the macaques (source or recipients) contracted fulminate *Pneumocystis* pneumonia (PcP) during the study. Determination of Pc colonization status was performed by detection of Pc DNA in BAL samples by nested PCR and by anti-Pc serology (29, 184). Pc colonization was defined as a positive nested PCR of BAL fluid and >3 fold change in plasma anti-Pc KEX1 titers (184). Additionally, BAL samples were stained for organisms by modified Giemsa and silver staining (29).

2.3.5 Peripheral blood collection

Peripheral blood was collected and processed as described (269). T cells were analyzed as described (184).

2.3.6 Cytokine and chemokine analysis

Quantitative analysis of cytokines and chemokines in BALF was performed with Beadlyte Human Multi-Cytokine Flex Kit (Upstate, Temecula, CA) according to manufacturer's instructions. Thirteen of the analytes shown in Table 2-3 were chosen based on cross-reactivity with non-human primate proteins(111). IL-10 and IL-13 levels were analyzed using monkey-specific ELISA kits (BioSource, Camarillo, CA and Cell Sciences, Canton, MA respectively). Dilution effect of BALF samples was normalized based on plasma urea concentrations (287).

2.3.7 Gelatin zymography

Detection of matrix metalloproteinase activity in BALF was performed by identifying proteins with gelatinolytic activity as previously described (312). Before performing zymography, 500 µL of each BALF sample was concentrated using Microcon Centrifugal filter devices with a 50,000 MW cutoff (Millipore, Billerica, MA) according to manufacturer's instructions. Final concentration factor was determined by the sample that could be concentrated the least due to inability to pass any more sample through the concentration device. This resulted in a final concentration factor of 3.3 fold. Samples that concentrated to a larger degree were diluted appropriately with sterile 0.9% sodium chloride (Sigma, St. Louis, MO) which was used for the initial BAL procedure. After concentration, 23 µL of BALF was added to non-denaturing loading buffer (40% glycerol, 200mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue) and separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.1% gelatin. SDS was then removed by two 30 minute washes with 2.5% Triton X-100 (Sigma, St. Louis, MO) followed by incubation for 24 hours at 37°C in developing buffer

(50 mM Tris-HCl pH7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$). Gels were then stained with Coomassie blue followed by destaining with destaining buffer (7.5% acetic acid, 5% methanol). MMP-2 and MMP-9 activity appeared as clear bands against a blue background that were quantitated by densitometry.

2.3.8 Pulmonary function testing

Pulmonary function tests (PFT) were performed at baseline and every other month after SHIV infection using whole body plethysmography and forced deflation technique. Monkeys were anesthetized with intravenous propofol and the oropharynx desensitized with 2% lidocaine followed by intubation. Endotracheal tube placement was verified by chest X-ray and monitored using a CO_2 detector (Nellcor Pedi-cap, Boulder, CO). PFTs were performed using a Buxco whole body plethysmograph (Buxco Electronics, Inc., Sharon, CT), and BioSystems for Maneuvers Software (Buxco Electronics, Inc.) was used to collect data on flow rates and flow volumes. Tests were considered valid when three measurements for forced vital capacity were within 10% of each other.

For bronchodilator challenge, standard PFTs were performed, followed by administration of one pediatric dosette of nebulized albuterol (3 ml of 0.083% albuterol) (Nephron Pharmaceuticals Corp., Orlando, FL). Fifteen minutes after administration, PFTs were repeated and compared to baseline values.

2.3.9 Quantitative computed tomography (CT)

Conventional, non-contrast CT scans were performed on 10 of the 12 animals in a GE 9800 Highlight Advantage CT scanner (General Electric Medical Systems, Milwaukee, WI). Anesthetized, intubated animals were mechanically ventilated to 20 cm H_2O to fully inflate the lung to ensure scan-to-scan volume uniformity. Axial slices (1.25 mm) were acquired during end-inspiratory breath-hold. Calculation of densities used for determination of lung properties in Table 2 was performed for animals at baseline and repeated post-SHIV infection as described (64). Briefly, mean CT scan attenuations of the lung were calculated and converted to density measurements (mg/mL) which was then multiplied by lung volume to obtain lung mass approximation. Actual lung weights were measured at necropsy. These weights correlated with lung weights calculated from endpoint scans by Pearson correlation analysis ($p = 0.01$). CT scan analysis was performed in a blinded manner using custom software (Emphylx: Department of Radiology/iCAPTURE Laboratory, University of British Columbia, Vancouver, BC, Canada) (273). Small airway dimensions were calculated using the PV-Wave software package (Visual Numerics, Boulder, CO) (252).

2.3.10 Lung tissue preparation and morphometry

Right lungs removed at necropsy were inflated to 25 cm H_2O with 10% buffered formalin. Paraffin-embedded, serial mid-sagittal sections from each lobe were then stained. Modified Harris hematoxylin-stained (Sigma) tissue was used to estimate alveolar size by determination of mean chord lengths (387). H&E-stained tissue sections were examined for the presence of

bronchial-associated lymphoid tissue, defined by the presence of non-encapsulated lymphoid tissue within outer airway walls. At least 100 airways per monkey were examined.

2.3.11 Statistical analysis

Pulmonary function data analysis was performed using the R environment for statistical analysis and graphics in which mixed linear models were used to estimate and test the relationship among pulmonary function profiles (dependent variable), Pc colonization (independent variable), and time (independent variable). Differences in profiles over time were tested using restricted maximum likelihood. All other data were analyzed using Prism software, (GraphPad, La Jolla, CA) using paired or unpaired, two-tailed Student's *t* test, where appropriate. A *p* value less than 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 Pc colonization of SHIV-infected macaques results in pulmonary obstruction

Twelve cynomolgus were infected with $SHIV_{89.6P}$ (286) and exposed to Pc via co-housing with Pc-infected macaques (184). Peripheral blood CD4+ T cell levels declined to $\leq$ 50% of baseline values by four weeks post-SHIV infection in all monkeys and remained depressed throughout the study (Figure 2-1). Peak viremia ranged from 3.4×10^6 to 2.3×10^8 RNA copies/ml by week two

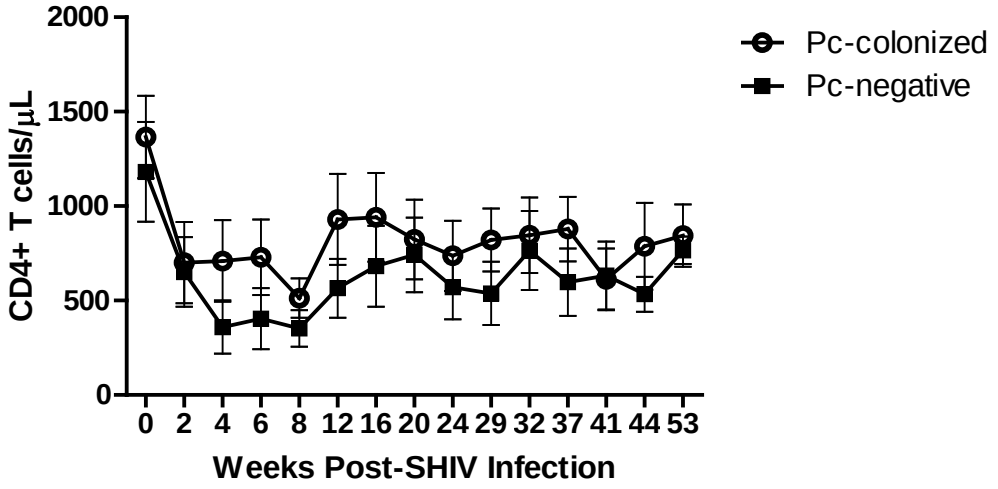

Figure 2-1 Peripheral blood CD4+ T cell levels are not different for SHIV/Pc+ animals versus SHIV/Pc- animals. Peripheral blood mononuclear cells isolated from whole blood were stained with anti-CD4 antibody and analyzed by flow cytometry. Open circles represent SHIV/Pc+ animals and closed squares represent SHIV/Pc- animals. $p = 0.79$ by two-way repeated measures ANOVA for SHIV/Pc+ ($n = 8$) versus SHIV/Pc- group ($n = 4$).

post-infection (not shown). Serial bacterial and fungal cultures of BALF were negative throughout the study. Eight of 12 monkeys became colonized with Pc by nine weeks post-SHIV infection (SHIV/Pc+), as determined by nested-PCR of bronchoalveolar lavage fluid and Pc serology (184), while four remained Pc-negative throughout the study (SHIV/Pc-). After initial exposure to Pc, anti-Pc titers in the SHIV/Pc+ animals remained above baseline throughout the study (not shown). None of the monkeys tested positively by PCR at every time point which was most likely due to low level organism burden and sampling of different areas of the lung at each time point. Among these animals, two to nine time points were positive by PCR during the

period studied. Modified Giemsa and silver staining were also performed on BAL samples but were not found to be positive for organisms at any time point. There was no significant difference in peak viral titers (mean peak viral titers (viral RNA copies/ml plasma): SHIV/Pc+: $4.42 \times 10^7 \pm 7.50 \times 10^7$, SHIV/Pc-: $3.19 \times 10^7 \pm 2.88 \times 10^7$; $p = 0.76$) or peripheral blood CD4+ T cells levels at any time post-SHIV infection between the groups (Figure 2-1).

To assess airway obstruction, pulmonary maneuvers using whole body plethysmography (280) were performed at baseline and every other month after SHIV infection. Peak expiratory flow (PEF), forced expiratory volume in 0.4 seconds (FEV0.4) and maximum mid-expiratory flow (MMEF) are the pulmonary function parameters chosen to evaluate obstructive disease. PEF is a measurement of the greatest rate of air flow during forced expiration, FEV0.4 is the volume of air expired in 0.4 seconds during forced expiration and MMEF is the average expiratory flow over the middle half of the forced vital capacity (FVC). No significant differences in baseline physical characteristics and pulmonary function parameters between groups were observed (Table 2-1).

Table 2-1

Baseline values for height, weight and pulmonary function parameters in SHIV/Pc- and SHIV/Pc+ animals.

Parameter	Pc negative*	Pc positive	p value
Height, cm	58.4 (53.3 – 61.0)	61.0 (45.7 – 63.5)	0.94
Weight, kg	4.7 (3.3 – 5.8)	5.8 (3.8 – 7.6)	0.19
Pulmonary Function Parameters[†]			
$FEV_{0.1}$, ml	39.1 (36.6 – 42.6)	37.3 (32.9 - 44.8)	0.66
$FEV_{0.2}$, ml	89.4 (86.2 – 96.7)	88.4 (77.3 - 101.2)	0.80
$FEV_{0.4}$, ml	187.2 (168.4-197.9)	186.2 (163.6 - 204.4)	0.97
FVC, ml	396.7 (223.8 - 454.6)	415.9 (227.8 - 527.8)	0.61
$FEV_{0.1}$/FVC, %	10.4 (8.0 - 17.2)	8.4 (7.2 - 19.7)	0.74
$FEV_{0.2}$/FVC, %	23.8 (19.0 - 38.6)	19.9 (16.8 - 44.4)	0.77
$FEV_{0.4}$/FVC, %	49.3 (39.8 - 75.3)	42.0 (35.3 - 85.4)	0.78
$FEF_{25\%}$, ml/s	504.7 (483.6 - 527.5)	497.3 (438.6 - 572.6)	0.95
$FEF_{50\%}$, ml/s	453.9 (430.6 - 483.0)	456.2 (404.1 - 542.7)	0.72
$FEF_{75\%}$, ml/s	382.2 (349.8 - 430.6)	391.7 (367.3 - 477.0)	0.36
$FEF_{90\%}$, ml/s	230.8 (180.7 - 376.2)	299.1 (170.8 - 370.5)	0.53
$FEF_{25-75\%}$, ml/s (MMEF)	450.5 (423.3 - 480.6)	450.6 (402.3 - 535.6)	0.71
PEF, ml/s	517.7 (492.9 - 546.8)	512.7 (447.1 - 576.2)	0.89

*Comparison of baseline values of animals that were subsequently infected with $SHIV_{89.6P}$ and were colonized with Pc (SHIV/Pc+, n = 8) or remained uncolonized (SHIV/Pc-, n = 4). No significant differences were observed in any of the parameters by unpaired *t* test.

[†]$FEV_{0.1}$, $FEV_{0.2}$, $FEV_{0.4}$, forced expiratory volume in 0.1, 0.2 and 0.4 seconds respectively; FVC, forced vital capacity; $FEF_{25\%}$, $FEF_{50\%}$, $FEF_{75\%}$, $FEF_{90\%}$, forced expiratory flow through 25%, 50%, 75% and 90% of forced vital capacity respectively; $FEF_{25-75\%}$ (MMEF), forced expiratory flow from 25% to 75% of forced vital capacity or maximum mid-expiratory flow; PEF, peak expiratory flow.

Values are medians with ranges shown in parentheses.

Six of eight SHIV/Pc+ animals developed airway obstruction as determined by decreased pulmonary function. All parameters evaluated decreased significantly in these animals compared to SHIV/Pc- monkeys (Figure 2-2A-C). Median change in peak expiratory flow from baseline to 10 months post-SHIV infection was -58.5 ml/sec and +2.5 ml/sec for SHIV/Pc+ and SHIV/Pc- animals, respectively ($p=0.004$). Median change from baseline forced expiratory volume in 0.4 seconds in SHIV/Pc+ animals was -16.0 ml versus +4.0 ml for SHIV/Pc- animals ($p=0.001$). For maximum mid-expiratory flow, median change from baseline for SHIV/Pc+ animals was -47.5 ml/sec versus +24.5 ml/sec for SHIV/Pc- monkeys. ($p=0.001$). Although the forced expiratory volume in 0.4 seconds to forced vital capacity ratio, another measure of COPD, declined in SHIV/Pc+ animals, the change was not significant by 10 months post-infection (Figure 2-2D).

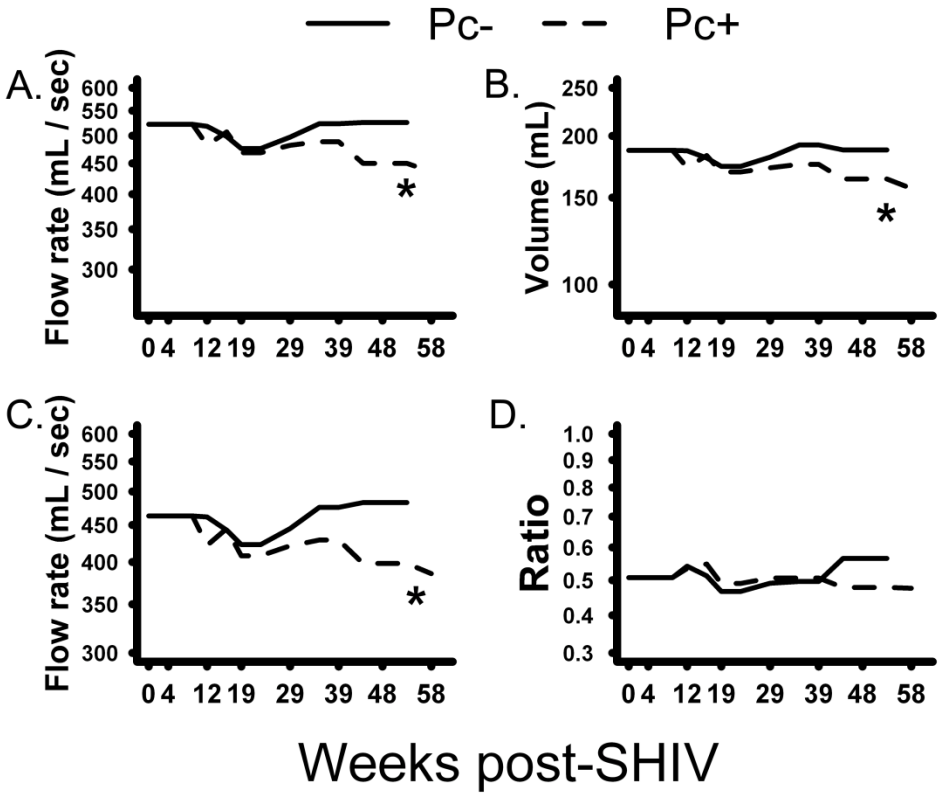

Figure 2-2 *Pneumocystis* colonization results in progressive pulmonary function decline. Whole body plethysmography was used to evaluate serial measurements of: (A) Peak expiratory flow, *p = 0.003. (B) Forced expiratory volume in 0.4 seconds, *p = 0.003. (C) Maximum mid-expiratory flow, *p = 0.002. (D) Forced expiratory volume in 0.4 seconds to forced vital capacity ratio, p = 0.32. For all graphs, SHIV/Pc+ animals are represented by dashed lines and SHIV/Pc- animals are represented by solid lines. Each p value is for the interaction between time and group (Pc-colonized (n = 8) versus non-colonized (n = 4)).

Since airflow limitation in COPD is poorly reversible in response to bronchodilator treatment, we tested the effect of administration of the bronchodilator, albuterol. No significant differences were observed post-treatment (not shown).

2.4.2 *Pneumocystis* colonization results in radiographic and pulmonary emphysema but not small airway thickening in SHIV-infected monkeys

Emphysema is associated with increased lung and airspace volumes, usually coupled with decreased lung weight. Quantitative computed tomography (CT) morphometry has been used to evaluate extent of emphysema in humans (64). We applied this technique to evaluate baseline and post-infection lung CT scans by performing tissue density analysis based on a density mask cut-off of $\leq$ -910 Hounsfield units (HU), which is similarly used to identify emphysema in humans (64). There was a significant increase compared to baseline values in lung percent at $\leq$ -910 HU in SHIV/Pc+ monkeys compared to SHIV/Pc- monkeys (Figure 2-3A). Lobe by lobe comparison of percent change in $\leq$ -910 HU during the course of infection revealed significant increases in upper and middle lobes of SHIV/Pc+ monkeys but not in lower lobes. No significant changes were observed in individual lobes of SHIV/Pc- monkeys (Figure 2-3B).

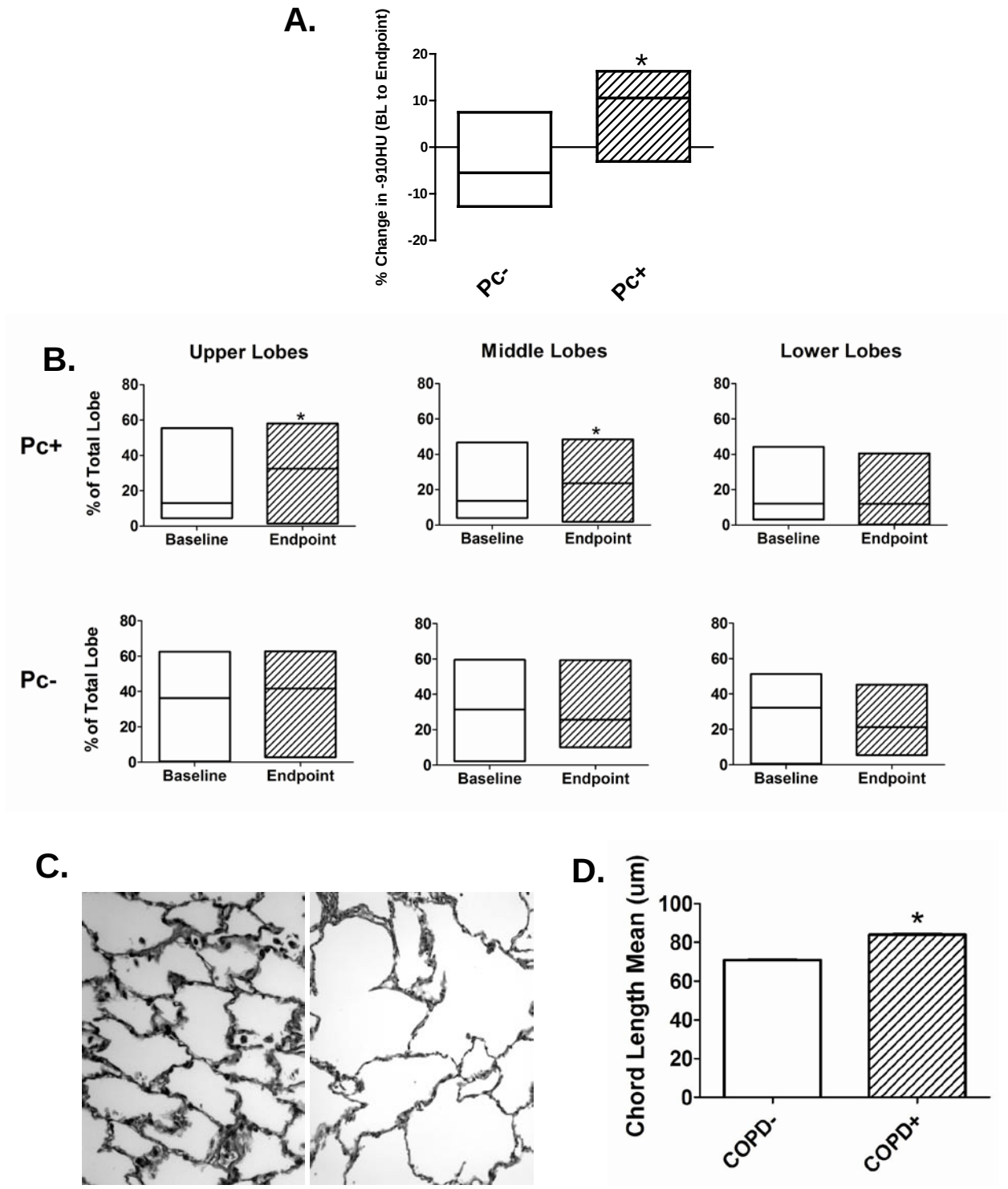

Figure 2-3 *Pneumocystis* colonization leads to an increase in the proportion of emphysematous tissue in the lungs. Quantitative computed tomography (CT) scans were

performed at 20 cm H_2O lung inflation pressure at baseline (BL) and post- SHIV infection. The cutoff mask of ≤ -910 Hounsfield units (HU) was used to assess amount of emphysematous lung tissue present at each scan. Boxes represent the range of values for the specified group with the median value represented by the line within the box. (A) Change in the proportion of emphysematous lung tissue for the animal groups; * p = 0.04 for SHIV/Pc- (n = 4) versus SHIV/Pc+ (n = 6[†]) animals by unpaired *t* test. (B) Comparison of the proportion of emphysematous lung tissue present at baseline and endpoint scan by lobe. For SHIV/Pc+ animals[†]: * p = 0.04 by paired *t* test, for the proportion of lung tissue that is emphysematous in both the upper and middle lobes for baseline versus endpoint scans; p = 0.78 by paired *t* test for proportion of lung tissue that is emphysematous in the lower lobe for baseline versus endpoint scans (n = 6). For SHIV/Pc- animals: p = 0.55, 0.80 and 0.11 by paired *t* test for proportion of lung tissue that is emphysematous in the upper, middle and lower lobes respectively for baseline versus endpoint scans (n = 4). (C) Representative hematoxylin-stained lung tissue sections from SHIV-infected monkeys; left: SHIV/Pc- and right: SHIV/Pc+. (D) Chord length analysis (mean ± SEM) of airspaces for animals exhibiting clinical type (≥ 12% decline in pulmonary function from baseline level) obstruction (COPD+, n = 5) versus non-obstructed animals (COPD-, n = 7), *p = 0.0001.

[†]Two SHIV/Pc+ animals were not included in either the pre- or post-infection analyses because baseline scans were not performed. Both of these animals developed airway obstruction based on pulmonary function testing.

Consistent with an increase in percentage of emphysematous tissue, total tissue volume and lung weight were significantly decreased from baseline in SHIV/Pc+ monkeys (Table 2-2), but not in SHIV/Pc- monkeys. No significant changes in small airway wall dimensions, including thickness, were observed for either group (not shown).

Airspace enlargement was also evaluated in lung tissue sections by determination of mean chord length, the average distance between opposing walls of a single alveolus. Figure 2-

3C shows representative lung tissue sections from both groups. In support of our radiologic findings, mean chord length was significantly larger in obstructed versus non-obstructed monkeys (Figure 2-3D).

Table 2-2

Quantitative CT analysis of the lungs pre- and post-infection

	SHIV/Pc+[‡]		SHIV/Pc-	
	Baseline*	Endpoint	Baseline	Endpoint
Total Lung volume, ml	352 ± 40	358 ± 49	372 ± 22	371 ± 34
Airspace volume, ml	300 ± 36	309 ± 44	321 ± 18	322 ± 28
Tissue volume, ml	52 ± 5	49 ± 5[§]	51 ± 6	49 ± 7
Lung weight, g	55 ± 5	52 ± 5[§]	54 ± 6	52 ± 7
% Voxels > -910 HU[†]	82 ± 6	74 ± 6[§]	68 ± 13	71 ± 12
% Voxels ≤ -910 HU	18 ± 6	26 ± 6[§]	32 ± 13	29 ± 12

*Values (mean ± SEM) were calculated in monkeys before SHIV infection (baseline) and following SHIV infection in SHIV/Pc+ (n = 6) and SHIV/Pc- (n = 4) animals at the termination of the experiment (10-12 months post-SHIV infection).

[†]HU: Hounsfield units

[§]Different from baseline (p = 0.04 by paired *t* test analysis)

[‡]Two SHIV/Pc+ animals were not included in either the pre- or post-infection analyses because baseline scans were not performed. Both of these animals developed airway obstruction based on pulmonary function testing.

2.4.3 *Pneumocystis* colonization results in increased bronchial-associated lymphoid tissue in SHIV-infected monkeys

As an indicator of inflammation due to increased pathogen burden, lung tissue was examined for presence of bronchial-associated lymphoid tissue. SHIV/Pc+ monkeys had significantly higher bronchial-associated lymphoid tissue frequency compared to SHIV/Pc- monkeys, indicating persistent pulmonary inflammation in these animals (Figure 2-4A, B).

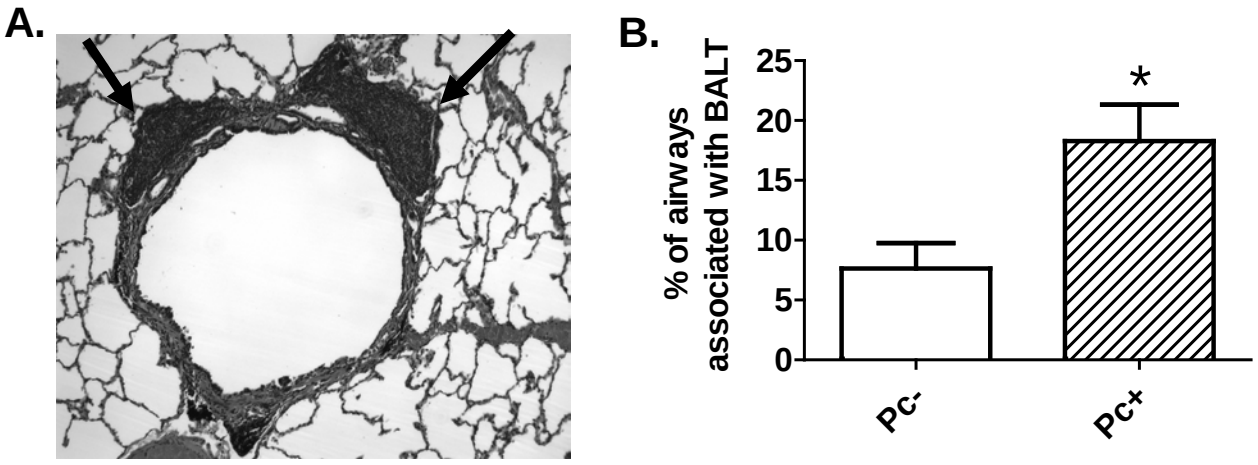

Figure 2-4 *Pneumocystis* colonization results in increased bronchial-associated lymphoid tissue formation. (A) Representative hematoxylin and eosin-stained lung tissue section from a SHIV/Pc+ animal showing an airway associated with lymphoid follicles (indicated by arrows). (B) Analysis of percent of airways with bronchial-associated lymphoid tissue in SHIV/Pc- versus SHIV/Pc+ monkeys. For SHIV/Pc- (n = 4) and SHIV/Pc+ (n = 8) animals, an average of 114 ± 43 and 103 ± 17 airways per animal were evaluated respectively, p = 0.04 by unpaired *t* test.

2.4.4 *Pneumocystis* colonization induces inflammatory and Th2-associated cytokines in the bronchoalveolar lavage fluid of SHIV-infected monkeys

Because COPD and PcP have been associated with vigorous inflammatory responses (119, 308), we evaluated inflammation indicators in serial BALF samples following SHIV infection and Pc colonization. Interestingly, there were no significant changes in absolute number or percentage of T cells, macrophages, neutrophils, or CD4+/CD8+ T cell ratios, in BALF of infected monkeys regardless of Pc status (up to 12 months post-SHIV infection) (not shown). Serial cytokine and chemokine analysis of BALF revealed changes from baseline in SHIV/Pc+, but not SHIV/Pc-, monkeys (Table 2-3). Assays were performed at baseline, four weeks post-SHIV infection (after significant CD4+ T cell decline, but prior to detectable Pc colonization), and weeks 16 and 35 (after detection of persistent Pc colonization). Increases at weeks 16 and 35 in interleukin (IL)-4, IL-5, IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF) and lymphotoxin-α and transient increases in IL-8, IL-13, interferon (IFN)-γ, CCL3 and tumor necrosis factor (TNF)-α were observed in SHIV/Pc+ monkeys. Conversely, SHIV/Pc- animals did not exhibit increases in cytokine levels, except for TNF-α at 35 weeks. These results demonstrate that SHIV infection alone had little effect on induction of inflammatory mediators in alveolar spaces, while Pc colonization induced a pro-inflammatory and Th2-skewed cytokine response, which was coincident with declining pulmonary function (Figure 2-2).

Table 2-3

Serial analyses of BAL cytokines and chemokines in SHIV-infected monkeys

Cytokine/ Chemokine	Weeks After SHIV Infection	SHIV/Pc+ Mean (pg/mL)	SHIV/Pc+ Std Dev	SHIV/Pc+ p (vs BL)	SHIV/Pc- Mean (pg/mL)	SHIV/Pc- Std Dev	SHIV/Pc- p (vs BL)
IL-4	BL	71.25	30.7		70.21	28.9	
	4	78.7	30.5	0.549	96.07	39.7	0.310
	16	146.8	42.9	*0.004*	72.13	33.8	0.230
	35	134.3	40.6	*0.026*	94.74	61.2	0.390
IL-5	BL	65.72	26.2		64.3	31.5	
	4	68.44	25.4	0.774	77.83	31.4	0.642
	16	143.5	40	*0.003*	66.41	27.5	0.343
	35	153.6	48.5	*0.007*	83.51	48.8	0.221
IL-13	BL	11.8	4.4		12.6	6.1	
	4	11.0	7.3	0.797	18.3	4.8	0.148
	16	21.6	4.8	*0.005*	10.7	4.0	0.595
	35	15.0	6.4	0.247	8.5	1.0	0.213
IL-10	BL	583.5	303.6		573.3	442.6	
	4	774.4	357.2	0.193	718.5	283.7	0.507
	16	752.7	279.1	0.150	474.1	298.9	0.309
	35	442.8	155.6	0.312	384.8	410.8	0.652
IFN-γ	BL	62.5	34.5		53.1	28.5	
	4	59.7	43.3	0.881	104.4	72.3	0.346
	16	179.2	71.8	*0.005*	53.9	45.6	0.528
	35	106.6	58.3	0.176	70.5	27.2	0.067
IL-12 (p40)	BL	23.0	52.3		82.6	131.0	
	4	9.4	22.6	0.266	8.5	17.0	0.351
	16	305.3	388.8	0.058	78.2	81.6	0.367
	35	241.0	462.6	0.239	80.2	96.0	0.982
Lymphotoxin	BL	117.0	43.5		124.0	68.1	
	4	128.1	61.5	0.579	137.9	39.8	0.782
	16	241.6	56.5	*0.002*	98.7	27.4	0.793
	35	248.9	93.9	*0.019*	152.3	84.2	0.241
TNF-α	BL	85.9	45.7		36.5	31.1	
	4	109.2	51.9	0.309	78.4	36.4	0.204
	16	232.6	89.2	*0.006*	83.7	37.2	0.115
	35	153.2	61.4	0.079	66.4	30.7	*0.013*
IL-1β	BL	81.3	31.2		83.2	43.9	
	4	93.0	49.5	0.476	100.2	17.9	0.508
	16	160.9	46.4	*0.006*	86.0	25.0	0.325
	35	192.0	81.4	*0.018*	120.7	76.2	0.177

Table 2-3 (continued)

IL-6	BL	113.7	43.7		114.5	54.1	
	4	120.8	58.5	0.776	144.6	28.9	0.499
	16	221	50.7	*0.004*	95.53	36.1	0.865
	35	196.4	69	*0.04*	137.4	71.4	0.296
IL-8	BL	161.1	83.4		185.4	141.0	
	4	251.3	258.2	0.327	174.2	45.7	0.893
	16	304.5	105.8	*0.013*	142.8	66.3	0.464
	35	385.8	248.4	0.059	400.2	416.2	0.224
GM-CSF	BL	82.0	51.1		72.2	44.4	
	4	80.8	22.6	0.951	76.9	40.0	0.909
	16	198.1	73.0	*0.007*	66.8	26.9	0.584
	35	173.2	70.8	*0.040*	121.7	55.9	0.096
CCL3	BL	148.8	172.3		0.0	0.0	
	4	109.1	144.0	0.541	155.9	192.8	0.204
	16	579.7	300.0	*0.010*	214.4	196.4	0.199
	35	307.1	219.1	0.181	1068	1931	0.350
CCL2	BL	2006.0	1695.0		1951.0	2249.0	
	4	1549.0	1222.0	0.508	2736.0	2414.0	0.427
	16	3905.0	6292.0	0.325	725.4	184.7	0.607
	35	4947.0	5785.0	0.177	13150.0	22628.0	0.352
CCL5	BL	91.2	86.8		176.4	136.2	
	4	55.5	32.2	0.318	70.8	25.5	0.248
	16	124.3	55.2	0.151	67.8	40.2	0.203
	35	438.4	471.9	0.060	122.5	36.1	0.528

BL: baseline

Pc colonization was detected by 8 weeks post-SHIV infection.

p values, analyzed by paired *t* test, are for baseline measurements versus measurements for the indicated week post-SHIV infection in SHIV/Pc+ (n = 8) and SHIV/Pc- (n = 4) animals. Timepoints where significant changes in cytokine levels were detected are shaded light gray.

2.4.5 Matrix metalloproteinase (MMP) activity increases early after Pc colonization and rapidly falls off

Since one of the prevailing theories of emphysema pathogenesis is a protease/anti-protease imbalance in the lungs skewed toward excessive proteolytic activity, we performed zymographic analysis of BALF at time points corresponding approximately to those used to evaluate cytokine secretion (weeks 12, 20, 29 post-SHIV infection). Our analysis showed that there was an early spike in MMP-9 activity at 12 weeks post-SHIV infection while MMP-2 activity remained relatively stable (Figure 2-5). Subsequent time points revealed that this increase in activity had declined to baseline levels by week 20 post-SHIV infection (not shown).

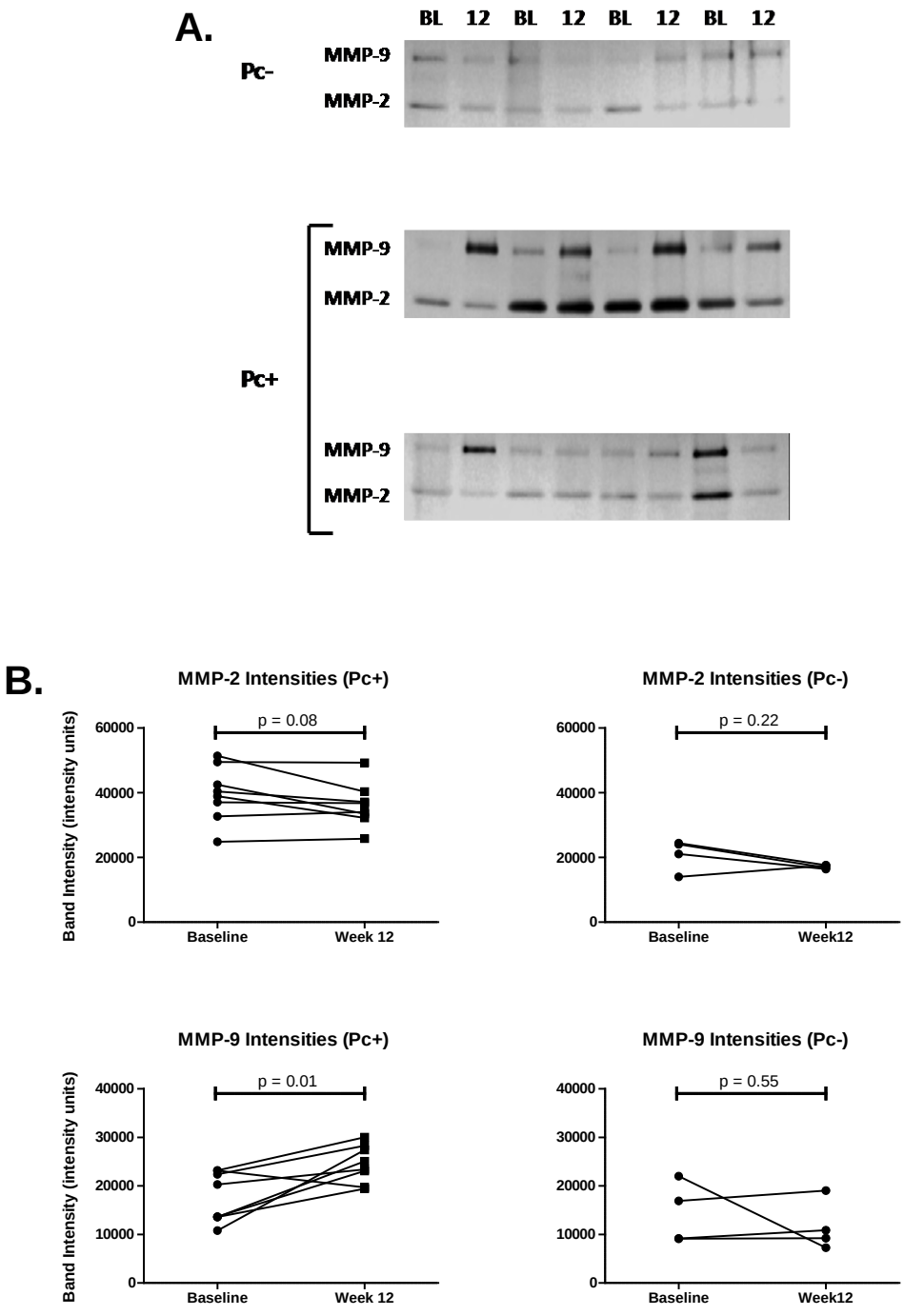

Figure 2-5 MMP-9 activity increases early after Pc colonization. (A) Inverted image of zymographic gelatin gel showing MMP-2 and MMP-9 activity in BALF harvested 12 weeks post-SHIV infection; BL=baseline, 12=12 weeks post-SHIV infection (B) Densitometric analyses of zymograms.

2.5 DISCUSSION

The results presented here support the concept that Pc colonization contributes to the development of COPD in a non-human primate model of HIV infection. SHIV-infected monkeys that became naturally colonized with Pc developed progressive pulmonary obstruction that was unresponsive to bronchodilator treatment. Additionally, Pc colonization correlated with anatomic evidence of emphysema, increased bronchial-associated lymphoid tissue frequency, and increased levels of pro-inflammatory mediators and Th2-type cytokines in BALF. In contrast, SHIV infection alone did not exert such effects. These data support the hypothesis that in HIV-associated COPD, persistent Pc carriage, common among HIV+ subjects (147, 242), induces lung inflammation, possibly promoting tissue damage and COPD development.

COPD is a complex disorder resulting from a combination of genetic and environmental factors associated with persistent lung inflammation (308). While cigarette smoking is the main risk factor, other factors likely influence disease progression, as only ~25% of smokers develop COPD (210). HIV infection is also associated with increased COPD risk, particularly in smokers. Diaz *et al*. reported 37% of HIV- infected smokers had emphysema by pulmonary function or chest CT scan, in contrast to no demonstrable emphysema in HIV-negative controls (81). Crothers *et al*. showed that HIV+ subjects are more likely to have a COPD diagnosis compared with HIV-negative controls, and that HIV infection was an independent predictor of COPD (66). Reports of high Pc colonization frequency in HIV-infected subjects and HIV-negative COPD patients (245, 279), suggests persistent Pc carriage may promote pulmonary function decline and COPD development. The primate model of HIV infection described here supports these clinical findings and enables longitudinal characterization of factors associated with development of COPD pathogenesis.

Simian immunodeficiency virus (SIV) and SHIV have been used extensively in rhesus and cynomolgus macaques as models of HIV (9). As in HIV infection, PcP is common in SIV- and SHIV-infected macaques and susceptibility correlates with peripheral blood CD4+ T cell decline (65, 164). In contrast to SIV, SHIV produces rapid decline in blood CD4+ T cells, facilitating long-term studies of persistent infection. We previously described SIV infection/Pc colonization in macaques using both intrabronchial inoculation and airborne transmission of Pc (29, 184). Airborne transmission is more likely representative of natural Pc transmission, and eliminates potential transient inflammatory responses associated with intrabronchial inoculations (29), allowing examination of inflammatory responses associated with persistent colonization.

In serial pulmonary function studies, we found significant obstruction in six of eight Pc-colonized monkeys, but not in monkeys infected with SHIV alone. These data suggest that viral infection is insufficient to induce emphysema in this timeframe, but SHIV-induced immunosuppression may increase Pc carriage susceptibility, which may result in obstructive changes. Interestingly, one SHIV/Pc+ monkey without measurable pulmonary function decline showed evidence of emphysema based on increased lung volume and percentage of lung tissue $\leq$ -910 HU (not shown). This suggests that SHIV infection and Pc colonization may result in emphysema without airflow obstruction, a COPD phenotype described in humans (107). No significant changes between baseline and endpoint small airway wall dimensions were observed in either group, suggesting the observed pulmonary obstruction was an emphysema-dominant phenotype with minimal small airway involvement (182).

Several studies have examined inflammatory responses in COPD patients, with conflicting results likely due to disease heterogeneity, variability in disease severity, and lung region sampling differences (57, 181). Neutrophils have been implicated in COPD pathogenesis.

Severe COPD patients have neutrophilic infiltration of airway walls, and increased neutrophil counts in BALF and sputum samples that correlate with disease severity (145, 333). In contrast, mild emphysema is not commonly associated with BAL neutrophilia (26). Similarly, studies have shown T cell infiltration in small airway walls and alveolar spaces of COPD patients with general increases in CD8+ T cell proportions though their role in COPD-associated inflammatory damage is unknown. CD4+ and CD8+ T cells can elaborate pro-inflammatory cytokines that may contribute to lung damage. Th1-skewed cytokine production has been reported in COPD patients in several studies (68), although a mixed or Th2-dominant response has also been reported (16).

We detected inflammatory changes in airspaces only after Pc colonization was evident, with little evidence of inflammation due to persistent SHIV infection. Increases in IL-4, IL-5, and IL-13 with minimal increases in IFN-γ and no detectable IL-12 in SHIV/Pc+ monkeys suggested a Th2-skewed response. Although Th2 cytokines are more commonly associated with asthma (290), these results support reports of increased IL-4 in emphysematous human lung tissue (388). Additionally, Ma *et al*. demonstrated that in mice genetically predisposed toward a Th1 response, over-expression of IL-4 resulted in emphysematous pulmonary destruction and reduced protease inhibitor levels in the lung (215).

Increased IL-13 observed in Pc-colonized monkeys is interesting in light of reports that emphysema was associated with IL-13 expression in a transgenic mouse model (146), and in murine models of *Nippostrongylus brasiliensis* (224) and persistent viral infection (180). Although its role in alveolar destruction progression is unclear, increased IL-13 in transgenic mice correlated with increased matrix metalloprotease and cathepsin production in lung tissue (387), which may promote lung tissue degradation in emphysema (60). In light of this report, it

is interesting to note that the increase in IL-13 roughly correlated with the increased MMP-9 activity which has been implicated in COPD pathogenesis in both murine (6, 95) and human (36, 312) studies. Additionally, the increases in both MMP-9 activity and IL-13 dropped off rapidly. This observation reinforces a key role for IL-13 in mediating COPD pathogenesis by the induction of an imbalance between proteases and anti-proteases in the lungs.

IL-1β, IL-6 and GM-CSF, pro-inflammatory cytokines associated with macrophage activation, were also increased in BALF of SHIV/Pc+ monkeys. These results are consistent with reports of increased levels of these cytokines in pulmonary and plasma samples from COPD patients (103) and in animal models of emphysema (193), indicating a key role for macrophage activation in the early process of lung damage in this model.

Contrary to studies reporting inflammatory cellular infiltration associated with human and animal COPD, we found no significant changes in absolute numbers or proportions of T cells or neutrophils in BALF of either monkey group, even after significant pulmonary function decline was evident. This may be due to the fact that in human studies, patients have had clinical disease for years whereas the primate model is capturing early events in disease progression. Unlike our previous study showing infiltration of CD8+ T cells and neutrophils in intrabronchially infected macaques (29), the Pc doses associated with natural colonization reported here were likely much lower. It is likely that as Pc burden increases, a CD8+ T cell- and neutrophil-dominant response may develop and amplify inflammation-mediated pulmonary damage.

Innate inflammatory responses initiated by alveolar macrophages or other cells such as mast cells, NK or NKT cells are likely activated early in response to Pc colonization thus elaborating pro-inflammatory cytokines prior to detectable activation of adaptive responses and

subsequent cellular infiltration. This hypothesis is consistent with studies suggesting macrophages and NKT cells are key effectors in murine models demonstrating IL-13-mediated emphysematous destruction (180, 224). Additionally, a role for mast cells in human COPD has been suggested (120), possibly via IL-4 upregulation (17). We further postulate that persistent Pc colonization is associated with an adaptive immune response, as indicated by increased frequency of bronchial-associated lymphoid tissue in Pc-colonized monkeys with COPD. These results are consistent with the finding of an increased frequency of bronchial-associated lymphoid tissue in COPD patients, and support the concept that persistent infection and host immune response is associated with COPD development (144, 145).

This study establishes a novel model for HIV-associated COPD and provides evidence supporting a role for Pc colonization in obstructive disease development. These results support the paradigm that infectious agents, directly or indirectly, can promote COPD pathogenesis (144, 224). A detrimental inflammatory response may be amplified by continuous or repeated colonization by various pulmonary pathogens leading to disease progression, as has been clinically shown (319). As in human COPD pathogenesis, it is likely that COPD development in SHIV-infected macaques is multifactorial and that genetic and environmental factors contribute to susceptibility. This study supports the concept that Pc colonization contributes to COPD pathogenesis in SHIV-infected macaques, but does not exclude a role for other factors. The non-human primate model allows for serial examination of various parameters associated with development of obstructive changes and should help define host responses that promote tissue destruction at early disease stages. Additionally, these results identify Pc as a potentially treatable risk factor for COPD development in HIV-infected and non-infected individuals.

2.6 AUTHOR CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Timothy W. Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) designed and supervised the research, generated the majority of the data, performed analyses and prepared the manuscript. Heather M. Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) provided assistance with nonhuman primate sample processing and analysis. Alison Morris (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) provided input on research design and analysis/critique of the project, data and the manuscript. Sangita Patil (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with sample collection and analysis, and generated PCR and flow cytometry data. Jan Kristoff (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with nonhuman primate sample processing and analysis. Siobhan E. Guyach (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with BALT analysis. Jessica E. Murphy (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with BALT analysis. Xiuping Shao (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with cytokine analysis and MMP zymography. Frank C. Sciurba (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) assisted in data analysis. Robert M. Rogers (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) assisted in data analysis. Thomas Richards (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) performed statistical analyses. Paul Thompson (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) assisted in data analysis. Ronald C. Montelaro (Department of Molecular Genetics and Biochemistry University

of Pittsburgh School of Medicine) provided technical advice. Harvey O. Coxson (The James Hogg iCAPTURE Center for Cardiovascular and Pulmonary Research) assisted in data analysis. James C. Hogg (The James Hogg iCAPTURE Center for Cardiovascular and Pulmonary Research) assisted in data analysis. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine) as the mentor and principal investigator on the project, provided extensive scientific knowledge, insight and critique of the project and manuscript. All authors, especially Karen A. Norris, contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

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3.0 PULMONARY GENE EXPRESSION ANALYSIS IN A PRIMATE MODEL OF HIV-RELATED COPD REVEALS NOVEL GENES ASSOCIATED WITH EARLY DISEASE PATHOGENESIS

A version of this chapter is being prepared for submission to the *American Journal of Respiratory Cell and Molecular Biology*. The authors are Timothy W. Shipley, Heather M. Kling, Alison Morris, and Karen A. Norris.

3.1 ABSTRACT

HIV-infected persons are at increased risk for developing pulmonary diseases including chronic obstructive pulmonary disease (COPD), and the fungal opportunistic pathogen, *Pneumocystis jirovecii* (Pc) has been implicated in the pathogenesis of HIV-related COPD. We previously developed a non-human primate model of HIV-related COPD using simian-human immunodeficiency virus (SHIV) and Pc co-infection in cynomolgus macaques. In the present study we examined gene expression profiles in lung tissue from SHIV/Pc co-infected monkeys with COPD and compared them to SHIV-infected monkeys infected with normal lung function. Microarray technology was used to develop gene profiles, and differential gene expression was determined by a comparative evaluation of competing normalization methods applied to our expression data set followed by validation using quantitative real-time polymerase chain reaction analysis for select genes. Of over 52,000 transcripts representing more than 20,000 genes analyzed, the SHIV/Pc infected macaques with COPD exhibited 243 differentially expressed (DE) genes compared to SHIV-infected monkeys with normal lung function. DE genes fell into a number of functional categories which may be important in COPD development including: inflammation (pulmonary surfactants A2, B, C, D, upregulated; alternative macrophage activation-associated CC chemokine, upregulated), protease/antiprotease balance (cathepsin H, upregulated; alpha-1-chymotrypsin and secretory leukocyte peptidase inhibitor, downregulated), redox balance (glutathione peroxidase 4 and mitochondrial aldehyde dehydrogenase 2, upregulated) and tissue homeostasis (connective tissue growth factor, downregulated; ornithine decarboxylase antizyme, upregulated). Furthermore, analysis of impacted molecular pathways revealed that the apoptosis-relevant VEGF signaling pathway was significantly affected. These

results identify factors and pathways involved in early development of *Pneumocystis* and SHIV-associated COPD and reveal several novel, potential therapeutic targets.

3.2 INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease of global importance and is predicted to become the third leading cause of death worldwide by the year 2020 (251). Although smoking is the primary risk factor for development of COPD, only about 15% of smokers develop the disease (93), suggesting that other factors are important in disease development and progression. Evidence has emerged supporting the concept that microbial colonization of the lower airways contributes to the pathogenesis of COPD, either directly or indirectly, through the induction of a persistent and detrimental inflammatory response. It has been suggested that a dysregulated inflammatory response results in structural damage in the lungs and promotes disease progression, but fails to clear the inciting pathogen (319). Several infectious agents have been associated with progression or exacerbations of COPD, including *Haemophilus influenza* and adenovirus (144, 320).

The role of pathogen-related COPD may be particularly important in HIV infection, where HIV-infected individuals are at increased risk for an accelerated form of emphysema (80, 81) and a high prevalence of COPD (66). Of particular interest is the fungal opportunistic pathogen, *Pneumocystis jirovecii* (Pc), which has been implicated in the pathogenesis of HIV-related COPD (241) and in non-HIV infected patients with COPD (244, 245). Animal models have also demonstrated an association between Pc colonization and the development of emphysema and COPD (55, 246, 258, 326). In a nonhuman primate model of HIV infection,

macaques infected with simian immunodeficiency virus-HIV (SHIV) and colonized with Pc develop significant airway obstruction and emphysematous lung tissue destruction, while animals infected with virus alone do not, but the exact mechanism by which Pc colonization contributes to COPD development is unknown (258, 326). To identify potential pathways important in COPD in this model, we compared gene expression profiles from lung tissue of SHIV-infected/Pc-colonized macaques with COPD to monkeys with normal lung function that were infected with SHIV alone. We identified a comprehensive profile of gene expression patterns by microarray analysis, with confirmation of gene expression by quantitative real time-polymerase chain reaction (qRT-PCR). From these results, several possible mediators of lung tissue destruction associated early stages of Pc and SHIV-related COPD were identified.

3.3 METHODS

3.3.1 Animals

Twelve adult, Chinese origin cynomolgus macaques (*Macacca fasicularis*), weighing between 5-8 kg, were used. All animals were purchased from National Primate Centers or vendors approved by the University of Pittsburgh, Department of Laboratory Animal Research. Prior to admission study entry, all animals underwent complete physical examination (pulmonary and cardiac auscultation, thoracic radiographs, computer tomography scanning, tuberculin skin testing, complete blood count, chemistry panel, urinalysis, and flow cytometric analysis of peripheral blood mononuclear and BAL cells) and were screened for simian retroviruses (simian immunodeficiency virus (SIV), simian retrovirus (SRV), and simian T-cell leukemia virus

(STLV)) to verify that they were free of any pre-existing disease that may confound the study. There were no significant differences in age, height or weight or pulmonary function of the monkeys prior to the start of the study. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited, biosafety level 2+ primate facility at the University of Pittsburgh. Animal husbandry and experimental procedures were conducted in accordance with standards set forth by the Guide for the Care and Use of Laboratory Animals (2) and the Provisions of the Animal Welfare Act. Prior to the initiation of this study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.3.2 SHIV and Pc infection and development of COPD in cynomolgus macaques

SHIV inoculation and Pc infection were performed as previously described (326). Briefly, monkeys were intravenously inoculated with $1x10^{4.9}$ $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{89.6P}$ (gift of Dr. Opendra Narayan, University of Kansas), which induces CD4+ T cell lymphopenia and AIDS-like disease with wasting and opportunistic infections (269, 286). Monkeys were monitored for disease progression by monthly quantitation of viral load (29) and by analysis of peripheral blood CD4+ T cell levels by flow cytometry (65).

To facilitate natural transmission of Pc, immediately following SHIV-inoculation, monkeys were co-housed with other SIV or SHIV/Pc co-infected macaques (184). Monthly evaluations for Pc colonization were performed by nested-PCR on DNA extracted from BAL samples and by increases in Pc-kexin-specific plasma antibodies (29, 184).

Spirometry, quantitative high resolution computed tomography (HRCT) scanning, and tissue morphometry were used to evaluate the progression of obstructive changes in SHIV and

SHIV/Pc monkeys, as previously described (326). Pulmonary function was evaluated at baseline and every other month after SHIV infection using whole body plethysmography and forced deflation technique (326). The forced expiratory volume in 0.4 seconds ($FEV_{0.4}$), peak expiratory flow (PEF), and mid-maximal expiratory flow (MMEF) was determined at baseline (uninfected monkeys) and then every other month up to 12 months post-infection. By termination of the experimental infection, 8 of 12 monkeys were colonized with Pc and had evidence of COPD (Pc+/COPD+). In contrast, 4 monkeys remained Pc-negative and had normal lung function throughout the study (Pc-/COPD-).

3.3.3 RNA isolation

Non-perfused lung tissue was recovered at necropsy, 10-12 months post-SHIV infection. Tissue was immediately immersed in RNAlater (Qiagen, Germantown, MD) and stored at -80° C until processing. For RNA isolation, 30-45 mg of left upper lobe was immersed in liquid nitrogen followed by pulverization with a pestle. The pulverized material was homogenized with a QIAshredder spin column (Qiagen) and total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA was submitted to the University of Pittsburgh Genomics and Proteomics Core Laboratory (GPCL) for assessment of RNA quality and concentration by Agilent Bioanlyzer (Agilent Technologies, Santa Clara, CA) and spectroscopy, respectively.

3.3.4 Preparation of biotinylated cRNA

One sample of biotinylated cRNA for each animal was prepared from total RNA according to the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, the One-Cycle cDNA Synthesis Kit (Affymetrix) was used to reverse transcribe one microgram of total RNA to single-stranded cDNA containing a T_7 RNA polymerase promoter sequence that was then converted to double-stranded cDNA. After clean up of the double-stranded cDNA using the Sample Cleanup Module (Affymetrix), the entire amount was converted to biotinylated RNA with a GeneChip IVT Labeling Kit (Affymetrix). The biotin-labeled RNA was purified with the Sample Cleanup Module (Affymetrix) and quality of the product was assessed by the GPCL with an Agilent Bioanalyzer (Agilent Technologies).

3.3.5 Microarray assay and chip analysis

The GeneChip Expression Analysis Technical Manual was used for microarray assay and chip analysis. Briefly, 20 μg of biotinylated cRNA was fragmented into segments of 35 to 200 bases (confirmed by Agilent Bioanalyzer). Fifteen micrograms of the fragmented RNA was added to hybridization cocktail and applied to the GeneChip Rhesus Macaque Genome Array (Affymetrix) followed by overnight incubation at 45ºC with rotation. The Affymetix 450 Fluidics Station was then used for subsequent washing and staining with streptavidin-phycoerythrin. A GeneArray 3000 scanner with 7G upgrade (Affymetrix) was used to scan the developed chip. Basic absolute analysis was performed using Microarray Analysis Suite (MAS) 5.0 (Affymetrix) with each chip scaled to a median signal intensity of 150. MAS 5.0 calculates a detection *p*-value providing a measure of the probability that the gene is present in the

transcriptome of the sample and therefore a measure of the reliability of the calculated signal value. Comparative measures between chips were performed by analyzing the signal log ratios reflecting the level of change in gene expression between samples, and a change in *p*-value thus estimated the likelihood that the expression levels were truly different between the samples.

3.3.6 Gene expression analysis

Gene expression data analysis was performed at the University of Pittsburgh's GPCL Bioinformatics Analysis Core. Alternative tests for identifying differentially expressed (DE) genes are known to exhibit different amounts of internal consistency, and alternative methods for normalization and transformation are also known to influence the reproducibility of various tests for differential expression (166). Therefore, various combinations of tests, normalization and transformation methods were performed on the raw gene expression profile data using caGEDA (cancer gene expression data analyzer) (http://bioinformatics2.pitt.edu/GE2/GEDA.html). Efficiency analysis of these competing normalization methods and tests was subsequently performed as previously described (166). Efficiency analysis compares the internal reproducibility of gene lists for various methods of analysis by comparing the percentage of overlapping genes found at various test thresholds in independently analyzed random splits (subsamples) of the datasets. Efficiency analysis was used to identify the optimal test, normalization method + threshold of differential expression for our study using 30 splits. Pathways analysis was performed with Ingenuity Pathway Analysis software (Ingenuity, Mountain View, CA).

3.3.7 Quantitative RT-PCR (qRT-PCR)

To validate microarray results, RNA isolated from lung tissue was first reverse transcribed to produce cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD) according to manufacturer's instructions. Twenty five nanograms of cDNA were then used for qRT-PCR in triplicate reactions for each primer set in the QuantiTect SYBR Green PCR kit (Qiagen) according to manufacturer's instructions. Reproducibility of the assay was further confirmed by performing a second assay with separate RNA preparations. Primers were designed using Primer-BLAST found on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). The primers (Table 3-3) were selected from NCBI database entries for individual *Macaca mulatta* genes. Amplicon specificity was confirmed by performance of a post-PCR melt curve analysis. Primer efficiency was validated by performing the assay for individual targets with serial dilutions of cDNA template. Efficiency was confirmed by correlation of increasing threshold cycle (C_T) (the first cycle number with detectable fluorescence above background) values with decreasing concentrations of template.

Relative quantitation of gene expression levels obtained by qRT-PCR was performed using the delta (Δ) C_T method. C_T values of the Pc+/COPD+ group and the Pc-/COPD- group were both normalized to the endogenous housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) followed by determination of relative expression according to the following equations:

$$\underline{\text{Normalization}}: -\Delta C_T = (\text{mean } C_T(\text{GAPDH}) - \text{mean } C_T(x))$$

$$\underline{\text{Relative Expression}}: 2^{(-\Delta\Delta C_T)}$$

where mean C_T(GAPDH) is the mean GAPDH C_T value for a group of animals (Pc+/COPD+ or Pc-/COPD-) and mean C_T(x) is the mean C_T value for the gene of interest for the same group of animals.

Fold increase or decrease of the Pc+/COPD+ animals relative to the Pc-/COPD- animals was then calculated by dividing the relative expression of the Pc+/COPD+ group by that of the Pc-/COPD- group.

3.3.8 Statistical analyses

Differences in gene expression profiles over time were tested using restricted maximum likelihood.

Linear correlation analysis of the microarray and qRT-PCR data was performed using Prism software (GraphPad, La Jolla, CA).

Changes in cytokine levels in the bronchoalveolar lavage fluid (BALF) were compared between baseline and various time points after SHIV infection by Student's paired *t* test (GraphPad).

Tests for pulmonary function were performed using the R environment for statistical analysis and graphics in which mixed linear models were used to estimate and test the relationship among pulmonary function profiles (dependent variable), Pc colonization (independent variable), and time (independent variable).

Significance in pathway analysis was calculated by Fisher's exact test. In this method, user-specified genes of interest (all of the differentially expressed genes) were entered into the Ingenuity Pathway Analysis database (www.ingenuity.com). A *p* value associated with a particular canonical pathway was determined by comparing the number of genes of interest that

occur in the pathway to the total number of genes in the pathway. Pathways containing more genes of interest than expected by chance were considered significantly affected.

In all analyses, a *p* value less than 0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Differentially expressed genes between COPD and non-COPD macaques as revealed by microarray analysis

We previously developed a nonhuman primate model of HIV-related COPD using SHIV and Pc co-infection in cynomolgus macaques (326). In the present study, we used these same animals to examine gene expression profiles in lung tissue from SHIV/Pc co-infected monkeys with COPD (Pc+/COPD+) and compared them to monkeys infected with SHIV alone (Pc-/COPD-), which had normal lung function. Table 3-1 shows pulmonary function data from before and after SHIV infection grouped by those that became colonized with Pc and those that did not.

Table 3-1

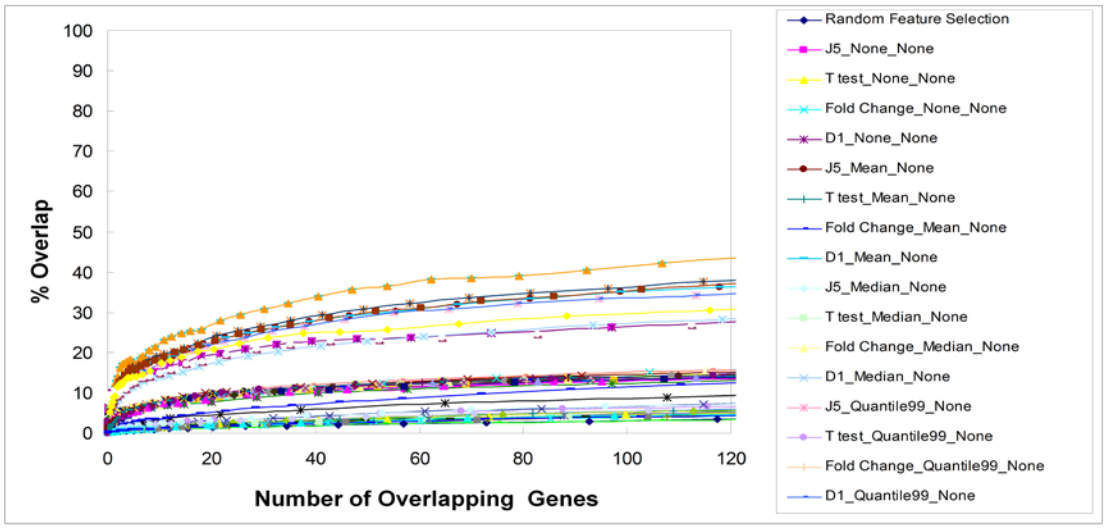
Pulmonary function measurements in monkeys pre- and post-SHIV infection

Parameter	Baseline	Endpoint	p value
Pulmonary Function*			
PEF, mL/s (SHIV/Pc+)	526.8 ± 19.0	452.9 ± 13.5	0.02
PEF, mL/s (SHIV/Pc-)	518.8 ± 13.2	522.0 ± 3.6	0.85
$FEV_{0.4}$, mL (SHIV/Pc+)	188.6 ± 5.9	165.8 ± 5.7	0.02
$FEV_{0.4}$, mL (SHIV/Pc-)	185.0 ± 6.7	185.3 ± 6.4	0.98
$FEF_{25–75\%}$, mL/s (MMEF) (SHIV/Pc+)	471.9 ± 18.8	405.5 ± 14.1	0.04
$FEF_{25–75\%}$, mL/s (MMEF) (SHIV/Pc-)	451.3 ± 15.5	470.3 ± 8.3	0.29

*PEF, Peak expiratory flow; $FEV_{0.4}$, forced expiratory volume in 0.4 seconds; FEF25-75% (MMEF), forced expiratory flow from 25% to 75% of forced vital capacity or maximum mid-expiratory flow. Values are means ± SEM followed by the p value for comparison of baseline (before SHIV infection) and endpoint values (study termination) for animals that became colonized with *Pneumocystis* (SHIV/Pc+) and those that remained non-colonized (SHIV/Pc-).

Microarray analysis was performed on cRNA prepared at the termination of SHIV infection. The array employed for this study contained 52,024 probe sets representing >20,000 genes. The microarray hybridization initially generated four non-normalized data sets: Probe Logarithmic Intensity Error (PLIER) Workflow Perfect Match (PM)-Mismatch (MM), PLIER Workflow PM-only, Robust Multi-array Analysis (RMA) PM-only and PM-only. These raw data were subjected to an efficiency analysis comparing the degree of overlap of DE genes between random subsets of the Pc+/COPD+ and Pc-/COPD- data sets over a range of testing methods to determine the normalization/feature selection combination that yielded the most internally consistent gene set (gene set identified to be differentially expressed by the greatest number of testing methods) (166). In the analyses of each of the raw data sets, the PM-only data yielded the most internally consistent results. Figure 3-1A shows plots of the various efficiency curves resulting from the 252 methods applied to the PM-only data. From these plots, we determined that the optimal method (i.e. the one demonstrating the highest internal consistency) was the one in which quantile 95 transformation and the J5 test were applied to the data without any normalization (Figure 3-1B). The optimal threshold for the J5 test was found to be 23.9. Consequently, when the absolute value of 23.9 was used as a minimum cutoff point, 243 genes in the Pc+/COPD+ animals were differentially expressed (DE) as compared to the Pc-/COPD- animals (Appendix B).

A.



B.

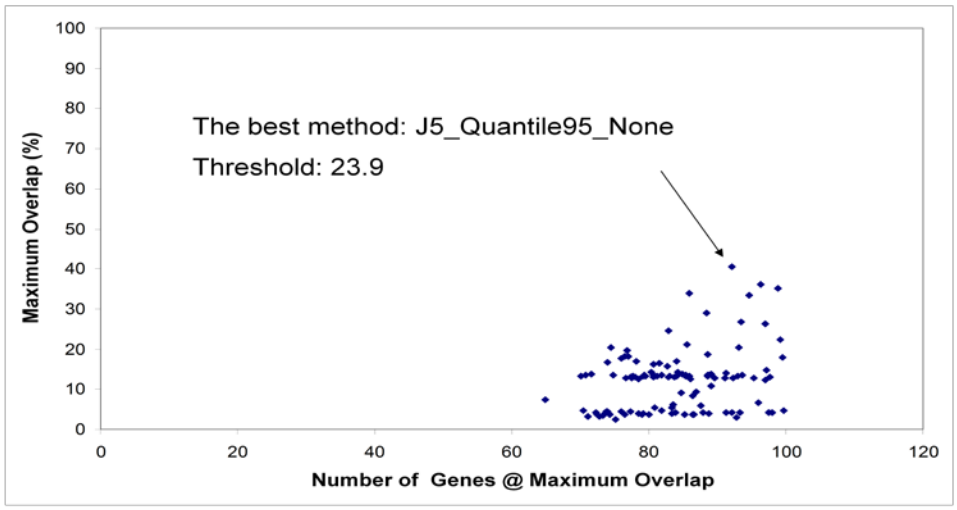


Figure 3-1 Efficiency analysis curves used to determine the most internally consistent test method for identifying differentially expressed genes between SHIV/Pc+ and SHIV/Pc-. Raw PM-only data obtained from microarray hybridization were subjected to all possible permutations of nine different normalizations, seven different transformations and four different tests for differential expression for a total of 252 possible methods. (A) Plot of all the methods for percent of overlap versus number of overlapping genes. (B) Plot of the maximum percent of overlap for the range 0-100 overlapping genes versus the number overlapping genes occurring at maximum overlap.

3.4.2 Immune response genes

Transcripts that may be associated with Pc infection or COPD pathogenesis were selected from the 243 DE genes for further analysis. These included transcripts encoding proteins associated with antigen presentation, β-2-microglobulin (β2m), a component of all major histocompatibility complex I molecules, and the invariant chain (CD74), a marker for MHC II molecules, which were both found to be upregulated in the Pc+/COPD+ macaques. These results are consistent with the fact that although both groups of monkeys were infected with SHIV, only the COPD+ monkeys had detectable lung colonization with Pc that might be expected to activate the acquired immune response in the local environment of the lung. Additionally, genes associated with inflammation and innate immunity were upregulated in Pc+/COPD+ macaques, including alternative macrophage activation-associated CC chemokine (AMAC-1, aka CCL18 or PARC) and surfactants, A2, B, C and D.

3.4.3 Protease/Anti-protease genes

A protease/anti-protease imbalance in lung tissue has been proposed to play a role in COPD lung injury (218). Microarray analysis revealed upregulation of the cysteine protease, cathepsin H (CatH), in the Pc+/COPD+ tissues and downregulation of protease inhibitors, alpha-1 antichymotrypsin (α1-ACT) and secretory leukocyte protease inhibitor (SLPI).

3.4.4 Tissue Homeostasis and oxidative stress genes

Repeated cycles of tissue destruction and repair and dysregulated apoptosis have been postulated to promote COPD pathology (382). Several genes associated with tissue homeostasis were differentially expressed in Pc+/COPD+ vs. Pc-/COPD- lung tissue, including upregulation of ornithine decarboxylase antizyme (ODC-Az), and downregulation of connective tissue growth factor (CTGF). Both of these molecules play key roles in tissue maintenance. Additionally, although it did not appear as differentially expressed by microarray analysis, pathways analysis using DE genes as input indicated that VEGF expression was significantly affected.

Oxidative stress has also been reported to contribute to COPD pathogenesis. Glutathione peroxidase 4 (GPX4), which has a role in protection from oxidative damage was upregulated as was expression of mitochondrial aldehyde dehydrogenase 2 (ALDH2), an enzyme important for aldehyde oxidation, in Pc+/COPD+ lung tissue.

3.4.5 Confirmation of microarray results by qRT-PCR

Independent analyses of expression levels of a subset of DE genes identified by microarray were performed using quantitative real time-PCR (qRT-PCR). Gene expression levels obtained by qRT-PCR were normalized to the GAPDH gene and expressed as fold increase or decrease relative to the COPD- group of animals. When microarray and qRT-PCR measurements were compared, the patterns of gene expression (up- or down-regulation) were similar for all the genes listed in Table 3-2 (Figures 3-2, 3-3).

Table 3-2

Differentially expressed genes between Pc+/COPD+ and Pc-/COPD- macaques

Category	Description	J5 Score	Expression Ratio (SHIV/Pc+:SHIV/Pc-)	
			Microarray	qRT-PCR
Antigen Presentation	major histocompatibility complex I (MHC I) β-2-microglobulin (β2m)	+55.5	1.17	1.05
	major histocompatibility complex II (MHC II) invariant chain (CD74)	+33.7	1.29	1.13
Inflammation	pulmonary surfactant A2	+85.1	1.42	2.69
	pulmonary surfactant B	+83.6	1.27	1.90
	pulmonary surfactant C	+110.9	1.46	1.99
	pulmonary surfactant D	+62.2	1.32	2.20
	alternative macrophage activation-associated CC chemokine (AMAC-1, aka PARC or CCL18)	+32.0	2.62	2.51
Protease	cathepsin H (CatH)	+25.0	1.19	2.19
Anti-protease	α1-antichymotrypsin (α1-ACT)	-35.8	0.35	0.29
	secretory leukocyte protease inhibitor (SLPI)	-39.5	0.41	0.86
Redox Balance	glutathione peroxidase 4 (GPX4)	+29.3	1.33	1.90
Apoptosis	ornithine decarboxylase antizyme (ODC-Az)	+54.1	1.25	1.28
	connective tissue growth factor (CTGF)	-55.1	0.47	0.35
	vascular endothelial growth factor (VEGF)	*ND	*ND	0.36

*ND, not significantly different

Table 3-3

Genes and primers used in qRT-PCR

Gene	Amplicon (bp)	Primer Sequence
α1-antichymotrypsin (α1-ACT)	209	Forward 5'-gtctgaggagggcacagaag-3'
		Reverse 5'-tactgagagccccactgctt-3'
cathepsin H (catH)	222	Forward 5'-ctttgccttcgaggtgactc-3'
		Reverse 5'-aggccacacatgttctttcc-3'
connective tissue growth factor (CTGF)	239	Forward 5'-atccgtacccccaaaatctc-3'
		Reverse 5'-aagatgtcattgtctcccgg-3'
glutathione peroxidase 4 (GPX4)	219	Forward 5'-gtaaccagttcgggaagcag-3'
		Reverse 5'-agccgttcttgtcaatgagg-3'
β2-microglobulin (β2m)	200	Forward 5'-tggaggtttgaagatgccgcatttgg-3'
		Reverse 5'-gccctcctaaagctagctgccca-3'
invariant chain (CD74)	238	Forward 5'-aagcactccttggagcaaaa-3'
		Reverse 5'-taccactgcagttctggtgc-3'
secretory leukocyte protease inhibitor (SLPI)	243	Forward 5'-cttcaaagccggagtctgtc-3'
		Reverse 5'-tggccatccatctcacagta-3'
pulmonary surfactant A2 (SPA2)	201	Forward 5'-gcctaggcctctagggaaga-3'
		Reverse 5'-atcctaagacctggcacacg-3'
pulmonary surfactant B (SPB)	203	Forward 5'-gacactgcacactctggcat-3'
		Reverse 5'-agctgggctttgagcagata-3'
pulmonary surfactant C (SPC)	218	Forward 5'-ccgcagtgcctacctctaag-3'
		Reverse 5'-tctgcaaaagctgcaaaaga-3'
pulmonary surfactant D (SPD)	219	Forward 5'-ttgcaacagctggtcatagc-3'
		Reverse 5'-gaccacgagacgcttttctc-3'
ornithine decarboxylase antizyme (ODC-Az)	177	Forward 5'-tcacccacccctgaagcccc-3'
		Reverse 5'-ctgtgagcccggactggaggt-3'
alternative macrophage activation-associated CC chemokine (AMAC1, aka CCL18 or PARC)	161	Forward 5'-gccttgcagctgccctcctt-3'
		Reverse 5'-tggtttggtgcactgggggc-3'

Table 3-3 (continued)

vascular endothelial growth factor (VEGF)	157	Forward	5’-tgcatgccacgggaggtgtg-3’
		Reverse	5’-tgctgaggtagctcgtgctggt-3’
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	233	Forward	5’-gaaggtgaaggtcggagtcaa-3’
		Reverse	5’-gctcctggaagatggtgatg-3’

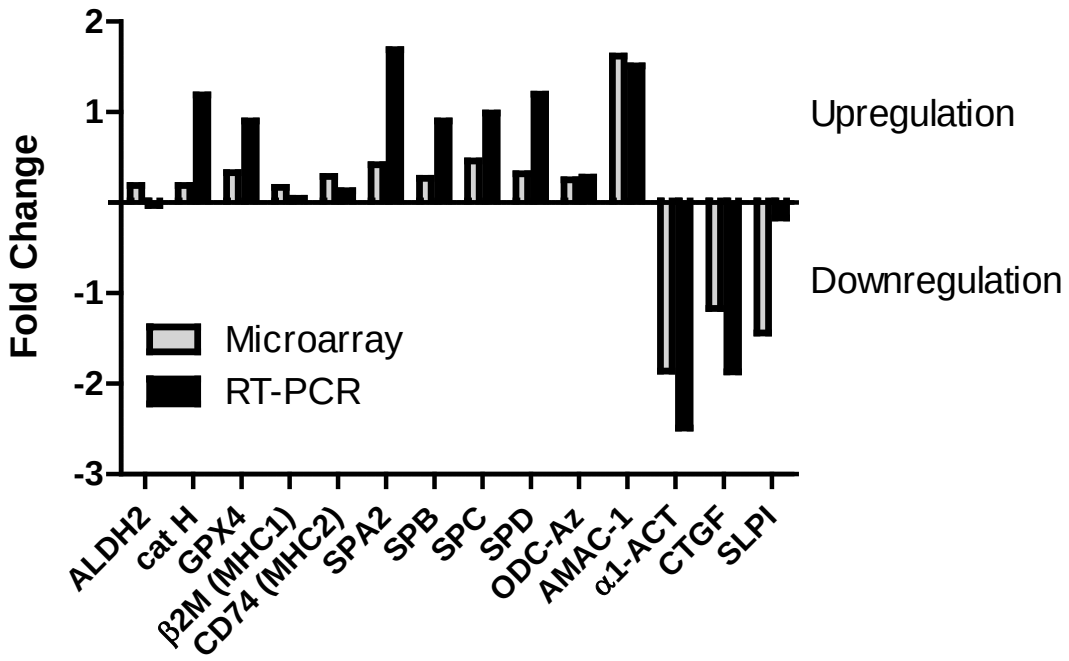


Figure 3-2 Quantitative real time-PCR of select genes validates microarray results. Fold change expression ratios with background subtracted for microarray (gray bars) and qRT-PCR (black bars) are shown aligned next to one another for the indicated genes. Fold change is expressed as the ratio of expression in the SHIV/Pc+ animals to expression in the SHIV/Pc- animals minus one (background) for ratios greater than one. For ratios less than one (gene is underexpressed in SHIV/Pc+ compared to SHIV/Pc- animals), one is subtracted from the reciprocal value and expressed as negative.

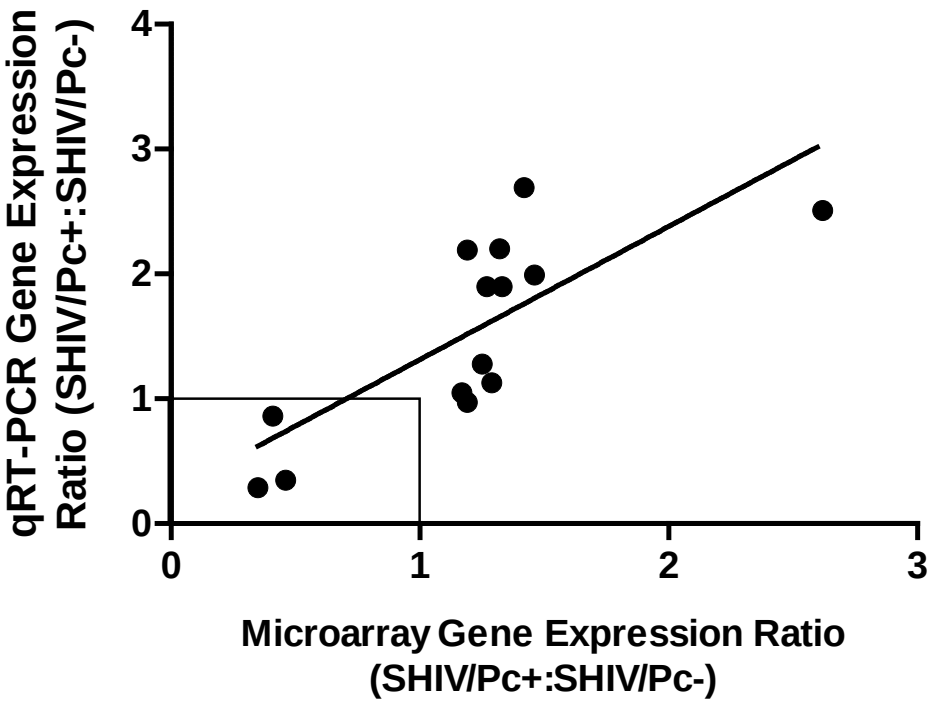


Figure 3-3 Linear regression analysis of expression ratios reveals correlation between the two data sets. Correlation between expression ratios derived from microarray analysis and qRT-PCR were analyzed by Pearson correlation test (p= 0.0013). The boxed area demarcates genes that were downregulated.

3.5 DISCUSSION

In this study, we used a nonhuman primate model of HIV infection to compare pulmonary gene expression in SHIV-infected, Pc-colonized monkeys with COPD to gene expression in SHIV-infected monkeys with normal lung function in order to determine DE genes important in COPD pathogenesis in this model. Analyses revealed 243 DE genes (Appendix B), of which a subset was further analyzed by qRT-PCR. Several classes of genes were differentially expressed in

Pc+/COPD+ vs non-COPD lung tissue, including several not previously associated with COPD. These gene classes included those related to host immune response, protease/antiprotease balance, tissue homeostasis and redox balance.

3.5.1 Immune response genes

Upregulation of the major antigen presenting gene complexes, MHC I and MHC II, was observed in lung tissue of the Pc+/COPD+ animals compared to the Pc-/COPD- animals. Previous histologic and morphometric analyses of airways of this SHIV-infected cohort revealed a significant increase in bronchus-associated lymphoid tissue (BALT) in the Pc+/COPD+ monkeys compared to Pc-/COPD- monkeys (326). The upregulation of genes associated with antigen presentation and the increased development of BALT in the Pc+/COPD+ tissue suggests the development of an adaptive immune response likely driven by persistent or repeated colonization with Pc or other infectious agents. These results are consistent with the findings of increased BALT associated with advanced COPD in humans (145) and the hypothesis that persistent microbial colonization may contribute to COPD pathogenesis or exacerbations (224, 288, 316, 326).

Multiple studies have shown an important link between inflammation and COPD (145, 178, 288, 360). While the pathogenesis of immune-mediated damage in COPD is generally associated with T helper (Th) 1-type effector mechanisms (127, 141, 220), evidence in experimental models of infection-associated COPD (180, 224) and in human COPD patients (180, 235, 388) implicate Th2-skewed responses, with increased levels of interleukin (IL)-4 and IL-13. These cytokines, induced as a consequence of persistent pulmonary infection, may contribute to the pathogenesis of COPD via induction of alternatively activated macrophages,

which express products associated with small airway remodeling (180). Consistent with these studies, our results show that the alternatively activated macrophage marker (AMAC-1, aka CCL18 or PARC) is upregulated in Pc+/COPD+ lung tissue. Furthermore, cytokine analysis of BAL fluid previously performed in the cohort described here demonstrated increased levels of IL-4, IL-5 and IL-13 in Pc+/COPD+ macaques compared to Pc-/COPD- monkeys (326).

The complex interplay between the surfactant system and Pc colonization in a SHIV-infected host likely influences multiple aspects of the host-pathogen interaction including immune responses necessary for clearance of the pathogen and modulation of inflammatory-mediated tissue damage. In an immunocompetent host, attachment of Pc to type I alveolar epithelial cells induces cell damage, leads to upregulation of surfactant proteins A and D, and an increase in production of inflammatory mediators (11, 51, 53, 153, 265, 355). These events promote macrophage activation and cytokine production leading to enhanced uptake and killing of the organisms, and balanced pro- and anti-inflammatory effects of surfactants A and D on alveolar macrophages and lymphocytes (30, 31, 67, 189, 293). In the SHIV immunosuppressed host, failure to clear colonizing Pc (or repeated Pc colonization) may promote persistent upregulation of surfactant proteins, potentially leading to dysregulated proinflammatory events and host tissue damage.

3.5.2 Protease/Anti-protease genes

A disruption in the normal balance between proteases and their inhibitors has been proposed as a key pathway in the breakdown of lung parenchyma resulting in emphysema (218). Much evidence has accumulated implicating neutrophil elastase (NE), other serine proteases and matrix

metalloproteases in COPD-related tissue destruction (211, 264, 312). The role of cathepsins in COPD is less clear; however, Zheng *et al*. have shown that IL-13-dependent induction of cathepsins (B, S, L, H and K) was associated with emphysema in an experimental murine model (387). The observation of increased expression of cathepsin H in Pc+/COPD+ tissue in the present study, along with evidence of IL-13 induction and Th2 skewing in this model (326) is consistent with the IL-13-dependent sub-type of emphysema (180, 224, 387). This sub-type may be particularly important in emphysema described in HIV-infected individuals (81), as changes from a Th1- to Th2-skewed response have been associated with progression to AIDS in HIV-infected individuals (183).

Consistent with a shift in protease/anti-protease balance in promotion of lung tissue destruction, we also observed downregulated expression of the serine protease inhibitors, SLPI and α1-ACT. In addition to its role in the inhibition of NE-mediated tissue destruction, SLPI has anti-microbial and anti-inflammatory properties (163, 384). Reduction in expression level of SLPI may not only promote increased proteolytic damage in the lung, but also impede Pc clearance and further disrupt the pro-/anti-inflammatory balance. While α1-antitrypsin is a major inhibitor of lung serine proteases and genetic variants are associated with early-onset emphysema (306), less is known about the role of α1-ACT in COPD. Alpha1-ACT may have a similar role as α1-antitrypsin in controlling protease-mediated lung tissue destruction as allelic variants are associated with increased frequency of COPD (152).

3.5.3 Tissue homeostasis and oxidative stress genes

Oxidative stress is caused by an imbalance in the generation of reactive oxygen species (ROS) and antioxidant mechanisms, leading to cellular damage. Increasing evidence suggests that

oxidative stress is an important contributor to the progression of COPD (217). While cigarette smoke is a key source of ROS (58), microbial interaction with inflammatory cells leads to activation and increased production of ROS (169, 285). The present analysis revealed differential expression of two genes associated with oxidative stress responses in Pc+/COPD+ vs. non-COPD tissue. Mitochondrial aldehyde dehydrogenase 2 (ALDH2) was modestly upregulated in Pc+/COPD+ tissues based on microarray analysis. ALDH2 is an enzyme involved in aldehyde oxidation and whose activation was recently shown to correlate with reduced ischemic heart damage in rodent models (49). Although primarily involved in alcohol detoxification, ALDH2 is emerging as an important cryoprotectant that is upregulated in response to oxidative damage (35). GPX4 was also upregulated in Pc+/COPD+ lung tissue. Upregulation of this molecule, which acts to protect cells from oxidative damage, may represent a response to oxidative stress highlighting a potential role for ROS-mediated tissue damage in early emphysema pathogenesis in Pc+/COPD+ monkeys.

Repeated cycles of tissue destruction and repair and a dysregulated apoptotic response have been postulated to promote COPD pathology (142, 150, 382). Several genes associated with tissue homeostasis and apoptosis were differentially expressed in Pc+/COPD+ vs. Pc-/COPD- lung tissue, including ornithine decarboxylase antizyme (ODC-Az), which inactivates ornithine decarboxylase (ODC) leading to its degradation in a ubiquitin-independent manner. ODC is the rate-limiting enzyme in the biosynthetic pathway for polyamines, which are required for cell growth and proliferation, and depletion of polyamines results in increased apoptosis (257). Thus, increased levels of ODC-Az may indirectly promote increased apoptosis in the Pc+/COPD+ lung. In addition, Pc+/COPD+ tissue had reduced expression of the gene

encoding CTGF, a key molecule associated with extracellular matrix (ECM) production and maintenance of lung architecture (72, 157).

Interestingly, decreased CTGF has been linked to decreased expression of vascular endothelial growth factor (VEGF) (157), a molecule implicated in apoptosis-mediated COPD pathogenesis (347). Although VEGF was not differentially expressed by microarray analysis, qRT-PCR showed decreased expression of VEGF in Pc+/COPD+ tissue. Additionally, biosynthetic pathways analysis showed that the VEGF pathway was significantly affected in expression of upstream molecules in Pc+/COPD+ tissue. These results are consistent with findings of decreased VEGF levels in the lung tissue of emphysema patients (347) and that blockage of VEGF leads to emphysema via apoptosis (176, 177).

3.5.4 Proposed model of early emphysema pathogenesis

In totality, these findings suggest a series of events that lead to the initial stages of emphysema. We, therefore, propose the following model for early stage COPD pathogenesis in immunocompromised individuals who become Pc-colonized (Figure 3-4).

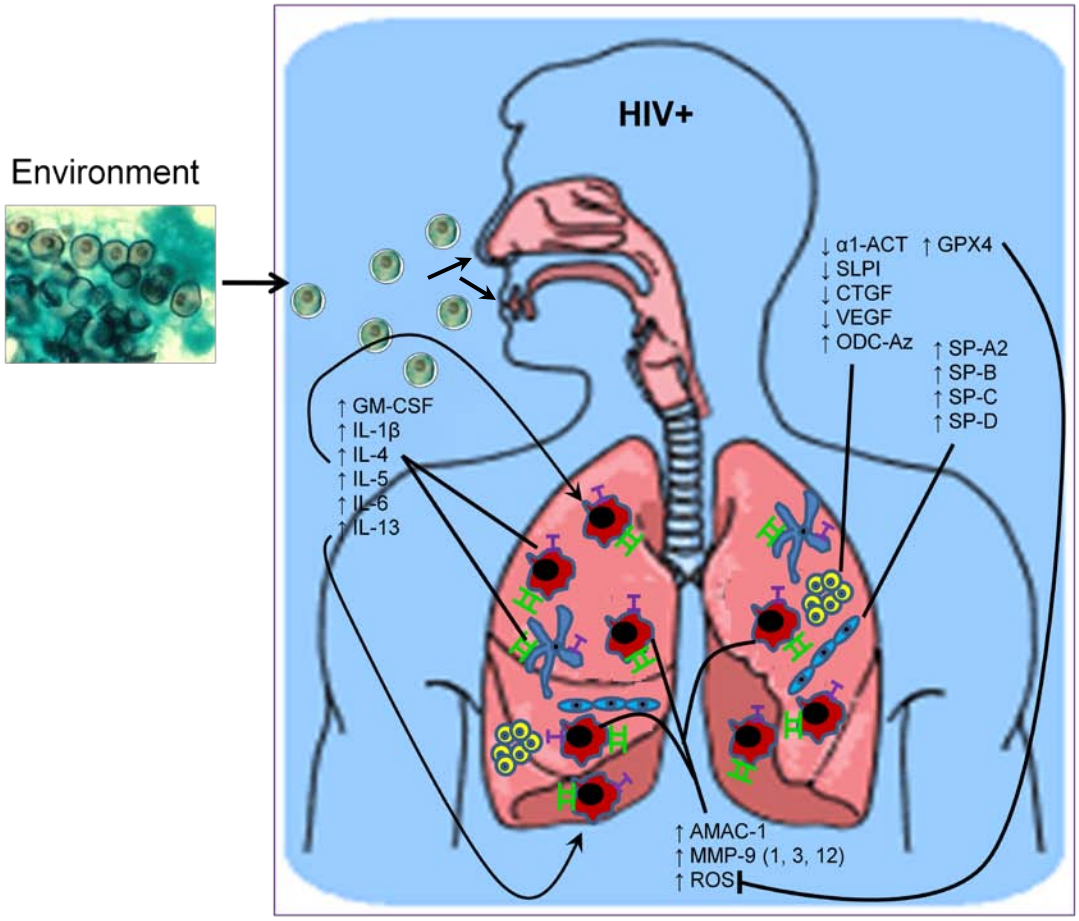


Figure 3-4 Early COPD pathogenesis model

Initially, a cascade of innate immune responses is set in motion as a result of Pc invasion of the lungs including upregulation of pulmonary surfactant expression by alveolar type II epithelial cells and downregulation of VEGF, CTGF, α1-ACT and SLPI by general structural lung cells, such as smooth muscle and endothelial cells. These same cells also upregulate ODC-Az. These events result in an immediate tilt of the balances involving apoptosis/proliferation and

protease/anti-protease activity. As the resident dendritic cells and ubiquitous macrophages encounter Pc and ingest/process it, they upregulate expression of MHC I and MHC II antigen presenting molecules and secrete several cytokines including: GM-CSF, IL-1β, IL-4, IL-5, IL-6, and IL-13. Exposure to these cytokines leads the macrophages to assume an alternatively activated phenotype leading to upregulation of MMPs and/or ROS. The presence of ROS results in increased apoptotic activity which structural cells attempt to counterbalance by upregulating GPX4. However, this event is overwhelmed by simultaneous overexpression of ODC-Az which further stimulates cellular apoptosis of lung tissue. The increased apoptosis combined with tissue degradation from the enhanced MMP secretion consequently result in early COPD development.

3.5.5 Concluding remarks

A number of studies have examined differentially expressed genes associated with COPD (27, 121, 256), but the present study is the first to characterize global pulmonary gene expression in a model of HIV-associated COPD. Thus, a number of previously unreported pathways emerged, most notably immune response genes associated with innate and acquired immune responses. There is a growing body of literature implicating Pc colonization with the development of COPD in human studies and animal models (55, 245, 258, 279), and the results of this study support the concept that amplification of the host innate and acquired immune responses to persistent Pc colonization likely promotes dysregulation of inflammatory responses, disruption of tissue homeostasis, and protease/anti-protease imbalance, ultimately leading to tissue destruction and altered lung mechanics. Although this study does not capture the initial stages of the Pc/SHIV-associated COPD, the primate model allows for future longitudinal genomic and proteomic

studies that will address the dynamic process of COPD pathogenesis and identify specific, novel therapeutic targets.

3.6 ACKNOWLEDGEMENTS AND AUTHOR CONTRIBUTIONS

3.6.1 Acknowledgements

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3.6.2 Author contributions

Timothy W. Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) designed and supervised the research, generated the majority of the data, performed analyses and prepared the manuscript. Heather M. Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) provided assistance with

nonhuman primate sample processing and analysis. Alison Morris (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) provided input on research design and analysis/critique of the project, data and the manuscript. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine) as the mentor and principal investigator on the project, provided extensive scientific knowledge, insight and critique of the project and manuscript. All authors, especially Karen A. Norris, contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

4.0 KINETICS OF EARLY PULMONARY FUNCTION DECLINE AND COPD ASSOCIATED WITH PNEUMOCYTSIS COLONIZATION IN A SIMIAN MODEL OF HIV INFECTION

4.1 INTRODUCTION

There is a vital need for effective treatment regimens for chronic obstructive pulmonary disease (COPD) as it is on course to become the third leading cause of death worldwide by the year 2020 (251). Despite enormous efforts directed to this end, beneficial therapies remain elusive due to the complexity of the disease (42, 57). Smoking is widely accepted as the primary risk factor for development of COPD, but only about 15% of smokers develop the disease (93) suggesting involvement of other factors that may be genetic or environmental. In particular, mounting evidence points to a role for infectious agents in development of COPD. For example, *Haemophilus influenzae* and adenovirus have both been implicated in exacerbations and/or COPD development (144, 320).

The contribution of infectious pathogens may be especially relevant in HIV+ subjects who are at increased risk for development of COPD and an accelerated form of emphysema (66, 80). *Pneumocystis jirovecii*, the causative agent of *Pneumocystis* pneumonia (PcP), is an opportunistic fungal lung pathogen that has been linked to the development of COPD in HIV+ patients when present in the lungs at subclinical levels (colonization) (241). Even with the

introduction of powerful antiretroviral drugs and anti-*Pneumocystis* prophylaxis, colonization can still be detected in the HIV+ population at rates as high as 69% (147). Furthermore, Pc colonization has been associated with COPD in non-HIV-infected patients (245). Animal models of *Pneumocystis* (hereafter, "Pc") infection and colonization also support a role for Pc in development of obstructive lung disease (55, 258).

In a simian model of HIV infection, we previously showed development of airway obstruction and radiologic and histologic evidence of emphysema in cynomolgus macaques infected with a chimeric simian/human immunodeficiency virus (SHIV) that became naturally colonized with Pc (326). These results identify Pc as a potentially treatable risk factor in COPD pathogenesis. Therefore, in the current study, we used this model to evaluate the kinetics of pulmonary function decline and determine whether clearance of Pc colonization by treatment with trimethoprim-sulfamethoxazole (TMP-SMX) altered the progression of disease.

4.2 METHODS

4.2.1 Animals

Seventeen adult, Chinese origin cynomolgus macaques (*Macacca fasicularis*), weighing between 5-8 kg, were used in this study. All animals were purchased from National Primate Centers or vendors approved by the University of Pittsburgh, Department of Laboratory Animal Research. Prior to admission to the study, all animals underwent complete physical examination (pulmonary and cardiac auscultation, thoracic radiographs, computer tomography scanning, tuberculin skin testing, complete blood count, chemistry panel, urinalysis, and flow cytometric

analysis of peripheral blood mononuclear and BAL cells) and were screened for simian retroviruses; SIV, SRV, and STLV to verify that they are free of any pre-existing disease that may confound the study. There were no significant differences in age, height or weight or pulmonary function of the monkeys prior to the start of the study. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited, biosafety level 2+ primate facility at the University of Pittsburgh. Animal husbandry and experimental procedures were conducted in accordance with standards set forth by the Guide for the Care and Use of Laboratory Animals (2) and the Provisions of the Animal Welfare Act. Prior to the initiation of this study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.2.2 SHIV and Pc infection and development of COPD in cynomolgus macaques

SHIV and Pc infection of the monkeys used in this study was previously described (326). Briefly, monkeys were intravenously inoculated with $1x10^{4.9}$ $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{89.6P}$ (gift of Dr. Opendra Narayan, University of Kansas), which induces CD4+ T cell lymphopenia and AIDS-like disease with wasting and opportunistic infections (269, 286). Monkeys were monitored for disease progression by monthly quantitation of viral load (269) and by analysis of peripheral blood CD4+ T cell levels by flow cytometry (184).

To facilitate natural transmission of Pc, immediately following SHIV-inoculation, monkeys were co-housed with other SIV or SHIV/Pc co-infected macaques (184). Monthly evaluations for Pc colonization were performed by nested-PCR on DNA extracted from BAL samples and by increases in Pc-kexin-specific plasma antibodies (29, 184).

Whole body plethysmography and forced deflation technique were used to evaluate progression of obstructive changes in SHIV/Pc+ and SHIV/Pc- monkeys, as previously described (326). As a readout for airway obstruction, the forced expiratory volume in 0.4 seconds ($FEV_{0.4}$) and peak expiratory flow (PEF) were determined at baseline (uninfected monkeys) and at monthly intervals up to 18 months post-infection.

4.2.3 TMP-SMX treatment

To eradicate Pc, TMP-SMX was administered daily (TMP: 20 mg/kg, SMX: 100 mg/kg SMX) for the duration of the study to a subset of the monkeys that had exhibited significant airway obstruction due to Pc colonization. Additionally, the group of animals that did not become colonized with Pc also received TMP-SMX treatments for the remainder of the study.

4.2.4 Statistical analyses

All analyses were performed using Prism software (GraphPad, La Jolla, CA). For all analyses, a *p* value less than 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 Baseline characteristics were not different between groups

Once animals' colonization statuses and dispositions regarding TMP-SMX treatment were known, we were able to perform statistical analyses to determine if there were differences

between physical characteristics or pulmonary function in the groups at the onset of the study that might factor into experimental differences that may arise during the course of the study. We found no differences in weight, height or pulmonary function between the animals that became colonized with Pc and those that remained non-colonized, nor did we discover any differences between the Pc+ macaques that were treated with TMP-SMX versus those that received no treatment (Tables 4-1, 4-2).

Table 4-1

Physical characteristics and pulmonary function of SHIV/Pc- and SHIVPc+ groups at baseline

Parameter	Pc negative*	Pc positive	p value
Height, cm	66.0 (63.5 – 76.2)	63.5 (61.0 – 78.7)	0.49
Weight, kg	7.3 (6.3 – 8.9)	7.0 (5.5 – 10.4)	0.94
Pulmonary Function Parameters[†]			
$FEV_{0.4}$, ml	195.5 (157.7 – 214.9)	201.1 (164.7 - 214.9)	0.47
PEF, ml/s	530.4 (436.8 – 563.8)	563.4 (455.7 – 604.8)	0.11

*Comparison of baseline values of animals that were subsequently infected with $SHIV_{89.6P}$ and remained uncolonized (Pc negative) or became colonized with Pc (Pc positive). All comparisons were by unpaired *t* test.

[†]$FEV_{0.4}$, forced expiratory volume in 0.4 seconds; PEF, peak expiratory flow.

Values are medians with ranges shown in parentheses.

Table 4-2

Baseline physical characteristics and pulmonary function of Pc-colonized animals divided into TMP-SMX treatment and no TMP-SMX treatment groups.

Parameter	TMP-SMX negative*	TMP-SMX positive	p value
Height, cm	64.8 (61.0 – 66.0)	63.5 (61.0 – 78.7)	0.49
Weight, kg	7.4 (6.8 – 9.5)	7.0 (5.5 – 10.4)	0.53
Pulmonary Function Parameters[†]			
$FEV_{0.4}$, ml	204.2 (189.4 – 214.9)	201.1 (164.7 – 213.4)	0.41
PEF, ml/s	571.8 (527.7 – 604.8)	563.4 (455.7 – 596.4)	0.44

*Comparison of baseline values of animals subsequently infected with $SHIV_{89.6P}$ that became colonized with Pc and divided into no TMP-SMX treatment (TMP-SMX negative) and TMP-SMX treatment (TMP-SMX positive) groups. All comparisons were by unpaired *t* test.

[†]$FEV_{0.4}$, forced expiratory volume in 0.4 seconds; PEF, peak expiratory flow.

Values are medians with ranges shown in parentheses.

4.3.2 SHIV disease was not different between Pc-colonized and non-colonized monkeys

In order to confirm that immune deficiencies arising as a result of SHIV infection were not different between animals that became colonized with Pc and those that did not become colonized, we evaluated parameters relevant to SHIV disease. To do this, we assessed peak viral loads and peripheral blood CD4+ T cell counts (Figure 4-1).

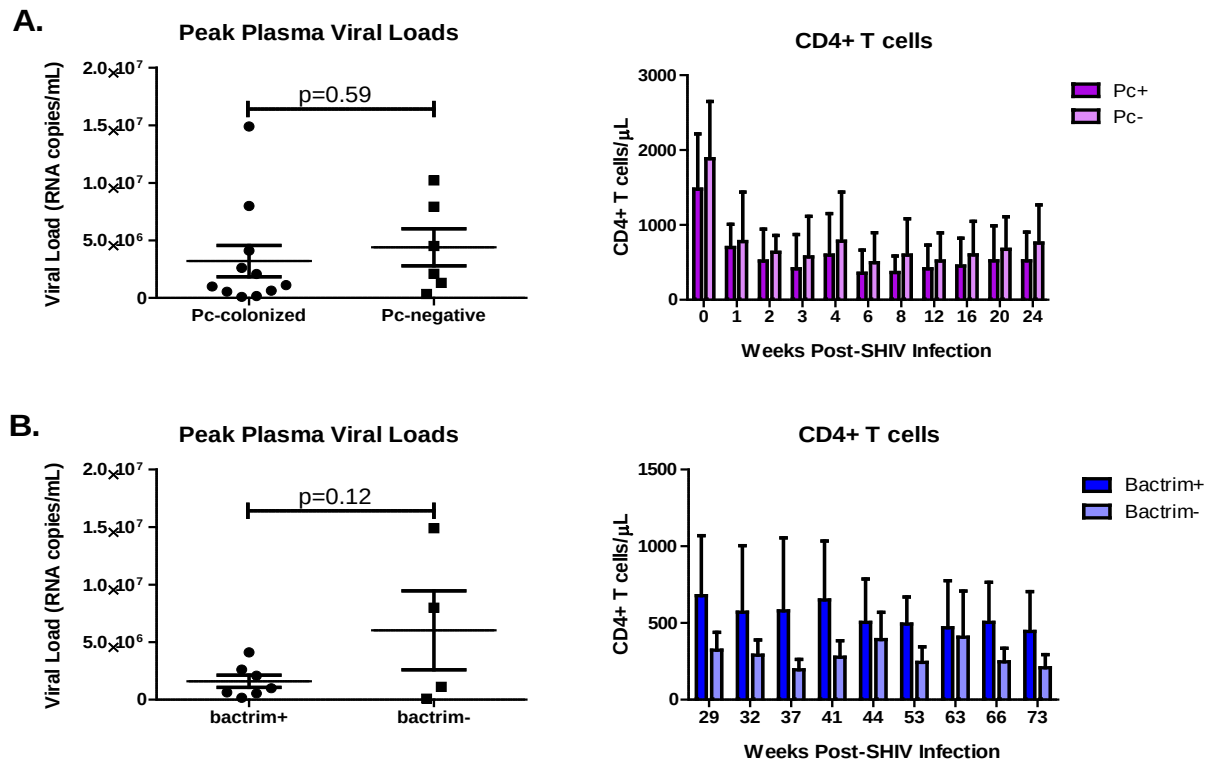


Figure 4-1 Disease progression between groups is not different. Peak plasma viral load and CD4+ T cell counts were monitored to evaluate disease progression. Up to initiation of TMP-SMX treatment (A) There were no differences between groups separated by Pc colonization status in peak plasma viral loads (p=0.59 by unpaired *t* test) or peripheral blood CD4+ T cell counts (p=0.89 by two-way repeated measures ANOVA). After TMP-SMX treatment began for the Pc-colonized animals (B) There were no differences between TMP-SMX groups in peak plasma viral loads (p=0.12 by unpaired *t* test) or peripheral blood CD4+ T cell counts for the duration of the study (p=0.78 by two-way repeated measures ANOVA).

4.3.3 Pc colonization in SHIV-immunosuppressed macaques results in airway obstruction

Recapitulating results from our previous study, animals that became colonized with Pc demonstrated airway obstruction (326) (Figure 4-2). By 25 weeks post-SHIV infection,

significant declines in pulmonary function were observed in the monkeys that had become colonized with Pc, but not the non-colonized animals.

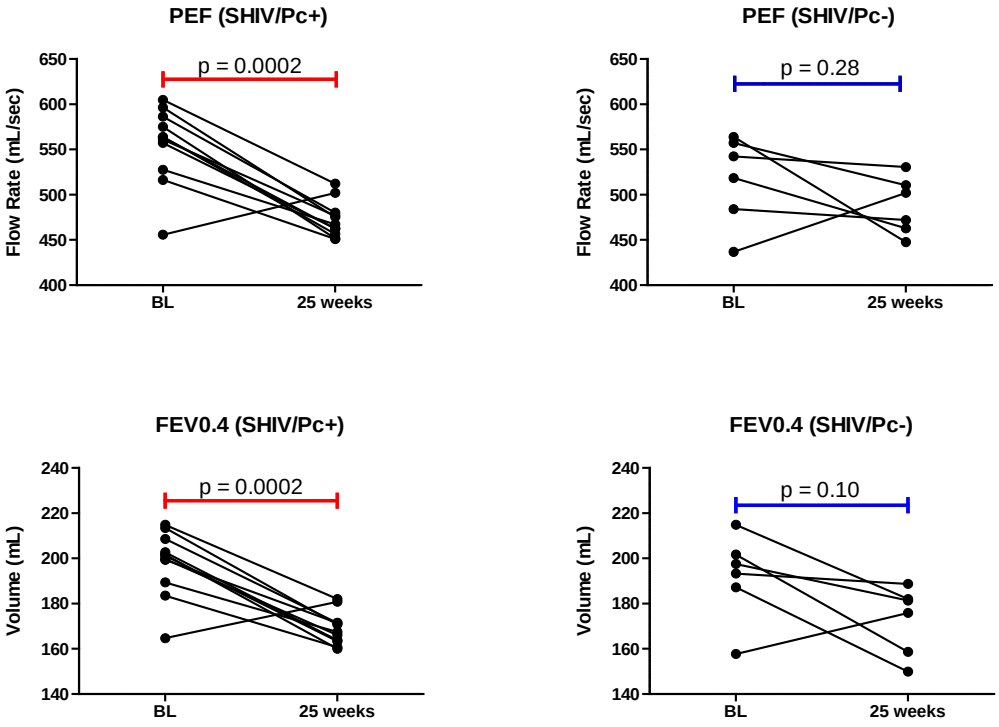


Figure 4-2 *Pneumocystis* colonization results in pulmonary function decline. Whole body plethysmography was used to evaluate peak expiratory flow (top) and forced expiratory volume in 0.4 seconds (bottom) in cynomolgus macaques that became colonized with Pc (left) or remained non-colonized (right) after SHIV infection. Displayed p values were obtained by performing paired *t* test on baseline data versus week 25 post-SHIV infection data for each of the shown parameters for each group (SHIV/Pc+ or SHIV/Pc-).

4.3.4 TMP-SMX treatment results in clearance of Pc colonization

To determine whether antibiotic clearance of Pc affected pulmonary function decline, TMP-SMX was administered to a subset of the SHIV/Pc+ monkeys and the SHIV/Pc- control group. Figure 4-3 shows IgG antibody profiles and PCR results of one representative animal each from the TMP-SMX treatment group (left) and the untreated group (right). The decline in anti-KEX1 antibody titers combined with the absence of positive Pc DNA PCR results after the start of TMP-SMX treatment indicate that the drug therapy was effective in clearing the Pc (184).

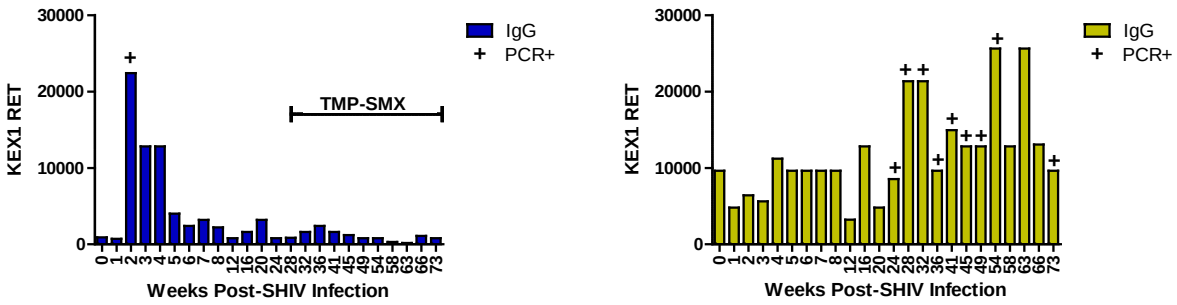


Figure 4-3 TMP-SMX treatment clears Pc colonization in SHIV-infected macaques. To arrest pulmonary function decline, a subset of the SHIV/Pc+ animals received TMP-SMX. Anti-KEX1 IgG antibody profiles and PCR data are shown for representative animals from the TMP-SMX treatment group (left) and the untreated group (right).

4.3.5 Administration of TMP-SMX 25 weeks post-SHIV infection does not arrest further pulmonary function decline

As expected, neither the originally non-colonized animals on TMP-SMX nor the previously Pc-colonized animals on TMP-SMX demonstrated any further development of airway obstruction up to 12 months after initiation of TMP-SMX (Figure 4-4).

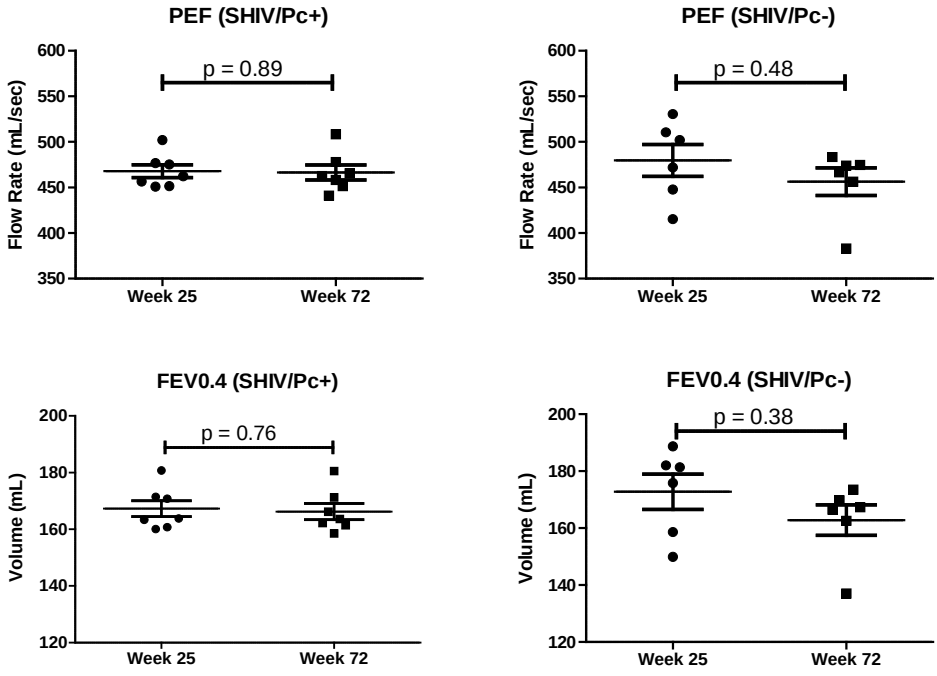


Figure 4-4 Eradication of *Pneumocystis* colonization results in leveling off of pulmonary function. Whole body plethysmography was used to evaluate PEF (top) and $FEV_{0.4}$ (bottom) in cynomolgus macaques that became colonized with Pc and then treated with TMP-SMX (left) or remained non-colonized (right) after SHIV infection. Displayed p values were obtained by performing paired *t* test on week 25 post-SHIV infection (TMP-SMX treatment initiated) data versus week 72 post-SHIV infection data for each of the pulmonary function parameters for each group (SHIV/Pc+ or SHIV/Pc-).

Unexpectedly, the group of animals not on TMP-SMX but that was Pc-colonized also did not develop any further airway obstruction (Figure 4-5).

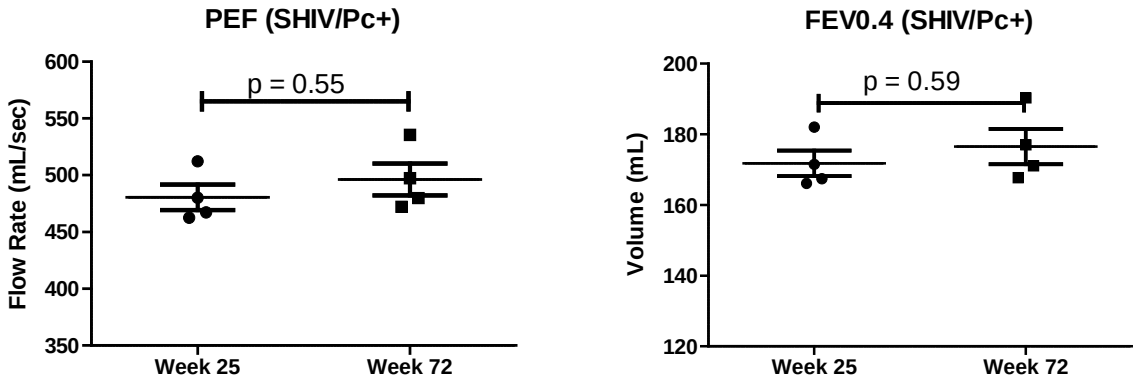


Figure 4-5 *Pneumocystis* colonization has no further effect on pulmonary function after initial induction of pulmonary function declines. Whole body plethysmography was used to evaluate PEF (left) and $FEV_{0.4}$ (right) in cynomolgus macaques that became colonized with Pc and allowed to continue in Pc-associated airways disease progression. Displayed p values were obtained by performing paired *t* test on week 25 post-SHIV infection data versus week 72 post-SHIV infection data for each of the pulmonary function parameters.

4.4 DISCUSSION

To establish a definitive link between Pc colonization and development of airway obstruction, we infected cynomolgus macaques with SHIV to induce immunosuppression and susceptibility to Pc colonization. As in our previous study (326), animals that became colonized with Pc, but not those that remained uncolonized, showed significant declines in pulmonary function by week 25 post-SHIV infection. Treatment of a subset of the SHIV/Pc+ macaques eradicated Pc in these

animals. However, no effect was noted because Pc-associated pulmonary function decline appears to only take place early after colonization followed by a plateau in function as evidenced by the lack of continued progression in airway obstruction in the untreated Pc-colonized animals.

The possibility that infectious agents may play a role in the development of airway obstruction has been explored (144, 316). It has been hypothesized that their persistence in the lungs results in chronic inflammation and lung destruction in COPD (316). In particular, Pc has been linked to COPD pathogenesis in both human and animal studies (55, 241, 245, 258). Development of COPD resulting from pulmonary infection represents a highly treatable condition for this disease which is the cause of significant morbidity and mortality throughout the world. We, therefore, aimed to determine if pulmonary function decline could be arrested after significant obstruction had already occurred due to Pc colonization. To do this, we administered TMP-SMX therapy to immunosuppressed nonhuman primates that were colonized with Pc and had already exhibited significant airway obstruction. When Pc-colonized animals were treated with TMP-SMX starting at 25 weeks post-SHIV infection after animals had been colonized an average of 8 weeks, we observed that Pc colonization-associated damage appeared to be self-limiting taking place very rapidly after first detection of colonization and then ceased to progress. This was evidenced by the unexpected lack of further pulmonary function decline in the untreated SHIV/Pc+ macaques. We, therefore, conclude that Pc induces airway obstruction very early after colonization in the context of immunosuppresion followed by an extended period of relative inactivity in the absence of some other insult.

Our observation is not entirely without precedent. Multiple studies on the acute effects of Pc infections have suggested that declines in pulmonary function observed after PcP may not return to baseline (236, 237, 323). However, Morris and coworkers reported significant declines

in pulmonary function in patients who had PcP only one month earlier that persisted for years (248). These studies demonstrate that Pc infection can result in permanent airway damage after a very short period of time of exposure to the pathogen.

The reason damage resulting in further decline in pulmonary function does not continue is not known. It is conceivable that host immune responses are able to gain some control over the pathogen and the damage it causes via antibody-mediated mechanisms. Two observations indicate that this may be true. First, despite immunosuppression, monkeys are able to mount a Pc-specific antibody response (184). Second, screening of monkeys for anti-Pc IgG titers before SHIV infection revealed that animals with higher titers against Pc were protected from Pc colonization and associated airway obstruction (Kling, unpublished results, manuscript submitted). These observations suggest that humoral responses against Pc, even when generated in an immunocompromised state, can be protective. This is further supported by the report showing that undetectable anti-Pc antibody titers was an independent predictor of more severe airway obstruction (244). The higher rate of emphysema reported in HIV patients (81) may be the result of loss of this control as patients' immune systems deteriorate and progress towards AIDS. It is also possible that prevalence of smoking among HIV+ individuals, which is at least 2-3 times higher than the 19.8% smoking rate of the general population (4, 124, 234, 376), plays a role in their increased risk for emphysema by amplifying the inflammatory response in patients colonized with Pc.

This study confirms previous results that Pc colonization results in airway obstruction in a simian model of AIDS. When drug treatment was administered to arrest pulmonary function decline, no effect was observed because lung damage associated with Pc colonization occurred early after the onset of colonization and plateaued quickly thereafter. For this reason, use of

TMP-SMX to control the progression of COPD is not appropriate for the prevention of pulmonary decline.

5.0 SUMMARY AND CONCLUSIONS

As the causative agent of PcP, the leading AIDS-defining illness (165), Pc has held a prominent position in modern medicine since the outbreak of the AIDS epidemic in the 1980s. Even with the advent of ART to aid in maintaining CD4+ T cell counts combined with the use of anti-Pc prophylaxis to prevent Pc infections, subclinical levels of this pathogen can still be detected at rates as high as 69% in HIV+ populations (147). The consequences of long-term carriage of Pc at levels too low to cause PcP (colonization) have not been well studied, but mounting evidence implicates this organism in the pathogenesis of chronic obstructive pulmonary disease (COPD). First, multiple studies have reported increased incidence of emphysema and COPD among HIV+ individuals (66, 79, 80). Other reports have shown declines in pulmonary function after PcP episodes (236, 237, 323). Moreover, these declines have been shown to last for years after resolution of the pneumonia (248) mimicking the permanent nature of airway obstruction in COPD. Still more evidence is found in epidemiological studies reporting high rates of Pc colonization in COPD patients as compared to healthy subjects or patients with other types of lung disorders (39, 279, 328). Additionally, increased Pc colonization that correlates with severity of COPD has been reported (245). Finally, there are many similarities in the inflammatory responses observed in COPD and Pc colonization. These similarities involve influx of the same types of cells into the lungs of COPD and PcP patients (77, 300, 330, 346). Closely associated with this inflammation is excessive proteolytic activity which has been

hypothesized to be at the root of COPD pathogenesis (357). Evidence of excessive proteolytic activity has also been observed in both COPD and Pc infections (136, 238, 264, 282, 345, 352). Although these studies do not prove the existence of a causal relationship between Pc colonization and COPD development, the strong association warrants further investigation.

The central goal of this research was to assess the role of sub-clinical infection with Pc in the context of HIV co-infection on the development of COPD. To this end, the first aim was to test the hypothesis that Pc colonization in a primate model of AIDS leads to progressive loss of pulmonary function and development of COPD. In fulfillment of this aim, cynomolgus macaques were infected with SHIV in order to induce immunosuppression that would allow natural Pc colonization. The macaques that became colonized with Pc exhibited significant airway obstruction accompanied by anatomic changes indicative of emphysema development including increased lung and airspace volumes in the upper lung lobes and decreases in total lung tissue and weight. We also observed histologic evidence of emphysema in the form of a greater mean chord length in the lung tissue of SHIV/Pc+ versus the SHIV/Pc- monkeys. Another histologic finding of significance was that the Pc-colonized monkeys had a higher frequency of BALT in their lungs than the non-colonized animals lending weight to a role for infectious agents in COPD development. Evaluation of cytokine levels in the BALF of the animals suggested that a Th2 response and macrophage activation are important in the development of emphysema in the simian model of AIDS. These results identify Pc as a potentially treatable risk factor for COPD development in HIV-infected and non-infected individuals.

The second aim of this research was to identify key immune mediators of SHIV/Pc-associated obstructive lung disease. To accomplish this, RNA was isolated from lung tissue taken at necropsy from the monkeys used in aim 1 and used for microarray analysis. Of over

52,000 transcripts analyzed, 243 genes were found to be differentially expressed in the SHIV/Pc+ animals as compared to the SHIV/Pc- animals. The differentially expressed genes that may be important to COPD pathogenesis spanned a wide array of categories. In support of infectious agent involvement, antigen presentation genes were upregulated. As expected in COPD, several genes associated with lung inflammation were also found to be differentially expressed. There was a combination of over- and underexpression of genes involved in protease-antiprotease balance in the lungs that could result in a net proteolytic phenotype. We also observed upregulation of one antioxidant gene which could indicate host response to oxidative stress elicited by Pc colonization. Up- and downregulation of a number of tissue homeostasis genes provided evidence that excessive apoptosis may also be playing a role in emphysema development. This was supported by a pathways analysis of the differentially expressed genes that indicated that VEGF gene expression, a protein that appears to be central in maintenance of lung tissue (177, 347, 356), was significantly affected. Although all of the gene groups that showed differential expression in our model have surfaced in other microarray studies of COPD, they have never all appeared simultaneously as possible mediators of the disease suggesting that the simian model of AIDS/COPD pathogenesis provides an excellent resource in the study of mechanisms and mediators important to COPD development.

The third aim of this research was to test the hypothesis that pulmonary function decline can be arrested by administering therapeutic doses of TMP-SMX in order to eliminate Pc colonization, thus directly correlating pulmonary function decline with the presence of Pc. Recapitulating the results of aim 1, we observed the development of significant airway obstruction in the new cohort of animals that became colonized with Pc. The animals that were Pc-colonized were then divided into TMP-SMX treatment/no treatment groups to show that

pulmonary function decline could be arrested in Pc-colonized animals. However, the drug therapy had no effect on further pulmonary function decline as was evidenced by the observation that the untreated group of animals did not demonstrate any further development of airway obstruction despite remaining colonized with Pc. This led us to conclude that Pc colonization-induced airway obstruction happens very rapidly and early after the onset of colonization followed by a plateau that can last for an extended period in the absence of some other inciting factor.

The research described here suggests a number of new directions in elucidating the role of Pc in COPD pathogenesis. For example, the microarray study performed in fulfillment of aim 2 utilized terminal lung tissue samples for identification of mediators involved in development of airway obstruction. However, the MMP assay showing increased activity at week 12 followed by a steep dropoff, and the pulmonary function data generated from the studies fulfilling aims 1 and 3, respectively, suggest that Pc colonization-induced lung damage happens rapidly after Pc colonization first takes place followed by a period of control over further impairment of pulmonary function despite continued Pc colonization. Therefore, microarray analysis of interim samples may shed light on other mediators involved in emphysema pathogenesis and development of airway obstruction. Since multiple surgeries to remove lung tissue samples is not reasonable, this work can be performed on RNA isolated from BAL cells harvested serially which were collected during the aim 3 study. If MMPs do play a significant role as expected, microarray of BAL cells will likely reveal this because the majority of these cells are macrophages which express a wide array of these proteases. Serial microarray data can then be correlated to data generated from cytokine/chemokine analysis of BALF and pulmonary function data.

To address the importance of alternatively activated macrophages in COPD pathogenesis as was implicated in the aims 1 and 2 studies, flow cytometry to detect markers for cells of this phenotype can be performed. We evaluated both alternatively and classically activated macrophages in the aim 3 study, but found no differences for either one of the macrophage types in the magnitude of the response between the SHIV/Pc+ and SHIV/Pc- groups for the period in which Pc-mediated declines in pulmonary function occurred (Appendix C). Further work is required to elucidate the role of macrophages in development of COPD.

Since the damage induced by Pc appears to be self-limiting and takes place early after onset of colonization of the lungs in the simian model of HIV infection (as shown in the aim 3 study), and since the HIV+ population has a considerably higher rate of smoking than the general population (4, 124, 234, 376), introduction of the effect of smoking to the model may allow us to gain a better understanding of why smokers who develop COPD experience enormous pulmonary function declines that continue over many years. Addition of this further insult would allow us to uncover other mediators of COPD pathogenesis. Furthermore, since it is plausible that the combined damaging effects of Pc and smoking may be longer lasting than what we observed in the simian AIDS/Pc model, TMP-SMX therapy administered well after the onset of Pc colonization may still provide some benefit. Thus, highly relevant and potentially far-reaching information can still be gleaned from the simian model by modifying how it is currently used through the addition of smoking, the leading risk factor for COPD development.

These studies establish a novel model for HIV-associated COPD and provide evidence supporting a role for Pc colonization in obstructive disease development. Nevertheless, a direct pathogenic link between Pc and COPD still remains to be demonstrated. Conventionally, establishment of etiology of disease has been by application of Koch's postulates which include:

1) isolating an organism from diseased hosts and growing it in culture, 2) re-introducing the cultured organism into healthy hosts to cause the same disease and 3) re-isolating the organism from diseased hosts that received the originally isolated organism and identifying it to be identical to the original causative agent.

There are limitations to fulfilling Koch's postulates in order to definitively establish Pc as the causative agent of COPD. For example, Pc cannot be cultured continuously. This precludes fulfillment of the second postulate of re-introducing the cultured organism into healthy hosts to cause disease that, in turn, makes fulfillment of the third postulate problematic. However, use of the simian AIDS/Pc model has allowed us to show multiple times that there is a link between Pc colonization and COPD development, consequently, providing a preponderance of evidence that may circumvent the necessity of applying Koch's postulates. First, Pc has been detected by the combination of a rise in anti-Pc serum antibody titers and PCR in every diseased (obstructed) host. As a caveat, due to the host specificity of the pathogen (101, 115) the Pc in the monkey model is specific for simian hosts and does not cause disease in humans. Notwithstanding, it has been shown that monkey Pc is evolutionarily close to human Pc compared to that used in other animals models (259). In the model used in the current research, organisms were not manually introduced into healthy hosts but, rather, natural colonization was allowed to take place after inducing immunosuppression through the introduction of an immunodeficiency virus. Once again, since Pc cannot be cultured, this was the only option available. As a second caveat, the opportunistic nature of Pc requires that the host be immunosuppressed, thus precluding the possibility of using "healthy" hosts.

Many organisms accepted to be the etiologic agents of various diseases do not fulfill Koch's postulates. For example, *Treponema pallidum, Mycobacterium leprae, Rickettsia sp*. and

Chlamydia trachomatis are accepted as causative agents for syphilis, leprosy, Rocky Mountain spotted fever and trachoma, respectively. However, these organisms do not fulfill Koch's postulates because they cannot be purely cultured *in vitro*. Other exceptions to Koch's postulates include pathogens for which an adequate animal model is unavailable because they only cause disease in humans. HIV is an example of such an exception. Yet, all of these pathogens have been accepted by the scientific and lay community alike as causative agents of disease due to evidence consistently linking them to their respective disease. In a like manner, we predict that Pc will also come to be accepted as an etiologic agent in COPD.

APPENDIX A

PUBLICATIONS

Shipley T.W., Kling, H.M., Morris A., Patil. S, Kristoff J., Guyach. S.E., Murphy J.M., Shao, X., Sciurba F.C., Rogers R.M., Richards T., Thomson P., Montelaro R.C., Coxson H.O., Hogg J.C., and K.A. Norris. Persistent *Pneumocystis* colonization leads to the development of chronic obstructive pulmonary disease in a non-human primate model of AIDS. *Journal of Infectious Diseases* 2010:202(2): 302-12.

Shipley T.W., Kling H.M., Morris A., Kristoff J., Shao X., and K.A. Norris. Comparison of lung tissue gene expression profiles from SHIV-infected cynomolgus macaques with and without *Pneumocystis* colonization-induced COPD. *In preparation.*

Kling, H.M., **Shipley T.W.**, and K.A. Norris. SHIV-infected cynomolgus macaques exhibit abnormalities in peripheral blood B cell populations. *In preparation.*

Kling, H.M., **Shipley T.W.**, Patil S., Kristoff J., Montelaro, R., Morris A., and K.A. Norris. Relationship of *Pneumocystis* Humoral Immunity to Prevention of Colonization and COPD in a Primate Model of HIV Infection. *Submitted.*

George, M.P., Brower, A., Kling, H.M., **Shipley, T.W.**, Kristoff, J., Reinhart, T.A., Murphey-Corb, M., Gladwin, M.T., Champion, H.C., Morris, A., and Karen, A. Norris. Pulmonary Vascular Lesions are Common in SIV- and SHIV-Env-infected Macaques. *AIDS and Human Retroviruses. In review.*

Kling H.M., **Shipley T.W.**, Patil S., Morris A., and K.A. Norris. *Pneumocystis* Colonization in Immunocompetent and Simian Immunodeficiency Virus-Infected Cynomolgus Macaques. *Journal of Infectious Diseases* 2009:199(1): 89–96.

Morris A., Netravali M., Kling H.M., **Shipley T.**, Ross T., Sciurba F.C., K.A. Norris."Relationship of *Pneumocystis* Antibody Response and Severity of Chronic Obstructive Pulmonary Disease." *Clinical Infectious Disease*. 2008: 47(S2):e64–e68.

APPENDIX B

Once published, this table will appear as an online supplement to the data contained in the third chapter of this thesis.

Table 1

Differentially expressed lung tissue genes due to Pc colonization

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
1	surfactant, pulmonary-associated protein C	110.94	MmugDNA.26420.1.S1_at	SFTPC	707696	0007585 // respiratory gaseous exchange // inferred from electronic annotation /// 0050828 // regulation of liquid surface tension // inferred from electronic annotation	0005576 // extracellular region // inferred from electronic annotation /// 0005578 // proteinaceous extracellular matrix // inferred from electronic annotation	---
2	---	102.37	MmuSTS.3295.1.S1_at	---	---	---	---	---
3	surfactant, pulmonary-associated protein C	95.996	MmugDNA.32454.1.S1_s_at	SFTPC	707696	0007585 // respiratory gaseous exchange // inferred from electronic annotation /// 0050828 // regulation of liquid surface tension // inferred from electronic annotation	0005576 // extracellular region // inferred from electronic annotation /// 0005578 // proteinaceous extracellular matrix // inferred from electronic annotation	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
4	Similar to beta globin	94.837	MmugDNA.2571.1.S1_at	LOC715559	715559	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding
5	similar to Uteroglobin-related protein 2 precursor (Cytokine HIN-1) (High in normal-1) (Secretoglobin family 3A member 1) (Pneumo secretory protein 2) (PnSP-2)	-88.43	MmugDNA.18903.1.S1_at	LOC716331	716331	---	---	---
6	surfactant, pulmonary-associated protein A2	85.101	MmugDNA.10271.1.S1_at	SFTPA2	701715	0006817 // phosphate transport // inferred from electronic annotation /// 0007585 // respiratory gaseous exchange // inferred from electronic annotation /// 0050828 // regulation of liquid surface tension	0005576 // extracellular region // inferred from electronic annotation /// 0005578 // proteinaceous extracellular matrix // inferred from electronic annotation /// 0005737 // cytoplasm // inferred from electronic annotation	0005488 // binding // inferred from electronic annotation /// 0005509 // calcium ion binding // inferred from electronic annotation /// 0005529 // sugar binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
7	Similar to beta globin	84.526	MmugDNA.2571.1.S1_x_at	LOC715559	715559	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding
8	Surfactant, pulmonary-associated protein B	83.637	MmuSTS.3296.1.S1_at	SFTPB	696477	---	---	---
9	hemoglobin, theta 1	75.841	MmugDNA.32562.1.S1_s_at	HBQ1	693930	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
10	similar to ribosomal protein S18	73.284	MmugDNA.43260.1.S1_at	LOC706414	706414	---	---	---
11	Transcribed locus, strongly similar to NP_990439.1 ribosomal protein S4 [Gallus gallus]	70.272	Mmu.5392.1.S1_at	---	---	---	---	---
12	Eukaryotic translation elongation factor 1 alpha 1	68.043	AFFX-Mmu-ef1a-3_x_at	EEF1A1	716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // inferred from electronic annotation /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding // inferred from electronic annotation
13	similar to Translationally-controlled tumor protein (TCTP) (p23) (Histamine-releasing factor) (HRF) (Fortilin) /// tumor protein, translationally-controlled 1	67.383	MmugDNA.35111.1.S1_s_at	LOC696376 /// TPT1	696376 /// 702155 /// 703941 /// 711798	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
14	---	66.868	MmugDNA.5184.1.S1_s_at	---	---	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation
15	Similar to beta globin	66.841	MmuAffx.949.1.S1_x_at	LOC715559	715559	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
16	Eukaryotic translation elongation factor 1 alpha 1	66.755	AFFX-Mmu-ef1a-M_s_at	EEF1A1	716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // inferred from electronic annotation /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding // inferred from electronic annotation
17	---	65.983	AFFX-r2-P1-cre-3_at	---	---	0006310 // DNA recombination // inferred from electronic annotation /// 0015074 // DNA integration // inferred from electronic annotation /// 0032196 // transposition // inferred from electronic annotation	---	0003677 // DNA binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
18	Eukaryotic translation elongation factor 1 alpha 1	64.135	AFFX-Mmu-ef1a-M_x_at	EEF1A1	716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // inferred from electronic annotation /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding // inferred from electronic annotation
19	---	-64.13	Mmu.6867.3.S1_s_at	---	---	0006810 // transport // inferred from electronic annotation /// 0009060 // aerobic respiration // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005743 // mitochondrial inner membrane // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation	0004129 // cytochrome-c oxidase activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0005507 // copper ion binding // inferred from electronic annotation /// 0009055 // electron carrier activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
20	similar to eukaryotic translation elongation factor 1 alpha 2 /// eukaryotic translation elongation factor 1 alpha 1	63.958	Mmu.12098.2.S1_x_at	EEF1A1 /// LOC702809	702809 /// 716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // inferred from electronic annotation /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding // inferred from electronic annotation
21	similar to ribosomal protein S3a /// similar to 40S ribosomal protein S3a (V-fos transformation effector protein)	63.136	MmugDNA.9700.1.S1_s_at	LOC693584 /// LOC701292 /// LOC703455 /// LOC709241 /// LOC711043 /// LOC712630 /// LOC714801	693584 /// 701292 /// 703455 /// 709241 /// 711043 /// 712630 /// 714801	---	---	---
22	---	-63.02	MmugDNA.35103.1.S1_at	---	---	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
23	---	-62.53	Mmu.11314.1.S1_x_at	---	---	0006810 // transport // inferred from electronic annotation /// 0009060 // aerobic respiration // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005743 // mitochondrial inner membrane // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation	0004129 // cytochrome-c oxidase activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0005507 // copper ion binding // inferred from electronic annotation /// 0009055 // electron carrier activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation
24	surfactant, pulmonary-associated protein D	62.272	MmuSTS.2751.1.S1_at	SFTPD	678657	0006817 // phosphate transport // inferred from electronic annotation /// 0007585 // respiratory gaseous exchange // inferred from electronic annotation /// 0050828 // regulation of liquid surface tension // inferred from electronic annotation	0005576 // extracellular region // inferred from electronic annotation /// 0005578 // proteinaceous extracellular matrix // inferred from electronic annotation /// 0005737 // cytoplasm // inferred from electronic annotation	0005488 // binding // inferred from electronic annotation /// 0005509 // calcium ion binding // inferred from electronic annotation /// 0005529 // sugar binding // inferred from electronic annotation
25	---	60.928	AFFX-Mmu-r2-P1-cre-3_s_at	---	---	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
26	MHC class I antigen /// hypothetical protein LOC720369	59.698	Mmu.6085.1.S1_x_at	LOC720369 /// MAMU-B	700391 /// 720369	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
27	similar to ribosomal protein S14	57.59	MmugDNA.3842.1.S1_s_at	LOC697734 /// LOC711090 1	697734 /// 710901	---	---	---
28	---	-57.17	Mmu.6867.1.S1_s_at	---	---	0006810 // transport // inferred from electronic annotation /// 0009060 // aerobic respiration // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005743 // mitochondrial inner membrane // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation	0004129 // cytochrome-c oxidase activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0005507 // copper ion binding // inferred from electronic annotation /// 0009055 // electron carrier activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation /// 0020037 // heme binding
29	---	57.108	AFFX-Mmu-r2-P1-cre-5_s_at	---	---	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
30	major histocompatibility complex, class I, B	57.05	Mmu.2177.1.S1_x_at	MAMU-B18	100141394	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
31	---	56.295	AFFX-r2-P1-cre-5_at	---	---	0006310 // DNA recombination // inferred from electronic annotation /// 0015074 // DNA integration // inferred from electronic annotation /// 0032196 // transposition // inferred from electronic annotation	---	0003677 // DNA binding // inferred from electronic annotation
32	similar to Epididymal secretory protein E1 precursor (Niemann-Pick disease type C2 protein) (hE1)	56.258	MmugDNA.19377.1.S1_at	LOC699881	699881	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
33	beta-2-microglobulin	55.518	MmugDNA.20334.1.S1_at	B2M	712428	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation	0005576 // extracellular region // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	0005515 // protein binding // inferred from electronic annotation
34	Similar to HLA class I histocompatibility antigen, Cw-14 alpha chain precursor (MHC class I antigen Cw*14) /// MHC class I antigen (Mamu-B gene), Mamu-B*28 allele /// Hypothetical protein LOC703106	55.369	Mmu.12385.4.S1_x_at	LOC703106 /// LOC720375	703106 /// 720375	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
35	connective tissue growth factor	-55.16	MmugDNA.27267.1.S1_at	CTGF	714520	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
36	---	54.879	Mmu.15402.10.S1_s_at	---	---	0006810 // transport // inferred from electronic annotation /// 0022900 // electron transport chain // inferred from electronic annotation /// 0022904 // respiratory electron transport chain // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005739 // mitochondrion // inferred from sequence or structural similarity /// 0005743 // mitochondrial inner membrane // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation	0004129 // cytochrome-c oxidase activity // inferred from electronic annotation /// 0005507 // copper ion binding // inferred from electronic annotation /// 0009055 // electron carrier activity // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation
37	similar to Ornithine decarboxylase antizyme (ODC-Az)	54.069	MmugDNA.278.1.S1_at	LOC721477	721477	---	---	---
38	S100 calcium binding protein A6	53.245	MmugDNA.17370.1.S1_s_at	S100A6	715169	---	---	---
39	similar to Translationally-controlled tumor protein (TCTP) (p23) (Histamine-releasing factor) (HRF) (Fortilin) /// tumor protein, translationally-controlled 1	52.813	MmugDNA.27184.1.S1_s_at	LOC702001 /// TPT1	702001 /// 702155 /// 706952 /// 711798	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
40	similar to 40S ribosomal protein S29	52.51	Mmu.6263.1.S1_s_at	LOC693820 /// LOC700392 /// LOC702289 /// LOC710674	693820 /// 700392 /// 702289 /// 710674	---	---	---
41	similar to thymosin, beta 4	51.566	MmunewRS.557.1.S1_s_at	LOC710959	710959	---	---	---
42	beta-2-microglobulin	51.258	MmugDNA.5628.1.S1_at	B2M	712428	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation	0005576 // extracellular region // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	0005515 // protein binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function

43	---	51.221	MmugDNA.2478.1.S1_at	---	---	0001516 // prostaglandin biosynthetic process // inferred from direct assay /// 0006461 // protein complex assembly // inferred from sequence or structural similarity /// 0006886 // intracellular protein transport // inferred from electronic annotation /// 0006886 // intracellular protein transport // inferred from sequence or structural similarity /// 0006955 // immune response // inferred from electronic annotation /// 0007165 // signal transduction // inferred from direct assay /// 0008283 // cell proliferation // inferred from direct assay /// 0016064 // immunoglobulin mediated immune response // inferred from sequence or structural similarity /// 0019882 // antigen processing and presentation	0005622 // intracellular // traceable author statement /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0016021 // integral to membrane // traceable author statement	0019955 // cytokine binding // inferred from physical interaction /// 0042289 // MHC class II protein binding // inferred from electronic annotation /// 0042289 // MHC class II protein binding // non-traceable author statement /// 0042802 // identical protein binding // traceable author statement

						// inferred from electronic annotation /// 0019883 // antigen processing and presentation of endogenous antigen // non-traceable author statement /// 0043030 // regulation of macrophage activation // non-traceable author statement /// 0043066 // negative regulation of apoptosis // inferred from direct assay /// 0045058 // T cell selection // non-traceable author statement		

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
44	similar to ribosomal protein S19	51.103	MmugDNA.28319.1.S1_s_at	LOC703774	703774	---	---	---
45	similar to 40S ribosomal protein S16	50.911	MmugDNA.24445.1.S1_s_at	LOC697219 /// LOC708154	697219 /// 708154	---	---	---
46	transgelin	-50.67	MmugDNA.30842.1.S1_s_at	TAGLN	697440	---	---	---
47	S100 calcium binding protein A6	50.419	MmugDNA.17370.1.S1_at	S100A6	715169	---	---	---
48	similar to ribosomal protein S11	50.331	Mmu.13450.1.S1_s_at	LOC718844	718844	0006412 // translation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
49	putative ISG12(c) protein	-49.87	MmunewRS.254.1.S1_at	IFI27	700513	---	---	---
50	similar to 60S ribosomal protein L32	48.964	MmugDNA.25831.1.S1_s_at	LOC694196 /// LOC695122 /// LOC699344 /// LOC699375 /// LOC702875	694196 /// 695122 /// 699344 /// 699375 /// 702875	---	---	---
51	---	-48.78	MmugDNA.12088.1.S1_at	---	---	---	---	---
52	similar to Apolipoprotein D precursor (Apo-D) (ApoD)	-48.4	Mmu.8637.1.S1_at	LOC709223	709223	---	---	---
53	fatty acid binding protein 4, adipocyte	48.187	MmugDNA.19691.1.S1_at	FABP4	701365	---	---	---
54	similar to ribosomal protein L27a	48.028	MmugDNA.15562.1.S1_s_at	LOC708863 /// LOC709769	708863 /// 709769	---	---	---
55	S100 calcium binding protein A4	47.999	MmugDNA.17365.1.S1_at	S100A4	715115	---	---	---
56	similar to 60S ribosomal protein L17 (L23)	47.837	MmunewRS.398.1.S1_at	LOC698967	698967	---	---	---
57	hypothetical protein LOC708858	47.306	Mmu.1278.1.S1_s_at	LOC708858	708858	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
58	---	46.572	AFFX-CreX-3_at	---	---	0006310 // DNA recombination // inferred from electronic annotation /// 0015074 // DNA integration // inferred from electronic annotation /// 0032196 // transposition // inferred from electronic annotation	---	0003677 // DNA binding // inferred from electronic annotation
59	similar to 40S ribosomal protein S28	44.811	MmugDNA.4116.1.S1_at	LOC707133	707133	---	---	---
60	similar to ribosomal protein S24	44.551	MmugDNA.6998.1.S1_at	LOC701477 /// LOC702961 /// LOC704054 /// LOC705596 /// LOC707085 /// LOC708965 /// LOC711145 /// LOC715668 /// LOC717801	701477 /// 702961 /// 704054 /// 705596 /// 707085 /// 708965 /// 711145 /// 715668 /// 717801	---	---	---
61	similar to ribosomal protein L21 /// similar to 60S ribosomal protein L21	44.504	MmugDNA.25790.1.S1_s_at	LOC699867 /// LOC710306 /// LOC711174 /// LOC712987 /// LOC713662	699867 /// 710306 /// 711174 /// 712987 /// 713662	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
62	---	-44.4	MmuMitochon.6.1.S1_s_at	---	---	0006810 // transport // inferred from electronic annotation /// 0006811 // ion transport // inferred from electronic annotation /// 0015986 // ATP synthesis coupled proton transport // inferred from electronic annotation /// 0015992 // proton transport // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0016469 // proton-transporting two-sector ATPase complex // inferred from electronic annotation /// 0031966 // mitochondrial membrane // inferred from electronic annotation /// 0045263 // proton-transporting ATP synthase complex, coupling factor F(o) // inferred from electronic annotation	0015078 // hydrogen ion transmembrane transporter activity // inferred from electronic annotation /// 0046933 // hydrogen ion transporting ATP synthase activity, rotational mechanism // inferred from electronic annotation /// 0046961 // hydrogen ion transporting ATPase activity, rotational mechanism // inferred from electronic annotation
63	LPLUNC1 protein	-44.32	MmugDNA.11702.1.S1_at	LOC710014	710014	---	---	---
64	similar to Vitelline membrane outer layer protein 1 homolog precursor	-43.86	MmugDNA.2066.1.S1_s_at	LOC709950	709950	---	---	---
65	similar to 60S acidic ribosomal protein P1	43.529	MmugDNA.25908.1.S1_at	LOC695442	695442	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
66	---	43.23	AFFX-CreX-5_at	---	---	0006310 // DNA recombination // inferred from electronic annotation /// 0015074 // DNA integration // inferred from electronic annotation // 0032196 // transposition	---	0003677 // DNA binding // inferred from electronic annotation
67	similar to ribosomal protein L35a	43.204	MmugDNA.23506.1.S1_s_at	LOC711011 /// LOC714858	711011 /// 714858	---	---	---
68	similar to eukaryotic translation elongation factor 1 alpha 2 /// eukaryotic translation elongation factor 1 alpha 1	43.13	Mmu.12098.1.S1_x_at	EEF1A1 /// LOC702809	702809 /// 716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // inferred from electronic annotation /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding
69	Eukaryotic translation elongation factor 1 alpha 1	43.125	AFFX-Mmu-ef1a-5_s_at	EEF1A1	716010	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity // 0003924 // GTPase activity // / 0005525 // GTP binding // inferred from electronic annotation
70	similar to 60S ribosomal protein L23	42.991	Mmu.5328.1.S1_x_at	LOC694719	694719	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
71	similar to ribosomal protein L5	42.925	MmugDNA.25868.1.S1_at	LOC704067 /// LOC706896 /// LOC707117	704067 /// 706896 /// 707117	---	---	---
72	---	42.74	Mmu.7883.1.S1_x_at	---	---	---	---	---
73	similar to ribosomal protein S3a /// similar to 40S ribosomal protein S3a /// similar to 40S ribosomal protein S3a (V-fos transformation effector protein) /// hypothetical protein LOC713060 /// hypothetical protein LOC721887	42.581	MmugDNA.26529.1.S1_at	LOC693584 /// LOC693844 /// LOC694471 /// LOC698301 /// LOC701292 /// LOC702892 /// LOC706910 /// LOC709241 /// LOC711043 /// LOC711135 /// LOC712630 /// LOC713060 /// LOC714801 /// LOC721887	693584 /// 693844 /// 694471 /// 698301 /// 701292 /// 702892 /// 706910 /// 709241 /// 711043 /// 711135 /// 712630 /// 713060 /// 714801 /// 721887	---	---	---
74	similar to annexin A2 isoform 1	42.553	MmugDNA.4914.1.S1_s_at	LOC706240	706240	---	---	---
75	similar to smooth muscle myosin heavy chain 11 isoform SM1A	-42.49	MmugDNA.33337.1.S1_s_at	LOC713882	713882	---	---	---
76	similar to ribosomal protein S15a	42.433	MmugDNA.25955.1.S1_at	LOC702174	702174	---	---	---
77	similar to Ig kappa chain V-III region HAH precursor	-42.43	MmugDNA.23504.1.S1_s_at	LOC701504	701504	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
78	similar to ribosomal protein L10	42.335	MmugDNA.19468.1.S1_s_at	LOC694799 /// LOC700795 /// LOC707414	694799 /// 700795 /// 707414	---	---	---
79	similar to ribosomal protein S3	41.919	MmugDNA.32744.1.S1_at	LOC695748	695748	---	---	---
80	similar to 40S ribosomal protein S16 /// hypothetical protein LOC710034	41.66	MmugDNA.25971.1.S1_at	LOC697219 /// LOC708154 /// LOC710034	697219 /// 708154 /// 710034	---	---	---
81	Similar to beta globin	41.629	MmugDNA.2571.1.S1_s_at	LOC715559	715559	0006810 // transport // inferred from electronic annotation /// 0015671 // oxygen transport // inferred from electronic annotation	0005833 // hemoglobin complex // inferred from electronic annotation	0005344 // oxygen transporter activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0019825 // oxygen binding // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation
82	similar to ribosomal protein S27	41.165	MmunewRS.940.1.S1_s_at	LOC694967	694967	---	---	---
83	similar to 60S ribosomal protein L11	40.95	Mmu.4594.1.S1_s_at	LOC705400	705400	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
84	hypothetical protein LOC693576 /// similar to 60S ribosomal protein L23a /// hypothetical protein LOC703315 /// hypothetical protein LOC704139 /// hypothetical protein LOC705849 /// hypothetical protein LOC709043 /// hypothetical protein LOC710889 /// hypothetical protein LOC713902 /// hypothetical protein LOC716039	40.736	MmugDNA.14184.1.S1_x_at	LOC693576 /// LOC695850 /// LOC702297 /// LOC703315 /// LOC704012 /// LOC704139 /// LOC704238 /// LOC705703 /// LOC705849 /// LOC706606 /// LOC706798 /// LOC708995 /// LOC709043 /// LOC709681 /// LOC710490 /// LOC710889 /// LOC713902 /// LOC714458 /// LOC716039 /// LOC718737 /// LOC721751	693576 /// 695850 /// 702297 /// 703315 /// 704012 /// 704139 /// 704238 /// 705703 /// 705849 /// 706606 /// 706798 /// 708995 /// 709043 /// 709681 /// 710490 /// 710889 /// 713902 /// 714458 /// 716039 /// 718737 /// 721751	---	---	---
85	---	-40.44	Mmu.15443.1.S1_x_at	---	---	---	---	---
86	dicarbonyl/L-xylulose reductase	40.303	MmugDNA.38889.1.S1_at	DCXR	715513	---	---	---
87	S100 calcium binding protein A10	39.871	MmuSTS.1770.1.S1_at	S100A10	574374	---	---	0005509 // calcium ion binding // inferred from electronic annotation
88	---	-39.54	MmugDNA.41975.1.S1_at	---	---	---	---	---
89	secretory leukocyte peptidase inhibitor	-39.02	MmugDNA.2108.1.S1_s_at	SLPI	711156	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
90	similar to 60S ribosomal protein L38	39.016	Mmu.1015.1.S1_s_at	LOC710665	710665	---	---	---
91	similar to 60S acidic ribosomal protein P2	38.879	MmugDNA.25921.1.S1_at	LOC700698	700698	---	---	---
92	similar to ribosomal protein S13	38.854	MmugDNA.25950.1.S1_at	LOC697513	697513	---	---	---
93	fatty acid binding protein 5 (psoriasis-associated)	38.647	MmunewRS.500.1.S1_at	FABP5	701009	---	---	---
94	similar to ribosomal protein S23	38.588	MmugDNA.26481.1.S1_at	LOC693947	693947	---	---	---
95	caveolin 1, caveolae protein, 22kDa	38.363	MmugDNA.19983.1.S1_s_at	CAV1	704449	---	---	---
96	similar to WAP four-disulfide core domain protein 2 precursor (Major epididymis-specific protein E4) (Epididymal secretory protein E4) (Putative protease inhibitor WAP5)	38.324	MmugDNA.36467.1.S1_s_at	LOC710469	710469	---	---	---
97	hypothetical protein LOC699632	38.019	MmunewRS.356.1.S1_s_at	LOC699632	699632	---	---	---
98	similar to HLA class I histocompatibility antigen, A-2 alpha chain precursor (MHC class I antigen A*2)	37.348	Mmu.12385.2.S1_x_at	LOC696824	696824	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
99	similar to Phospholipase A2 precursor (Phosphatidylcholine 2-acylhydrolase) (Group IB phospholipase A2)	37.139	MmugDNA.2316.1.S1_s_at	LOC696712	696712	---	---	---
100	metallothionein 2A	-36.97	MmugDNA.5794.1.S1_at	MT2A	700719	---	---	---
101	similar to ribosomal protein L24 /// hypothetical protein LOC699643	36.96	MmugDNA.26107.1.S1_s_at	LOC698942 /// LOC699643	698942 /// 699643	---	---	---
102	similar to caldesmon 1 isoform 4	-36.71	MmugDNA.39129.1.S1_at	LOC707050	707050	---	---	---
103	---	-36.59	MmugDNA.38698.1.S1_at	---	---	0006909 // phagocytosis // non-traceable author statement /// 0007155 // cell adhesion // inferred from electronic annotation /// 0007596 // blood coagulation // inferred from electronic annotation /// 0016337 // cell-cell adhesion // inferred from direct assay /// 0042116 // macrophage activation // non-traceable author statement	0005886 // plasma membrane // inferred from direct assay /// 0005886 // plasma membrane // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred by curator /// 0016023 // cytoplasmic membrane-bounded vesicle // inferred from electronic annotation	0001849 // complement component C1q binding // inferred from direct assay /// 0004872 // receptor activity // inferred from electronic annotation /// 0004872 // receptor activity // non-traceable author statement /// 0005488 // binding /// 0005509 // calcium ion binding // 0005515 // protein binding // inferred from electronic annotation /// 0005515 // protein binding // inferred from physical interaction /// 0005529 // sugar binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
104	similar to ribosomal protein S2	36.422	MmunewRS.522.1.S1_s_at	LOC700955	700955	---	---	---
105	similar to 60S ribosomal protein L32	36.261	MmugDNA.25831.1.S1_at	LOC694196 /// LOC695122 /// LOC699375 /// LOC702875	694196 /// 695122 /// 699375 /// 702875	---	---	---
106	similar to HLA class II histocompatibility antigen, DR alpha chain precursor (MHC class II antigen DRA)	36.069	MmugDNA.1028.1.S1_at	LOC720539	720539	0002504 // antigen processing and presentation of peptide or polysaccharide antigen via MHC class II // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042613 // MHC class II protein complex // inferred from electronic annotation	---
107	alpha-1-antichymotrypsin	-35.81	MmuSTS.2150.1.S1_at	LOC574106	574106	---	---	---
108	leukotriene A4 hydrolase	35.759	MmugDNA.19725.1.S1_at	LTA4H	713038	---	---	---
109	similar to 60S ribosomal protein L26 (Silica-induced gene 20 protein) (SIG-20)	35.62	MmunewRS.849.1.S1_at	LOC696134	696134	---	---	---
110	similar to ribosomal protein L10	35.441	MmugDNA.19468.1.S1_at	LOC700795	700795	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
111	---	-35.06	MmugDNA.34285.1.S1_at	---	---	0001558 // regulation of cell growth // inferred from electronic annotation /// 0007399 // nervous system development // traceable author statement	0005576 // extracellular region // inferred from electronic annotation /// 0005624 // membrane fraction // not recorded /// 0005886 // plasma membrane // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0016021 // integral to membrane // traceable author statement	0004857 // enzyme inhibitor activity // inferred from electronic annotation /// 0004867 // serine-type endopeptidase inhibitor activity // inferred from electronic annotation /// 0005010 // insulin-like growth factor receptor activity // traceable author statement /// 0005520 // insulin-like growth factor binding // inferred from electronic annotation
112	similar to HLA class II histocompatibility antigen, DR alpha chain precursor (MHC class II antigen DRA)	35.026	MmugDNA.1046.1.S1_s_at	LOC720539	720539	0002504 // antigen processing and presentation of peptide or polysaccharide antigen via MHC class II // inferred from electronic annotation /// 0006955 // immune response /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042613 // MHC class II protein complex // inferred from electronic annotation	---
113	hypothetical protein LOC718964	35.011	MmugDNA.39913.1.S1_at	LOC718964	718964	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
114	similar to Immunoglobulin lambda-like polypeptide 1 precursor (Immunoglobulin-related protein 14.1) (Immunoglobulin omega polypeptide) (Ig lambda-5) (CD179b antigen)	-34.92	MmugDNA.11907.1.S1_s_at	LOC708547 /// LOC708665	708547 /// 708665	---	---	---
115	similar to ribosomal protein L9	34.785	MmugDNA.25903.1.S1_s_at	LOC699362	699362	---	---	---
116	similar to ribosomal protein S12 /// ribosomal protein S12	34.746	MmugDNA.9537.1.S1_s_at	LOC700257 /// LOC700807 /// LOC706087 /// RPS12	700257 /// 700807 /// 706087 /// 708419	0006412 // translation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation /// 0030529 // ribonucleoprotein complex // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
117	similar to 40S ribosomal protein S6	34.682	MmugDNA.33829.1.S1_s_at	LOC712274 /// LOC718556	712274 /// 718556	---	---	---
118	mitochondrial aldehyde dehydrogenase 2	34.664	MmuSTS.3435.1.S1_at	ALDH2	713451	---	---	---
119	---	34.565	MmuAffx.78.1.S1_s_at	---	---	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
120	similar to ubiquitin and ribosomal protein S27a precursor	34.309	MmugDNA.26506.1.S1_at	LOC709143	709143	---	---	---
121	MHC class I antigen heavy chain	33.8	Mmu.10195.2.S1_x_at	MAMU-B	720372	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
122	similar to advanced glycosylation end product-specific receptor isoform 1 precursor	33.745	MmugDNA.30962.1.S1_at	LOC717296	717296	---	---	---
123	CD74 molecule, major histocompatibility complex, class II invariant chain	33.666	Mmu.9241.2.S1_at	CD74	710820	---	---	---
124	similar to 40S ribosomal protein S26	33.648	MmugDNA.26485.1.S1_s_at	LOC699166 /// LOC711319 /// LOC715370 /// LOC718588	699166 /// 711319 /// 715370 /// 718588	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
125	similar to ribosomal protein L13a	33.54	MmugDNA.16774.1.S1_s_at	LOC698713	698713	0006412 // translation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation /// 0015934 // large ribosomal subunit // inferred from electronic annotation /// 0030529 // ribonucleoprotein complex // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
126	similar to ribosomal protein L19	33.433	MmugDNA.25770.1.S1_at	LOC695340	695340	---	---	---
127	similar to ribosomal protein L34	33.376	MmugDNA.25833.1.S1_s_at	LOC696636 /// LOC702677 /// LOC704365 /// LOC708118 /// LOC709678 /// LOC716593	696636 /// 702677 /// 704365 /// 708118 /// 709678 /// 716593	---	---	---
128	similar to 60S ribosomal protein L12	33.289	MmugDNA.7346.1.S1_s_at	LOC707504	707504	---	---	---
129	similar to ribosomal protein L4	33.256	Mmu.8980.1.S1_at	LOC710590	710590	---	---	---
130	similar to 60S ribosomal protein L14 (CAG-ISL 7)	32.981	MmunewRS.1027.1.S1_at	LOC697476	697476	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
131	---	32.97	Mmu.3064.4.S1_s_at	---	---	0006120 // mitochondrial electron transport, NADH to ubiquinone // inferred from electronic annotation /// 0006810 // transport // inferred from electronic annotation /// 0042773 // ATP synthesis coupled electron transport // inferred from electronic annotation /// 0055114 // oxidation reduction	0005739 // mitochondrion // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation	0008137 // NADH dehydrogenase (ubiquinone) activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation
132	similar to ribosomal protein L30	32.866	MmugDNA.27693.1.S1_at	LOC703120	703120	---	---	---
133	ferritin H chain /// similar to ferritin H chain /// similar to Ferritin heavy chain (Ferritin H subunit) (Proliferation-inducing gene 15 protein) /// ferritin, heavy polypeptide 1	32.694	MmunewRS.750.1.S1_at	FTH1 /// LOC574118 /// LOC699053 /// LOC706802 /// LOC708254	574118 /// 699053 /// 706802 /// 707011 /// 708254 /// 714576	0006826 // iron ion transport // inferred from electronic annotation /// 0006879 // cellular iron ion homeostasis // inferred from electronic annotation	---	0005488 // binding // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0008199 // ferric iron binding // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation /// 0046872 // metal ion binding // inferred from electronic annotation /// 0046914 // transition metal ion binding

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
134	acidic ribosomal phosphoprotein PO /// similar to acidic ribosomal phosphoprotein P0 /// similar to 60S acidic ribosomal protein P0 (L10E)	32.597	MmugDNA.12980.1.S1_s_at	LOC574363 /// LOC705501 /// LOC718979	574363 /// 705501 /// 718979	0006414 // translational elongation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
135	similar to 60S ribosomal protein L26 (Silica-induced gene 20 protein) (SIG-20)	32.451	MmunewRS.849.1.S1_s_at	LOC717378	717378	---	---	---
136	similar to 60S ribosomal protein L8	32.305	Mmu.1393.1.S1_s_at	LOC708535	708535	---	---	---
137	similar to cytoplasmic polyadenylation element binding protein 1 /// similar to 40S ribosomal protein S17	32.117	MmugDNA.39039.1.S1_s_at	LOC694357 /// LOC694424 /// LOC700848 /// LOC701429 /// LOC701909 /// LOC706389 /// LOC708833 /// LOC713986	694357 /// 694424 /// 700848 /// 701429 /// 701909 /// 706389 /// 708833 /// 713986	---	---	---
138	similar to ribosomal protein S21	32.073	MmunewRS.307.1.S1_at	LOC699565	699565	---	---	---
139	chemokine CCL18/PARC	32.029	MmugDNA.616.1.S1_at	LOC574181	574181	0006935 // chemotaxis // inferred from electronic annotation /// 0006954 // inflammatory response // inferred from electronic annotation /// 0006955 // immune response	0005576 // extracellular region // inferred from electronic annotation /// 0005615 // extracellular space // inferred from electronic annotation	0005125 // cytokine activity // inferred from electronic annotation /// 0008009 // chemokine activity // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
140	similar to ribosomal protein L27	31.904	MmugDNA.25812.1.S1_s_at	LOC712352 /// LOC717053	712352 /// 717053	---	---	---
141	MHC class II antigen, Mamu-DRB5 /// MHC class II antigen /// similar to HLA class II histocompatibility antigen, DRB1-4 beta chain precursor (MHC class I antigen DRB1*4) (DR-4) (DR4) /// similar to HLA class II histocompatibility antigen, DRB1-1 beta chain precursor (MHC class I antigen DRB1*1) (DR-1) (DR1)	31.527	MmunewRS.436.1.S1_s_at	LOC705588 /// LOC716685 /// MAMU-DRB /// MAMU-DRB5	677701 /// 692100 /// 705588 /// 716685	0002504 // antigen processing and presentation of peptide or polysaccharide antigen via MHC class II // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042613 // MHC class II protein complex // inferred from electronic annotation	---
142	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	-31.49	MmugDNA.16478.1.S1_at	DDX5	677694	---	---	---
143	similar to HLA class I histocompatibility antigen, B-37 alpha chain precursor (MHC class I antigen B*37)	31.229	Mmu.6085.2.S1_x_at	LOC720309	720309	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
144	alpha-1-antichymotrypsin	-31.13	Mmu.10083.1.S1_s_at	LOC574106	574106	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
145	similar to solute carrier family 39 (zinc transporter), member 8	31.112	MmugDNA.19008.1.S1_at	LOC710142	710142	---	---	---
146	similar to caldesmon 1 isoform 4	-31.11	MmugDNA.27788.1.S1_at	LOC707050	707050	---	---	---
147	small EDRK-rich factor 2	31.028	MmugDNA.39687.1.S1_s_at	SERF2	711580	---	---	---
148	similar to 40S ribosomal protein S20	30.539	MmugDNA.28095.1.S1_s_at	LOC704640	704640	---	---	---
149	transgelin	-30.31	Mmu.15501.1.S1_s_at	TAGLN	697440	---	---	---
150	similar to Guanine nucleotide-binding protein beta subunit 2-like 1 (Receptor of activated protein kinase C 1) (RACK1) (Receptor for activated C kinase)	30.205	MmugDNA.36451.1.S1_at	LOC708526	708526	---	---	---
151	similar to ribosomal protein L34	30.137	MmugDNA.25833.1.S1_at	LOC696636 /// LOC702677 /// LOC704365 /// LOC716593	696636 /// 702677 /// 704365 /// 716593	---	---	---
152	similar to Thioredoxin (ATL-derived factor) (ADF) (Surface-associated sulphydryl protein) (SASP) /// thioredoxin	30.123	MmugDNA.4158.1.S1_at	TXN	693422 /// 693792 /// 712587	0006810 // transport // inferred from electronic annotation /// 0045454 // cell redox homeostasis // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005737 // cytoplasm // inferred from electronic annotation	---
153	similar to ribosomal protein L18	29.949	MmugDNA.25768.1.S1_at	LOC718136	718136	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
154	MHC class I antigen Mamu B*07	29.882	Mmu.1700.1.S1_x_at	---	---	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
155	similar to ribosomal protein L35a	29.872	MmugDNA.42687.1.S1_at	LOC714858	714858	---	---	---
156	similar to ribosomal protein L18a	29.761	Mmu.7476.1.S1_s_at	LOC719242	719242	---	---	---
157	hypothetical protein LOC718964	29.711	MmugDNA.36274.1.S1_at	LOC718964	718964	---	---	---
158	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)	29.681	Mmu.3361.2.S1_s_at	TCEB3	710467	---	---	---
159	similar to cytoplasmic beta-actin	29.622	MmugDNA.28776.1.S1_s_at	LOC711964	711964	---	---	0005515 // protein binding // inferred from electronic annotation /// 0005524 // ATP binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
160	similar to 60S ribosomal protein L23a /// hypothetical protein LOC703315 /// hypothetical protein LOC704139 /// hypothetical protein LOC709043	29.603	MmugDNA.25793.1.S1_x_at	LOC702297 /// LOC703315 /// LOC704012 /// LOC704139 /// LOC706606 /// LOC706798 /// LOC708995 /// LOC709043 /// LOC718737 /// LOC721751	702297 /// 703315 /// 704012 /// 704139 /// 706606 /// 706798 /// 708995 /// 709043 /// 718737 /// 721751	---	---	---
161	similar to ribosomal protein S8 /// hypothetical protein LOC708603	29.517	MmugDNA.26577.1.S1_s_at	LOC695670 /// LOC696015 /// LOC698297 /// LOC700262 /// LOC703957 /// LOC708603	695670 /// 696015 /// 698297 /// 700262 /// 703957 /// 708603	---	---	---
162	similar to 60S ribosomal protein L35	29.478	MmugDNA.2923.1.S1_at	LOC702847	702847	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
163	ribosomal protein SA /// similar to laminin receptor 1 (ribosomal protein SA) /// similar to 40S ribosomal protein SA (p40) (34/67 kDa laminin receptor) (Colon carcinoma laminin-binding protein) (NEM/1CHD4) (Multidrug resistance-associated protein MGr1-Ag) /// similar to 40S ribosomal protein SA (p40) (34/67 kDa laminin receptor)	29.361	MmuSTS.1434.1.S1_s_at	LAMR1 /// LOC695751 /// LOC697065 /// LOC697561 /// LOC698768 /// LOC700502 /// LOC701421 /// LOC701691 /// LOC708140 /// LOC710477 /// LOC716735 /// LOC717739	693293 /// 695751 /// 697065 /// 697561 /// 698768 /// 700502 /// 701421 /// 701691 /// 708140 /// 710477 /// 716735 /// 717739	0006412 // translation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation /// 0015935 // small ribosomal subunit // inferred from electronic annotation /// 0030529 // ribonucleoprotein complex // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
164	---	-29.36	Mmu.6048.1.S1_s_at	---	---	0006120 // mitochondrial electron transport, NADH to ubiquinone // inferred from electronic annotation /// 0006810 // transport // inferred from electronic annotation /// 0042773 // ATP synthesis coupled electron transport // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation	0008137 // NADH dehydrogenase (ubiquinone) activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
165	glutathione peroxidase 4	29.263	MmugDNA.16268.1.S1_s_at	GPX4	705333	---	---	---
166	similar to Uteroglobin precursor (Secretoglobin family 1A member 1) (Clara cell phospholipid-binding protein) (CCPBP) (Clara cells 10 kDa secretory protein) (CC10) (Urinary protein 1) (Urine protein 1) (UP1)	-29.25	MmugDNA.8056.1.S1_at	LOC718857	718857	---	---	---
167	similar to ubiquitin B precursor	29.151	MmugDNA.35404.1.S1_at	LOC696110	696110	---	---	---
168	---	29.121	MmuSTS.87.1.S1_at	---	---	---	---	---
169	Actin, beta	28.958	AFFX-Mmu-actin-3_s_at	ACTB	574285	---	---	0005515 // protein binding // inferred from electronic annotation /// 0005524 // ATP binding // inferred from electronic annotation
170	similar to Eukaryotic translation initiation factor 1 (eIF1) (Protein translation factor SUI1 homolog) (Sui1iso1) (A121) /// eukaryotic translation initiation factor 1	28.941	MmugDNA.3378.1.S1_s_at	EIF1 /// LOC704606	704606 /// 718407	---	---	---
171	similar to thioredoxin interacting protein	-28.91	MmugDNA.40204.1.S1_at	LOC698683	698683	---	---	---
172	similar to smooth muscle myosin heavy chain 11 isoform SM1A	-28.72	MmugDNA.33337.1.S1_at	LOC713882	713882	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
173	---	28.663	MmugDNA.31036.1.S1_at	---	---	0006629 // lipid metabolic process // inferred from electronic annotation /// 0006665 // sphingolipid metabolic process // inferred from electronic annotation /// 0007585 // respiratory gaseous exchange // inferred from electronic annotation /// 0007585 // respiratory gaseous exchange // traceable author statement /// 0009887 // organ morphogenesis // traceable author statement /// 0050828 // regulation of liquid surface tension	0005576 // extracellular region // inferred from electronic annotation /// 0005578 // proteinaceous extracellular matrix // inferred from electronic annotation /// 0005615 // extracellular space // inferred from electronic annotation /// 0005615 // extracellular space // not recorded /// 0005764 // lysosome // inferred from electronic annotation	---
174	heat shock 70kDa protein 8	28.659	MmugDNA.2144.1.S1_s_at	HSPA8	707989	---	---	---
175	myosin light chain kinase	-28.47	MmugDNA.37873.1.S1_at	MYLK	715422	---	---	---
176	actin, beta	28.466	MmunewRS.624.1.S1_s_at	ACTB	574285	---	---	0005515 // protein binding // inferred from electronic annotation /// 0005524 // ATP binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
177	similar to 40S ribosomal protein S10 /// hypothetical protein LOC715171	28.41	MmugDNA.1196.1.S1_s_at	LOC694687 /// LOC696921 /// LOC708539 /// LOC715171	694687 /// 696921 /// 708539 /// 715171	---	---	---
178	selenoprotein W, 1	28.35	MmugDNA.17715.1.S1_at	SEPW1	718370	0045454 // cell redox homeostasis // inferred from electronic annotation	0005737 // cytoplasm // inferred from electronic annotation /// 0005739 // mitochondrion // non-traceable author statement	0003954 // NADH dehydrogenase activity // non-traceable author statement /// 0008430 // selenium binding // inferred from electronic annotation
179	Similar to HLA class I histocompatibility antigen, A-74 alpha chain precursor (MHC class I antigen A*74) (Aw-74) (Aw-19)	-28.26	MmugDNA.2178.1.S1_s_at	LOC699243	699243	0002474 // antigen processing and presentation of peptide antigen via MHC class I // inferred from electronic annotation /// 0006955 // immune response // inferred from electronic annotation /// 0019882 // antigen processing and presentation // inferred from electronic annotation	0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation /// 0042612 // MHC class I protein complex // inferred from electronic annotation	---
180	hydroxyprostaglandin dehydrogenase 15-(NAD)	28.235	MmugDNA.18778.1.S1_at	HPGD	697864	---	---	---
181	similar to prosaposin	28.219	MmugDNA.11365.1.S1_at	LOC709510	709510	---	---	---
182	similar to Apolipoprotein D precursor (Apo-D) (ApoD)	-28.05	MmugDNA.10643.1.S1_s_at	LOC709223	709223	---	---	---
183	hypothetical protein LOC711872	-27.9	MmuSTS.4350.1.S1_at	LOC711872	711872	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
184	---	-27.88	MmugDNA.3680.1.S1_at	---	---	---	---	---
185	---	27.85	MmugDNA.37382.1.S1_s_at	---	---	0006629 // lipid metabolic process // traceable author statement /// 0006810 // transport // inferred from electronic annotation /// 0006869 // lipid transport // inferred from electronic annotation /// 0033344 // cholesterol efflux // inferred from direct assay /// 0033700 // phospholipid efflux // inferred from direct assay /// 0042157 // lipoprotein metabolic process	0005576 // extracellular region // inferred from electronic annotation /// 0005783 // endoplasmic reticulum // inferred from direct assay	0005319 // lipid transporter activity // inferred from electronic annotation
186	SPARC-like 1	-27.72	MmugDNA.28367.1.S1_at	SPARCL1	701468	---	---	---
187	similar to eukaryotic translation elongation factor 1 alpha 2 /// similar to eukaryotic translation elongation factor 1 alpha 1 /// eukaryotic translation elongation factor 1 alpha 1	27.716	MmugDNA.37793.1.S1_x_at	EEF1A1 /// LOC702809 /// LOC703715 /// LOC704199 /// LOC704438 /// LOC709017 /// LOC715351 /// LOC716010 /// LOC717003	702809 /// 703715 /// 704199 /// 704438 /// 709017 /// 715351 /// 716010 /// 717003	---	---	0000166 // nucleotide binding // inferred from electronic annotation /// 0003746 // translation elongation factor activity /// 0003924 // GTPase activity // inferred from electronic annotation /// 0005525 // GTP binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
188	similar to ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	27.573	MmugDNA.13288.1.S1_s_at	LOC699950	699950	---	---	---
189	similar to 60S ribosomal protein L21 /// similar to ribosomal protein L21 /// hypothetical protein LOC698492 /// hypothetical protein LOC699376	27.561	MmugDNA.25790.1.S1_at	LOC693767 /// LOC698377 /// LOC698492 /// LOC699376 /// LOC699398 /// LOC699867 /// LOC700271 /// LOC701710 /// LOC703876 /// LOC704538 /// LOC709045 /// LOC709144 /// LOC710306 /// LOC711174 /// LOC711760 /// LOC712987 /// LOC713294 /// LOC713662 /// LOC716588 /// LOC718134	693767 /// 698377 /// 698492 /// 699376 /// 699398 /// 699867 /// 700271 /// 701710 /// 703876 /// 704538 /// 709045 /// 709144 /// 710306 /// 711174 /// 711760 /// 712987 /// 713294 /// 713662 /// 716588 /// 718134	---	---	---
190	similar to High affinity immunoglobulin epsilon receptor gamma-subunit precursor (FceRI gamma) (IgE Fc receptor gamma-subunit) (Fc-epsilon RI-gamma)	27.531	MmugDNA.26925.1.S1_s_at	LOC720291	720291	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
191	thrombomodulin	-27.48	MmuSTS.4002.1.S1_at	THBD	702132	---	---	---
192	similar to acidic ribosomal phosphoprotein P0 /// similar to 60S acidic ribosomal protein P0 (L10E)	27.302	Mmu.13435.1.S1_at	LOC705501 /// LOC718979 /// LOC719112 /// LOC720470	705501 /// 718979 /// 719112 /// 720470	0006414 // translational elongation // inferred from electronic annotation	0005622 // intracellular // inferred from electronic annotation /// 0005840 // ribosome // inferred from electronic annotation	0003735 // structural constituent of ribosome // inferred from electronic annotation
193	similar to 60S ribosomal protein L29 (P23) /// similar to 60S ribosomal protein L29 (Cell surface heparin-binding protein HIP)	27.249	MmuSTS.2357.1.S1_s_at	LOC698130 /// LOC698602 /// LOC704510 /// LOC716320	698130 /// 698602 /// 704510 /// 716320	---	---	---
194	similar to ribosomal protein L15	27.124	MmugDNA.31525.1.S1_s_at	LOC701255 /// LOC716888	701255 /// 716888	---	---	---
195	similar to Actin, gamma-enteric smooth muscle (Smooth muscle gamma actin) (Alpha-actin-3)	-27.07	MmugDNA.30998.1.S1_at	LOC707447	707447	---	---	---
196	similar to 60S ribosomal protein L29 (Cell surface heparin-binding protein HIP)	26.992	MmuSTS.2357.1.S1_x_at	LOC698602	698602	---	---	---
197	similar to proteasome (prosome, macropain) subunit, alpha type 7	26.904	MmugDNA.22347.1.S1_at	LOC720175	720175	---	---	---
198	similar to 60S ribosomal protein L23	26.9	Mmu.4110.1.S1_s_at	LOC694719	694719	---	---	---
199	---	26.864	MmugDNA.23648.1.S1_at	---	---	---	---	---
200	similar to secretoglobin, family 3A, member 2	26.756	MmugDNA.13874.1.S1_at	LOC709138	709138	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
201	similar to ribosomal protein L24 /// hypothetical protein LOC699643	26.725	MmugDNA.25796.1.S1_s_at	LOC695715 /// LOC698942 /// LOC699643 /// LOC702381 /// LOC710502	695715 /// 698942 /// 699643 /// 702381 /// 710502	---	---	---
202	similar to dynein, cytoplasmic, light peptide	26.72	MmugDNA.2252.1.S1_at	LOC702360	702360	---	---	---
203	similar to Vitelline membrane outer layer protein 1 homolog precursor	-26.63	MmugDNA.2066.1.S1_at	LOC709950	709950	---	---	---
204	similar to Rho-GTPase-activating protein 6 (Rho-type GTPase-activating protein RhoGAPX-1)	26.558	MmugDNA.10026.1.S1_at	LOC705650	705650	---	---	---
205	---	26.524	MmugDNA.34186.1.S1_at	---	---	---	---	---
206	similar to diazepam binding inhibitor	26.518	MmugDNA.11287.1.S1_s_at	LOC698652	698652	---	---	0000062 // acyl-CoA binding // inferred from electronic annotation /// 0005488 // binding // inferred from electronic annotation
207	---	26.467	MmugDNA.35612.1.S1_x_at	---	---	0006281 // DNA repair // inferred from electronic annotation /// 0006974 // response to DNA damage stimulus /// 0007049 // cell cycle // inferred from electronic annotation	0005634 // nucleus // inferred from electronic annotation /// 0005694 // chromosome // inferred from electronic annotation	0003677 // DNA binding // inferred from electronic annotation /// 0005515 // protein binding // inferred from physical interaction /// 0005515 // protein binding // non-traceable author statement

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
208	---	26.384	MmugDNA.13186.1.S1_at	---	---	0006511 // ubiquitin-dependent protein catabolic process // inferred from electronic annotation /// 0006512 // ubiquitin cycle // inferred from electronic annotation /// 0007283 // spermatogenesis // traceable author statement	---	0004221 // ubiquitin thiolesterase activity // inferred from electronic annotation /// 0004843 // ubiquitin-specific protease activity // traceable author statement /// 0008233 // peptidase activity // inferred from electronic annotation /// 0008234 // cysteine-type peptidase activity // inferred from electronic annotation /// 0008234 // cysteine-type peptidase activity // traceable author statement /// 0016787 // hydrolase activity // inferred from electronic annotation
209	---	-26.24	MmuMitochon.10.1.S1_s_at	---	---	0006120 // mitochondrial electron transport, NADH to ubiquinone // inferred from electronic annotation /// 0006810 // transport // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation	0008137 // NADH dehydrogenase (ubiquinone) activity // inferred from electronic annotation /// 0016491 // oxidoreductase activity // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
210	similar to 60S acidic ribosomal protein P0 (L10E)	26.142	MmugDNA.32039.1.S1_x_at	LOC720470	720470	---	---	---
211	similar to N-acylsphingosine amidohydrolase (acid ceramidase) 1 preproprotein isoform a	26.13	MmugDNA.21971.1.S1_at	LOC703699	703699	---	---	---
212	similar to transgelin 2	26.124	MmugDNA.22158.1.S1_at	LOC719527	719527	---	---	---
213	similar to 40S ribosomal protein S15 (RIG protein)	26.086	MmugDNA.25954.1.S1_at	LOC707241	707241	---	---	---
214	tumor necrosis factor (ligand) superfamily, member 10	-26.08	MmugDNA.30129.1.S1_at	TNFSF10	694451	---	---	---
215	---	26.069	AFFX-Mmu-r2-Ec-bioD-3_at	---	---	---	---	---
216	similar to ribosomal protein L18a	25.943	Mmu.8777.1.S1_s_at	LOC719242	719242	---	---	---
217	glutathione peroxidase 4	25.893	MmugDNA.16268.1.S1_at	GPX4	705333	---	---	---
218	similar to troponin C, cardiac/slow skeletal	25.872	MmugDNA.6849.1.S1_at	LOC697047	697047	---	---	---
219	similar to 40S ribosomal protein S7 (S8)	25.831	MmugDNA.28023.1.S1_at	LOC721900	721900	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
220	putative 6-16 protein	-25.81	MmuSTS.4428.1.S1_at	16-Jun	716339	0001836 // release of cytochrome c from mitochondria // inferred from sequence or structural similarity /// 0006916 // anti-apoptosis // inferred from sequence or structural similarity /// 0043154 // negative regulation of caspase activity // inferred from sequence or structural similarity /// 0051902 // negative regulation of mitochondrial depolarization	0005739 // mitochondrion // inferred from sequence or structural similarity	0005515 // protein binding // inferred from sequence or structural similarity
221	similar to ribosomal protein L5	25.787	Mmu.11049.1.S1_s_at	LOC704067 /// LOC707117	704067 /// 707117	---	---	---
222	similar to ribosomal protein L13	25.565	MmugDNA.42536.1.S1_s_at	LOC700603	700603	---	---	---
223	similar to 40S ribosomal protein S6	25.493	MmugDNA.7002.1.S1_s_at	LOC712274 /// LOC718556 /// LOC722608	712274 /// 718556 /// 722608	---	---	---
224	similar to 15 kDa selenoprotein isoform 1 precursor	25.32	MmugDNA.5517.1.S1_at	LOC712469	712469	---	---	---
225	similar to ribosomal protein S5	25.233	MmugDNA.26535.1.S1_at	LOC711259	711259	---	---	---
226	similar to reticuloendotheliosis viral oncogene homolog B	25.198	MmuAffx.161.1.S1_at	LOC714677	714677	---	---	---

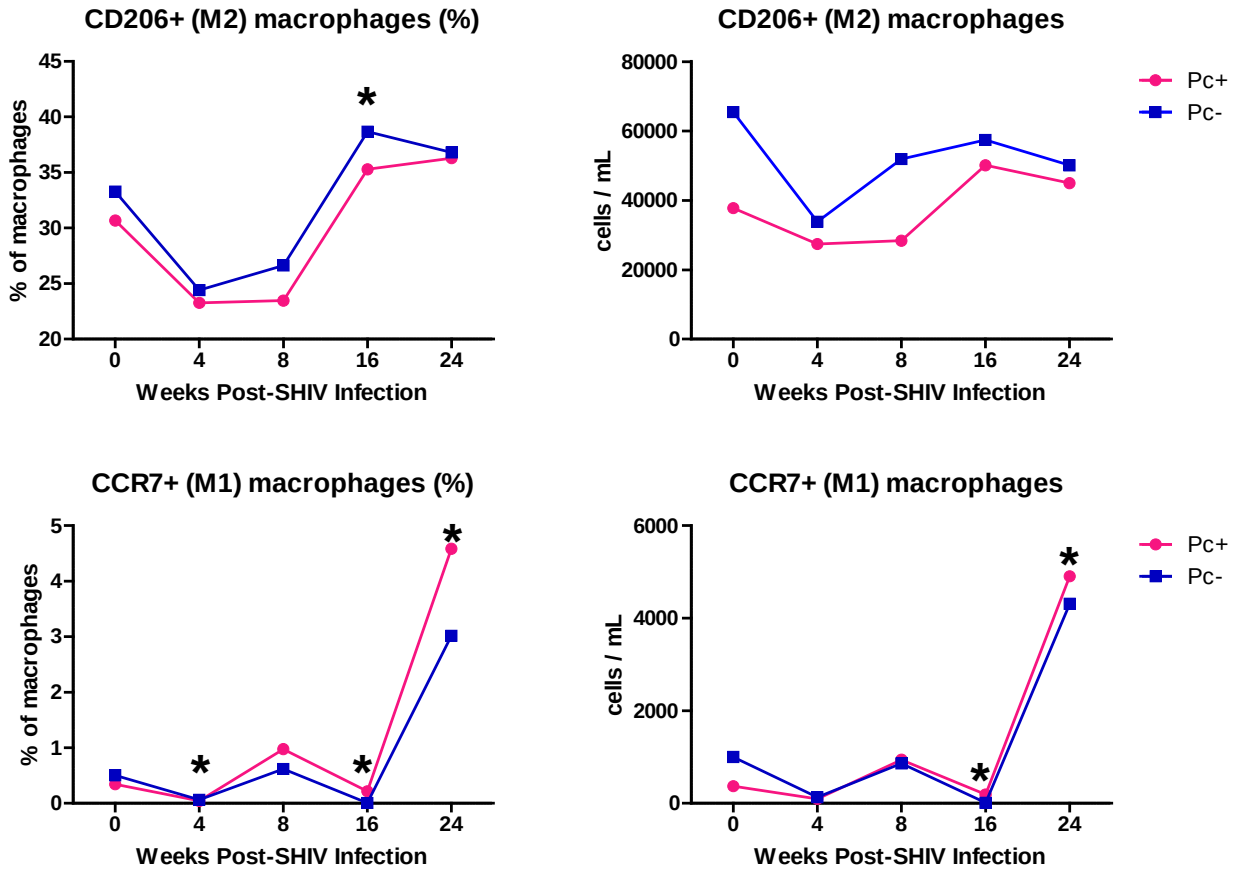
Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
227	deleted in malignant brain tumors 1	-25.09	MmugDNA.25527.1.S1_at	DMBT1	574192	---	0016020 // membrane // inferred from electronic annotation	0005044 // scavenger receptor activity // inferred from electronic annotation
228	similar to Galectin-3 (Galactose-specific lectin 3) (Mac-2 antigen) (IgE-binding protein) (35 kDa lectin) (Carbohydrate-binding protein 35) (CBP 35) (Laminin-binding protein) (Lectin L-29) (L-31) (Galactoside-binding protein) (GALBP)...	25.06	MmugDNA.32094.1.S1_at	LOC697290	697290	---	---	---
229	cathepsin H	25.014	MmuSTS.4176.1.S1_at	CTSH	711437	---	---	---
230	---	-25	MmuMitochon.4.1.S1_at	---	---	0006810 // transport // inferred from electronic annotation /// 0009060 // aerobic respiration // inferred from electronic annotation /// 0055114 // oxidation reduction // inferred from electronic annotation	0005739 // mitochondrion // inferred from electronic annotation /// 0005743 // mitochondrial inner membrane // inferred from electronic annotation /// 0005746 // mitochondrial respiratory chain // inferred from electronic annotation /// 0016020 // membrane // inferred from electronic annotation /// 0016021 // integral to membrane // inferred from electronic annotation	0004129 // cytochrome-c oxidase activity // inferred from electronic annotation /// 0005506 // iron ion binding // inferred from electronic annotation /// 0005507 // copper ion binding // inferred from electronic annotation /// 0009055 // electron carrier activity /// 0016491 // oxidoreductase activity // inferred from electronic annotation /// 0020037 // heme binding // inferred from electronic annotation

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
231	similar to Myosin regulatory light chain 2, smooth muscle isoform (Myosin RLC) (LC20) (Myosin regulatory light chain 9)	-24.76	MmunewRS.1055.1.S1_at	LOC709784	709784	---	---	---
232	---	24.682	Mmu.6730.1.S1_at	---	---	---	---	---
233	tropomyosin 2 (beta)	-24.64	MmugDNA.6549.1.S1_at	TPM2	696604	---	---	---
234	similar to Finkel-Biskis-Reilly murine sarcoma virusubiquitously expressed	24.597	MmugDNA.39247.1.S1_at	LOC716761	716761	---	---	---
235	similar to ribosomal protein L13	24.558	MmugDNA.37060.1.S1_at	LOC700603	700603	---	---	---
236	placenta-specific 8	24.443	MmugDNA.13757.1.S1_at	PLAC8	693310	---	---	---
237	similar to N-acylsphingosine amidohydrolase (acid ceramidase) 1 preproprotein isoform a	24.292	MmugDNA.13035.1.S1_s_at	LOC703699	703699	---	---	---
238	caveolin 1, caveolae protein, 22kDa	24.266	MmugDNA.13156.1.S1_at	CAV1	704449	---	---	---
239	Actin, beta	24.223	AFFX-Mmu-actin-M_at	ACTB	574285	---	---	0005515 // protein binding // inferred from electronic annotation /// 0005524 // ATP binding // inferred from electronic annotation
240	similar to Cytochrome c oxidase polypeptide VIIa-liver/heart, mitochondrial precursor (Cytochrome c oxidase subunit VIIa-L) (VIIaL)	24.163	MmugDNA.16710.1.S1_at	LOC703896	703896	---	---	---
241	similar to 40S ribosomal protein S29	24.129	MmugDNA.26508.1.S1_at	LOC702289	702289	---	---	---

Rank	Gene Title	J5	Probe Set ID	Gene Symbol	Entrez Gene	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
242	similar to diazepam binding inhibitor	24.044	MmugDNA.8184.1.S1_s_at	LOC698652	698652	---	---	0000062 // acyl-CoA binding // inferred from electronic annotation /// 0005488 // binding // inferred from electronic annotation
243	similar to 40S ribosomal protein S25	23.963	MmugDNA.41046.1.S1_s_at	LOC702593	702593	---	---	---

APPENDIX C

Method: Stained, fixed cells from BAL fluid were analyzed by flow cytometry (65). The following antibodies were used: mouse anti-human CD206- allophycocyanin (clone 19.2) (alternative activation (M2) marker) (BD Pharmingen, San Diego, CA), mouse anti-human CCR7 (CD197)-phycoerythrin (clone 150503) (classical activation (M1) marker) (R&D Systems, Minneapolis, MN). Acquisition was performed on BD LSRII flow cytometer using BD FacsDiva software. Forward/side scatter dot plot was used to gate the live macrophage population. All analyses were performed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).



There are no differences in frequency of BAL macrophages that are alternatively or classically activated between Pc-colonized versus non-colonized monkeys. Flow cytometry was used to examine frequency of CD206 to evaluate M2 (alternatively activated) macrophages (top row) and CCR7 to evaluate M1 macrophages (bottom row). No differences were found. Asterisks indicate significant differences for both populations from baseline levels.

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