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ABSTRACT OF DISSERTATION

Kerry McGarr Empey

The Graduate School

University of Kentucky

2007

NEONATAL IMMUNE MODULATION TO IMPROVE PNEUMOCYSTIS
CLEARANCE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of doctoral philosophy in the
college of pharmacy at the University of Kentucky

By
Kerry McGarr Empey

Lexington, Kentucky

Director: Dr. Robert P. Rapp, Professor of Pharmacy and Medicine
Co-Directors: Dr. Beth A. Garvy, Associate Professor of Medicine
and Dr. Patrick McNamara, Professor of Pharmacy
and Dr. Val Adams, Professor of Pharmacy
and Dr. Hsin-Hsiung Tai, Professor of Pharmacy

Lexington, Kentucky

2007

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ABSTRACT OF DISSERTATION

NEONATAL IMMUNE MODULATION TO IMPROVE PNEUMOCYSTIS CLEARANCE

Pneumocystis carinii is an opportunistic fungal pathogen that causes life-threatening pneumonia in immunocompromised individuals. Infants appear to be particularly susceptible to *Pneumocystis* (PC) pulmonary infections. The higher incidence of PC as well as other pulmonary infections among infants is likely due to an immature immune system. The neonatal lung environment is deficient immunologically in preterm as well as term infants (1, 2). Decreased phagocytic capacity of macrophages in newborns may increase the risk of infection from inhaled pathogens (1, 2). We have previously demonstrated that there is approximately a 3-week delay in the clearance of PC organisms from pup mouse lungs compared to adults. Herein, we demonstrate that there is also a 1-week delay in the infiltration of AMs in pup compared to adult PC-infected mice. We go on to show that there is a delay in pup versus adult lung macrophage phenotypic expression and cytokine production in response to PC organisms. We demonstrated that pup AMs are competent to produce cytokine in response to LPS and that stimulation with zymosan generates cytokine production in pup AMs that is comparable to adult cytokine production. These data indicate that pup lung macrophages are specifically poorly responsive to PC organisms and likely require exogenous stimulation to mount a significant immune response and expedite clearance of the organism. We go on to show that heat-killed *Escheria*

coli improves cytokine response, cellular infiltration and reduces organism burden in PC-infected pup mice. The clinically relevant cytokine, GM-CSF, has been used to improve the clearance of several pulmonary infections, including PC in adult animal models. We show that monotherapy with GM-CSF is insufficient to improve PC clearance in pup mice; however, when combined with TMP/SMX it improves PC clearance and maintains a reduced PC burden following discontinuation of therapy. Furthermore, we have shown that GM-CSF improves the ability of human infant lung macrophages to phagocytose PC organisms without generating an increased inflammatory response. These data suggest that combination therapy with TMP/SMX and GM-CSF may be a viable treatment option for infants failing or intolerant to standard therapy.

KEYWORDS: *Pneumocystis*, alveolar macrophage, granulocyte macrophage-colony stimulating factor, neonate/infant, immunomodulation

Kerry McGarr Empey

April 13, 2007

NEONATAL IMMUNE MODULATION TO IMPROVE PNEUMOCYSTIS
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I dedicate this work to my husband, Philip Empey, for whom this would not otherwise have been possible, and to our beautiful twins, Piper and Caden, who make this world a better place because they are here.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AM	Alveolar macrophage
APC	Antigen presenting cell
BALF	Bronchial alveolar lavage fluid
BGR	Beta-glucan receptor
CDC	Centers for Disease Control
CLR	C-type lectin receptor
CR	Complement receptor
DC	Dendritic cell
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HKEC	Heat-killed E. coli
IL	Interleukin
IFN γ	Interferon gamma
LM	Lung macrophage
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
MMR	Macrophage mannose receptor
MDC	Myeloid dendritic cell
NK	Natural killer

OI	Opportunistic infection
PC	Pneumocystis
PCP	Pneumocystis pneumonia
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
PMN	Polymorphonuclear cell
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
SIDS	Sudden infant death syndrome
SR	Scavenger receptor
TIP	Type I pneumocyte
TIIP	Type II pneumocyte
TCR	T cell receptor
TEM	Transmission electron microscopy
T _H	T helper
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha

CHAPTER 1. Introduction

A. Overview

Pneumocystis carinii f. sp. *hominis* is an opportunistic fungal pathogen known to cause life-threatening pneumonia in immunocompromised individuals. Infants, predominantly those that are immunocompromised, appear to be particularly susceptible to primary PC infection. Among HIV-infected children who develop pneumonia, *Pneumocystis carinii* f. sp. *hominis* is one of the most common causative pathogens (1). Furthermore, HIV-infected children less than 1 year of age have a higher incidence and a more fulminate course of *Pneumocystis* pneumonia (PCP) than older children with AIDS (2). More recently *Pneumocystis* (PC) colonization and infections have been identified in immunocompetent individuals, particularly in young children (3-6).

The higher incidence of PC as well as other pulmonary infections among infants is likely due to an immature immune system. The neonatal lung environment is deficient immunologically in preterm as well as term infants (7, 8). Decreased phagocytic capacity of polymorphonuclear (PMN) cells, monocytes, and macrophages, low immunoglobulin levels, decreased expression of complement receptors and possible defects in T lymphocyte immunoregulation in newborns may increase the risk of infection from inhaled pathogens (7, 8).

It is well known that T lymphocytes are crucial in host defense against PC as made evident by the Human Immunodeficiency virus (HIV) epidemic; a disease which kills CD4 T cells. We have previously demonstrated that there is approximately a 3-week delay in the clearance of *Pneumocystis* organisms from pup mouse lungs compared to adults which corresponds with a delay in T cell infiltration into the neonatal versus adult lungs (9). However, when T lymphocytes from pup mice were adoptively transferred into adult mice, they were shown to clear PC as efficiently as adult T cells (10) suggesting that the delayed PC clearance in pups can not be attributed to T cells alone. We have further shown that there is approximately a 1-week delay in alveolar macrophage (AM)

activation in pup versus adult mice (11). Macrophages are resident in the lungs and thus make up the first line of defense against inhaled pathogens. They appear to be the primary effector cells responsible for killing and clearance of PC (12) and are therefore important cells to consider when studying the failure of pup lungs to clear the organism. It is therefore, the goal of this project to determine the possible delays or defects present in pup lung macrophages (LMs) compared to adult LMs and to further identify a clinically relevant treatment option that would help stimulate LMs and expedite the clearance of PC organisms from infant lungs. Throughout this dissertation, lung macrophages (LMs) will be used to refer to both alveolar and lung tissue macrophages; alveolar macrophages (AMs) will be used when specifically referring to macrophages residing in the alveolar spaces.

According to the Center for Disease Control (CDC) guidelines, Trimethoprim/sulfamethoxazole (TMP/SMX) is currently the drug of choice in both adults and children for treatment and prophylaxis of PCP (13). Some of the adverse reactions that occur in children secondary to TMP/SMX include, but are not limited to, rash, hematological abnormalities, and interstitial nephritis (13-15). Furthermore, infants less than 4 weeks of age are at increased risk of jaundice when exposed to TMP/SMX due to its bilirubin displacing properties (1995 MMWR Revised guidelines for prophylaxis against PCP), (Brito 2006, Science Direct). If intolerance to TMP/SMX occurs, adults have several other treatment options, including pentamidine, atovaquone, clindamycin/primaquine, dapsone/trimethoprim, or trimetrexate glucuronate/leucovorin (13). Treatment options for the 15% of children who develop substantial adverse reactions to TMP/SMX (16), however, are severely limited with pentamidine being the only alternative agent with available data for use in children (13). Pentamidine is not a benign drug with 17% of children experiencing a substantial adverse drug reaction (17). Severely limited options, significant adverse reactions, and increasing resistance to TMP/SMX (18) (19) (20) (21), obviate and accentuate the need for further treatment options in infants suffering from PCP.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein produced by several pulmonary cells including macrophages, activated T cells, and epithelial cells (22). It is a hematopoietic growth factor that regulates the proliferation and release of progenitor cells and modulates the function of mature monocytes and macrophages. Among the functions of GM-CSF are its ability to influence the number of macrophages at a site of infection and activate macrophages for enhanced activity against invading pathogens (23) (24). GM-CSF is a commercially available agent indicated for use in leukemia following cancer chemotherapy, myeloid reconstitution following bone marrow transplants and mobilization for peripheral blood stem harvesting (Immunex Co.). Unlike G-CSF, GM-CSF is a potent inhibitor of neutrophil migration which may be beneficial during pulmonary infections known to mount a large, damaging inflammatory response, such as PC infection. GM-CSF has also been shown to enhance the clearance and improve outcomes of several different pulmonary infections, including but not limited to group B streptococcus infection (24), *Pseudomonas aeruginosa* (25), *Histoplasma capsulatum* (26, 27), *Mycobacterium tuberculosis* (28), and *Pneumocystis carinii* (29). However, there is little data on the effects of GM-CSF on pulmonary infections in infants and no data regarding the addition of GM-CSF for the treatment of PCP in the infant population. These aforementioned attributes and the ability of GM-CSF to improve outcomes in pulmonary infections in adult animal models suggest that this clinically relevant agent may be of therapeutic benefit for infants with pulmonary infections failing to respond to antimicrobial therapy alone. HIV-infected infants with PCP are a particularly fragile population requiring more treatment options to improve outcomes. However, the potential role of GM-CSF in the lung for host defense against PC has not been investigated in the neonatal population and will therefore be addressed within the scope of this project.

B. Neonatal Lung Environment

1. Immature neonatal immune function

Neonates are highly susceptible to infectious diseases during the early postnatal period, particularly at the mucosal surface of the respiratory tract (30, 31). Maturation deficiencies in both the innate (32) and adaptive (31) immune functions are thought to contribute to this increased vulnerability (33) (34). Studies in both humans and mice have demonstrated that neonatal immune cells are qualitatively distinct from adult cells. Among neonates and adults, subsets of cells exist in different proportions; furthermore cells of the same subtypes exhibit different phenotypes. Many studies throughout the literature have demonstrated deficiencies or alterations in neonatal T cells, B cells, and antigen-presenting cells (APCs) compared to adult cells. (30, 31, 33-35). These aforementioned immune deficiencies occur in the lung as well as throughout the neonatal immune system and likely contribute to the increased risk of respiratory infections observed in newborns. LMs, in particular, constitute an important first line of defense against inhaled pathogens (36) (30), therefore underdeveloped or functionally inefficient LMs must be considered when studying increased susceptibility to pulmonary infections in the neonatal population.

2. Alveolar macrophages

Phagocytosis

Tissue-fixed macrophages in the lung are instantly available to respond to inhaled pathogenic organisms. They do so through phagocytosis followed by death of the organisms intracellularly. Some organisms, such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, are able to escape death and live within a cell following phagocytosis by evading the killing capacity of the phagolysosome (37). Most organisms, such as *Pneumocystis carinii*, however, are extracellular and succumb readily to the acidic environment composed of oxygen radicals generated within the phagosome upon ingestion. Some phagocytosis assays performed on neonatal and adult macrophages have

demonstrated that neonatal macrophages are as competent as adult macrophages at phagocytosing both bacterial (38) and fungal pathogens (39). However, other studies done specifically on alveolar macrophages have shown that there is a strong correlation between impaired phagocytosis and infancy (40). One possibility for this stark discrepancy is the difference in the local environment. The phagocytosis assays performed by Karlsson et al. and Marodi et al. were done on cord blood derived monocytes, whereas those done by Martin et al. were performed on alveolar macrophages. The neonatal lung environment is thought to be immunosuppressant during its vulnerable post-natal development period which may, therefore, contribute to the lower phagocytic function demonstrated by Martin et al. Similar to phagocytic function; research done on the generation of oxygen radicals within activated macrophage phagosomes differs according to where the cells were derived. Neonatal and adult monocyte-derived macrophages from either cord or peripheral blood have been shown to produce similar levels of oxygen radicals in response to various stimuli (41, 42). Delacourt and colleagues, however, demonstrated that oxygen radical production is significantly reduced in neonatal rat LMs compared to adult LMs upon stimulation (43).

In addition to phagocytosis, upon interaction with foreign particles normally functioning LMs perform several duties which have been shown to be less efficient in neonates compared to adults, including recognition and binding through pattern recognition receptors (PRRs) (44), secretion of cytokines and chemokines (45) (44, 46, 47), up-regulation of complement receptors or integrins (45), and up regulation of co-stimulatory molecules (48) and antigen presenting molecules (49).

Pattern recognition receptors

Macrophages express several PRRs that recognize many different microbial components and which can discriminate between the surface molecules displayed by a pathogen versus one displayed by the host. Some important PRRs found on macrophages include the macrophage mannose

receptor (MMR), beta-glucan receptors (BGRs) (Dectin-1, CD11b, etc.), scavenger receptor (SR), LPS receptor (CD14), toll-like receptors (TLR), and CD11b/CD18 (complement receptor 3; CR3) (50). The up regulation of these receptors can often tell us if the macrophage has become activated or stimulated by a pathogenic organism. For example, CD14 recognizes and binds to the lipopolysaccharide component of the *Escherichia coli* (*E. coli*) cell wall. Additionally, macrophages are thought to identify PC through recognition of its cell wall components, MMRs (51) (52) and BGRs (53) (54) (55).

Of particular importance in regards to identifying the presence of PC are MMR and the beta-glucan receptor, dectin-1. These 2 receptors are known as C-type lectin receptors (CLR), which are defined as carbohydrate binding molecules that bind ligands in a calcium-dependent manner (56). The MMR binds both endogenous and exogenous ligands bearing mannose, fucose, N-acetylglucosamine, and sulphated sugars via its cysteine-rich domain (56). It is most commonly known for its ability to bind pathogen-associated mannose structures found on bacteria, yeast, and fungi (56). Many studies have suggested that the MMR is important in the recognition and clearance of PC (51, 52). Interestingly, however, other studies performed found that MR^{-/-} mice were no more susceptible to fungal pathogens, including PC, than their wild-type counterparts as measured by mortality and dissemination of infection (57) (58).

Like the MMR, dectin-1 is also a CLR involved in the clearance of glycosylated antigens present in fungal cell walls such as PC, particularly β -1,3- and β -1,6-linked glucans (56, 59). Unlike MMR, however, dectin-1 elicits pro-inflammatory mediators such as TNF α in response to fungal pathogens (60). It does this in collaboration with TLR2 (60) (61) which interacts with dectin-1's immunotyrosine activation motif (ITAM). Furthermore, dectin-1 has recently been shown to stimulate both CD4 and CD8 T lymphocytes, thereby inducing co-stimulatory molecule expression and IFN γ production (62). The MMR (51, 63) and BGRs (64) (54, 65) are both thought to play important roles in the recognition of PC and the subsequent stimulation of alveolar macrophages in the adult lung. Currently, however, there is no available data demonstrating whether or not

differences exist in the expression levels of MMR or dectin-1 on lung macrophages following bacterial or fungal stimuli in neonates versus adults.

The family of PRRs known as TLRs is unique from other PRRs in that they appear not to recognize and bind pathogens directly like MMR and dectin-1 (50). Instead, they associate with other receptors that directly recognize and bind pathogens. Once associated, the TLRs initiate an intracellular signaling cascade resulting in translocation of the transcription factor, NF κ B, to the nucleus. This results in the subsequent production of several important mediators of innate immunity such as cytokines and chemokines. Additionally, TLR signaling is responsible for generating CD80 and CD86, two important molecules in adaptive immunity. The presence of these co-stimulatory molecules along with pathogenic antigens presented on the surface of antigen presenting cells (APC), including macrophages, is an important method by which CD4 T lymphocytes are activated and thus tie TLRs to the initiation of the adaptive immune response (50). Specific agonists for nine of the ten human TLR members have been described thus far (66). Among these are the previously mentioned TLR2 which is known to associate with the BGR following recognition of the fungal pathogen PC. One of the first TLRs to be described was TLR4, which is known to associate with the LPS receptor CD14 following recognition of the *E. coli* cell wall component, LPS. Previous research has suggested that the deficiency of the innate immune response in neonates is due, in part, to impaired responses of neonatal macrophages to TLR ligands (67) (68).

The final PRR that will be discussed in regards to lung macrophages is CR3. Like many mediators of the immune system, CR3 is a multifunctional receptor performing roles in pathogen recognition as well as leukocyte adhesion and extravasation. CR3 is one of 4 complement receptors found on phagocytes. It is responsible for binding pathogens opsonized with complement components and stimulating phagocytosis (50). CR3 also belongs to a family of cell-adhesion molecules called integrins which are responsible for binding to cell-adhesion molecules and promoting the extravasation, whereby phagocytic cells expressing CR3 or other integrins are transported through endothelial membranes to the site

of infections (50). The third function of CR3 is as a PRR which recognizes and ligates β -1,3-glucans leading to cell priming for ingestion of the fungal pathogen (69-71). The ability of CR3 to recognize the β -1,3-glucan component of fungal cell walls as well as its constitutive expression on LMs makes it a useful tool in assessing LMs activation status following PC infection. There is no data in the literature describing whether or not differences exist in the expression level of CR3 in neonates versus adults. Thus information on the expression level of this receptor in the presence of PC infection in pup versus adult LMs is also lacking. Evidence from data collected in our lab demonstrates that clear differences exist between the expression level of the CD11b component of CR3 between pup and adult mice (11). Following intranasal PC inoculation of both pup (24-48hrs old) and adult mice (\geq 8 weeks old), CD11b expression was significantly lower on pup LMs compared to adult LMs by 6 days post-infection (11).

While other PRR exist, such as the scavenger receptor, for the purposes of this introduction, I have chosen to focus on the ones that are pertinent for my work with PC infection in the neonatal lung. One final PRR that I will briefly mention is CD11c/CD18 (CR4). CR4 is another complement receptor in the same family as CR3 which also plays a dual role as an integrin. Unlike CR3, CR4 has demonstrated no ability to recognize any pathogenic organisms outside the realm of the complement pathway. Throughout this research, however, we use antibodies specific for both the CD11b and CD11c components of CR3 and CR4, respectively, as a means of identifying LMs.

Cytokines and chemokines

Once a tissue macrophage recognizes and binds a pathogenic organism it is activated to release cytokines and chemokines, which in turn increase the state of inflammation by recruiting more immune cells and eventually initiating the cellular immune response (50). Cytokines are small proteins released by many different immune cells, including macrophages. The release of cytokines from their respective cells is generally initiated through the ligation of specific receptors as described above. Once these soluble proteins are released they can

act in an autocrine, paracrine, or endocrine manner thereby affecting the behavior of the host cell, adjacent cells, or distant cells, respectively (50). Chemokines are a distinct subset of cytokines with chemoattractant properties. These proteins are responsible for recruiting mainly monocytes, neutrophils, and other blood-derived cells with the appropriate receptors to sites of infection where the chemokine was initially released (50). The cytokines secreted by macrophages in response to pathogenic stimuli include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), $\text{TNF}\alpha$, and the chemokine interleukin-8 (IL-8) (50). IL-1 functions include activation of the vascular endothelium and local tissue destruction thereby mediating an increase in access of additional effector cells. Additionally, IL-1 is involved in lymphocyte activation and therefore is yet another link between the innate and adaptive immunity. IL-6 is also involved in B lymphocyte activation leading to increased antibody production. IL-12 induces the differentiation of CD4 T lymphocytes into T_H1 cells, thereby driving a more cellular response. IL-8, MIP-1, and MCP-1 are all chemokines responsible for recruiting other leukocytes to the site of infection; MCP-1 also acts to activate macrophages in an autocrine fashion. $\text{TNF}\alpha$ is an extremely potent cytokine involved in vascular endothelium activation and increased permeability, allowing an increased flux of immune cells and molecules to the site of infection and an increase in fluid drainage to regional lymph nodes (50). $\text{IFN}\gamma$ is a cytokine that is produced and secreted by T lymphocytes and natural killer (NK) cells. While it is not produced by macrophages in significant amounts, it is important in that it activates macrophages to further boost the immune response by increasing phagocytosis, increasing the expression of PRRs and co-stimulatory molecules, and increasing the production of cytokines and chemokines.

The two cytokines $\text{TNF}\alpha$ and $\text{IFN}\gamma$ have been shown to be very important in the clearance of PC (72) (73) (74, 75). Additionally, upon infection with PC, neonatal mice demonstrated a much weaker $\text{TNF}\alpha$ and $\text{IFN}\gamma$ mRNA response compared to adult mice (10). As previous research in our lab has demonstrated, the exogenous administration of $\text{TNF}\alpha$ is capable of stimulating a T cell

response in neonatal mice and thus may expedite the clearance of PC in neonatal mice (73). In my research, I have demonstrated that the administration of inhaled IFN γ alone does not improve clearance in PC infected neonatal mice. However, the administration of heat-killed *E. coli* (HKEC), a known stimulator of TNF α production, demonstrated a significantly increased clearance in PC-infected neonatal mice compared to their untreated counterparts. As will be made evident in the results section of my research, TNF α is an important cytokine in the pathogenesis of PC in a neonate. Furthermore, I will show that the production of TNF α in a neonate differs from that of an adult and may be due in part to the vulnerable state of the underdeveloped lung environment of a neonate.

Table 1.1 Cytokines/Chemokines

Cytokine	Effect on Macrophages and other cells	Affect on PC infection
TNF α	Activates Macrophages; Activates vascular endothelium and increases vascular permeability	PC is not cleared from reconstituted SCID mice in the absence of TNF α ; must be present early in disease (74)
IL-1	Activates vascular endothelium and lymphocytes	In the absence of IL-1, clearance of PC is almost completely inhibited (76)
IL-6	Activates lymphocytes leading to increased antibody production	PC clearance is not dependent on IL-6; during infection IL-6 regulates inflammation and antibody response (77)
IL-12p70	Promotes differentiation of CD4 T cells to T _H 1 cells	Improves proliferation of immune cells during PC infection but does not improve clearance (78)
IL-8	Recruits T cells and neutrophils	IL-8 promotes lung pathogenesis secondary to PC leading to a poorer prognosis; PC-mediated IL-8 release from AMs requires MRs and TLR2 (79)
MIP-1	Recruits monocytes, T cells, and DCs	Not specifically studied in PCP infection
MCP-1	Recruits monocytes, T cells, and DCs	Contributes to the pathologic T cell response leading to progression of PCP; may increase epithelial cell repair (80)
IFN γ	Activates macrophages	IFN γ does not play a significant role in PC clearance when given exogenously (74)
GM-CSF	Increases differentiation and proliferation of granulocytes; activates macrophages	Critical role in inflammatory response to PC through granulocyte proliferation and macrophage activation (81)

Co-stimulatory molecules

The co-stimulatory molecules and signals that are required for lymphocyte activation provide an extremely important function in maintaining a form of checks and balances in the initiation of the immune response. Activation of T lymphocytes by APCs are controlled by specialized ligands and receptors found on the APCs and the T lymphocytes. The co-stimulatory molecules I will focus on for the purposes of this research are the two structurally related glycoproteins CD80 and CD86 (also known as B7.1 and B7.2 respectively) found on APCs and CD40 ligand (CD40L) found on T lymphocytes. Once an APC, such as an LM, recognizes a pathogen (often by a PRR), it phagocytoses it, internally processes it and then presents an antigenic component of that pathogen on its surface in the context of a major histocompatibility class II (MHC II) molecule. This peptide: MHC II complex on the APC can now be recognized by naïve T lymphocytes. The peptide: MHC II complex on the APC binds to the T-cell receptor (TCR) and its co-receptor (ex. CD4) thereby delivering the first required signal in T lymphocyte activation. This one signal is not enough, however; a second stimulatory signal is required for full T lymphocyte activation. The second signal is delivered through binding of the co-stimulatory molecules CD80 and CD86 on the APC to the CD28 receptor found on the T lymphocyte. Only now will clonal expansion and the subsequent differentiation into a T_H1 or T_H2 T lymphocyte occur. The requirement of this second signal ensures that naïve T lymphocytes won't react to self antigen that is sometimes presented in an MHC II molecule (50). This description of the APC and T lymphocyte interaction is a basic one; there are several caveats that bear mentioning. Before a macrophage phagocytoses a pathogenic organism, it expresses little to no MHC II, CD80, or CD86 on its surface. The PRR that initially recognizes the pathogen is what provides the necessary signals for the expression of MHC II and the co-stimulatory molecules on its surface. At this point, macrophages are still in a relatively unstimulated state and may still function as APCs, but lack the capacity to be highly effective phagocytic cells which are often needed to clear many extracellular organisms such as PC. Like naïve T lymphocytes, macrophages

also require two signals in order to become activated. The first signal is provided by $\text{IFN}\gamma$, which can come from a number of sources, but the primary source is from armed effector T lymphocytes (CD4 or CD8 T lymphocytes). In the absence of T lymphocytes, $\text{IFN}\gamma$ may also be provided by NK cells. The second signal, which can be provided by a variety of means, is required to sensitize the macrophage to respond to $\text{IFN}\gamma$ (50). Perhaps the most common source of this second signal is the CD40L found on the surface of T_H1 cells as previously described, thereby allowing T_H1 cells to be the primary source of both signals required for macrophage activation. Without these two signals and the subsequent activation of macrophages, one can see how organism such as PC, which requires macrophage activation, can cause fulminate infections. This scenario is best exemplified by HIV, in which CD4 T lymphocytes are destroyed, subjecting the infected host to a wide range of opportunistic infections (OI) such as PC. Although T_H1 cells provide the most convenient source of both macrophage activating signals, there are other sources. As already described, $\text{IFN}\gamma$ can be provided by CD8 T lymphocytes or NK cells. In addition to CD40L, the secondary signal may also come from $\text{TNF}\alpha$, LPS, or other bacterial or fungal antigenic components. Once activated, macrophages undergo several changes that greatly increase their antimicrobial effectiveness leading to an enhanced immune response. For example, activated macrophages increase their expression of CD40 receptor, MHC II, CD80 and CD86, as well as inducing the production of oxygen radicals (50). In my research, I will be discussing the differences of some of these markers of macrophage activation as it pertains to neonates versus adults that have been infected with PC organisms. Additionally, I will discuss my findings regarding the up regulation the PRRs (CR3, TLR2, MMR, and BGRs) and cytokines ($\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-6, and MCP-1) thought to be important in PC infection.

The role of lung macrophages in Pneumocystis clearance

LMs are known to play a significant role in host defense against PC. Using a rat model depleted of LMs, Limper and colleagues demonstrated that PC

infection could not be controlled in adult rats in the absence of LMs (82). There are many organisms that come into contact with LMs that are cleared without the need for LM activation. PC organisms however, have been shown to require T lymphocytes for adequate clearance, suggesting that LM activation, and therefore, T lymphocyte: LM interaction, is important for clearance (83). While LMs are believed to be the effector cells that are directly involved with killing the PC organisms, LMs are not capable, on their own, to adequately control the infection. This has been clearly demonstrated in experiments in which T lymphocyte-deficient mice, with fully functional LMs, are susceptible to PC infection (83). Using an adoptive transfer model, our lab has previously demonstrated that neonatal T lymphocytes are fully functional and work as efficiently as adult T lymphocytes in clearing PC organisms (10). Thus, while it is understood that T lymphocytes continue to play a crucial role, the goal of my research is to define the differences between neonatal and adult LMs that lend to the inefficient clearance of PC from neonatal lungs. Furthermore, my research will focus on evaluating clinically relevant means by which to improve neonatal LM function to help fight PC infection as well as other pulmonary infections in the neonatal host.

3. Neutrophils

Following macrophages, neutrophils (polymorphonuclear cells; PMNs) comprise the second major group of phagocytes. There are many similarities and differences between these two phagocytic families; Table 1. Unlike macrophages, neutrophils are not tissue-fixed. They have a short life cycle which is spent primarily in the blood until called upon to help fight off an invading pathogen. While macrophages are the first to respond to an infectious process in the tissues, they are soon reinforced with large numbers of neutrophils to help with phagocytosis. Like macrophages, neutrophils recognize foreign pathogens through their large repertoire of PRRs. Neutrophils, however, differ from macrophages in that once they phagocytose a foreign particle they die soon thereafter, whereas a macrophage proceeds to present antigenic material on its

surface and thus acts as an intermediary between innate and adaptive immunity. The most important difference between these two phagocytic cells, for the purposes of my research, is that neutrophils do not appear to play a major role in the clearance of PC organisms compared to macrophages which are a key effector cell in clearing this organism (84). There are many differences between these two phagocytic cells that may contribute to the apparent lack of neutrophil involvement in PC clearance; Table 1.1. While a specific reason for this difference has not been fully elucidated, one could speculate that because neutrophils do not reside in the tissues, unlike macrophages (LMs in this case) they are less involved in the response to PC.

Table 1.2 Macrophages Versus Neutrophils

Function	Macrophages	Neutrophils
Life span	Long-lived	Short-lived
Phagocytic capacity	Continual	Once
MMR	Present	Absent
BGR	Present	Present
CD14 (LPS receptor)	Predominant	Little to no expression
Location	Tissues	Blood
CD4 T cell activation	Yes	No
Complement receptors	Yes	Yes

4. Dendritic cells

The function of a dendritic cell (DC) resembles that of a tissue macrophage, with some distinct exceptions. DCs are considered the primary professional APC. Like tissue-fixed macrophages, DCs take up antigenic material within infected tissue via PRRs, such as those previously described. Once this has occurred, intracellular signals trigger the DCs to lose their phagocytic abilities and become potent APCs. Like LMs, once a DC has become activated, they increase their expression of the co-stimulatory molecules, CD80 and CD86 as well as MHC II molecules on their surface. Unlike LMs, the activated DCs are no

longer phagocytic; instead they are signaled to migrate to the spleen or regional lymph nodes where they exhibit highly efficient T lymphocyte activation.

DCs exist as two major subsets in the human system. Myeloid DCs (MDC) are postulated to originate from circulating monocytes. Depending on the surrounding signals, monocytes can be stimulated to develop into either macrophages or MDCs (85). Monocytes, in the presence of granulocyte macrophage – colony stimulating factor (GM-CSF), develop into macrophages; however, in the presence of both GM-CSF and interleukin-4 (IL-4), monocytes differentiate into MDCs (86). The role of GM-CSF will be discussed in great detail later in the introduction. The other major subset of DCs are the plasmacytoid DCs (PDC), which look more like plasma cells, but retain some MDC characteristics (87). Structurally, when we think of DCs, we often picture follicular DCs (FDC) with their characteristic finger-like protrusions called dendrites. When we look at these cells under a standard light microscope, however, they are difficult if not impossible, to differentiate from macrophages. Additionally, both of these cells express the same surface markers, albeit at different expression levels, making these two cells even more difficult to discriminate; DCs and LMs are notoriously difficult to decipher from one another (88). For my *in vitro* experiments that I will discuss later in this dissertation, I rely on the fact that macrophages differ from DCs in their level of adherence. When cells are lavaged from an uninfected mouse lung, the majority of the cells are macrophages, with a small percentage of them being DCs. When incubated in a tissue culture plate, macrophages stick readily. DCs, on the other hand, are washed off, leaving a relatively pure population of macrophages to work with. Our *in vivo* work, which involves *in vivo* stimulation followed by flow cytometric analysis, discriminates between LMs and DCs based on the level of CD11c expression. Cells that express a high level of CD11c are considered LMs, whereas cells that express low levels of CD11c are considered DCs (89, 90).

5. T lymphocytes

T lymphocytes play a crucial role in the clearance of PC, as made evident by the HIV epidemic. HIV is a virus that targets CD4 T cells leaving its host vulnerable to opportunistic pathogens such as Cytomegalovirus (CMV), Toxoplasmosis, Candida albicans, and PC (13). T cells can be distinguished based on their distinct functions and expression of cell-surface proteins. T cells that express the co-receptor CD4 recognize antigens presented within MHC class II molecules, while T cells that express CD8 recognize antigens presented within MHC class I molecules (50). As discussed in the macrophage portion of the introduction, APCs associate with T cells via a peptide:MHC complex and receive 2 signals in order to become activated. Once an APC has engulfed a pathogenic organism, processed it internally and presented an antigenic component of it on its surface via an MHC molecule, it is ready to interact with a naïve T cell. During antigen recognition, CD4 or CD8 co-receptors associate on the T cell surface along with the T-cell receptor (TCR) and bind to the peptide:MHC II or I complex respectively, on the APC. As previously described, a second signal is required in order for the T cells to become activated. This second signal is provided by the co-stimulatory molecules, CD80 and CD86, expressed on the surface of APCs, which in turn bind to the CD28 receptor found on the surface of T cells. Once activated, T cells proliferate and differentiate to perform their respective effector functions (50).

CD8 T cells are known for their direct interactions with APCs bearing recognizable antigens. Antigens derived from replicating virus or other pathogens are displayed, in the context of MHC class I molecules, on the surface of an infected cell, where they are recognized by cytotoxic T cells (CD8 T cells). The CD8 T cells kill the infected cell by releasing perforin (which punches holes in the target cell membrane) and granzymes (proteases that trigger apoptosis intracellularly). CD8 T cells, also termed cytotoxic T cells, are important in the clearance of viral pathogens (50). CD4 T cells, however, appear to be the most crucial component of cell-mediated immunity in terms of PC infection. As previously stated, CD4 cells are the primary target in HIV disease, which leave

the host vulnerable to opportunistic infections (OI) such as PC. Also as previously pointed out however, in the absence of CD4 T cells, CD8 T cells are capable of providing LMs with $IFN\gamma$, 1 of the 2 signals required for macrophage activation. Furthermore, it has been demonstrated that while CD8 T cells alone are insufficient to clear PC organisms, they are associated with increased pathology in the lungs of PC-infected mice (91). When mice are depleted of both CD4 and CD8 T cells, they developed a less fulminate infection compared to mice depleted of CD4 T cells alone (92).

Upon activation, CD4 T cells can differentiate into either T_H1 or T_H2 cells, which differ in their cytokine production and thus in the functions they perform. Although the precise factors that determine whether a CD4 T cell will differentiate into a T_H1 or T_H2 cell are not fully understood, it is thought that the environment under which they differentiate may guide them, including the cytokines elicited by pathogenic organisms, the co-stimulatory molecules used to drive the activation, and the nature of the peptide:MHC molecule. Upon interacting with their specific peptide:MHC molecule complex, naïve CD4 T cells first respond by making IL-2 and proliferating. Subsequently, these cells will then differentiate into a T_H1 or T_H2 cell. This differentiation is extremely important in the type of immune response that will be elicited as each type of cell performs distinctly different functions. T_H1 cells function to activate macrophages, enabling them to destroy organisms, such as PC, more efficiently. Additionally T_H1 cells can activate B cells to produce opsonizing antibodies which aid in the recognition and uptake of certain microorganism by phagocytic cells. T_H2 cells, alternatively, drive a primarily humoral response characterized by the differentiation of B cells and their production of immunoglobulins. Additionally, T_H2 cells are responsible for activating naïve B cells to proliferate and secrete IgM. In our lab we have demonstrated that opsonization of PC by PC-specific antibody results in increased clearance of the organism from neonatal lung (11). Thus, B cell activation, in addition to CD4 T cell activation also plays an important role in the clearance of PC from neonatal lungs.

6. B lymphocytes

It is widely accepted that both T lymphocytes and LMs play a critical role in the host defense against PC, however, the role of B lymphocytes has been somewhat overlooked. In fact, B lymphocytes appear to play a significant role in PC infection as demonstrated by B cell deficient mice which were shown to be susceptible to PC (93). Furthermore, it is known that over 90% of people have PC-specific antibody by two years of age, suggesting that B lymphocytes have responded to PC organisms by at least the age of two (94). B lymphocytes play several roles in host defense in general, including antibody production, antigen presentation, and regulating the expansion of antigen-specific CD4 T cells (95). In regards to PC, it has been previously reported that B lymphocytes are capable of enhancing clearance through the production of PC-specific antibodies by promoting macrophage phagocytosis (96-98). The importance of PC-specific antibody production by B lymphocytes is reiterated when we look at children afflicted with X-linked hyper-IgM syndrome that are susceptible to PC infection. This disease is characterized by a mutation in the CD40L gene, which results in deficient T lymphocyte – dependent antibody responses (99). Additionally, further research has demonstrated that B lymphocytes can aid in PC clearance through an antibody-independent mechanism (96, 98). Although, the precise role(s) that B lymphocytes play in PC clearance has not been fully elucidated, it appears to be multifactorial. Lund and colleagues looked at the role that B lymphocytes play in CD4 T cell-mediated immunity against PC. Using a chimeric mouse model in which MHC class II molecules were expressed on all APCs except B lymphocytes, these researches demonstrated the importance of antigen presentation by B lymphocytes as the mice lacking MHC class II molecules on their B cells failed to clear PC compared to wild-type mice. To further assess the role of B cells on CD4 T cell-mediated immunity, this same group used an adoptive transfer mouse model in which they transferred CD4 T cells from either B-cell-deficient or wild-type mice into severe combined immunodeficient mice (SCID) mice. The mice that received CD4 T cells primed in B-cell-deficient mice were unable to affect PC clearance due to their inability develop into effector T

cells and migrate into the lung (96). Based on this evidence it is obvious that B cells play multiple roles in the clearance of PC. Although my research does not directly look specifically at the role of B cells in PC clearance, it is necessary to consider the whole host immune response to PC organisms when studying a particular aspect of it such as LMs.

C. *Pneumocystis carinii*

1. History

The history of *Pneumocystis* began in the early 1900's when it was erroneously characterized as stages in the life cycle of *Trypanosoma cruzi* (TC). In 1909 Carlos Chagas, a young Brazilian scientist studying at the Oswald Cruz Institute in Brazil published a 60-page article describing these newly identified cyst-like structures (100). This discovery was thought to be of such great importance that the article included color drawings of the 109 stages of TC's life cycle, an extremely rare occurrence at that time. Stages 41 through 44 of Chagas' proposed TC life cycle are believed to actually be the cyst forms of PC in which one can see eight spores (also called intracystic bodies), which Chagas described as "daughter cells" (100). Antonio Carini, an Italian scientist also studying in Brazil, became interested in Chagas' findings and began to search for these new life cycle stages of TC. Like Chagas, he used a rat model which he infected with TC. Following removal of the lungs and mounting tissue slices onto glass slides, Carini also observed the same cystic forms described by Chagas. This brought about the second article, published 1910, which mistakenly characterized PC as belonging life cycle stages of TC (101). Fortunately, Carini began to doubt whether the cysts he and Chagas were observing truly belonged to the life cycle of TC when he noticed the cysts were absent in some rats despite extensive infection with TC. Subsequently, he sent data and specimens to the Pasteur Institute in Paris where careful studies by Pierre and Mme Delanoe demonstrated that the cysts described by Chagas and Carinii could be found in TC-naïve rats, thereby proving it to be a separate species. They named

this new organism *Pneumocystis carinii*; *Pneumocystis*, because the organisms were lung-specific and *carinii*, in honor of the scientist who provided them with the specimens (102). Thus, in 1912 the first accurate description of this newly identified organism was published in a French article by Pierre and Mme Delanoë, in which they described the morphology of PC as can be observed upon Giemsa staining to this day (103). The Delanoës proceeded to publish one more paper describing PC in guinea pigs, a rabbit, a frog, and eels in 1914, after which time neither the Delanoës, Carini, nor Chagas pursued further research of the organism assuming it to be a trivial parasite found in rodents (104). Aside from the articles published by the three key players in the discovery of PC, only four other descriptions of PC in various species were published from 1913-1917 after which time almost three full decades passed without a single scientific publication regarding the organism we now know as PC (105-108).

In 1942, around the time of World War II, PC made its second debut in the history books appearing as a mysterious new type of pneumonitis in human infants in Europe (109). The disease was primarily afflicting infants between the ages of 2 to 8 months, born prematurely or suffering from malnourishment in infant nursing wards. Clinically, the infants would present with a subtle decrease in feeding, increased restlessness, poor weight gain, and sometimes diarrhea. Subsequently, the infants developed labored breathing which became increasingly worse. Eventually their skin turned a characteristic grey-blue color along with blue lips and eyes. Over the course of 1-2 weeks the condition would worsen until they either died from exhaustion or eventually recovered after a couple more weeks. The caregivers of the time, while they suspected an infectious etiology, could only provide comfort measures as none of the antimicrobial agents they tried improved the infants' outcomes (109) (102). The first article to report this mysterious infant-specific disease was published in Berlin in 1938 and described it as an interstitial plasma cell pneumonitis (110). Subsequently, over 700 cases of this illness were reported in Switzerland alone between 1941 and 1949 (111). It was also commonly reported throughout Europe, including Czechoslovakia, Italy, Hungary, Germany, Yugoslavia, Austria,

Denmark, Sweden, France, and Finland in the early 1950s (109). Two physicians from the Netherlands, van der Meer and Brug, had published an article in the very beginning of this pneumonitis epidemic in 1942 implicating PC as the etiological organism responsible for the much feared illness. Included in the article were photomicrographs showing the characteristic PC cysts and trophozoites retrieved from the autopsy of 3 infants that had succumbed to the disease (112). Sadly, it was published in a Dutch medical journal during World War II and went unnoticed for many years. It wasn't until 1951 when a Czechoslovakian doctor by the name of Joseph Vanek discovered PC in the lungs of 16 infants who had died of the mysterious disease. Upon microscopic examination the alveoli of these infants were found to be full of PC organisms (113). Although van der Meer and Brug had made this same discovery a decade earlier, it was Vanek and his colleagues who, by publishing their work in Czech and German medical journals, disseminated this important information to the medical community (114). Their hypothesis naming PC as the culprit of the interstitial plasma cell pneumonitis epidemic was soon well accepted by physicians throughout Europe (114).

The first case of PC was reported in the United States in Connecticut in 1956 by Dauter and colleagues (115). Four months later, two infants with pneumonitis in Oklahoma City were described (116). Following this, several cases of PC were reported in Canada (117, 118) and Chile (119). In 1959, some important contributions were made in our quest to learn more about PC by Dr. Walter Sheldon, a pathologist at Emory University in Atlanta, Georgia. Sheldon demonstrated PC to be the cause of two fatal cases of pneumonitis. The first was in an otherwise healthy 3-month-old infant found dead in her crib, the second was in a 10-year-old girl being treated with corticosteroid for glomerulonephritis. These were the first cases to which PC was associated with both sudden infant death syndrome (SIDS) and immunosuppressive therapy, respectively (102). Subsequently, Sheldon began to use experimental animals to try to reproduce the disease, which added to our understanding of PC and the eventual use of animal models in order to research PC organisms (120, 121). Sheldon and a

German scientist named Weller were the first to generate cortisone-treated animal models to study PC organisms (120, 122, 123). Unfortunately, no culture system has ever been developed that has been able to sustain PC organisms, thus the work done by Sheldon and Weller, built the foundation by which PC is still studied today.

Eventually the PC pneumonitis epidemic in European infants subsided along with World War II. Perhaps it had something to do with the improved economics and living conditions leading to less malnourishment among infants. Once again, PC was forgotten until it emerged again in the 1960s with the introduction of cancer-related chemotherapy and radiation. This time the organism was not targeting infants in European nursing wards, but rather children and adults in major medical centers with cancer or congenitive immunodeficiencies. Although no epidemiological reporting system for PC was in place during this time in history, a resourceful researcher by the name of Peter Walzer took advantage of the fact that Pentamidine was the only drug available for the treatment of PC and it was only available upon special request from the Centers for Disease Control (CDC) (102). Walzer determined that 579 requests for pentamidine were made from 1967 to 1970; of these, the diagnosis of PC was confirmed histologically in 194 patients. The underlying diseases in these cases included leukemia, Hodgkin's disease and lymphomas, solid tumors, congenitive immunodeficiency disorders, and organ transplant recipients (124). As the use of immunosuppressive cancer chemotherapy became more common, the incidence of PC pneumonitis increased leading to epidemic proportions at cancer centers throughout the United States. The problem had become so alarming that in 1973, the first international symposium on PC pneumonitis was held at the NIH leading to the publication of important events in the evolution of the disease (125). Two more important articles were published during this decade which demonstrated the association of PC with immunosuppressive therapy and immunodeficiency disorders. In both articles, severe combined immune deficiency disease (SCID) was the most common underlying illness associated with the presence of PC pneumonitis (126, 127). While PC pneumonitis was routinely discovered in

immune deficient syndromes and malnourished infants, reports of the organism in normal, healthy people were not reported until the late 1960s (128). In 1977 and 1978, important serological studies performed on normal populations in the Netherlands (129) and the United States (130) provided evidence to suggest that PC organisms were common in the human host. Results from these serologic studies indicated that most people become infected with PC organisms by 6 months of age and that by the age of 2 years basically all children have detectable antibody titers (129). From the 1960s up to the 1980s many histological and morphological studies were undertaken in both animals and humans to help further elucidate the organism that was still thought to be a protozoan at that time (131-134). In 1972 the first article to demonstrate species-specificity between PC organisms was published comparing PC isolated from rats versus those isolated from humans (135). It was the following year, in 1973 that the name *P. jiroveci* was suggested and came to be used to designate the human form of the organism (102).

The history of PC now becomes more familiar after 1980 and the global AIDS epidemic. It was in 1981 when young men in both New York and California were found to have PC pneumonitis without any known risk factors, which led some shrewd physicians to search for some kind of underlying immune deficiency to help explain the appearance of PC. Among the 11 men in New York (136) and the 4 men in California (137) reported to have PC pneumonitis; they had in common a homosexual lifestyle and/or intravenous drug abuse along with abnormally low T cell numbers. These reports aroused suspicion at the CDC and within the year, AIDS was defined and PC was to become known as an “opportunistic infection” and an “AIDS defining illness”. In 1983 a review by Jaffee and colleagues revealed that 50% of the first 1000 cases of AIDS also had PC pneumonitis (138). In addition to reports of PC in the context of AIDS, there have been some reports of PC occurring in immunocompetent individuals. Specifically, in 1999 Vargas and colleagues published an article demonstrating a correlation between SIDS and PC organisms. The study, performed in Santiago, Chile and Oxford, United Kingdom, looked at autopsies from SIDS cases and

identified PC in 35.1% and 14.8% of the cases respectively, suggesting a link between PC infection and SIDS (6).

Within the United States, PC infection remains primarily a disease of the immunocompromised host, whether the immune deficiency is chemotherapy-induced, AIDS-induced, or congenitally acquired, PC is common among all. In underdeveloped countries such as South Africa, where the HIV is poorly controlled, PC pneumonitis is still a major cause of morbidity and mortality, particularly in young children. Among HIV-infected children who develop pneumonia, PC is one of the most common causative pathogens (1). Infants appear to be particularly susceptible to primary PC infection, likely due to an immature immune system and a lung environment that protects against damaging inflammation early after birth (5). PC pneumonia (PCP) has been identified in 10-49% of children in Africa (139, 140). An autopsy study of Zambian children with HIV infection that died due to respiratory illness showed that PCP was present in 27% of cases. Furthermore, in the same study, PCP was found more commonly in infants aged 0-5 months (51%) than in those aged 6-11 months (26%) (141). The history of PC began as a case of mistaken identity; it was thought of as a miniscule, harmless organisms found in rodents. Today we know it to be one of the deadliest and most prominent infections among the immune deficient population. Research which furthers our understanding of this organism and new drugs designed to target the infection this organism causes remains an extremely important task.

2. Taxonomy

The history of PC would not be complete without some discussion about PC's taxonomy. Up through the 1960s, PC was considered to be a protozoan based on the fact that visually, they did not look like typical fungi (142), they failed to grow in culture, and antifungal therapy, while it reduced the organisms' viability somewhat, did not eradicate PC infections (143). The three morphological forms that PC undergoes throughout its life-cycle also led people to believe that it was a protozoan: Cysts, which are round or crescent shaped;

sporozoites, which are found within the cysts, and trophozoites, which are the free-floating forms of the organisms. A mature cyst can contain up to 8 sporozoites or intracystic bodies. The trophic form of PC tends to be pleomorphic and found in clusters. Furthermore, drugs that were used against protozoal infections, such as pentamidine and trimethoprim/sulfamethoxazole, were also effective against PC. The protozoan theory remained until the late 1980s when the sequence of the 18S ribosomal RNA (rRNA) gene of PC from rats was found to more closely resemble those of fungi, such as *Saccharomyces cerevisiae* and *Candida albicans*, than the 18S rRNA genes found in protozoa (144, 145). Since this time more than 1400 PC genes have been partially sequenced, with most of these genes closely resembling fungal gene orthologues (143). Despite the genetic resemblance of PC to other fungal organisms, other characteristics of PC are known to be very different from typical fungal characteristics. For instance, we know that fungal organisms such as *S. cerevisiae* and *C. albicans* grow rampantly in growth media, whereas PC cannot be sustained in culture. Additionally, ergosterol, the characteristic fungal cell membrane component which is the target of many antifungal drugs, is lacking in PC; instead, PC contains cholesterol in its membrane (146).

3. Pathogenesis

The damaging effects of PCP within the lung can be divided into two different categories; PC-mediated and immune-mediated lung injury. Direct PC-mediated damage to the host lung is caused by virulence factors associated with PC organisms. The first detectable event in PCP disease progression is the attachment of the trophic forms of PC to type I pneumocytes (TIP) within the lung (147) (148). Following attachment, there is an increase in permeability of the alveolar-capillary membrane, degeneration and necrosis of the TIP, and finally erosion of the basement membrane (147). Two factors were noted during this process of TIP damage. The first was an increase in the proliferation of Type II pneumocytes (TIIP), seemingly in an effort to replace the TIPs and repair the damaged alveoli (149) (150). The second important factor noted in these studies

of early PC infection was the lack of an inflammatory response despite ongoing alveolar damage (147) (150). Animal studies suggest that the interaction of PC organisms with TIIPs and the subsequent capillary leak may serve as a mechanism by which the organisms acquire nutrients needed for continued growth and survival (151, 152). Although less common, other studies have shown the adherence of PC organisms to TIIPs and their subsequent degradation, are likely due to the increased proliferation of TIIPs seen following TIP loss (149). Furthermore, experts in the area suspect, based on animals studies (149), that the interaction of PC with TIIPs may contribute to the pathology caused by PC organisms by preventing the proliferation and migration of TIIPs to repair the damaged alveoli (152). In addition to directly adhering to and destroying pneumocytes, PC organisms have other virulence factors that may contribute to PCP's pathology, including the production of proteolytic and glycolytic enzymes. While the primary function of PC-derived enzymes likely involves the acquisition of nutrients or the evasion of host defense mechanisms, they may also be causing collateral lung damage (152). A number of different potentially PC-derived enzymes capable of causing alveolar damage have been reported in the literature, including chymase (153), collagenase (151), protease (154), elastase (154), cysteine protease (155) (156), and enolase (157). In addition to these enzymes, a fungal kexin-like molecule has been identified in mouse-, rat-, and human-derived PC (158) (159, 160). Although not thought to be a virulence factor in human-derived PC, mouse- and rat-derived PC kexin is localized to the surface of the organism and has been suggested to play a role in attachment to the host, nutrient acquisition, or host defense evasion and thus possibly also lung injury. Within mouse- and human-derived PC, the kexin protein is encoded by single-copy genes; this unique characteristic has enabled its use in quantifying PC organisms via real-time PCR (159) (3). Lastly, PC is known to disrupt surfactant homeostasis within the lung leading to respiratory impairment during PC infection (152). Specifically, PC is thought to inhibit the secretion of surfactant components from TIIPs or by binding to certain surfactant components and sequestering them in inactive forms (161, 162).

In the clinical setting, the presentation of PCP is highly dependent on the immune status of the patient, suggesting that the host immune response to PCP is contributing to its pathogenesis by initiating immune-mediated lung injury in addition to the direct effects of PCP on T1Ps and T2Ps. The initial observation of PCP in cancer patients often occurs once immunosuppressive steroid therapy is being tapered (163) (164); in bone marrow transplant recipients it typically occurs after engraftment and immune function restoration (165) (166); and in AIDS patients it generally occurs following rapid recovery of CD4 T lymphocytes secondary to initiating highly active antiretroviral therapy (HAART) (167, 168). Additionally, it has been well documented that lung injury during moderate to severe PCP is minimized with the administration of adjunctive steroid therapy, reiterating the detrimental effects of the host immune response to PC in the infected lung (169) (170). More specifically, it has been suggested that the adaptive immune response causes the majority of lung injury compared to the innate response following PC infection. This theory is generated from the fact that it is difficult to prove involvement of the innate immune response, whereas data obtained from studying PC-infected SCID mice point to a significant role of the adaptive immune response in causing lung injury (152). The accumulation of PMNs in the lungs of PC-infected patients has been associated with decreased oxygenation and a poor prognosis (171) (172). Additionally, *in vitro* experiments have suggested that PC organisms are capable of directly stimulating macrophages (173) (47). The dilemma with these experiments, however is that its impossible to determine if PMNs are directly causing lung injury or are recruited in response to lung injury. Likewise, it is indeterminate whether PC-stimulated macrophages contribute to lung injury or are merely a conduit to the adaptive immune response, which has been undeniably shown to cause lung damage in PC-infected lungs (174, 175). Both CD4 and CD8 T lymphocytes have been implicated in immune-mediated lung damage, the best example of which can be observed among the AIDS patients infected with PC. The role of CD4 T cells is obviated following HAART and the rapid recovery of CD4 T cells. While an AIDS patient with low CD4 T cell counts will often have a high PC burden,

they also tend to have a subacute onset of PCP. Upon CD4 T cell restitution following HAART, however, these patients develop a rapid decline in pulmonary function, suggesting a significant role for CD4 T cells in PC pathogenesis (167, 168). As I describe my research throughout the remainder of this document, I will discuss both the activation of neonatal versus adult LMs as well as the infiltration of CD4 and CD8 T lymphocytes following PC infection and the role that each plays in clearing the infection.

4. Identifying and quantifying Pneumocystis organisms

For the purpose of identifying and studying PC organisms, it is important to discuss the stages PC's life-cycle and the different morphological methods available for studying each of these stages. What is known about the life-cycle of PC has been derived primarily through the use of transmission electron microscopy (TEM). Based on this data, the transition from a trophozoite to a mature, eight-spore-containing cyst is thought to occur through 3 consecutive stages of the sporocyte (176). The cycle begins with a pleomorphic, thin-walled mononuclear trophic form of the organism. This form is thought to attach to type I epithelial cells in the alveoli. These trophic forms are then thought to evolve into a thick-walled sporocytic and cystic stage. At this point, multiple nuclear divisions are thought to generate eight spores within the cyst (176). It is hypothesized that PC growth results primarily from cyst development rather than from trophic binary division based on the growth retardation imparted by antifungal echinocandins which function to inhibit beta-1,3 glucan synthesis (a component of the cyst wall). In a PC-infected rat model, treatment with therapeutic doses of an echinocandin (L0671,329) resulted in the selective elimination of PC cysts. Subsequently, the administration of lower, prophylactic doses of L-671,329 prevented the appearance of the trophic forms of the organism (177). Understanding the morphology and life-cycle stages of PC are important for both quantification and targeting drug therapy for this organism, both of which will be discussed herein.

Human-derived PC organisms are retrieved from either BALF or induced sputum samples. Most commonly, they are then stained with methenamine silver

nitrate and can then be observed under a light microscope. While this method is sensitive for identifying the presence of PC, it only stains the cyst cell walls, leaving the PC trophozoites unstained (176) (178), and thus is not ideal for PC quantification. PC can also be stained with polychrome methylene blue; however this method stains only the intracystic bodies and not the cell wall (178). For the purpose of PC quantification it is important to use a method that will identify PC throughout its different life-cycle stages. Diff-Quik[®] is one such stain (179). This staining method is able to discriminate between nuclei (stains pinkish purple) and cytoplasm (stains blue). In this manner one can not only count PC nuclei for quantitative purposes, but can also quantify the number of cysts, intracystic bodies, and trophozoites. More recently, polymerase chain reaction (PCR) (180) and real-time PCR (181) (182) have been introduced as effective methods for the diagnosis and quantification of PC organisms. This method has been shown to be highly sensitive and will likely become more widely used in the study of PC organisms. In my research, I used real time PCR to identify and quantify PC organisms when detection by light microscopy was determined to be inefficient and minimally sensitive. The methods used for PC quantification during my research will be discussed further in the methods section of this document.

D. Drug Therapy

1. History

When PC first started killing infants in Europe in the early 1940s, little could be done in the way of treatment. At that time, it wasn't even known that PC was the culprit of this mysterious pneumonitis, thus caregivers could only provide supportive care, while they watched infant after infant succumb to the disease. It wasn't until the early 1950s that PC was identified as the source of the infectious pneumonitis, which by this time, was causing disease in chemotherapy-related and other immunodeficiency disorders. Because little was known about PC organisms, the identification of targeted drug therapy was difficult.

The first drug to show effectiveness against PC was pentamidine isethionate, manufactured in England by May and Baker, Ltd. Because PC was thought to be a protozoon, drugs that were effective against other protozoa, such as pentamidine, were studied for the treatment of PC. One of the first studies to look at changes in mortality with this drug was published in 1958 by Ivady and Paldy (183). Subsequent studies looking at outcomes in PC-infected children receiving pentamidine demonstrated a decrease in mortality from 50% to approximately 20% (184) (185). While pentamidine was the only drug known to decrease mortality from PC, it was certainly not an ideal drug. It required parental administration, it couldn't be used for prophylaxis, and it had many adverse effects, including cardiovascular, hematologic, dermatologic, and endocrine reactions, most notably, severe hypoglycemia (186) (187) (188). Furthermore, in the 1960s through the early 1980s, pentamidine had not been approved by the U.S. Food and Drug Administration (FDA), and thus was not available for general use. If a case of PC was diagnosed, physicians had to contact the Parasitic Disease Drug Branch of the CDC, provide data confirming their diagnosis of PC pneumonitis, and wait for CDC approval. If approved, the drug was sent by commercial airline to the city where it had been requested. Physicians and patients could only hope that the drug made it there before it was too late (102). While other antimicrobial agents have shown effectiveness against PC organisms, it was the discovery of the combination of trimethoprim and sulfamethoxazole in 1974 that dramatically changed how we treat and prophylax against PC. Hughes and colleagues demonstrated that this combination drug therapy was highly effective for both the prevention and treatment of PC pneumonitis in a corticosteroid-treated rat model (189). Subsequently, their research was extrapolated to clinical trials where they demonstrated therapeutic safety and efficacy in PC-infected cancer patients (190, 191). After this discovery, several studies followed suit which confirmed both the safety and efficacy of TMP/SMX for the treatment of PC pneumonitis in both adults and children (192) (193, 194).

2. Current Recommendations

Guidelines for both the treatment and prevention of opportunistic infections were devised for both adults and children following the increase in opportunistic infections (OIs) associated with HIV infections. Being that PCP is the most common OI associated with HIV, it is included within these recommendations. While the occurrence of PCP is not limited to HIV-infected individuals, the guidelines for treatment of PCP associated with HIV can be extrapolated to all cases of PC infection. Outlined below are the most recent therapeutic recommendations put forth by the CDC for both adults and children infected with PC.

Trimethoprim/sulfamethoxazole

As previously described, the CDC recommends TMP/SMX as the first line treatment and prophylaxis for PCP in both adults and children (13, 195). TMP/SMX is a combination of two antimicrobial agents, each of which act on two different enzymes to synergistically inhibit folate metabolism; TMP blocks dihydrofolate reductase (DHFR) and SMX blocks dihydropteroate synthase (DHPS) (196). Ultimately, the inhibition of these enzymes leads to the inhibition of thymidine synthesis and subsequently DNA synthesis within the target organism. Since the discovery of its effectiveness against PCP in the early 1970s (190, 197), TMP/SMX has been shown to be as efficacious as IV pentamidine as well as other alternative agents (191) (189). Its ease of administration, relatively fewer side effects, and reasonable cost have kept it the drug of choice for PCP since it was first officially endorsed as first-line treatment by the CDC in 1989 (198).

Two major factors obviate the need for newer alternative agents for the treatment of PCP; the first is the high rate of adverse reactions associate with TMP/SMX. Some of the adverse reactions that occur in children secondary to TMP/SMX include, but are not limited to, rash, hematological abnormalities, and interstitial nephritis (13-15). Furthermore, infants less than 4 weeks of age are at increased risk of jaundice when exposed to TMP/SMX due to its bilirubin

displacing properties (1995 MMWR Revised guidelines for prophylaxis against PCP), (Brito 2006, Science Direct) (199-201). If intolerance to TMP/SMX occurs, adults have several other treatment options, including pentamidine, atovaquone, clindamycin/primaquine, dapsonе/trimethoprim, or trimotrexate glucuronate/leucovorin (13). Treatment options for the 15% of children who develop substantial adverse reactions to TMP/SMX (16), however, are severely limited with pentamidine being the only alternative agent with available data for use in children (13). The rate of toxicity is higher among HIV-infected patients though the reason for this is not understood (202). Over 80% of HIV-infected individuals experience an adverse reaction to TMP/SMX; in more than 50% of these patients, the adverse reaction may be dose-limiting. The most common toxicities include gastrointestinal symptoms, rash, fever, cytopenia, hepatitis, nephritis, hyperkalemia, and pancreatitis (203) (204).

The second major factor necessitating newer treatments for PCP is the increase in *Pneumocystis jiroveci* resistance to SMX (the primary agent in the combination product) (205), undoubtedly brought about by the wide exposure of PC organisms to SMX following the start of the AIDS epidemic. While sulfa resistance has been well documented among a variety of different organisms such as *S. pneumoniae* (206), *N. meningitides* (207), and *E. coli* (208), drug resistance to PC cannot be determined by typical methods because patient isolates cannot be cultured in order to determine drug sensitivities. Thus, resistance detection relies on the identification of mutations within the DHPS gene. The PC DHPS gene was sequenced in 1997 from six patient isolates in which specific genetic polymorphisms were identified. Each of the polymorphisms resulted in changes in the respective encoded amino acids suggesting that an evolutionary selective pressure, perhaps imparted by exposure to SMX, occurred in PC isolated from these patients (209). Additional studies have been done looking at the existence of DHPS mutations in patients receiving TMP/SMX prophylaxis compared to those not receiving prophylaxis. In every study except one, the groups receiving TMP/SMX prophylaxis had significantly more DHPS mutations compared to their respective controls (18, 210-215). The one study

that did not show a difference had a very small sample size which may have contributed to the results (216). In addition to these studies, 4 other studies have been published which take a closer look at the correlation between the DHPS mutation and clinical treatment failure. The first study, published in 1999 showed an increased 3-month mortality rate in patients with mutant DHPS compared to patients with wild-type PC infections. The other three studies looked at associations between mutant DHPS and treatment failure rates; of these, 2 of the studies found a correlation between the mutant DHPS and treatment failure (212) (217) while the third study found no correlation (218). While the conflicting results of these studies leave us questioning their clinical applications, it is clear that with continued selective pressure by the wide-spread use of TMP/SMX treatment and prophylaxis, we will begin to see clinically significant PC mutations leading to resistance.

Alternative agents available for use in children

IV pentamidine is the only alternative agent recommended in children infected with PC. While aerosolized pentamidine has been assessed for AIDS-related PC pneumonia, it was not as effective as IV pentamidine or TMP/SMX and is therefore not recommended for treatment (219, 220). It is generally only administered to children who cannot tolerate TMP/SMX or who demonstrate clinical failure after 5-7 days of TMP/SMX therapy. Approximately 17% of children who receive pentamidine experience a severe adverse reaction, including renal toxicity, severe hypotension, severe endocrine abnormalities, and pancreatitis (17) (195). No evidence exists for any synergistic or additive benefits with the use of pentamidine together with TMP/SMX; in fact, the use of these two agents together should be avoided due to the additive adverse effects (195). Pentamidine is considered only as an alternative treatment to TMP/SMX due to its greater number of severe adverse effects, IV administration and lack of availability (191).

While other alternative agents are available for the treatment of PCP in adults and may actually be used in children, their use in children is not endorsed

due to the limited data available for their use in children. Although pentamidine is known to cause many serious adverse reactions, it has long history for treating PCP in children since it was the first known treatment for the disease. Due to the limited treatment options available for children intolerant to or failing TMP/SMX therapy, pentamidine remains one of the few available options left for children infected with PC.

Alternative agents available for use in adults

Dapsone plus TMP is a recommended alternative in adults for the treatment of mild-to-moderate PCP for those who are allergic or otherwise cannot tolerate TMP/SMX. Those who are allergic to TMP/SMX are generally allergic to the sulfa component (SMX) and thus are still able to take TMP. While dapsone contains a sulfa moiety, it is a sulfone drug compared to a sulfonamide drug; therefore patients allergic to sulfonamides such as SMX will generally tolerate dapsone (221). This regimen may have similar efficacy and fewer side effects than TMP/SMX, but is less convenient due to the large number of pills required (13).

Primaquine plus clindamycin is considered to be an effective alternative regimen for the treatment of mild-to-moderate PCP. Studies have shown this regimen to be as effective with equal or less side effects than TMP/SMX (222) (223). While clindamycin is readily available in both IV and PO formulations, primaquine is only available orally and thus is inappropriate for severe cases of PCP in which the patient is unable to take oral medications.

IV pentamidine is an alternative agent for treating severe disease in adults unable to take oral medications. As discussed previously in regards to treating PC-infected children, this drug has several serious adverse reactions associated with it, making it a second line treatment option to TMP/SMX (224) (191).

Atovaquone suspension has been shown to be less effective than TMP/SMX for the treatment of PCP. However, it was also shown to have fewer side effects than TMP/SMX and is therefore recommended as an alternative agent for mild-to-moderate disease in adults (225) (226). Trimetrexate and

leucovorin is an alternative regimen similar to atovaquone, except that it is available IV (227).

Corticosteroids

Studies in adults have demonstrated a reduction in mortality and a faster recovery time in patients with moderate-to-severe PCP who receive corticosteroids within 72 hours of starting PCP treatment (228) (229) (230). Studies in children have shown similar results with those receiving corticosteroid treatment having a reduction in acute respiratory failure, decreased need for mechanical ventilation, and a decrease in overall mortality (231) (232) (233). Indications for the use of corticosteroids include room air $pO_2 < 70$ mm/Hg or arterial-alveolar O_2 gradient > 35 mm/Hg (195) (13). The observation that steroid therapy improves the outcomes of PC infection further demonstrates the role that the host's immune system plays in the pathogenesis of PCP and underscores the need for therapeutic agents involved in immunomodulation.

Granulocyte macrophage colony-stimulating factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein produced by several pulmonary cells including macrophages, activated T cells, and epithelial cells (22). Among the functions of GM-CSF are its ability to influence the number of macrophages at a site of infection and activate macrophages for enhanced activity against invading pathogens (23) (24). GM-CSF is a commercially available hematopoietic agent indicated for use in leukemia following cancer chemotherapy, myeloid reconstitution following bone marrow transplants and mobilization for peripheral blood stem harvesting (Immunex Co.). GM-CSF has also been shown to enhance the clearance and improve outcomes of several different pulmonary infections, including but not limited to group B streptococcus infection (24), *Pseudomonas aeruginosa* (25), *Histoplasma capsulatum* (26, 27), *Mycobacterium tuberculosis* (28), and *Pneumocystis carinii* (29). However, there is little data on the effects of GM-CSF on pulmonary infections in infants and no data regarding the addition of GM-

CSF for the treatment of PCP in the infant population. These aforementioned attributes and the ability of GM-CSF to improve outcomes in pulmonary infections in adult animal models suggest that this clinically relevant agent may be of therapeutic benefit for infants with pulmonary infections failing to respond to antimicrobial therapy alone. HIV-infected infants with PCP are a particularly fragile population requiring more treatment options to improve outcomes. However, the potential role of GM-CSF in the lung for host defense against PC has not been investigated in the neonatal population and will therefore be addressed within the scope of this project.

E. Project Overview

Our lab has previously demonstrated that there is approximately a 3-week delay in the clearance of *Pneumocystis* organisms from pup mouse lungs compared to adults which corresponds with a delay in T cell infiltration into the pup versus adult lungs (9, 234). However, when T lymphocytes from pup mice were adoptively transferred into adult mice, they were shown to clear PC as efficiently as adult T cells (10) suggesting that the delayed PC clearance in neonates can not be attributed to T cells alone. We have further shown that there is approximately a 1-week delay in LM activation in pup versus adult mice (11). Resident macrophages in the lungs make up the first line of defense against inhaled pathogens such as PC and appear to be the primary effector cells responsible for killing and clearance of PC (12). Furthermore, the role of activated T lymphocytes during PC infections has been shown to be crucial in the clearance of this organism (97, 234), thus the activation role of LMs toward T lymphocytes is likewise crucial to PC clearance. It was therefore, the goal of this project to elucidate the role that lung macrophages play in delayed T lymphocyte infiltration and delayed PC clearance, to determine any deficiencies in pup LMs compared to adult lung macrophages and to further identify a clinically relevant treatment option that would help stimulate lung macrophages and expedite the clearance of PC organisms from pup lungs.

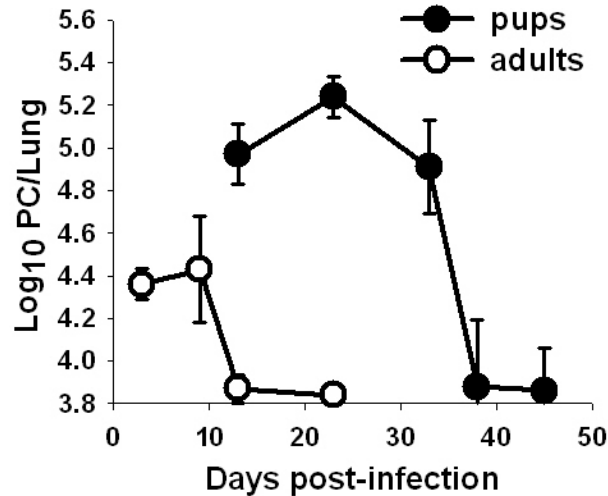


Figure 1.1 Delayed PC clearance in pup mice. Mice were infected with PC as neonates (24-72 hours after birth) or as adults (>8 weeks). Whole lungs were collected and processed into obtain single-cell suspensions. Aliquots were spun onto glass slides, stained with Diff-Quick[®], and PC nuclei were enumerated microscopically. Results represent the mean \pm SD of 4-5 mice per group and 4 separate experiments.

I have developed three hypotheses designed to evaluate the role of lung macrophages in the delayed clearance of PC and potential mechanisms for improving outcomes in PC-infected pup mice and human infants through the immunomodulation of LMs. These hypotheses and their respective aims are outlined below.

Hypothesis 1: Lung macrophage activation is delayed in *Pneumocystis*-infected pup compared adult mice.

Aim 1: Flow cytometry was used to compare the expression levels of activation markers on the surface of PC-infected pup and adult LMs. Additionally, the infiltration of T lymphocytes was assessed using flow cytometry. These markers of activation and T cell markers were evaluated over the time course of PC infection in both pup and adult lavage and lung tissue.

Aim 2: CBA and ELISA kits were used to assess cytokine production in pup versus adult alveolar macrophages. The production of cytokines secondary to PC, were also determined and compared for both pups and adults over time.

Hypothesis II: Exogenous immunomodulation with IFN γ , Heat-killed *E. coli* (HKEC), GM-CSF alone, or GM-CSF plus TMP/SMX will stimulate the activation of LMs and reduce the intensity of PC infection by augmentation of host defense in pup mice.

Aim 1: CBA and ELISA kits were used to assess cytokine production in pup versus adult alveolar macrophages *in vitro* and *in vivo* following stimulation with PC \pm IFN γ , HKEC (LPS *in vitro*), or GM-CSF. To determine if differences in NF κ B p65 nuclear translocation could be increased in pup mice following exogenous stimulation, a chemiluminescent plate-based assay was used.

Aim 2: To determine if pup macrophage infiltration into PC-infected lungs could be increased and their activation expedited through exogenous stimulation, LM activation markers were evaluated and compared via flow cytometry following different immunomodulatory treatment regimens. These treatment regimens were also evaluated for their ability increase T lymphocyte infiltration and activation in PC-infected pup lungs via flow cytometry to determine if exogenous LM activation played a role.

Aim 3: To determine if exogenous immunomodulatory treatment regimens could expedite the clearance of PC organisms from PC-infected pup lungs, lung tissue from both pup treatment and control groups were collected, digested, spun onto glass slides, stained, and analyzed microscopically. PC nuclei were manually counted and compared between groups.

Aim 4: To determine if T lymphocytes play a role in LM activation and PC clearance in HKEC-treated pups, HKEC-treated SCID and WT pups were compared in their ability to phagocytose DiO-labeled PC organisms.

Hypothesis III: Human infant LMs have a delayed activation profile compared to adult LMs following exposure to antigenic particles and can be stimulated exogenously to expedite their activation.

Aim 1: CBA and ELISA kits were used to assess cytokine production in infant versus adult AMs *in vitro*. The production of cytokines secondary to PC along

with immunomodulatory agents, were determined and compared for both infants and adults over time.

Aim 2: Phagocytosis assays were performed on LMs isolated from uninfected infants and adults. An *in vitro* model was used in which cells were isolated from human subjects and stimulated with PC ± rhGM-CSF. This method enabled me to determine the utility of rhGM-CSF as a clinically relevant immunomodulator to improve infant LM phagocytosis of PC organisms.

CHAPTER 2. Materials and Methods

A. Materials

1. Equipment

FACSCalibur cytofluorimeter (BD Biosciences, San Jose, CA), Sorvall RT7 centrifuge, Sorvall Biofuge, Fresco (Kendro Labs, Newton, CT), Nikon Eclipse E400 with spot camera, E600 (Tokyo, Japan), μ Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT), Shandon Cytospin 3, cytocentrifuge, (IMEB, inc. San Marcos, CA), Nalgene[®] Mr. Frosty freezing container (Sigma-Aldrich, St. Louis, MO).

2. Animals

Five-6 wk-old BALB/c mice (National Cancer Institute, NCI); 8-wk-old B6xDBA2F1 mice (Jackson Laboratory, Bar Harbor, ME); BALB/c and B6xDBA2F1 (F1) were bred and maintained at the Veterinary Medical Unit (VMU) of the Veterans Administration Medical Center (VAMC) under specific pathogen-free conditions. For the purposes of this research, neonatal mice are considered to be ≤ 7 days old and pups are considered to be ≤ 1 month old. C.B-17 severe combined immune deficient (SCID) mice were used to maintain a source of PC and were also bred at the VAMC VMU in microisolator cages containing sterilized food and water. Protocols for the usage of mice were approved by the VMU Institutional Animal Care and Use Committee.

3. Cell Line

Murine macrophage cell line, J744A.1 was generously provided by Dr. Susan Straley, Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, KY.

4. Chemicals

Chemicals/kits

Hank's Balanced Salt Solution (HBSS) and Roswell Park Memorial Institute (RPMI) 1640, Gentamicin, and Penicillin-Streptomycin were purchased from Gibco in Carlsbad, CA. Collagenase A, DNase, Ethylenediaminetetraacetic acid (EDTA), Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), Dimethyl sulfoxide (DMSO), glutathione and sodium azide were purchased from Sigma, in St. Louis, MO. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals in Lawrenceville, GA. Diff-Quik Solution I/II was purchased from Dade Behring in Newark, DE. Ficoll-Paque Plus was purchased from GE Healthcare Bio-Sciences in Piscataway, NJ. RosetteSep Human Monocyte Enrichment Cocktail was purchased from StemCell Technologies, Inc. in Vancouver, B.C. Vybrant DiO Solution and Carboxyfluorescein succinimidyl ester (CFSE) were purchased from Molecular Probes in Carlsbad, CA. Murine GM-CSF, human GM-CSF, and murine IFN γ were purchased from PeproTech in Rocky Hill, NJ. TMP/SMX was purchased from Alpharma in Fort Lee, NJ. Halothane was purchased from Halocarbon Labs in River Edge, NJ. Trypan Blue was purchased from Fisher Scientific, Houston, TX. Mouse and Human Inflammation and TH1/TH2 Cytometric Bead Arrays (CBA) were purchased from BD Biosciences, San Diego, CA. Mouse rmGM-CSF ELISA kit was purchased from eBiosciences, San Diego, CA. Nuclear Extraction Assay and NF κ B p65 Transcription Factor Assay (Human/Mouse/Rat) kits were purchased from Chemicon, Inc., Temecula, CA. Nuclear Extraction and TransBinding NF κ B Assay Kits (Human) were purchased from Panomics, Inc., Redwood City, CA. DC Protein Assay was purchased from Bio-Rad, Hercules, CA. PE-conjugated Human Mannose Receptor was purchased from Immunotech, Marseille, France. Anti-human Dectin-1/CLECSF12 was purchased from R&D, Minneapolis, MN.

Prepared media and solutions

Lavage fluid (HBSS + 0.02% EDTA), Lung/spleen collection media (RPMI 1640 + 5% FBS), ACK (8.29 g NH $_4$ Cl + 1 g KHCO $_3$ + 37.2 mg Na $_2$ EDTA in 1 L

water, pH 7.2-7.4), PBA (PBS+ 0.1% BSA+ 0.002% sodium azide), Mouse AM culture media (MACM) [RPMI 1640 + 5% heat-inactivated FBS (HIFBS) + 1% penicillin and streptomycin (P/S) + 55mM of 2-mercaptoethanol (2-ME) at 1:2000 + 0.5% gentamicin)], J744A.1 culture media (JCM) (DMEM + 1% p/s + 10% FBS), Dulbecco's Modified Eagle's Medium (DMEM) (ATCC), Human Culture Media (HCM) (RPMI + 10mcg/ml gentamicin + 12.5% human serum), J744A.1 freezing media (JFM) (40% RPMI 1640 + 50% FBS + 10% DMSO).

Antibodies

Hypothesis I:Aim1: Biotinylated CD11c, PE-Cy5-conjugated streptavidin (SA), FITC-conjugated Ia^d, PE-conjugated Ia^b, PE-conjugated CD40, and PE-TLR-4 (BD Pharmingen, San Diego, CA); APC-conjugated CD11b, FITC-conjugated F4/80, APC-TLR-2, FITC-conjugated CD68, and PE-Cy5-CD4, APC-CD8, PE-CD44, FITC-CD62, FITC-CD4, PE-Cy7-CD44 (eBiosciences, San Diego, CA), FITC-CD68, FITC-Dectin-1, PE-CD206 (Serotec, Oxford, UK).

Hypothesis II:Aim 1: Biotinylated CD11c, PE-Cy5-SA, FITC-Ia^d, PE-Ia^b, PE-CD40, APC-CD11b, FITC-F4/80, FITC-CD68, FITC-Dectin-1, PE-CD206 (Serotec, Oxford, UK), APC-TLR-2 (eBiosciences), PE-TLR-4 (BD Pharmingen).

Hypothesis II:Aim 2: PE-Cy5-CD4, APC-CD8, PE-CD44, FITC-CD62, FITC-CD4, PE-Cy7-CD44 (eBiosciences).

Biologicals

Pneumocystis murina (PC) was maintained in C.B-17 SCID mice that were bred and maintained at the VMU, HKEC was made from lyophilized cells of strain W(9637; ATCC) *Escherichia coli* (Sigma), LPS 100ng/ml (Sigma), Zymosan A 200µg/ml (Sigma).

B. Methods

1. Preparation of Pneumocystis for inoculations

PC-infected C.B-17 SCID mice were killed via CO₂ narcosis, their lungs were excised, collected in sterile HBSS + P/S (1:100) + gentamicin (1:1000) (5ml/mouse), and pushed through stainless steel mesh using a glass plunger in a sterile hood. The tissue was spun at low speed (300RPM) for 3 minutes to remove heavy debris. The supernatant was collected, the volume noted, and the sample was placed on ice after taking a small aliquot. The aliquoted sample was spun onto a glass slide (a 1:20 or 1:50 dilution is usually required), fixed in methanol, and stained with Diff-Quick[®]. PC nuclei were enumerated by microscopy and the final concentration was adjusted to 1×10^7 PC nuclei/100 μ l by spinning down the original sample at 1300 x g for 15 minutes and resuspending in the appropriate volume of HBSS + P/S + gentamicin. BALB/c or C57BL/6 x DBA/2J (B6D2F1/J) mice were lightly anesthetized with halothane and inoculated intranasally at 8-wks-old with 1×10^7 PC organisms in 50 μ l and at 48- to 72-hrs-old with 1×10^6 PC organisms 10 μ l. In some experiments, mice were infected with DiO-labeled PC. Live purified PC was incubated with DiO at a ratio of 5×10^6 PC:5 μ l DiO in 1ml PBS for 30 minutes at 37°C prior to inoculating the mice.

2. Collection and preparation of tissue/BALF for flow cytometry

All mice were killed immediately prior to tissue/BALF collection with either Halothane or Isoflurane by placing the animals in a bell jar with Halothane- or Isoflurane-soaked paper towels. Protocols for the usage of mice were approved by the VMU Institutional Animal Care and Use Committee.

Serum

Whole blood was obtained by severing the abdominal aorta and collecting it using a disposable glass pipette. The whole blood was spun at 2000RPM for 10 minutes to separate the serum, which was then decanted, placed in fresh Eppendorf tubes and stored at -80°C.

BALF

A flexible syringe-tip needle was inserted into the trachea of both pups and adults. Two-5ml of lavaged fluid was instilled into the lungs in 3-5 divided aliquots. Each aliquot of lavaged fluid was massaged in the lung and pulled back out into a fresh syringe via a 3-way stop-cock mechanism. The first aliquot was collected separate from the rest of the BALF, spun at 1200RPM for 10 minutes and the supernatant collected and frozen at -80°C. The cell pellet was resuspended and combined with the remainder of the BALF. An aliquot of the BALF was reserved for cytopins and cell counts; the remainder was spun down at 1200RPM for 7 minutes, the supernatant was removed and approximately 1ml of ACK was added to lyse any remaining RBCs. Once the ACK was added, the cells were vortexed briefly; the ACK was left on the cells for 30-60 seconds before diluting it with twice the volume of HBSS. The cells were then spun at 1200RPM for 7 minutes, washed one more time with HBSS and resuspended in 0.5-1ml of HBSS for flow cytometric analysis. Ten micro liters of the reserved aliquot of BALF (dilutions were made based on concentration) was mounted on a hemocytometer and counted under a 40x objective. These cell counts were used to determine the volume required for each flow cytometry polystyrene tube (1×10^6 cells/tube) and for later analysis. When enough cells were available, 100 μ l of the reserved aliquots were spun onto glass slides, fixed in methanol, and stained with Diff-Quik[®] for cell differential counts.

Lung tissue/spleen

Right lung lobes were excised and collected in 2ml of lung collection media. The lungs were then prepared for flow cytometry by processing them to achieve a single-cell suspension. They were minced and enzyme treated at 37°C for 1hr in lung collection media, 50U/ml Dnase, and 1 mg/ml collagenase A. Digested lung tissues were pushed through 70 μ m cell strainers with 3ml syringe plungers to obtain a single cell suspension. At this point, an aliquot was reserved to be spun onto glass slides and stained as previously described for BALF. For pup experiments in which the mice were \leq 13 days post-infection, the lung digest

was diluted 1:10; for experiments in which the mice were > 13 days post-infection, the dilution was 1:20. The glass slides were then fixed in methanol and Diff-Quik[®] stained. For the remaining lung digest, RBCs were lysed with ACK, resuspended in 0.5-1ml HBSS and counted on a hemocytometer as previously described for BALF. Once the appropriate volume per tube was determined, the cells were filtered into polypropylene tubes through 80µm mesh prior to processing for flow cytometry.

Tracheobronchial lymph nodes

The TBLNs were collected in 1ml of HBSS. To obtain a single-cell suspension they were pushed through 70µm cell strainers with 3ml syringe plungers. As previously described for BALF, the cells were treated with ACK, resuspended in 0.5-1ml HBSS and aliquoted into polypropylene tubes for flow cytometry. The spleen, often used as a one-color control for T lymphocyte antibodies, was processed in the same manner as the TBLN.

Staining

Once all the cells were aliquoted into the appropriate tubes, PBA was added to achieve approximately the same volume for all tubes. They were then spun down at 1200RPM for 10 minutes and the supernatants dumped. Fluorochrome-conjugated antibodies, previously titrated for optimal staining of 1×10^6 cells, were diluted in PBA; added to the pelleted cells and incubated for 20 minutes on ice (the antibodies used are outlined in the Materials section). At this time, 1 ml PBA was added to all the cells and spun at 1200RPM for 10 minutes. The supernatant was dumped and the cells were resuspended in 200µl of HBSS to be run on the FACSCalibur or CSR II cytofluorimeter. Cells being stained with a biotinylated Ab were incubated a second time with streptavidin before being resuspended in HBSS. 50,000 events were routinely required.

3. Pneumocystis enumeration and differential cell counts

As previously described, BALF and lung digest were spun onto glass slides and stained for later microscopic evaluation. Both BALF and lung digest samples were spun onto the glass slides via cytopspin at 900 RPM for 5 minutes. Each slide was allowed to air dry before fixing in methanol for 1 minute. Once again the slides were air dried and then Diff-Quik[®] stained by submerging them for 1 minute in Diff-Quik[®] Solution I, followed immediately by 5 minutes in Diff-Quik[®] Solution II. The slides were then washed 3 times by submerging them in water. They were air dried and cover slipped and allowed to dry overnight. The slides were analyzed microscopically under a 60x oil emersion lense. For each BALF sample slide, the number of macrophages, monocytes, neutrophils, and lymphocytes were determined. For each lung digest sample slide, PC nuclei were enumerated and reported as Log₁₀ PC per lung.

4. Cell culture

Murine macrophage cell line, J744A.1

Cells were stored in vapor-phase liquid nitrogen until ready for use. To freeze, cells were pelleted at 1200RPM for 10 minutes and resuspended in JFM to a concentration of 1×10^6 cells/ml in cryotubes. The 1ml aliquots were then submerged in an isopropyl alcohol-filled freezing container (Mr. Frosty[®]) and placed in the -80°C freezer for two days before transferring them to the liquid nitrogen tank. To thaw, cells were removed from liquid nitrogen and placed in a 37°C water bath just long enough for the media to begin to change back to a liquid phase. At this time 37°C media was added directly onto the cells, they were transferred to a fresh Eppendorf[®] tube and then immediately spun at 1200RPM for 10 minutes. The pelleted cells were resuspended in 1ml of JCM and transferred to a 75cm² tissue culture flask. An additional 19ml of JCM was added to bring the total volume to 20ml. The cells were then placed in a 37°C, 5% CO₂ incubator and were allowed to grow to confluence. Once the cells became confluent they were either used for an experiment or they were scraped with a

rubber spatula and split at no more than 1:6 and replated for future experiments. All J744A.1 cell manipulations were performed under sterile conditions.

Culture of murine alveolar macrophages

The BALF from uninfected pup and adult mice was collected as previously described in section 2.2 of Methods, except that the collection process was performed under sterile conditions. All BALF cells collected from pups were pooled together as was the BALF cells from adults. Aliquots of both pup and adult BALF cells were reserved to be spun onto glass slides, stained, and evaluated microscopically for AM purity. The remainder of the BALF was spun at 1200RPM for 10 minutes. To lyse any contaminating RBCs, ACK was added at one tenth the original BALF volume, vortexed, and allowed to sit for 60 seconds. HBSS was added at 6 times the volume of ACK and the cells were spun again at 1200RPM for 10 minutes. The cells were washed a second time and resuspended in 1/10 the original BALF volume with MACM warmed to 37°C. Both pup and adult cells were counted using a hemacytometer, as previously described, and their volumes were adjusted to achieve concentrations of 1×10^6 cells/ml. On average, the BALF yielded approximately 2×10^5 Ams per pup and 6×10^5 Ams per adult. Cells were then plated in either 96-well tissue culture plates in aliquots of 200 μ l (2×10^5 cells/well), or in 6-well tissue culture plates in aliquots of 2ml (2×10^6 cells/well) depending on the experiment being performed. The cells were rested overnight before being used experimentally.

Human airway macrophages

Human neonatal suctioned sputum samples were obtained in accordance with the University of Kentucky, Institutional Review Board (IRB) approved research protocol entitled “Immunomodulation to Improve Neonatal Clearance of *Pneumocystis*” (Protocol # 04-0086-P1B); informed consent was waived. Human adult BALF samples were obtained in accordance with the IRB approved research protocol entitled “Control, Immunoregulation, and expression of Innate Host Defenses” (Protocol # 05-0796-P2G). Subjects were excluded if they had

HIV or tuberculosis. All other demographic information was excluded and informed consent was waived as no patient identifiers were used. Each human sample was collected in hospital issued specimen cups and maintained on ice till they could be picked up; no samples were left on ice for more than 1 hour before being retrieved and sample processing begun. All human samples were handled with biosafety level (BSL) 2 precautions under sterile conditions. Each sample was immediately washed with equal volumes of HBSS and spun at 1200RPM for 10 minutes. The supernatants were dumped, resuspended in HCM (warmed to 37°C), and counted on a hemacytometer under a 40x objective. The samples were adjusted with HCM to a final concentration of 1×10^6 cells/ml and added in 200 μ l (2×10^5 cells) aliquots to 96-well tissue culture plates. The cells were rested overnight before being used experimentally.

Throughout this text the term “neonate” will be used to refer to infants less than or equal to 4 weeks of age and “adults” include persons who are 18 years or older.

Human peripheral blood monocyte-derived macrophages

Human peripheral blood was obtained in accordance with the University of Kentucky, IRB approved research protocol entitled “Immunomodulation to Improve Neonatal Clearance of *Pneumocystis*” (Protocol # 04-0086-P1B). Peripheral blood was obtained from enrolled healthy volunteers with whom informed consent was obtained. Between 5 and 15ml of blood per session was collected into sterile Buffered Sodium Citrate Plus Blood Collection Vacutainer tubes (BD) using 23G3/4 x12” Vacutainer Brand Safety-Lok Blood Collection Sets (Becton Dickinson, Franklin Lakes, NJ). Monocytes were isolated using the RosetteSep Human Monocyte Enrichment Cocktail according to the manufacturer’s instructions. Per the manufacturer’s protocol, 10 μ l of room temperature (RT) 100mM EDTA per 1ml of whole blood was mixed gently with the blood sample in a 50ml conical vial. Next, 50 μ l of RosetteSep Cocktail per ml of whole blood was added, mixed, and incubated for 20 minutes at RT. The sample mixtures were then diluted with equal volumes of PBS + 2% FBS + 1mM

EDTA (wash buffer) and gently mixed. Separately, Ficoll-Paque (volume specified in protocol based on volume of whole blood retrieved) was placed in a new, sterile 50ml conical vial. Subsequently, the blood solution layered on top of the Ficoll-Paque very carefully so as not to disrupt the Ficoll-Paque layer. The layered 50ml conical vial was then spun at 1200xg at RT for 20 minutes with the brake turned off. The RosetteSep cocktail crosslinked all unwanted cells (leaving the monocytes free) to multiple RBCs, forming immunorosettes. This caused the density of the unwanted or rosetted cells to increase so that they were pelleted along with the RBCs when they were centrifuged through the Ficoll-Paque. Following the 20 minute spin, 4 layers were formed, including (starting from the bottom): RBCs/unwanted cells (red), Ficoll-Paque (clear), enriched monocytes (white), and the top plasma layer (yellow). The top plasma layer was removed and discarded to allow better access to the white monocyte layer. A bulk pipette was used to remove the monocyte layer, which was then placed in a fresh 50ml conical vial. To this, 20ml of PBS + 2% FBS + 1mM EDTA was added and the cells spun at 1200RPM for 7 minutes. The supernatant was removed and the cells were resuspended in 2ml of wash buffer to be counted on the hemocytometer. On average, 3×10^5 monocytes per 1ml of whole blood are retrieved via this method. The wash step is repeated one more time before resuspending the cells in a volume of HCM to achieve a final concentration of 1×10^6 cells/ml. The cells were then placed in sterile, autoclavable, 6-well Teflon liners at 0.5-1ml per well (5×10^5 – 1×10^6 cells/well, respectively) and cultured for 5-7 days to allow them to differentiate into macrophages. Upon differentiation, the peripheral blood, monocyte-derived macrophages are ready for stimulation experiments to be described later in the Experimental Design section.

Cell Viability

An equal volume of 0.4% Trypan Blue was added to a cell suspension of approximately 1×10^6 cells/ml in PBS or serum-free media. The suspension was incubated for 3 minutes at RT before being mounted on a hemocytometer and observed under a 40x objective. Non-viable, deep-blue cells and clear, viable

cells were both enumerated. The percent viability was calculated as follows:
Percent viable = (number of viable cells/number of total cells) x 100.

Cell Stimulation

If cytokine analysis was the goal of the experiment, once the Ams were isolated or thawed (as described above) they were plated in sterile 96-well tissue culture plates. If the Ams were to be used to compare NFkB levels or phagocytosis, the cells were cultured in sterile 6-well tissue culture plates. All cultured Ams were allowed to rest over night following initial plating before any experimental stimulation or manipulations were performed and fresh media added the following day. In all culture experiments, cells were suspended in a final concentration of 1×10^6 cells/ml and plated at 200 μ l (2×10^5 cells) per well; if 6-well plates were being used, cells were plated at 1ml (1×10^6 cells) per well. Table 2.1 describes the agents used to stimulate or block AM activity in culture.

Table 2.1 Cell Stimulation

<u>Stimulant/Block</u>	<u>Dose</u>
PC	50 PC per 1 macrophage
IFN γ	165ng/ml
LPS	100ng/ml
rmGM-CSF	100ng/ml
rhGM-CSF	100ng/ml
Opsonized Zymosan	250 μ g/ml
Unopsonized Zymosan	250 μ g/ml
Dectin-1 Receptor Antibody	25 μ g/ml
Mannose Receptor Antibody	40 μ l/ml

5. *Pneumocystis* purification

PC-infected C.B-17 SCID mice were killed via CO $_2$, their lungs were excised, collected in sterile-filtered HBSS + 0.5% glutathione (pH 7.3) (5ml/mouse), and pushed through stainless steel mesh using a glass plunger in a

sterile hood. The Tissue was then aspirated through a 22G needle 2 times, a 26G needle once and transferred to a 50ml conical vial. The lungs were then spun at 300RPM for 3 minutes to remove the heavy sediment and the supernatant was transferred to a fresh 50ml conical vial and spun at 2600RPM for 15 minutes. The supernatant was removed and discarded, the pellet dissociated and resuspended/washed in 2ml of sterile deionized water for 30 seconds, followed by 2ml of 2x PBS, 6ml of HBSS + 0.5% glutathione (pH 7.3), and 200U Dnase (in 500 μ l HBSS). The lungs were incubated in this solution for 30minutes at 37°C and then aspirated once again through a 26G needle 2 times. The tissue was spun at 300RPM for 3 minutes and the supernatant was transferred to a new 50ml conical vial before being spun once again at 1300g for 15 minutes. The supernatant was removed and the pellet was resuspended in 1ml HBSS. A 1:100 dilution is usually made before spinning an aliquot of the purified PC onto a glass slide, fixed in methanol, stained with Diff Quik and enumerated microscopically. The resultant purified PC can then be adjusted to the desired concentration with HBSS.

6. Phagocytosis Assay

Human and mouse macrophages were acquired as described in sections 4.2 and 4.3 respectively. Each sample was washed by adding 2x the volume of HBSS and spinning at 1200RPM for 10 minutes. When necessary, a RBC lysing buffer was added to the pelleted cells and washed twice with HBSS. The final pellets were resuspended in HBSS to a final concentration of 1×10^6 cells/ml. One 10 x 75-mm snap-top tube was prepared for each condition being studied. To each tube, 100 μ l of macrophage suspension (1×10^5 cells), 100 μ l (1×10^6) freshly thawed, purified, murine PC (as described above), and 2 μ l of freshly thawed ice-cold human serum was added. For each sample, tubes were prepared as describe above with and without the addition of GM-CSF (100ng/ml); duplicate or triplicate tubes were set up for each sample and condition. The tubes were capped tightly, parafilmmed, and rotated end over end in a 37°C water bath for 25 minutes.

Following this incubation, the tubes were spun at 1200RPM for 10 minutes, the supernatant was dumped, and 1ml of ice-cold HBSS was added and the pellet was resuspended by pipetting up and down with a Pastuer Pipette. This wash step was repeated 2 more times for a total of 3 washes. The pellet from the last wash was resuspended in 1ml of ice-cold HBSS to a final concentration of 1×10^5 cells/ml. A 100 μ l aliquot (1×10^4 cells) of each sample was removed and cytocentrifuged onto a microscope slide by spinning 5 minutes at 900 rpm in a Cytospin 3. The slides were stained with Diff-Quik[®] as described in section 3. Phagocytosis was quantified using oil-immersion microscopy (1000x) by examining at least 200 cells and counting the number of internalized PC organisms in each one. The amount of phagocytosis was calculated according to the following formula: phagocytic index (PI) = (percent macrophages containing at least 1 organism) x (mean number of PC per positive cell)(235-237).

7. *In Vivo* Treatment Protocols

IFN γ

Recombinant murine IFN γ was purchased as a lyophilized protein and reconstituted in sterile deionized water to a stock concentration of 100 μ g/ml. This stock was further diluted into working aliquots with sterile PBS + 0.5% mouse serum and stored at -20°C. Prior to administering IFN γ , the mice were lightly anaesthetized with a mixture of 5% halothane in O₂. A frozen aliquot of IFN γ was brought to RT and then administered intranasally to the anaesthetized mice. Experiments using IFN γ treatment were designed in one of two ways. The first experimental design included 4 groups of mice in which 2 groups were infected with PC and two groups were uninfected. One subset from the infected and one subset from the uninfected group received 16ng/g of i.n. IFN γ daily every 72hrs starting on day 1 post-infection and through the completion of the experiment. The second experimental design involved a dose escalation study to determine if differences in outcomes could be altered by increasing dose. Doses included 16ng/g, 80ng/g, and 160ng/g of body weight; control mice received vehicle only (sterile PBS + 0.5% mouse serum).

Heat-killed E. coli

In order to heat-kill the *E. coli*, 1 gm of the lyophilized cells were suspended in 100ml of sterile, deionized water and divided into two separate 50ml conical vials. They were then boiled for 45 minutes in a double boiler (in a chemical hood) to remove the capsular antigens and expose the “O” antigens. The cells were allowed to cool, centrifuged at 3500g for 15 minutes, and the supernatant was removed and placed in a beaker of bleach. The pelleted cells were resuspended in 80ml (40ml per conical vial) of sterile deionized water and spun once again at 3500g for 15minutes. This wash step was repeated 2 more times for a total of 3 washes. The pelleted cells from the last wash were resuspended in 20ml (10ml per conical vial) of sterile, deionized water and the two vials were then combined for a total volume of 20ml (238). The resultant HKEC solution was then transferred into cryotubes in 1ml aliquots and stored at -80°C.

When dosing mice with HKEC, 1 vial would be thawed to RT and brought up to 5ml with sterile, deionized water. Mice were placed in a 20 x 20 x 40cm Plexiglas chamber attached to a T Updraft Nebulizer as depicted in Figure (2.1). The 5ml *E. coli* solution was placed in the nebulizer and aerosolized into the chamber till all of the solution had been aerosolized (~ 30-45min). The nebulizer and chamber were contained in a chemical hood during the aerosolization process. For all HKEC experiments, mice were dosed once daily beginning 48hrs post-PC-infection and continuing through day 20 post-infection. Control mice were aerosolized with sterile, deionized water on the same schedule as the treatment mice.

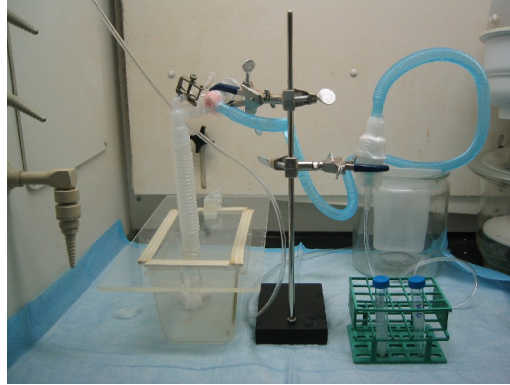


Figure 2.1 HKEC aerosolization chamber.

Granulocyte Macrophage-Colony Stimulating Factor

Recombinant murine GM-CSF (rmGM-CSF) was purchased as a lyophilized powder; it was reconstituted with sterile, deionized water to a stock concentration of $1\mu\text{g}/\mu\text{l}$ and was stored at -20°C . Further dilutions were made by adding sterile normal saline (NS) + 1% mouse albumin to the desired concentration. If the rmGM-CSF was to be used within 1 week's time, it was stored at 4°C in order to avoid frequent freeze-thaw cycles. If the route of administration was intraperitoneal (i.p.) injection into the abdomen, no anaesthetization was required. The dose used for i.p. experiments was $0.5\text{mcg}/\text{g}$ daily in two divided doses beginning day 7 post-PC-infection and continuing for a total of 5 days (239). If the rmGM-CSF was to be administered intranasally, the mice were first lightly anaesthetized in 5% halothane in O_2 . The i.n. dose of rmGM-CSF ranged between $5\text{-}50\text{ng}/\text{g}$ in $10\mu\text{l}$ of diluent daily (to minimize halothane exposure) beginning on day 7 post-PC-infection and continuing for a total of 5-7 days. All control mice received diluent only on the same schedule and by the same route of administration as the treatment mice in the same experiment.

Trimethoprim/Sulfamethoxazole

TMP/SMX was purchased as a cherry-flavored, oral suspension. Each milliliter contained 8mg of TMP and 40mg of SMX. For mice receiving TMP/SMX,

it was administered orally using a pipette at a dose of 40mg/kg TMP/200mg/kg SMX body weight twice daily (240); no anaesthetization was required. For all experiments involving treatment with TMP/SMX, dosing began 7 days post-PC infection and continued for 14 days (21 days post-infection).

8. Cytokine analysis

Cytokine production in pup versus adult mouse BALF and cultured AM supernatants was determined using a Mouse Inflammation CBA[®] kit as outlined in the manufacturer's instructions. Briefly, CBA[®] kits are bead-based assays that utilize flow cytometry to measure soluble analytes. The standards, provided in each kit, are used to make a standard curve, which then allows the measured proteins to be quantified. Each bead in a CBA[®] kit provides a capture surface for a specific protein; the capture bead mixture provided in each CBA[®] kit is in a suspension to allow for the detection of multiple analytes in a small volume sample, which is crucial when working with the small volumes collected from pups. The Mouse Inflammation CBA[®] kit includes the following analytes: IL-12p70, TNF α , IFN γ , MCP-1, IL-10, and IL-6. Data analysis was performed using the BD CBA[®] Software according to the manufacturer's instructions. An ELISA kit was used to determine the level of GM-CSF in pup and adult BALF and cultured AM supernatants according to the manufacturer's instructions.

Cytokine production in human neonate versus adult LM supernatants was determined using either a Human Inflammation or TH1/TH2 CBA[®] kit as outlined in the manufacturer's instructions and as described above. The Human Inflammation kit includes the following analytes: IL-8, IL-1 β , IL-6, IL-10, TNF α , and IL12p70; the Human TH1/TH2 kit includes the following analytes: IL-2, IL-4, IL-5, IL-10, TNF α , and IFN γ .

In all cell culture experiments, supernatants were collected from treated or control cells and frozen at -80°C until ready for cytokine analysis. Depending on the experiment, supernatants were collected at 6, 12, 18, 24, 48, or 72 hours post-stimulation.

9. NFkB analysis

NFkB p65 transcription factor levels within the nucleus of Ams were compared between pups and adults using Chemicon's Nuclear Extraction Assay and NFkB p65 Transcription Factor Colorimetric Assay according to the manufacturer's instructions. Briefly, cells were lysed using a detergent-based lysis buffer and a 27 gauge needle. They were subsequently centrifuged at high speed to create a pellet which contained the nuclear portion of the lysed cells. The pellet was resuspended in Nuclear Extraction Buffer, 0.5mM dithiothreitol (DTT), and 1/1000 protease inhibitor (PI) provided with the kit. The DTT was added as a reducing agent to stabilize enzymes with free sulfhydryl groups and the PI was added to inhibit protease enzyme activity. I used a 27 gauge needle to pull up each sample and a 30 gauge needle to expel them to further disrupt the nuclei. The samples were gently agitated in the Extraction Buffer on an orbital shaker and centrifuged at high speed once again; the supernatant contained the nuclear extracts. The protein from each nuclear extract sample was quantified using Bio-Rad's DC Protein Assay kit according the manufacturer's instructions. The samples were then snap-frozen in liquid nitrogen and stored at -80°C.

The samples were later thawed to be used in the NFkB Transcription Factor, plate-based, colorimetric assay. Briefly, a Streptavidin-coated 96-well strip plate was incubated with the following reagents and samples in the order listed: Blocking Reagent dissolved in Transcription Factor Assay (TFA) buffer containing sonicated salmon sperm DNA to block non-specific DNA binding activity was added first, followed by the NFkB Capture Probe (5'-GGGACTTCC-3') or TFA Negative Control Probe (non-specific oligonucleotide), and lastly the nuclear extract samples or HeLa Whole Cell Extract provided with the kit as a positive control were added. Following a two-hour incubation at RT, the wells were washed 3 times, then incubated with the primary anti-NFkB p65 antibody (incubated for 1hr) and the secondary anti-HRP antibody (incubated for 30 minutes). At this time, TMB substrate was added to each well and incubated for 5-10 minutes while monitoring for color change. Once the color change was

adequate, the provided Stop Solution was added and the absorbances read on a spectrophotometer.

10. Medical Institutional Review Board

Two Expedited Review Applications were submitted to the IRB requesting approval to collect both adult and neonatal human samples. Both protocols were approved and samples were subsequently collected in accordance with each protocol as described in the methods section. The protocol entitled: *Immunomodulation to Improve Neonatal Clearance of Pneumocystis* (Protocol number 04-0086-P1B) was approved to collect neonatal BALF and adult peripheral blood. The second protocol, entitled “*Control, Immunoregulation, and expression of Innate Host Defenses*” (Protocol # 05-0796-P2G) was approved to collect adult BALF samples.

11. Statistical analysis

Data are expressed as the means \pm standard deviations (SD) for three to six mice per group. Differences between experimental groups with treatment as the only variable were determined using the Student's *t* tests (comparing 2 groups) or 1-way analysis of variance (1-way ANOVA) (comparing more than 2 groups) followed by Student Neuman Kuels post-hoc test where appropriate. Differences between experimental groups with two variables (treatment and time) were analyzed using a 2-way ANOVA. If data was not normally distributed it was analyzed using the Holm-Sidak test. Differences were considered statistically significant when *p* was < 0.05 . SigmaStat statistical software (SPSS, Inc., Chicago, Ill) was used for all statistical analysis.

CHAPTER 3: Alveolar macrophage activation markers and T cell infiltration are delayed in PC-infected pups

It has been previously demonstrated that there is a 3 week delay in the clearance of PC from mice infected as neonates compared to mice infected as adults (Figure 1.1) (9), (234), (10). To determine whether AM infiltration and activation is also delayed in pups in response to PC we used flow cytometry to examine macrophage markers at various times post-infection. Mice were infected with PC within 72 hrs of birth or as adults and their lungs lavaged at four different time points. Alveolar macrophage activation was examined using antibodies specific for CD11b, MHC class II, CD40, and CD80. There was a significant 2 week delay in the infiltration of macrophages into PC-infected pup lungs compared to adults (Figure 3.1). Adult mice infected with PC demonstrated an increase in the expression level (Figure 3.2) and the total number of cells expressing (Figure 3.3) CD11b, MHC class II, CD40 and CD80 compared to PC-infected pups (Data not shown for CD40 and CD80). These data demonstrate that adult mice respond to PC infection more efficiently than pups.

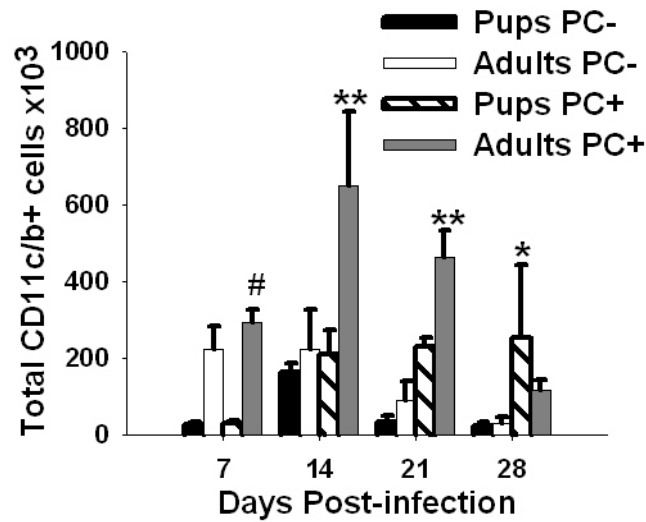


Figure 3.1 Delayed macrophage infiltration into pup lungs. Mice were infected with PC as neonates (24-72 hours after birth) or as adults (>8 weeks). Four to six mice per group were lavaged at each indicated time post-infection. The cells were stained with antibodies specific for CD11c and CD11b and then analyzed by flow cytometry. Results represent the mean \pm SD of 4-6 mice per group and 2 separate experiments. **Total number of CD11c+/CD11b+ cells are significantly greater than their control groups and PC-infected pup groups ($p < 0.05$). # Total number of CD11c+/CD11b+ cells are significantly greater than PC-infected pup group and uninfected adult group ($p < 0.05$). *Total number of CD11c+/CD11b+ cells is significantly greater than pup and adult control groups ($p < 0.05$).

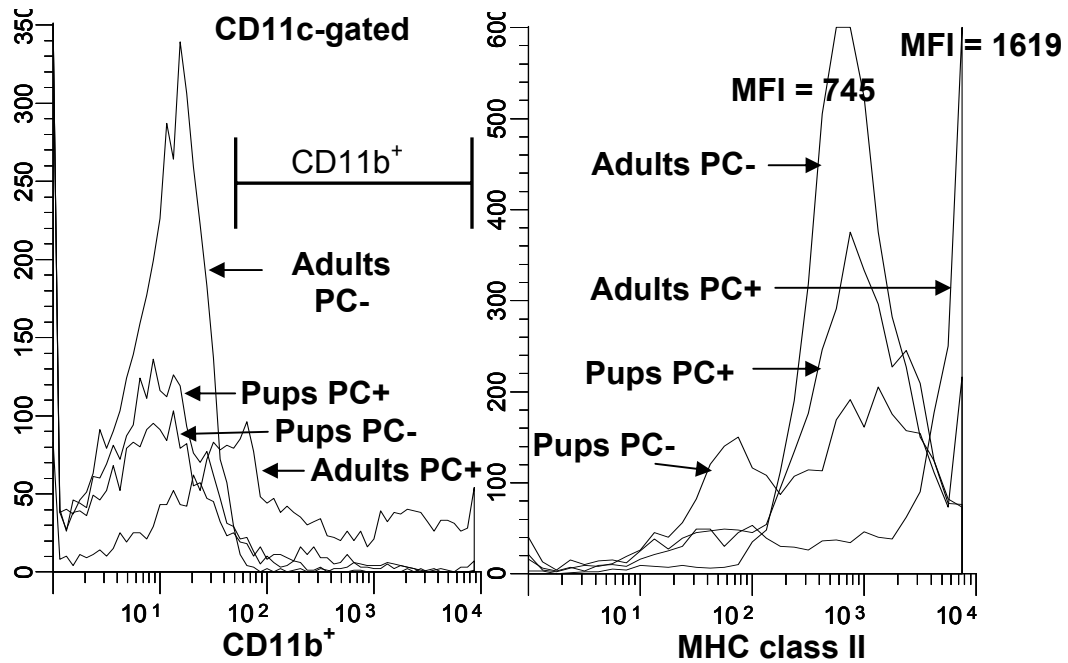


Figure 3.2 Delayed expression of CD11b and MHC class II molecules on pup AMs. Mice were infected with PC as neonates (24-72 hours after birth) or adults. Lungs were lavaged at day 10 post-infection to examine macrophage levels. Cells were stained with antibodies specific for CD11c and CD11b or MHC class II; phenotypes were examined by flow cytometry. Representative histograms of CD11b⁺ cells (gated on CD11c⁺ cells) and MHC class II⁺ cells (gated on large nonlymphocytes) are shown. Cells were gated for large nonlymphoid cells by using forward and side light scatter. Controls include BALF cells from uninfected neonates or adults. Data are representative of results for 3-5 mice per group.

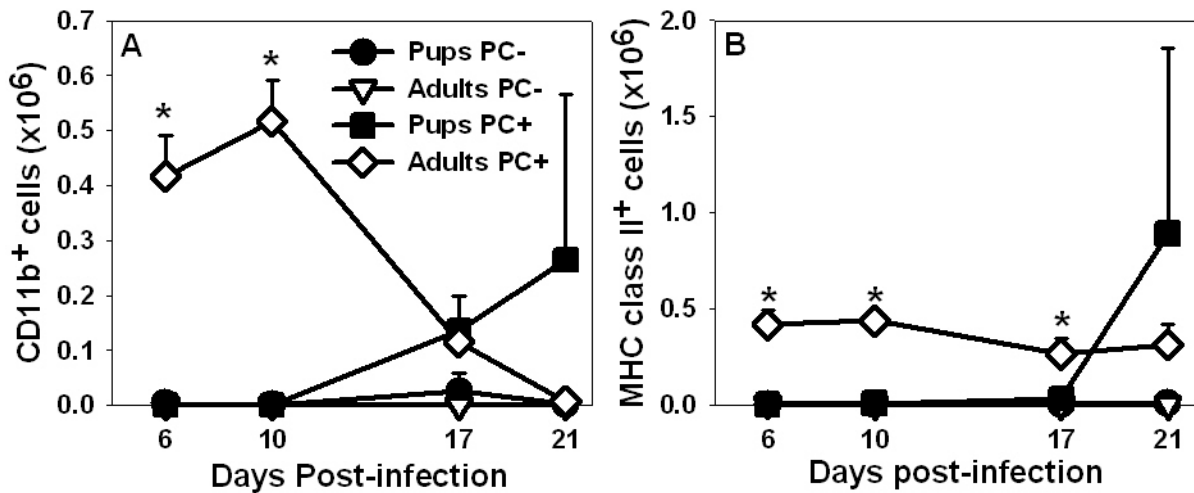


Figure 3.3 Pup AM activation is delayed following PC infection. Numbers of nonlymphoid cells expressing CD11b and MHC class II in the alveolar spaces of PC-infected neonates were significantly lower than those in adult lungs through 2 weeks post-infection. Mice were infected with PC as neonates (24-72 h after birth) or adults. Control mice received HBSS vehicle. Lungs were lavaged at the indicated time points, cells were stained with antibodies specific for CD11b and MHC class II, and cells were examined by using flow cytometry. Cells were gated on large nonlymphocytes and the number of cells expressing (A) CD11b and (B) MHC class II were determined. Data represent the means \pm SD for three to five mice per group. * $p < 0.05$ compared to all other groups at the same time point.

To further demonstrate an age-specific difference, separate phenotypic studies were performed in which pup mice were infected with PC as neonates and followed through day 50 post-infection. Expression of the macrophage markers CD11c, CD11b, Fc γ R, MHC class II, and CD40 following infection were compared to those of PC-infected adult mice at similar time points. As expected, the expression of CD11c did not change based on infection status (Figure 3.4a-b). The total number of cells expressing CD11b, MHC class II, CD40, and Fc γ R was increased among all infected animals compared to controls; however, this expression was delayed up to 2 weeks in pups compared to adults (Figure 3.4c-j). This data describes the kinetics of AMs expressing activation markers following PC infection in pups versus adults and demonstrates the delayed expression in PC-infected pups compared to adults.

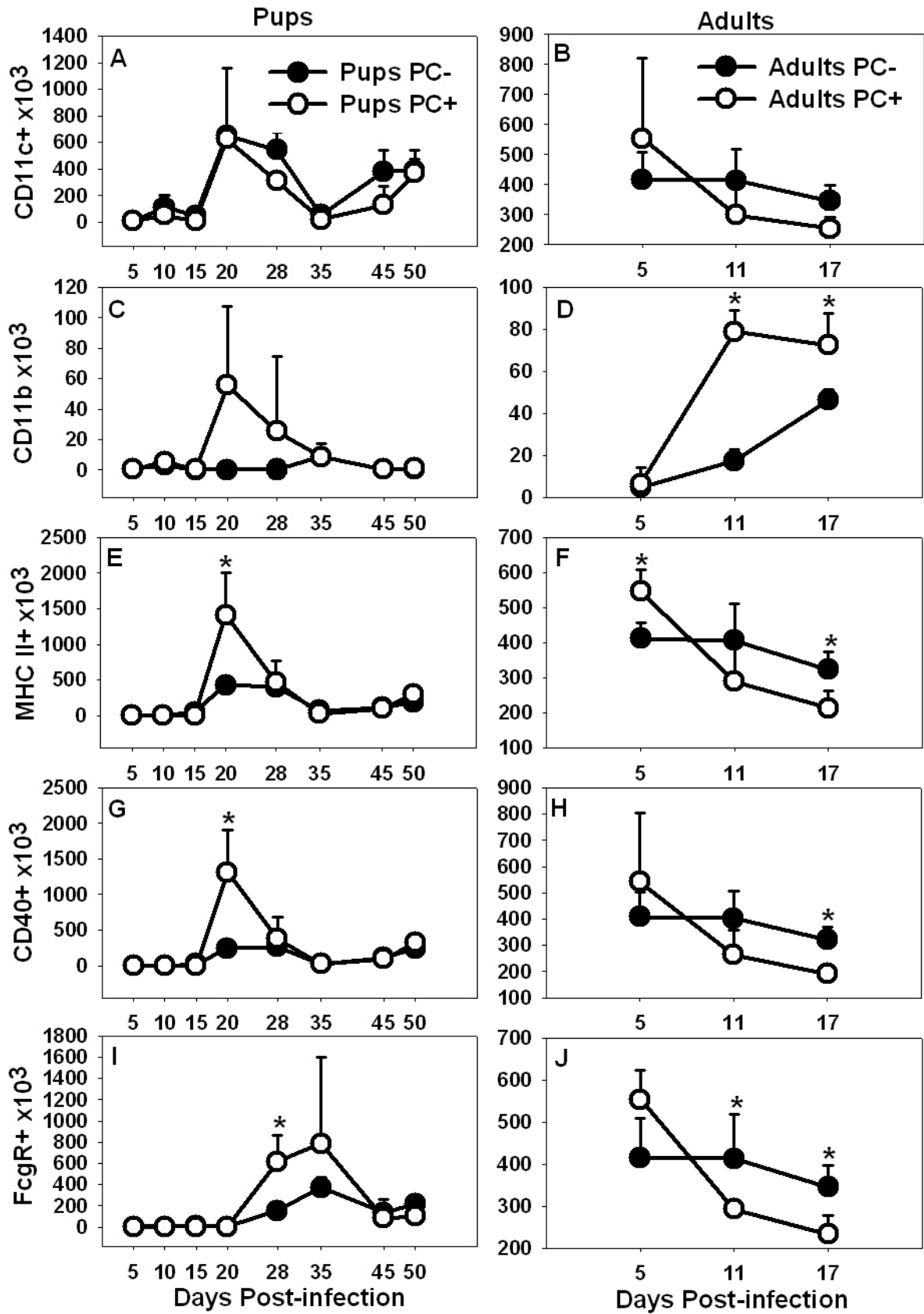


Figure 3.4 Delayed activation markers on pup AMs. The number of cells expressing activation markers following PC infection was delayed on pup AMs compared to adults. Mice were infected with PC as neonates (24-72 h after birth) or adults. Control mice received HBSS vehicle. Lungs were lavaged at the indicated time points. Cells were stained with antibodies specific for CD11c, CD11b, MHC class II, CD40, and Fc γ R and were examined by flow cytometry. Cells were gated on large nonlymphocytes and CD11c and the number of cells expressing (A-B) CD11c, (C-D) CD11b, (E-F) MHC class II, (G-H) CD40, and (I-J) Fc γ R were determined for mice infected with PC as neonates and as adults. Data represent the means \pm SD for four to five mice per group. * p <0.05 compared to the respective pup groups at the same time point.

The expression of two important chemokine receptors, chemokine receptors 2 and 5 (CCR2 and CCR5) on LMs were compared over time between PC-infected and PC-uninfected pups and adults. CCR2 and CCR5 are receptors for the ligands macrophage chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) respectively and function to increase the number of inflammatory cells, primarily macrophages, at the site of infection. The number of adult macrophages that are CCR2 positive are greater than pup macrophages regardless of infection status up to day 28 post-infection (Figure 3.5a). At this time, the number of CCR2-expressing macrophages increases in the PC-infected pups. A similar trend occurs in the CCR5-expressing macrophages except that the rise in pup cells occurs about 1 week earlier than CCR2-expressing cells (Figure 3.5b). The dual expression of CCR2 and CCR5 peaks by day 14 on macrophages from PC-infected adults but not till day 28 on pup macrophages infected with PC (Figure 3.5c). These data demonstrate a delay in the up-regulation of CCR2 and CCR5 on LMs from pups compared to adults infected with PC. Overall, the delayed expression of these key activation markers on PC-infected pups compared to adults may contribute to their inefficient clearance of the organism.

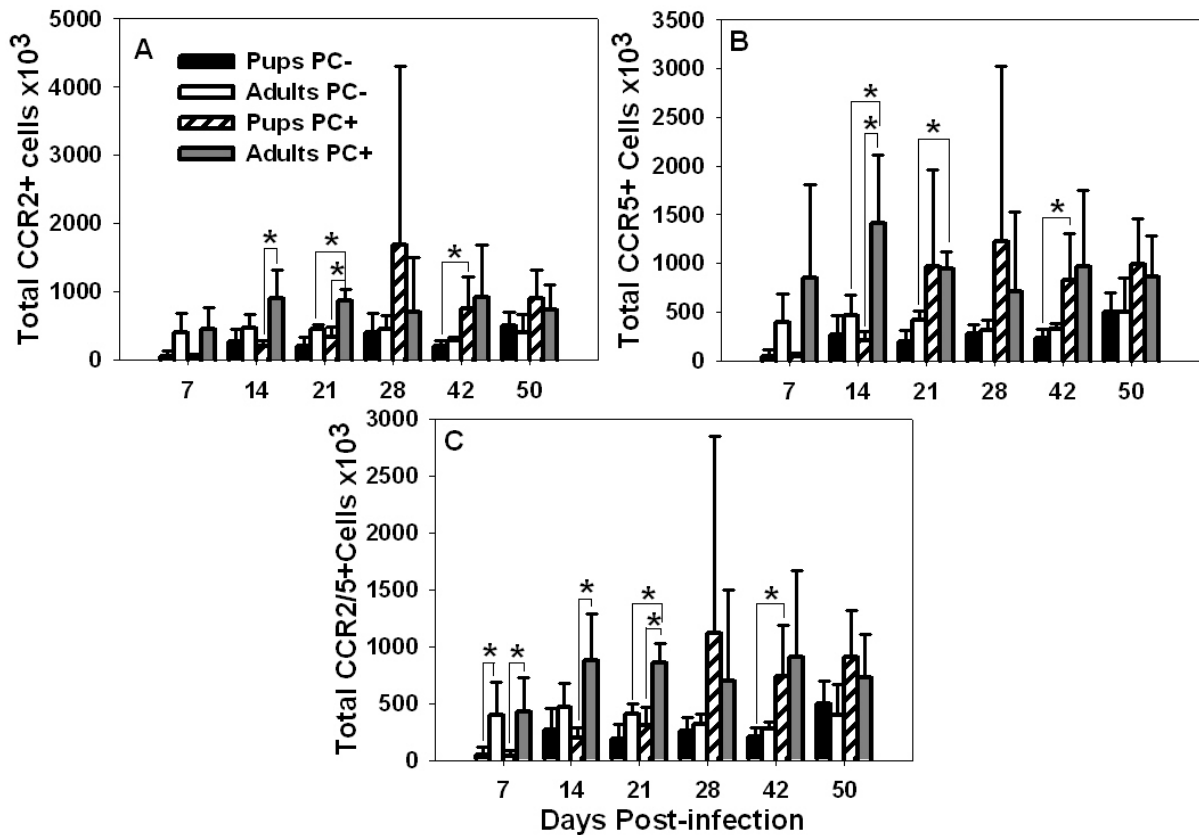


Figure 3.5 PC-infected pup AMs expressing CCR2 and CCR5 are delayed. The number of cells expressing CCR2 and CCR5 following PC infection was delayed on pup AMs compare to adults. Mice were infected with PC as neonates (24-72 h after birth) or adults. Control mice received HBSS vehicle. Lungs were lavaged at the indicated time points. Cells were stained with antibodies specific for CD11c, CCR2, and CCR5 and examined by flow cytometry. Cells were gated on large nonlymphocytes and CD11c and the number of cells expressing (A) CCR2, (B) CCR5, and (C) CCR2/5 were determined for mice infected with PC as neonates and as adults. Data represent the means \pm SD for four to five mice per group. * $p < 0.05$ compared to the indicated groups.

The delay in PC clearance observed in pups compared to adults correlates with a delay in CD4 T cell infiltration into the alveolar spaces of pups versus adults (3.6). It is well known that T cells are important for controlling PC infections (241), (242). As previously discussed in the introduction, it is known that T cells require activation in order to efficiently respond to PC organisms at the site of infection in the lung. This occurs through cell-to-cell signaling and

antigen presentation, both of which are important functions of LMs during PC infection. Likewise, LMs work more efficiently when they are in turn, stimulated by activated T cells. Thus, when discussing the role of LMs in the clearance of PC, it is important to assess part of the LM function based on T cell infiltration into the lungs. As shown in figures 3.6 a and b, the infiltration of both CD4 and CD8 T cells occurs much more rapidly in PC-infected adults versus PC-infected pups. At days 7 and 14 post-infection there are significantly more T cells in the infected adult group compared to the infected pup groups. The infiltration of T cells into infected pup lungs does not occur until day 21 post-infection. By looking at the time frame of T cell infiltration compared to PC clearance, one can see a correlation in which T cells enter the lungs of the infected mice approximately 1 week prior to the initiation of PC clearance (Figure 3.6). This data further demonstrates the importance of T cells in the clearance of PC organisms.

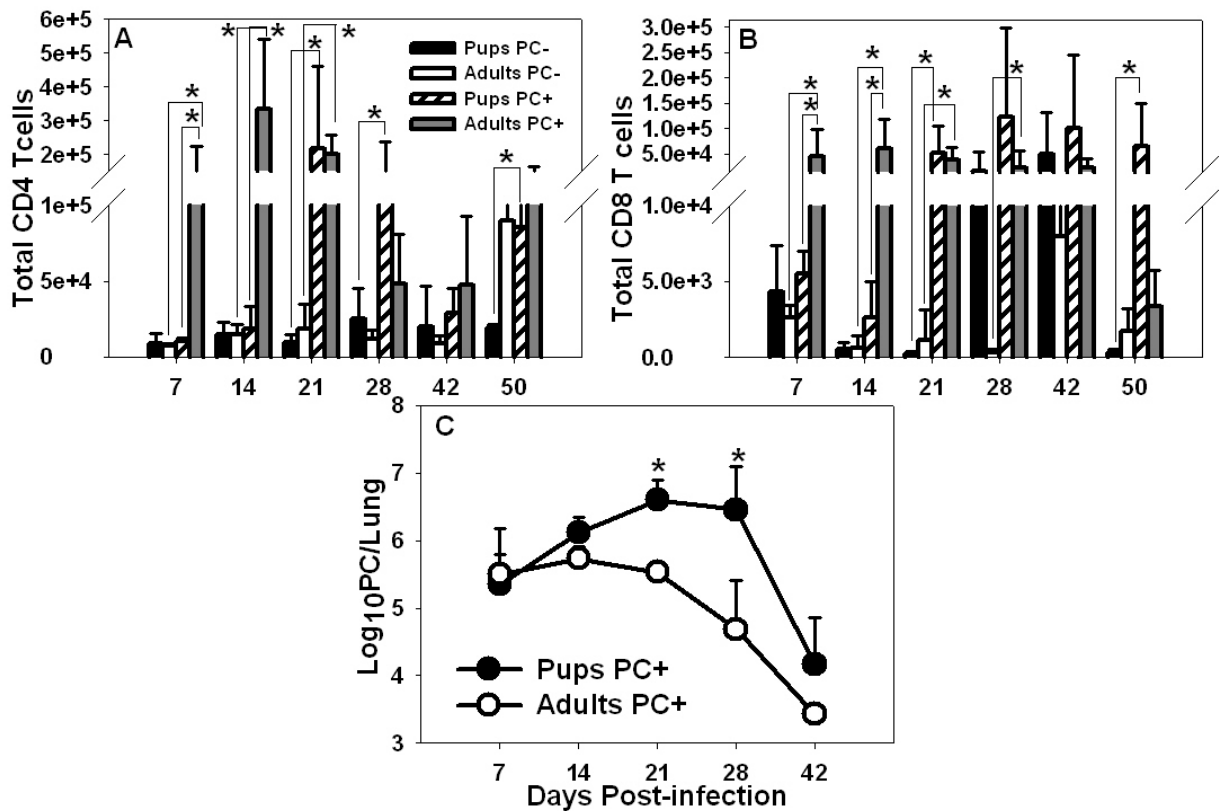


Figure 3.6 T cell infiltration is delayed in PC-infected pup mice. An increase in T cell infiltration occurs approximately one week prior to a reduction in PC burden and is delayed in PC-infected pup compared to adult mice. Mice were infected with PC as neonates (24-72 h after birth) or adults. For A-B, lungs were lavaged at the indicated time points. Cells were stained with antibodies specific for (A) CD4 and (B) CD8 and examined by flow cytometry. Cells were gated on small, non-granular lymphocytic cells. For panel C, whole lungs were collected at the indicated time points and processed into single-cell suspensions. Cells were spun onto glass slides, stained with Diff-Quick®, and enumerated microscopically. Data represent the means \pm SD of four to five mice per group. * $p < 0.05$ compared to the indicated groups.

CHAPTER 4: *In vitro* cytokine production is delayed in PC-stimulated pup compared to adult alveolar macrophages.

The initial experiments done to assess cytokine production in pup versus adult LMs were performed on cultured cells. As previously described, uninfected pup and adult mice were lavaged under sterile conditions and AMs were isolated from the BALF. The cells were allowed to rest overnight and were then stimulated in culture for various lengths of time depending on the specific experiment. One of the first experiments performed using this method involved stimulating the cells with sonicated PC, whole PC, or media for 24 hours. Following this incubation, the culture supernatants were retrieved and analyzed for TNF α , IL-6, and MCP-1 using a BD CBA kit[®]. These cytokines are important pro-inflammatory cytokines responsible for cell recruitment and lymphocyte activation (50) in PC infection (74, 77, 175). As illustrated in figure 4.1, PC was unable to stimulate pup AMs to produce TNF α , IL-6, or MCP-1 after 24 hours. While both PC formulations were able to stimulate some cytokine production among adult AMs, whole PC provided a much stronger stimulus than sonicated PC for IL-6 and MCP-1, but not TNF α (Figure 4.1a-c). This observation suggests that cytokine production may be linked to the simultaneous binding of multiple PC antigenic components which would be possible with whole PC and much less likely with sonicated PC. Overall, this data demonstrates that pup AMs do not respond as well as adult AMs to PC.

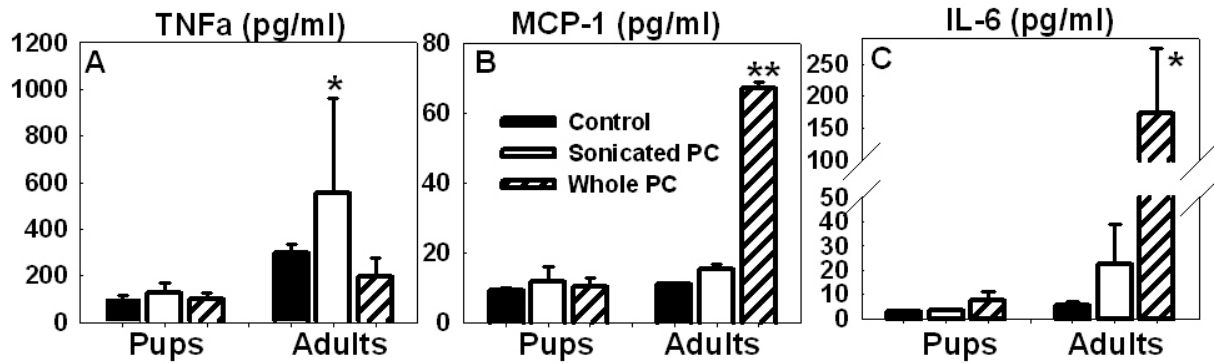


Figure 4.1 Pup AMs are specifically unresponsive to PC. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled and the AMs were allowed to adhere to a 96-well tissue culture plate. Culture media was replaced after 24 hours with fresh media containing media only (Controls), sonicated PC, or whole PC organisms. After 24 hours of stimulation, the levels of (A) TNF α , (B) MCP-1, and (C) IL-6 were determined by Cytometric Bead Array (BD Biosciences). Data represent the means \pm SD for 3 wells per group and are representative of 3 experiments. * p <0.05 compared to the respective pup groups. ** p <0.05 compared to respective pup group and the adult control group.

To evaluate the effect of time, similar experiments were performed as described above. Supernatant aliquots were evaluated for cytokine production following 1, 6, 12, 24, and 48 hours of incubation with PC. No increase in TNF α or IL-6 occurred after 1 hour of PC stimulation within pup or adult AMs relative to unstimulated cells. After 24 hours, PC stimulation induced a significantly increased production of TNF α and IL-6 compared to PC stimulated pup cells and adult unstimulated cells (Figure 4.2a-b). Alternatively, PC appeared to produce an early stimulation of MCP-1 production in pup and adult cells which was lost by 24 hours post-stimulation (Figure 4.2c). IL-10 production was relatively elevated in both pup and adult unstimulated cells suggesting that there is a constitutive production occurring in the absence of antigenic stimulation (Figure 4.2d). Within the first hour of PC-stimulation among pup AMs, IL-10 production was reduced to below detectable limits (Figure 4.2d). Overall, this data suggests that pup AMs have a poor pro-inflammatory response to PC compared to adult AMs, but they appropriately reduce production of the inhibitory cytokine IL-10.

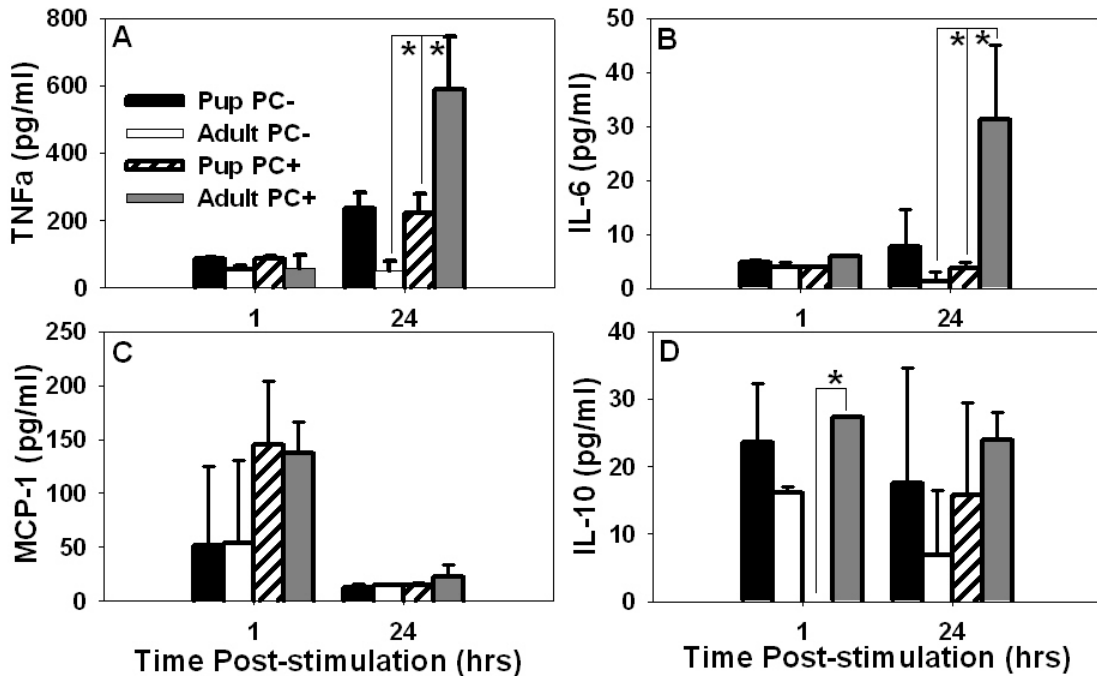


Figure 4.2 Pup and adult AM cytokine production following PC stimulation. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled and the AMs were allowed to adhere to a 96-well tissue culture plate. Culture media was replaced after 24 hours with fresh media containing media only (PC-) or sonicated PC (PC+). After 1 or 24 hours of stimulation the levels of (A) TNF α , (B) IL-6, (C) MCP-1, and (D) IL-10 were determined by Cytometric Bead Array (BD Biosciences). Data represent the means \pm SD for 3 wells per group and are representative of 3 experiments. * $p < 0.05$ compared to the indicated groups.

The production of TNF α and IL-6 among PC-stimulated pup AMs did not increase above their untreated control cells until 48 hours post-stimulation, compared to 6 and 24 hours respectively in adult cells (Figure 4.3 a-b). MCP-1 production in PC-stimulated pup cells was undetectable at 6 and 12 hours of incubation. After 24 hours, however, MCP-1 levels were equal to those of the adult AMs (Figure 4.3 c). No differences were observed in IL-10 production in pup or adult AMs regardless of PC stimulation (Figure 4.3 d), however overall levels were greater in these later time points compared to the levels after 1 hour (Figure 4.2 d). After 48 hours of incubation, there is a small increasing trend in IL-10 production in pup and adult cells stimulated with PC. Taken together, these data suggest that pup AM pro-inflammatory cytokine production is delayed compared to adult AMs.

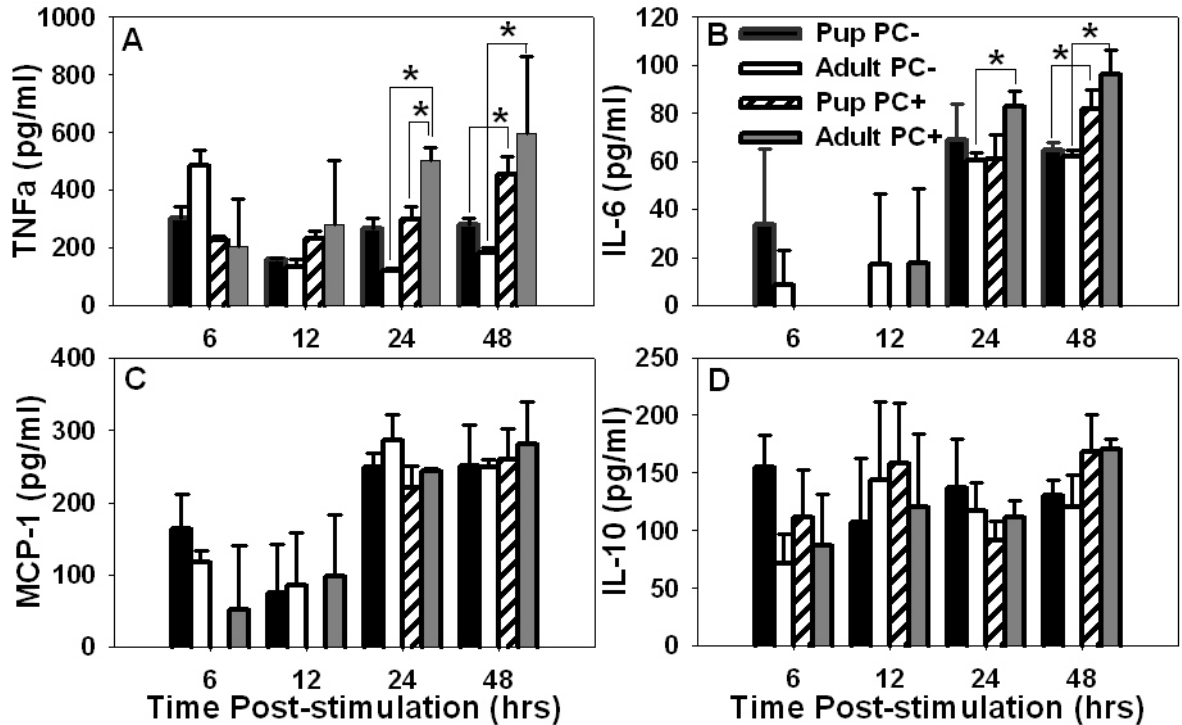


Figure 4.3 Cytokine production is delayed in PC stimulated pup AMs. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled and the AMs were allowed to adhere to a 96-well tissue culture plate. Culture media was replaced after 24 hours with fresh media containing media only (PC-) or sonicated PC (PC+). After the indicated times post-stimulation the levels of (A) TNF α , (B) IL-6, (C) MCP-1, and (D) IL-10 were determined by Cytometric Bead Array (BD Biosciences). Data represent the means \pm SD for 3 wells per group and are representative of 3 experiments. * $p < 0.05$ compared to the indicated groups.

NF κ B is a transcription factor involved in the synthesis of cytokines, including TNF α , IL-6, and MCP-1. To elucidate if NF κ B could be involved in the differential cytokine production between PC-stimulated pup and adult AMs, a nuclear translocation assay was performed on pup and adult AMs stimulated for 1 hour with PC. NF κ B p65 nuclear translocation was significantly increased in adult AMs stimulated with PC compared to their unstimulated control cells (Figure 4.4). No differences were observed between pup PC-stimulated and unstimulated cells due to the high baseline level of NF κ B p65 in the unstimulated pup control cells which were similar to the levels measured in adult PC-stimulated cells. This data reflects the increased pro-inflammatory cytokine

production observed in adult LM stimulated with PC compared to unstimulated control cells. The lack of increased NFkB p65 translocation to the nucleus observed in pup PC-stimulated cells also reflects the lack of cytokine production previously demonstrated. The high baseline level of nuclear NFkB p65 without a concomitantly high baseline cytokine production may indicate that pup mice have inefficient binding of NFkB p65 to the DNA binding site. This data further reflects the absent cytokine response from pup LMs following PC stimulation. This data also suggests that the cytokine response demonstrated by pup LMs following stimulation with other stimuli, such as LPS and zymosan, that will be described in future sections may utilize alternative signaling intermediates and or transcriptions factors.

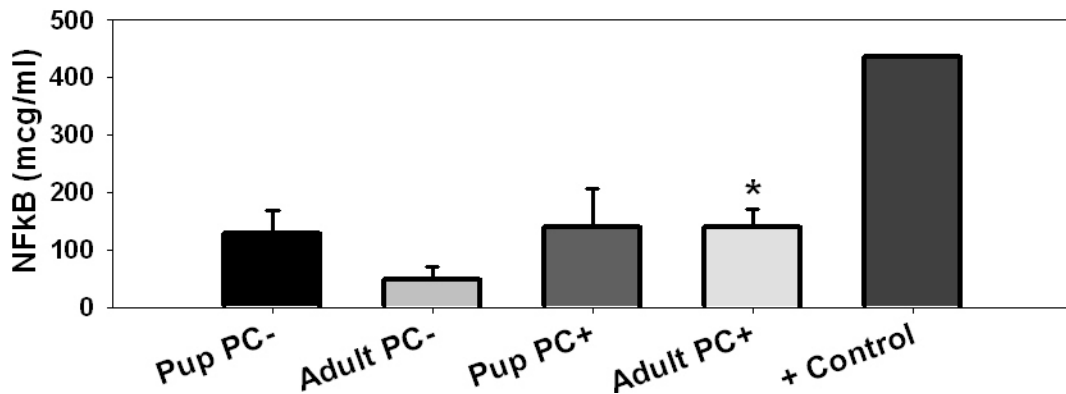


Figure 4.4 NFkB nuclear translocation is unchanged in pup AMs stimulated with PC. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled, stimulated with 50 PC organisms for every 1 AM (50:1) for 1 hour. The cells were lysed and the nuclear extract was isolated. Chemicon's NFkB p65 Transcription Factor Colorimetric Assay was used to quantify NFkB p65 in the nuclear extract. Data represent the means \pm SD for 3 samples per group and are representative of 2 experiments. * $p < 0.05$ compared to adult PC- group.

CHAPTER 5: Exogenous immunomodulation stimulates the activation of lung macrophages and reduces intensity of *Pneumocystis* infection in pup mice

A. Interferon gamma

We have previously shown that infant mice have a significant delay in mounting an immune response to PC compared to adult mice (9, 234). Delayed clearance is accompanied by a delay in the infiltration of T cells and activation of LMs as well as delayed cytokine up regulation, including IFN γ (9, 10, 234). It has been shown, moreover, that the delivery of IFN γ to the lungs of PC-infected adult SCID mice can stimulate clearance of the organism (243, 244). We therefore hypothesized that infected pup mice would clear their infection faster when treated with exogenous IFN γ .

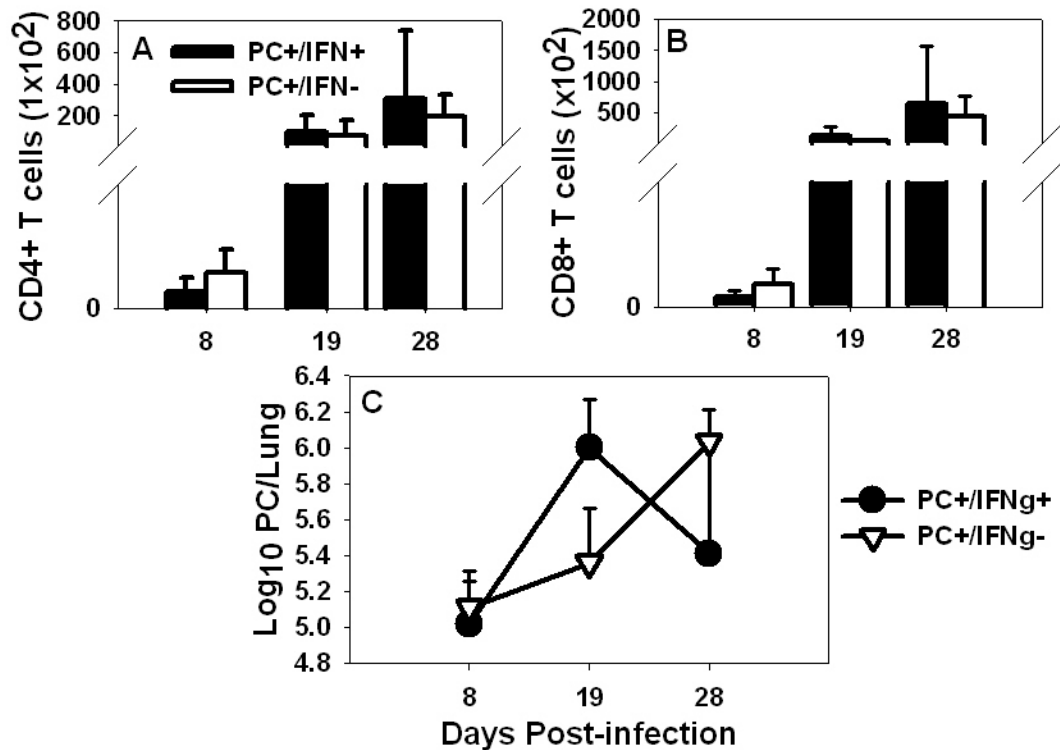


Figure 5.1 Effects of exogenous IFN γ on pup lung defenses against PC. Mice were infected with PC as neonates (24-72 h after birth) and then treated with either IFN γ or PBS intranasally every 72 h beginning 24 h post-infection. Lungs were lavaged and cells collected at the indicated times. Cells from BALF were stained with antibodies specific for CD4 or CD8 and then analyzed using flow cytometry. Total CD4 T cell (A) and CD8 T cell (B) numbers were determined. (C) Whole lungs were digested and aliquots stained with Diff-Quik[®]; PC nuclei were enumerated microscopically. Results represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. No statistical differences were found between groups.

Murine IFN γ (16ng/g) or vehicle (PBS) was administered intranasally to PC-infected pup mice every 48 hours beginning 24 hours post-infection and continuing throughout the course of the experiment. Mice from each group were killed at days 8, 19, and 28 post-infection. Whole lung and lung lavage were collected and flow cytometry was performed to examine the infiltration of lymphocytes in response to PC infection. Exogenous IFN γ had no effect on the infiltration of either CD4 T cells (Figure 5.1 a) or CD8 T cells (Figure 5.1 b) into the lungs of PC-infected neonates. Similarly, no statistically significant

differences were found in lung burden of PC organisms between the two groups by day 28 (Figure 5.1 c). However, there appeared to be a shift in the clearance kinetics between the two groups. The IFN γ -treated group actually had a greater PC burden than the control group at day 19 but by day 28 the burden among the two groups diverged in opposite directions with the IFN γ -treated group having less PC than the control group. Ultimately, no statistically significant difference in PC clearance was observed between the two groups; furthermore, no mice from either group were able to clear the PC completely by day 28 post-infection.

To determine if the IFN γ dosing strategy had an impact on initiating the immune response and PC clearance, this experiment was repeated with a dose escalation. PC-infected pup mice were divided into 4 groups. Three groups received 16ng/g, 80ng/g, or 160ng/g of intranasal IFN γ . The fourth group served as a control and received PBS only. Mice from each group were killed at days 15 and 29 post-infection. Despite increased doses of IFN γ , no differences in either T-cell or macrophage infiltration into pup PC-infected lungs were observed when compared to the control group (data not shown). Additionally, cytokine production in the BALF of each group was determined by CBA[®] analysis, and no statistically significant differences were found between any of the groups receiving IFN γ compared to the control group, suggesting that the resident LMs were not stimulated by the presence of PC plus IFN γ (data not shown). Lastly, PC clearance was assessed microscopically by counting the number of PC nuclei in each group treated with IFN γ as well as the control group (Figure 5.2). While the group receiving 16ng/g had more mice clearing the organism than mice in the control group, the results were highly variable and, as with the previous IFN γ experiment, no significant differences were detected. Furthermore, increased doses of IFN γ did not improve PC clearance. These data indicate that none of the intranasally administered doses of IFN γ tested significantly improved the clearance of PC in pup mice. Furthermore, no dose of IFN γ tested was able to stimulate the infiltration or activation of immune cells in the infected lung, nor did it increase the production of cytokines in PC-infected pup lungs.

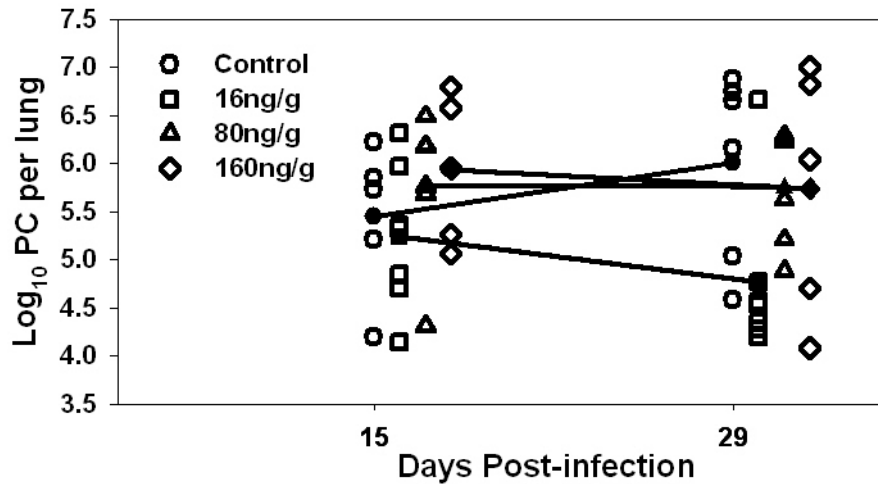


Figure 5.2 Low levels of exogenous IFN γ moderately reduced PC lung burden in pups. Mice were infected with PC as neonates (24-72 h after birth) and then treated with 16ng/g, 80ng/g, 160ng/g IFN γ or PBS intranasally every 72 h beginning 24 h post-infection. Whole lungs were collected and digested at days 15 and 29 post-infection. PC nuclei were enumerated microscopically. Filled symbols represent the means \pm SD for five to six mice per group.

B. Heat-killed *E. coli*

1. LPS increases cytokine production from pup alveolar macrophages *in vitro*

We have shown that LM activation is delayed in pup compared to adult mice in response to PC infection. To elaborate upon this finding, an *in vitro* model was used to determine whether pup AMs are capable of responding to exogenous stimulation with LPS compared to PC. AMs from uninfected pup and adult mice were isolated and cultured with PC, LPS, or media. The activation of AMs was assessed by quantifying their cytokine responses at 6, 12, 24, and 48 hours post-stimulation. At all time points both adult and pup AMs treated with LPS produced significantly more TNF α , IL-6, and MCP-1 than their corresponding control groups (Figure 5.3a-c). Moreover, LPS-stimulated adult AMs produce significantly more TNF α at 6 hours post-stimulation, more IL-6 at all time points, and more MCP-1 at 6, 12, and 24 hours post-stimulation compared to pup LPS-treated cells (Figure 5.3a-c). PC stimulation did not induce the same level of TNF α or IL-6 production as LPS in pup or adult cells. Overall, adult AMs

still produced more TNF α and IL-6 than pup cells stimulated with PC. These data demonstrate that pup AMs are capable of pro-inflammatory cytokine production following exogenous stimulation with LPS but produce less than adults overall and are specifically unable to respond PC.

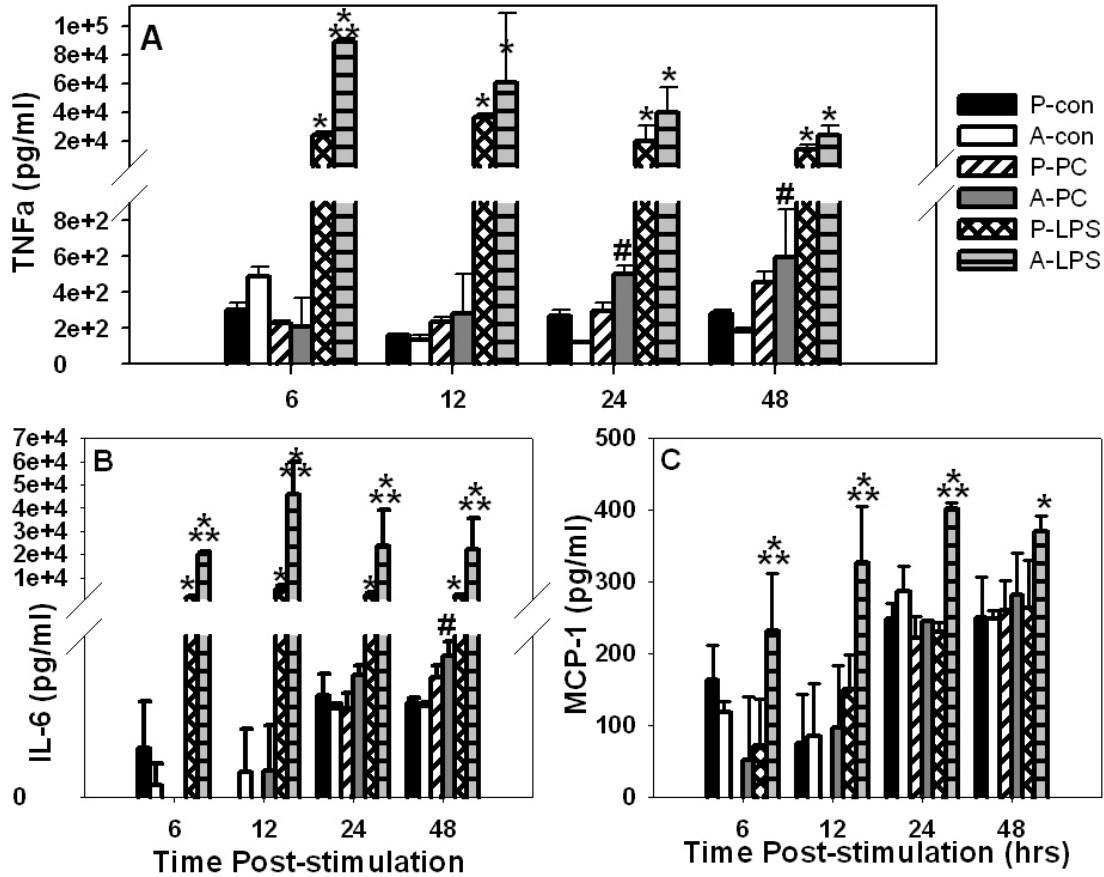


Figure 5.3 Pup and adult alveolar macrophages produce significant amounts of TNF α upon stimulation with LPS. Macrophages from 2-week old and adult mice were isolated from the BALF, placed in 96-well tissue culture plates and allowed to rest for 24 h. Subsequently, the cells were treated with either 100ng/ml of LPS, 4×10^5 PC organisms, or media alone for 6, 12, 24, or 48 h. Supernatant concentrations of LPS- and PC-stimulated cells were determined by Cytometric Bead Array.[®] P = pups, A = adults, con=unstimulated control. Data represent the means \pm SD for three wells per group. * Concentration of cytokine was significantly greater than the respective control and PC-infected groups per time point, $p < 0.05$. ** Concentration of cytokine was significantly greater than that produced by pup cells stimulated with LPS at the same time point, $p < 0.05$. # Concentration of cytokine was significantly greater than adult control cells at the same time point, $p < 0.05$. Comparisons are representative of 2 separate experiments.

Two other macrophage-produced cytokines, IL-12p70 and IL-10, were evaluated in their response to LPS and PC stimulation due to the effects their functions may have on delayed neonatal responses to PC. IL-12p70 induces CD4 T cell differentiation into T_H1 cells and IL-10 is a potent suppressor of macrophage function (50). Both PC and LPS failed to induce IL-12p70 production in pup and adult AMs at any time points tested (data not shown). Similarly, IL-10 production was not increased secondary to either stimulus; however, there was a constitutive elevated production among all groups (data not shown).

To determine whether the kinetics of cytokine production in pup AMs was delayed, this experiment was repeated looking at 72 hour post-stimulation. The cytokine response among both pup and adult AMs was similar to the responses observed at the earlier time points with the exception of IL-10. After 72 hours of stimulation, IL-10 production had increased among the LPS-treated pup compared to adult AMs ($P < 0.05$; data not shown). This observation suggests that with continuous stimulation, pup AMs respond by producing the anti-inflammatory cytokine, IL-10. The *in vitro* data described above demonstrates two important findings; the first being that pup macrophages are less responsive to LPS compared to adults and the second being that PC appears to be a relatively weak stimulus to adult AMs and pup AMs are unresponsive. These findings begged the question of whether or not differences exist in the expression level of the following three important receptors: dectin-1, a beta-glucan receptor (44, 65), TLR4 a receptor for LPS (44, 245), and TLR2 a receptor for ligands such as zymosan, peptidoglycan, lipoprotein, and more recently thought to be a receptor for the major surface glycoprotein found on PC (44, 246-249). To answer this question, the expression levels of dectin-1, TLR2, and TLR4 on pup and adult LMs were determined. As illustrated in figure 5.4, the expression of dectin-1 and TLR2 was the same on pup and adult LMs isolated from uninfected mice (Figure 5.4). These data suggest that the difference in production of TNF α between PC-stimulated pup and adult macrophages is not due to differences in dectin-1 or TLR2 pattern recognition receptor expression. Conversely, differences were detected in TLR4 expression between pup and adult LMs (Figure 5.4). It is

possible then, that the delayed expression of TLR4 on pup LMs may be at least partially responsible for the significantly reduced cytokine production compared to adult LMs stimulated with LPS.

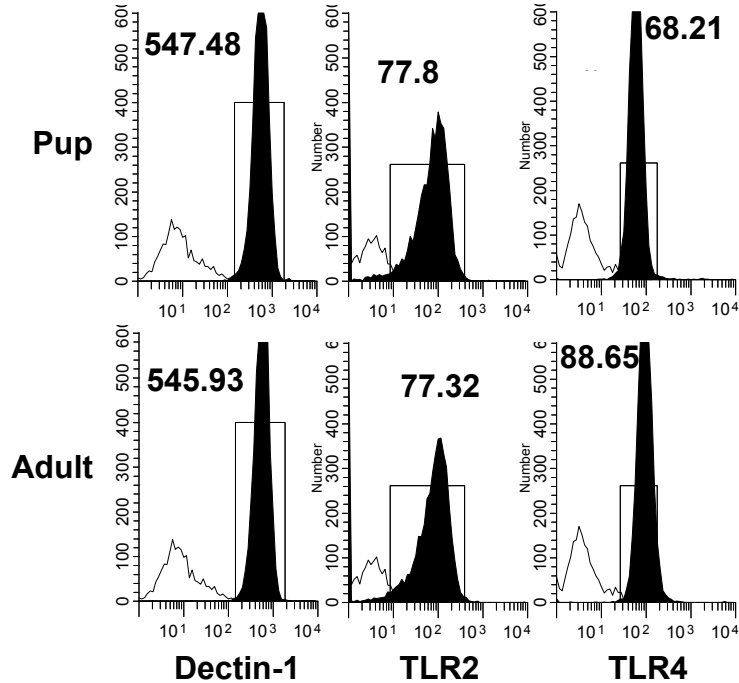


Figure 5.4 Adult LMs expressed significantly higher baseline TLR4 than pup LMs ($p < 0.05$). LMs were isolated from uninfected 14-day old pup and adult (≥ 8 weeks old) BALF. Cells were stained with antibodies specific for CD11c, dectin-1, TLR2, and TLR4 and analyzed by flow cytometry. Representative histograms of dectin-1, TLR2, and TLR4 positive cells (gated on CD11c⁺ cells) are shown. Cells were gated on large nonlymphoid cells by using forward and side light scatter. Data are representative of five mice per group and 2 separate experiments.

The transcription factor NF κ B p65 is responsible for the production of several cytokines, including TNF α . Considering the differences in TNF α production following stimulation in pup versus adult AMs, we examined the possibility that the nuclear translocation of NF κ B p65 may be delayed in pups versus adults stimulated with LPS. AMs were isolated from pooled BALF collected from uninfected pup and adult mice. These cells were cultured for 1 hour with LPS or media, the cells were collected, and the nuclear extracts were isolated. The amount of NF κ B p65 was determined for both pup and adult AMs. The level of NF κ B p65 was significantly greater in the adult cells compared to the

pup cells following 1 hour of LPS stimulation (Figure 5.5). This data suggests that the impaired $\text{TNF}\alpha$ production may be at least partially caused by poor NFkB p65 nuclear translocation, which may be related to an upstream impairment such as low TLR4 expression or the use of a MyD88-independent pathway.

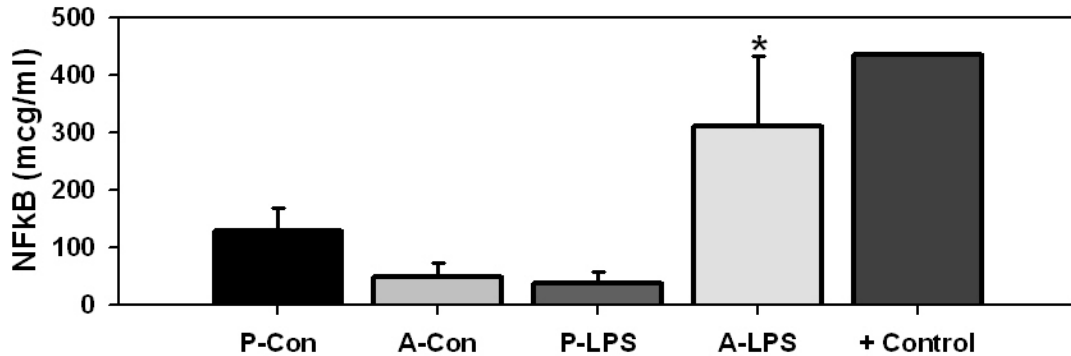


Figure 5.5 NFkB nuclear translocation increases in adult but not pup AMs stimulated with LPS. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled, the cells were lysed, and the nuclear extract was isolated. Chemicon's NFkB p65 Transcription Factor Colorimetric Assay was used to quantify NFkB p65 in the nuclear extract. Data represent the means \pm SD for 3 samples per group and are representative of 2 experiments. * $p < 0.05$ compared to adult control and pup LPS groups.

We next asked the question of whether pup AMs have a global unresponsiveness to other fungal ligands. To investigate this question an *in vitro* experiment was performed comparing the response of pup versus adult AMs to the *Saccharomyces cerevisiae* cell wall polysaccharide, zymosan. This yeast cell wall preparation is known to express substrates, such as beta-1,3-glucans and mannan, for several pattern recognition receptors present on LMs, including Dectin-1 and mannose receptors as well as TLR2 and TLR6 ligands (61, 250).

After 12 and 24 hours of stimulation with zymosan, both pup and adult AMs produced significantly more $\text{TNF}\alpha$ compared to their respective PC-stimulated and control wells (Figure 5.6a), suggesting that both pup and adult LMs have functional beta-glucan receptors and TLR2. Interestingly, pup AMs stimulated with zymosan also produced significantly more $\text{TNF}\alpha$ than their adult counterparts after 12 hours of stimulation. Consistent with the results shown in figure 5.3, stimulation of the AMs with PC, an organism known to contain beta-

1,3-glucan (47, 249), failed to induce the production of $\text{TNF}\alpha$ in pup AMs after 12 and 24 hours of stimulation. At 24 hours post-PC-stimulation, adult AMs produced slightly elevated levels of $\text{TNF}\alpha$ compared to unstimulated control cells as well as pup PC-stimulated AMs.

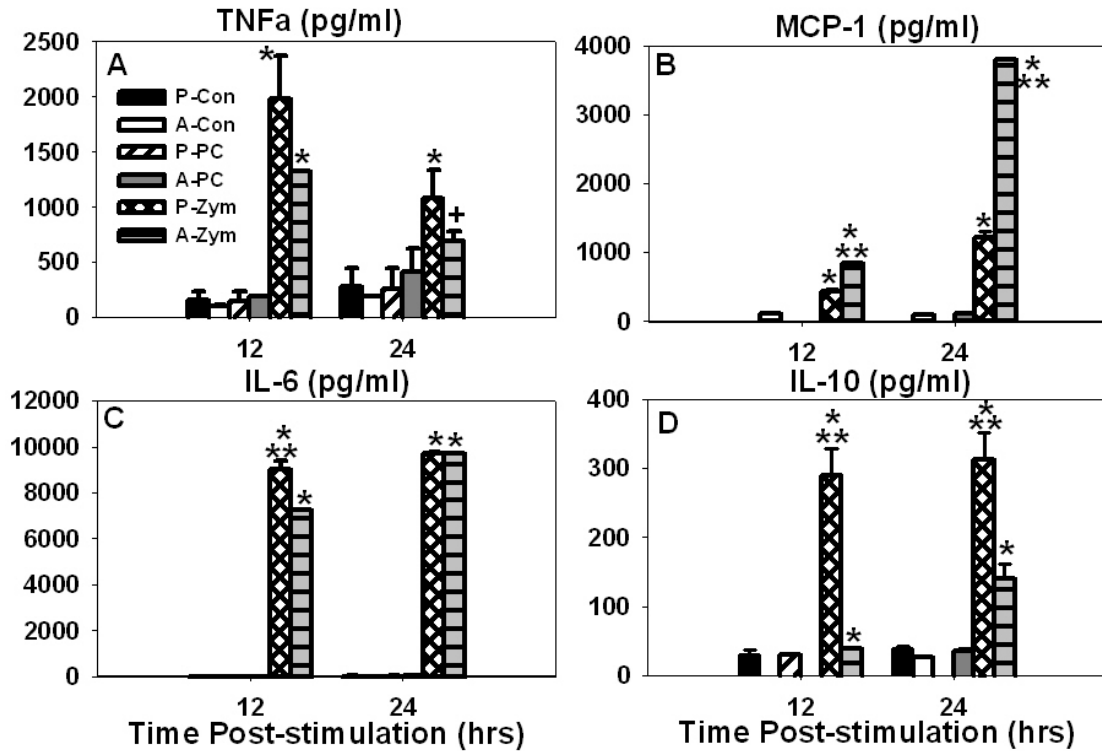


Figure 5.6 Zymosan stimulated significantly more AM cytokine production compared to PC. AMs were isolated from the pooled BALF of adult (A=adult) (≥ 8 wks) and 12-14 day old pup (P=pup) mice and cultured in a 96-well plate with PC, zymosan (zym), or media alone (con). Supernatants were removed at 12 and 24 hours post-stimulation and (A) TNF, (B) MCP-1, (C) IL-6, (D) and IL-10 were analyzed by Cytometric Bead Array analysis and flow cytometry. Data represent 3-4 wells per group. * Cytokine concentrations are significantly greater than their respective control and PC-treated groups, $p < 0.05$. + Cytokine concentrations are significantly greater than their respective control group only, $p < 0.05$. ** Cytokine concentrations are significantly greater than their comparator zymosan-treated groups, $p < 0.05$.

Production of the other pro-inflammatory cytokines tested, IL-6 and MCP-1, were significantly increased in both pup and adult AMs secondary to stimulation with zymosan (Figure 5.6b and c). Furthermore, MCP-1 production was significantly greater in adult AMs compared to pup AMs stimulated with

zymosan at both 12 and 24 hours post-stimulation. The levels of IL-6 and MCP-1 following PC stimulation were minimal or undetectable, further suggesting that AMs are specifically less responsive to PC organisms. Interestingly, IL-10, an inhibitory cytokine, was significantly increased in pup AMs following 12 and 24 hours of stimulation with zymosan, but not PC (Figure 5.6d). These data indicate that pup AMs respond differently than adult AMs to fungal antigens. Pup AMs are capable of producing significant amounts of pro-inflammatory cytokines when stimulated appropriately. These data suggest that PC is a significantly weaker stimulus than either zymosan or LPS for both pup and adult LMs.

2. Aerosolized heat-killed *E. coli* improves *Pneumocystis* clearance in pup mice

The resolution of PC in immunocompetent adult mice is associated with the up-regulation of pro-inflammatory cytokines IFN γ and TNF α (10, 234). Since our *in vitro* studies demonstrated that LPS, but not PC, stimulates pup AMs to produce significant levels of TNF α , we wanted to evaluate whether the TNF α produced secondary to LPS administration *in vivo* would improve PC clearance in pup mice. Reports in the literature further support the hypothesis that exogenously increasing the level of TNF α can improve PC clearance (251). Harmsen and Chen have reported that treatment of adult thymectomized and CD4-depleted mice with aerosolized HKEC also expedites the clearance of PC (238). They further showed that pretreatment of the animals with anti-TNF IgG minimizes the benefit imparted by the aerosolized HKEC suggesting that stimulation of TNF α plays a significant role in the clearance of PC from immunocompromised mice (238). Based on this information, we proceeded with experiments to administered aerosolized HKEC to PC-infected pup mice. Treatment with HKEC began 48 hours post-infection and continued through day 20 post-infection. Control mice received PC and aerosolized sterile water. By day 12 post-infection a divergence in PC burden between HKEC-treated and control mice could already be identified (Figure 5.7a). By day 21 there was a sharp decline in the HKEC-treated group and by day 32 post-infection, there was a

significant difference in PC burden in the HKEC-treated group compared to the control group. These data suggest that pup mice, as was found with CD4-depleted adult mice (238), can clear PC infection more efficiently when treated with aerosolized HKEC.

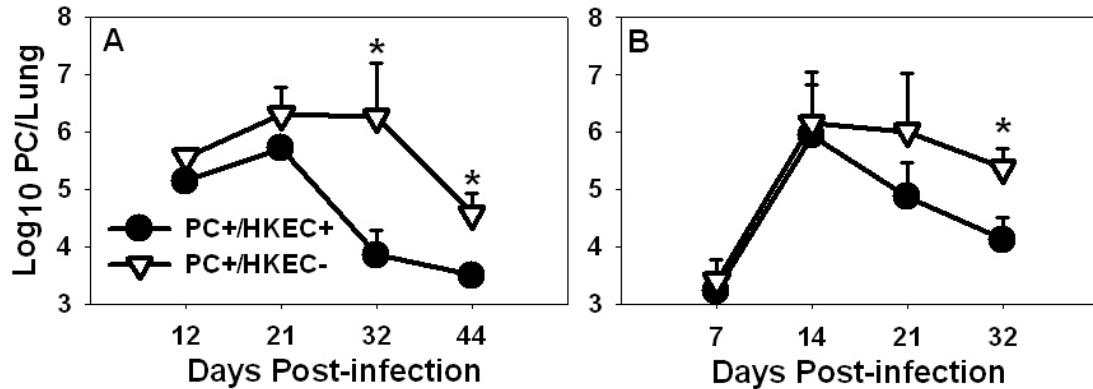


Figure 5.7 Mice treated with HKEC demonstrated a faster rate of PC clearance than control mice. Mice were infected as neonates (24 to 72 h after birth) and treated with aerosolized HKEC or sterile water 3 times per week. Lungs were collected at (A) days 12, 21, 32, and 44 post-infection or (B) days 7, 14, 21, and 32 post-infection, digested and spun onto glass slides. Slides were Diff-Quik® stained and enumerated microscopically. Data are representative of results of four to five mice per group and 3 separate experiments; * $p < 0.05$.

3. Aerosolized heat-killed *E. coli* influences the infiltration of immune cells in pup lungs

We were able to induce a more efficient clearance of PC organisms from the lungs of infected pup mice with the administration of aerosolized HKEC. In order to determine if this improved PC clearance was associated with an increase in cellular lung infiltration, BALFs were collected from mice treated with aerosolized HKEC and control mice treated with aerosolized sterile water. Cell differential counts were performed to determine macrophage, lymphocyte and neutrophil infiltration into the lungs of both the HKEC-treated mice and control mice. Overall minimal differences were noted, but most importantly, a divergence between the treated and untreated groups was observed among infiltrating macrophages into the lung by day 32 post-infection (data not shown). This correlates with the significant decrease in PC burden observed in the HKEC-

treated mice by 32 post-infection (Figure 5.7b). Neutrophil numbers appeared to follow HKEC treatment rather than PC infection as the neutrophil numbers rose early on and declined upon discontinuation of the HKEC treatment by day 21 (data not shown).

For both the HKEC-treated and untreated PC-infected mice, the infiltration of lymphocytes into the lung space, as determined by cell differential counts, sharply increased on day 21 post-infection and rapidly declined by day 32 post-infection (data not shown). The evaluation of lymphocytes, namely CD4+ T cells, by flow cytometry showed similar results with a rise in CD4+ T cells by day 21 post-infection and a subsequent drop thereafter (Figure 5.8a). By comparing the timing of CD4+ T cell infiltration into the lungs and PC clearance, one can see that the increase in CD4+ T cells in the alveolar spaces as well as the lung tissue immediately precedes the subsequent decline in PC burden in the HKEC-treated mice (Figures 5.7 and 5.8a-b). The infiltration of CD8+ T cells followed the same pattern as CD4+ T cells (data not shown). The decline in T cell numbers in the lungs falls rapidly upon discontinuation of HKEC treatments at day 20 post-infection suggesting that T cell infiltration was stimulated by HKEC.

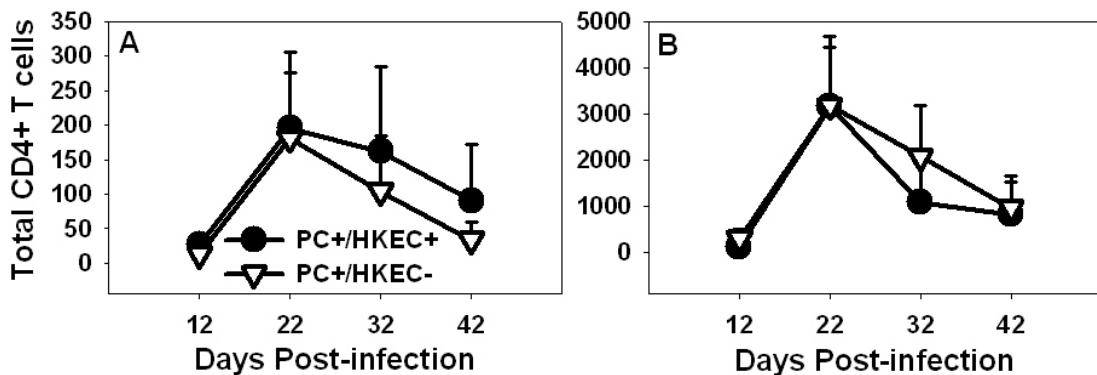


Figure 5.8 Treatment with HKEC induced CD4+ T cell infiltration into the lungs of PC-infected pups. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with either aerosolized HKEC or sterile water. BALF and whole lungs were collected on days 12, 21, 32, and 44 post-infection. No statistical difference in CD4+ T cells in the (A) BALF or (B) lung digest was observed between the groups treated with HKEC or the control group. Data represent the means \pm SD for five mice per group and are representative of 3 separate experiments.

4. Aerosolized heat-killed *E. coli* influences the activation of pup lung macrophages

We used flow cytometry to assess the activation status of PC-infected, pup LMs treated with aerosolized HKEC compared to those treated with aerosolized vehicle. Several different combinations of surface markers known to be signs of macrophage activation in adults were used. For all experiments involving flow cytometry, the cells were gated on non-lymphocytes using forward and side scatter.

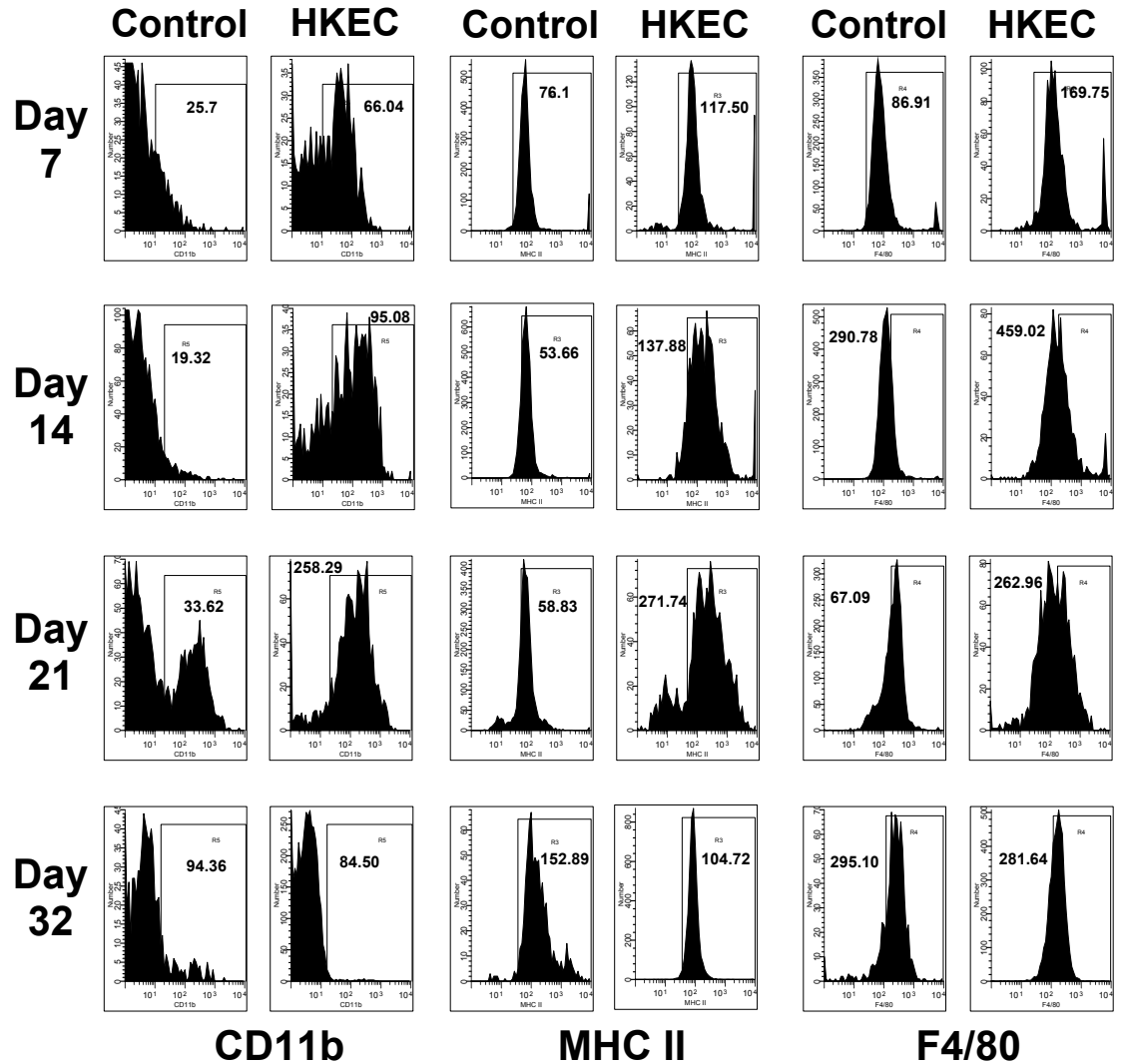


Figure 5.9 Expression levels of CD11b, MHC class II, and F4/80 on macrophages from alveolar spaces of PC-infected. HKEC-treated neonatal mice were increased at days 14 and 21 post-infection. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with either aerosolized HKEC or sterile, deionized water. BALF was collected at days 7, 14, 21, and 32 post-infection. Cells were stained with antibodies specific for CD11c and CD11b, MHC class II, or F4/80, and phenotypes were examined using flow cytometry. Representative histograms of CD11b, MHC class II, or F4/80 positive cells and the MFI for each (gated on CD11c+ cells) are shown. Cells were gated for large nonlymphoid cells by using forward and side light scatter. Data are representative of four to five mice per group and 2 separate experiments.

Figure 5.9 shows expression of the LM activation markers, CD11b, MHC II, and F4/80 at 4 time points after PC infection in representative pup mice from each group. The PC-infected group receiving HKEC showed an increase in activated LMs, as indicated by a right shift in the CD11b cell populations, compared to the infected group receiving aerosolized water only (Figure 5.9). This right shift could be seen in the HKEC group as early as day 7 post-infection and persisted through day 21 post-infection. These data correlated well with the decreased PC lung burden beginning at day 21 post-infection, suggesting that the activation of pup LMs by HKEC contributed to the expedited PC clearance (Figure 5.7b). The group that was PC-infected, but received aerosolized water only did not start to increase in CD11b expression until day 21 post-infection and both groups showed a return to baseline expression levels by day 32 post-infection. Similarly, the group receiving HKEC had increased expression of both MHC Class II and F4/80 molecules compared to untreated mice by day 7 post-infection. The difference in expression was most notable at days 14 and 21 post-infection and preceded PC clearance (Figure 5.7b). Overall, these data suggest that the treatment of PC-infected mice with HKEC increases the activation of LMs in pup lungs and contributing to the reduced PC lung burden.

5. Cytokine production was increased secondary to aerosolized heat-killed *E. coli*

We have previously reported that cytokines such as TNF α and IFN γ appear in the lungs of PC-infected pup mice much later than in the lungs of infected adult mice (234). We next examined cytokine production *in vivo* following treatment of PC-infected mice with aerosolized HKEC using Cytometric Bead Array[®] analysis. Similar to the results observed *in vitro* with LPS, neither IL-12p70 nor IL-10 levels were elevated in the HKEC group compared to the control group at any time points following infection with PC (data not shown). While no statistically significant differences in TNF α , IFN γ , or MCP-1 were found between groups in the BALFs, the group treated with HKEC tended to have greater levels of cytokines compared to the untreated group (Figure 5.10 a-c). Additionally, LMs

from the HKEC-treated group did produce significantly more IL-6 compared to the uninfected group (Figure 5.10 d). The production of IL-6 throughout this experiment was interesting in that it appeared to follow the HKEC treatment rather than the PC (Figure 5.10 d). At day 21 post-infection only those groups receiving HKEC had increased production of IL-6 regardless of PC infection. Furthermore, the production of IL-6 was minimal following the day 21 timepoint, which can likely be explained by the fact that the final HKEC treatment occurred on day 20 post-infection. Overall, this cytokine data reiterates the findings that LM-produced cytokines, particularly $TNF\alpha$, are involved in the expedited clearance of PC observed in the HKEC-treated groups. Although $IFN\gamma$ is not produced by LMs in significant quantities, LMs are known to stimulate $IFN\gamma$ -producing cells such as T lymphocytes, which would explain the increase seen herein (Figure 5.10 b).

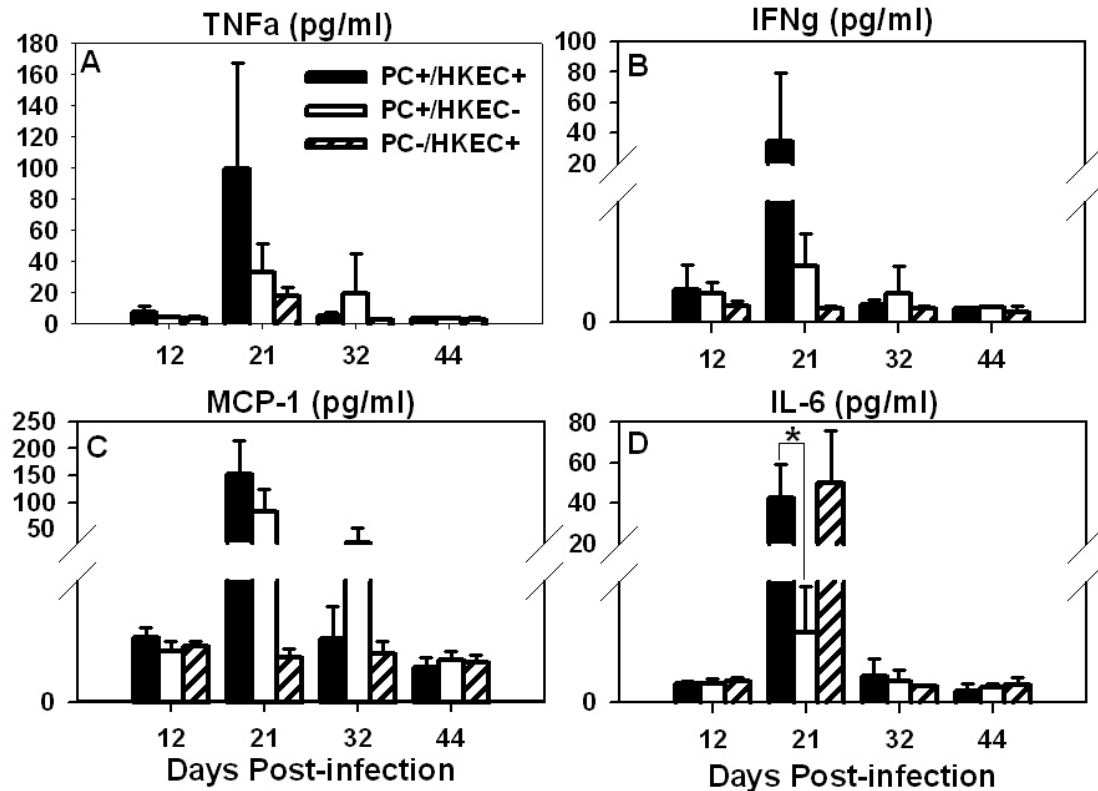


Figure 5.10 PC-infected mice treated with HKEC had elevated cytokine levels by day 21 post-infection. Mice were infected as neonates (24 to 72 h after birth) and then treated with either aerosolized HKEC or sterile, deionized water. BALF was collected on days 12, 21, 32, and 44 post-infection. Production of (A) TNF α , (B) IFN γ , (C) MCP-1, and (D) IL-6 was determined by Cytometric Bead Array[®] BD Biosciences, San Diego, CA. There were no significant differences between the group receiving HKEC and the group receiving only sterile water. Data represent the means \pm SD of five mice per group; * p <0.05.

6. Lung macrophage phagocytosis is less efficient in the absence of T lymphocytes

To determine if T lymphocytes are playing a role in the increased LM activation and PC clearance observed in HKEC-treated pups, HKEC-treated SCID and WT pups were compared in their ability to phagocytose DiO-labeled PC organisms. Six-7 day-old Balb/c or SCID mice were treated with aerosolized HKEC (as previously described) on days 4 and 2 prior to and once more on the day of PC infection (DiO-labeled). On day 1 post-infection the mice were lavaged. Some cells were reserved for differential counts and the rest were processed for analysis via flow cytometry. The total number of LMs

phagocytosing PC organisms was the same between SCID and WT pups (data not shown). However, treatment with HKEC drove more efficient phagocytosis of PC in LMs from treated compared to untreated WT pups as shown by the increased mean fluorescence intensity of DiO (Figure 5.11b). LMs from WT pups were significantly more efficient at phagocytosing PC compared to SCID pups, despite HKEC treatment in both groups (Figure 5.11a-b). The overall MHC class II expression was similar between the WT and SCID pups suggesting that the difference in phagocytosis was not likely due to lower activation in the SCID pups (data not shown). Although neutrophils are not thought to be important in PC clearance (84), we show that neutrophil infiltration in SCID pups was equal to WT pups but they were less capable of phagocytosing PC organisms compared to WT pups despite treatment with HKEC (Figure 5.11c-d). These data suggest that in the absence of T lymphocytes, HKEC-treated pup LMs are still capable of phagocytosing PC but are less efficient.

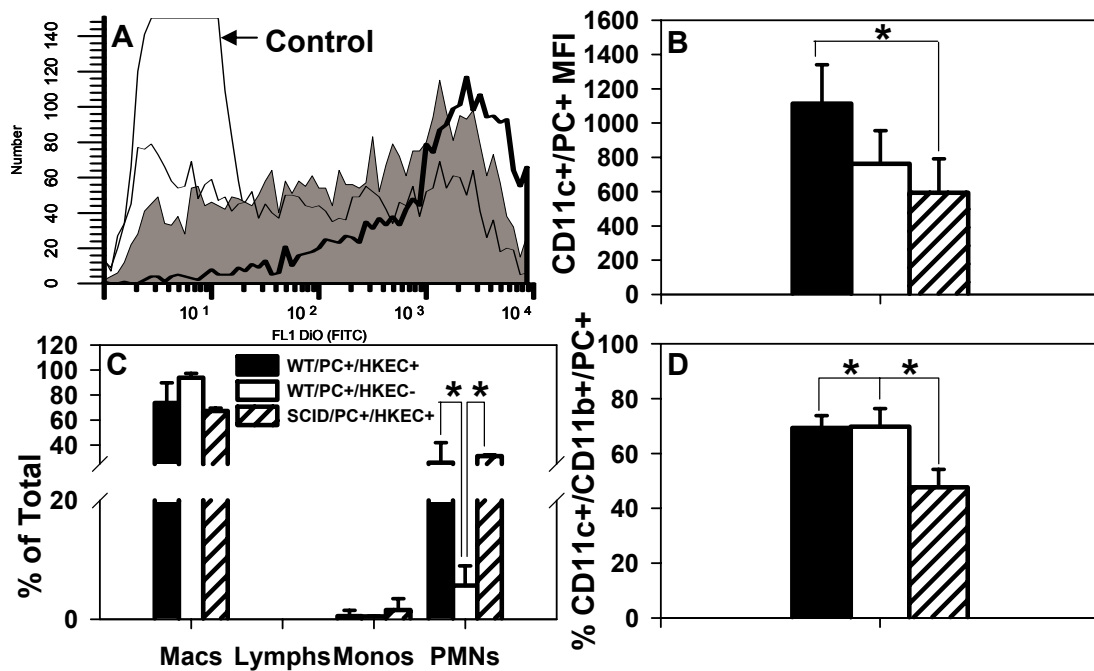


Figure 5.11 SCID pups treated with HKEC do not phagocytose DiO-labeled PC as efficiently as WT pups. SCID and WT pups were treated with aerosolized HKEC on days 4 and 2 previous to and on the day of DiO-labeled PC infection. BALF was collected on day 1 post-infection. Cells were stained with antibodies specific for CD11c, CD11b, and MHC class II and examined for co-expression with DiO-labeled PC. (A) Representative histogram showing DiO fluorescent/CD11c+ cells from HKEC-treated, WT pups (thick black line), HKEC-treated, SCID pups (medium line), untreated, WT pups (grey fill); (B) Mean fluorescence intensity of DiO in CD11c+ cells, * $p < 0.05$ compared to untreated WT and HKEC-treated SCID pups; (C) Differential counts of BALF cells, * $p < 0.05$ compared to untreated WT pups; (D) Mean fluorescence intensity of DiO in CD11c-, CD11b+ cells, * $p < 0.05$ compared to HKEC-treated SCID pups. Data represent the means \pm SD of four mice per group.

C. Granulocyte macrophage-colony stimulating factor

GM-CSF has been shown to enhance clearance and improve outcomes of several different pulmonary infections, including but not limited to group B streptococcus infection (24), *Pseudomonas aeruginosa* (25), *Histoplasma capsulatum* (26, 27), *Mycobacterium tuberculosis* (28), and *Pneumocystis carinii* (29). However, there is little data on the effects of GM-CSF on pulmonary infections in infants and no data regarding the addition of GM-CSF for the treatment of PCP in the infant population. The ability of GM-CSF to improve

outcomes in pulmonary infections in adult animal models suggests that this clinically relevant agent may be of therapeutic benefit for infants with pulmonary infections failing to respond to antimicrobial therapy alone. Thus, we hypothesized that treatment of PC-infected pup mice with rmGM-CSF (recombinant mouse GM-CSF); either alone or in combination with TMP/SMX, would improve PC clearance secondary to increased LM numbers and activation.

1. rmGM-CSF increases cytokine production in pup alveolar macrophages

Initial rmGM-CSF experiments were done *in vitro* to minimize cost while testing the potential benefit of this agent and its ability to stimulate LMs to produce cytokines. The first of such experiments was done by collecting the BALF of both adult and pup mice, isolating the AMs and culturing them with rmGM-CSF for 6, 24, 30, and 48 hours. Stimulation of both adult and pup cells with rmGM-CSF alone increased the production of TNF α , MCP-1, and IL-6 at 24 hours post-stimulation above that which was produced by the control cells; for MCP-1 this increase was significantly greater than that produced by the control cells for both pups and adults (Figure 5.12 a,b,d). For TNF α and IL-6, while the increased cytokine production stimulated by rmGM-CSF alone was not statistically significant, stimulation of both pup and adult cells with rmGM-CSF plus PC did generate a statistically significant increased cytokine production at 24 hours post-stimulation (Figure 5.12 a and d). After 30 and 48 hours post-stimulation, the production of TNF α in the pup cells secondary to rmGM-CSF alone was significantly greater than their control cells; while the adult cells also had an increase in TNF α after 30 and 48 hours, it did not reach statistical significance. However, for both pup and adult cells stimulated with rmGM-CSF plus PC, TNF α was still significantly greater than the control cells after 30 and 48 hours post-stimulation (Figure 5.12a). MCP-1 production was significantly greater in both pup and adult cells stimulated with rmGM-CSF alone at 48 hours post-stimulation, but only the adult cells stimulated with both rmGM-CSF and PC had significantly more MCP-1 production after 48 hours of stimulation; the pup cells had a large intra-group variability causing it to fail reaching statistical

significance (Figure 5.12b). IL-6 production was significantly increased in both pup and adult cells stimulated with both rmGM-CSF and PC together; IL-6 production was not significantly increased in those cells stimulated with rmGM-CSF alone (Figure 5.12d). The production of IL-10 was not affected by the addition of rmGM-CSF either alone or in combination with PC (Figure 5.12c). Overall these data suggest that the addition of rmGM-CSF to PC-infected pup cells increases the production of $\text{TNF}\alpha$, MCP-1, and IL-6, but not IL-10.

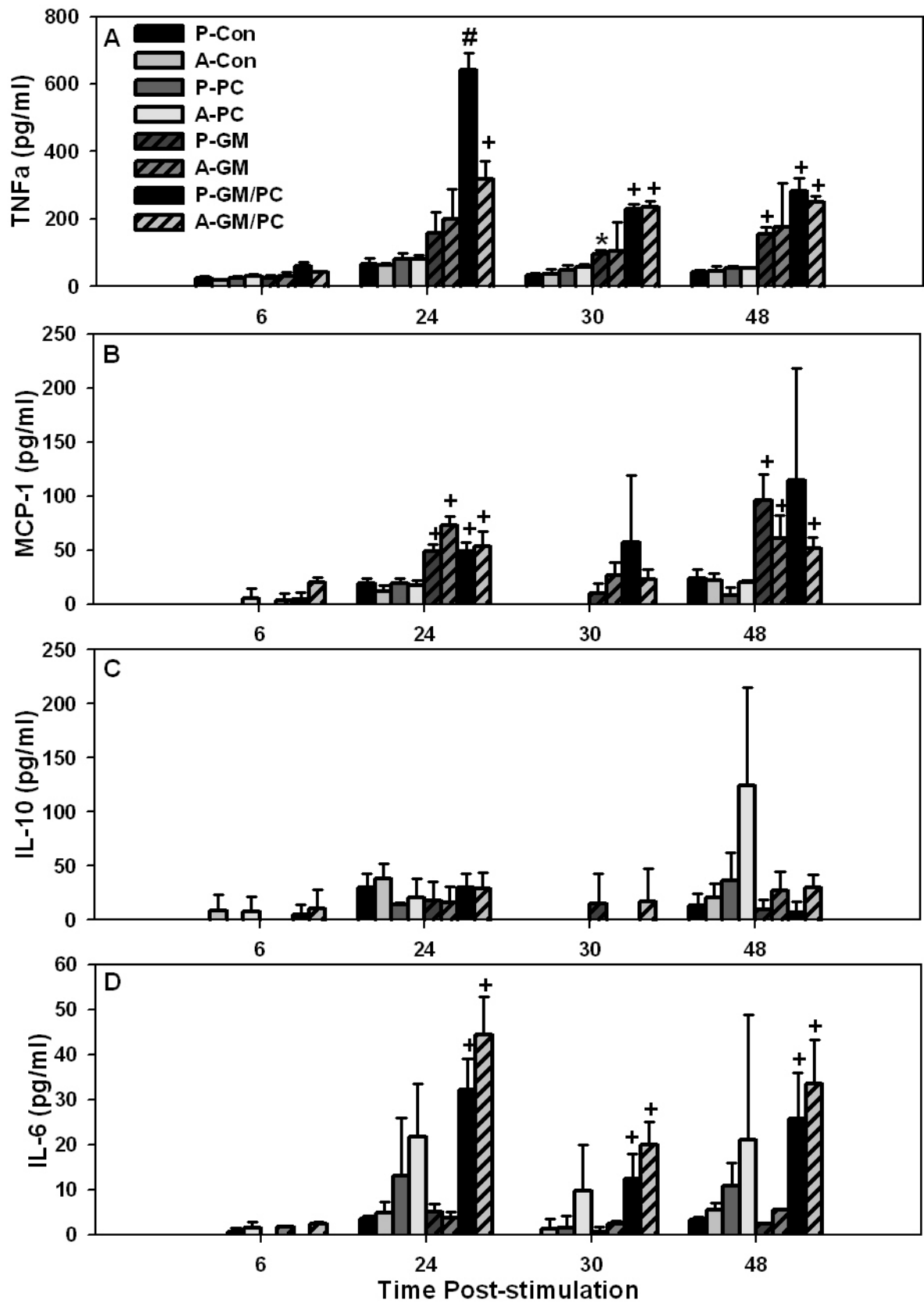


Figure 5.12 rmGM-CSF increases cytokine production in pup AMs. AMs were isolated from the pooled BALF of adult (≥ 8 wks) and 7-10 day old pup (P) mice and cultured in a 96-well plate with media alone (Con), PC, rmGM-CSF (GM), or PC plus rmGM-CSF (GM/PC). Supernatants were removed at 6, 24, 30, and 48 hours post-stimulation and (A) TNF, (B) MCP-1, (C) IL-10, (D) and IL-6 were analyzed by Cytometric Bead Array analysis and flow cytometry. Data represent the mean \pm SD of 3-4 wells per group. # $p < 0.05$, compared to all other pup groups and A-GM/PC; + $p < 0.05$, compared to the respective pup or adult control and PC groups; * $p < 0.05$, compared to the respective pup or adult control groups.

2. NFkB p65 nuclear translocation is stimulated by rmGM-CSF in adult but not pup alveolar macrophages

Similar to the assay performed following LPS stimulation, an NFkB p65 assay was performed to determine the level of translocation into the nucleus following 1 hour of stimulation with PC alone, rmGM-CSF alone, or PC and rmGM-CSF in combination. After one hour there was significantly more NFkB p65 nuclear translocation in the adult AMs stimulate with rmGM-CSF compared to the pup AMs (Figure 5.13). Both adult and pup AMs stimulated with PC antigen and rmGM-CSF had less NFkB p65 translocation than that which occurred with rmGM-CSF alone, suggesting that this combination may play some what of an inhibitory role on NFkB p65 nuclear translocation in both adult and pup AMs early in the AM-PC encounter. Upon prolonged exposure to PC antigen, however, it would appear that this possible suppression is overcome based on the cytokine data shown in figure 5.12a in which TNF α is largely produced secondary to rmGM-CSF alone and in combination with PC infection by 24 hours post-stimulation.

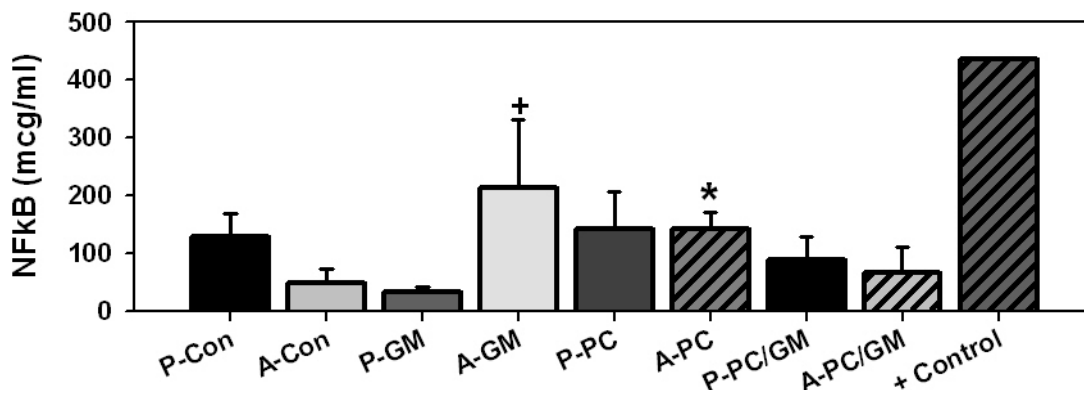


Figure 5.13 rmGM-CSF increases adult, but not pup NFkB p65 nuclear translocation. The lavage fluid from 7-10 day-old pup mice and adult mice were each pooled and then cultured with media alone (con), rmGM-CSF (GM), PC (PC), or PC plus rmGM-CSF (PC/GM). The cells were lysed and the nuclear extract was isolated and NFkB p65 quantified using Chemicon's NFkB p65 Transcription Factor Colorimetric Assay. Data represent the means \pm SD for 3 samples per group and are representative of 2 experiments. ⁺ $p < 0.05$ compared to adult control and pup rmGM-CSF groups. ^{*} $p < 0.05$ compared to adult control group.

3. rmGM-CSF delivered i.p. increases pup lung macrophage activation and T cell infiltration but not Pneumocystis clearance

Based on the data generated from the *in vitro* experiments, an rmGM-CSF pilot experiment was performed using a small number of mice. Pups were infected with PC, as previously described, at 24-72 hours old. Half the pups were then dosed with 0.25 μ g of rmGM-CSF per gram body weight by i.p. injection twice daily beginning on day 7 post-infection and continuing for a total of 5 days (ending day 11 post-infection). The mice were lavaged and their lungs collected at days 10 and 20 post-infection for flow cytometry and PC enumeration. While no statistically significant differences between the two groups were found during our data analysis, some interesting trends were observed. First, those mice receiving the rmGM-CSF treatment had an increase in AM infiltration as indicated by a rise in CD11c⁺ cells in the lavage (Figure 5.14a) as well as a rise in their activation level as indicated by an increase in CD11c⁺/CD11b⁺ and CD11c⁺/MHC class II⁺ cells (Figure 5.14b-c). Interestingly, no differences were observed in macrophages in the lung digest (Figure 5.15a-c). Additionally activated CD4 and CD8 T cells were elevated in the rmGM-CSF-treatment groups in the lavage and activated CD8 T cells were elevated in the treatment

group in the lung digest (Figure 5.16 a,c, and d). No changes were noted in the lung digest for CD4 T cells (Figure 5.16b). Lastly, no differences were observed in PC burden between the treated and untreated mice at either timepoint tested (Figure 5.17). While this data showed no definitive results, some important trends were observed. Although not statistically significant, the increase in AM and T cell infiltration and activation in the BALF of mice receiving rmGM-CSF was promising and encouraged us to proceed with experiments using rmGM-CSF in larger experiments with more mice. A change in both the dose and route of administration were applied for subsequent experiments; administration of rmGM-CSF by i.n. rather than by i.p. administration was instituted based on published literature demonstrating safety and efficacy in initiating a specific pulmonary immune response with aerosolized GM-CSF (252, 253).

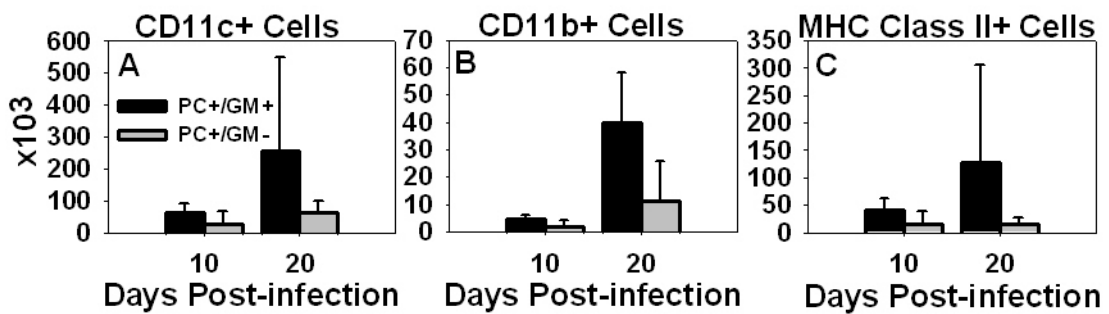


Figure 5.14 rmGM-CSF administered i.p. did not affect the number of pup AMs expressing CD11c, CD11b, or MHC class II molecules in the lavage. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 0.25µg/g i.p. rmGM-CSF or sterile, deionized water twice daily for 5 days beginning day 7 post-infection. BALF was collected on days 10 and 20 post-infection. Cells were stained with antibodies specific for CD11c and CD11b, or MHC class II and were examined using flow cytometry. Cells were gated for large nonlymphoid cells by using forward and side light scatter and CD11c+ cells. Data are representative of the mean ± SD of four to five mice per group and 2 separate experiments.

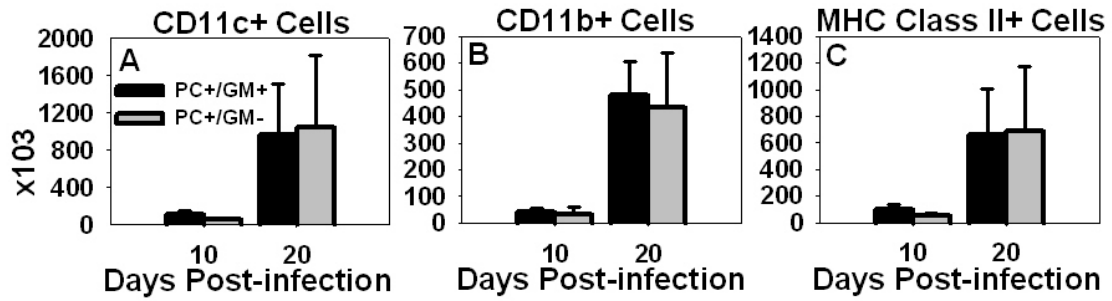


Figure 5.15 rmGM-CSF administered i.p. did not affect the number of pup macrophages expressing CD11c, CD11b, or MHC class II molecules in the lung digest. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 0.25 μ g/g i.p. rmGM-CSF or sterile, deionized water twice daily for 5 days beginning day 7 post-infection. Whole lungs were collected on days 10 and 20 post-infection and processed into single cell suspensions. Cells were stained with antibodies specific for CD11c and CD11b, or MHC class II and were examined using flow cytometry. Cells were gated for large nonlymphoid cells by using forward and side light scatter and CD11c+ cells. Data are representative of the mean \pm SD of four to five mice per group and 2 separate experiments.

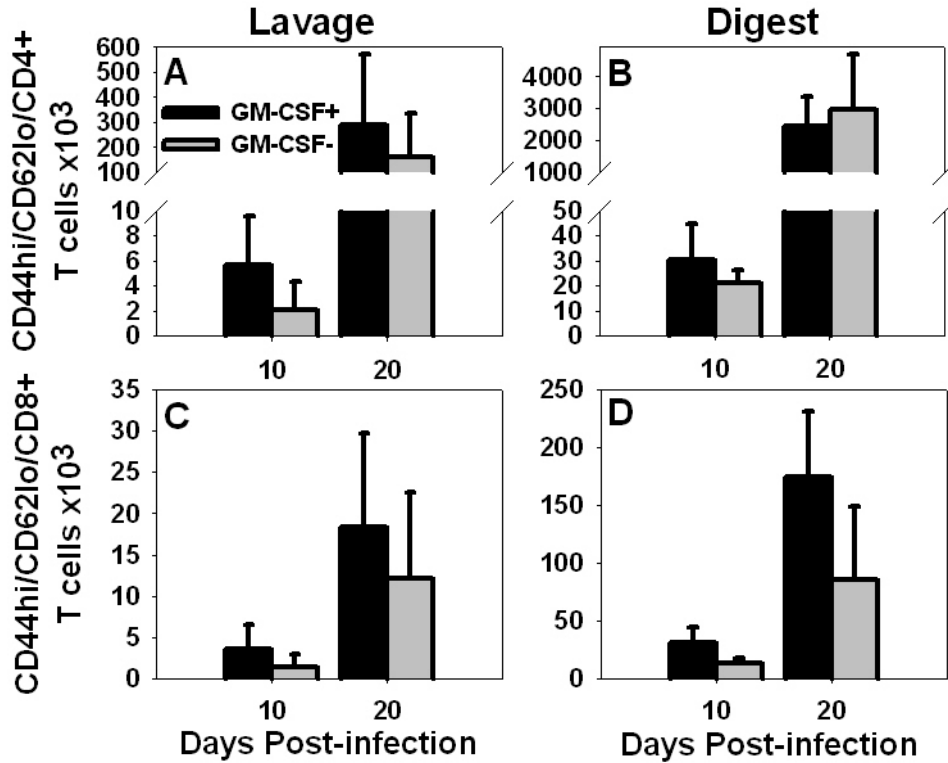


Figure 5.16 rmGM-CSF administered i.p. did not increase the infiltration of activated T cells in PC-infected pup lungs. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 0.25 μ g/g i.p. rmGM-CSF or sterile, deionized water twice daily for 7 days beginning day 7 post-infection. Lavage fluid and whole lungs were collected on days 10 and 20 post-infection and processed into single cell suspensions. Cells were stained with antibodies specific for CD4, CD8, CD44, and CD62 and examined using flow cytometry. Cells were gated for small non-ganular lymphoid cells by using forward and side light scatter. Data are representative of the mean \pm SD of four to five mice per group and 2 separate experiments.

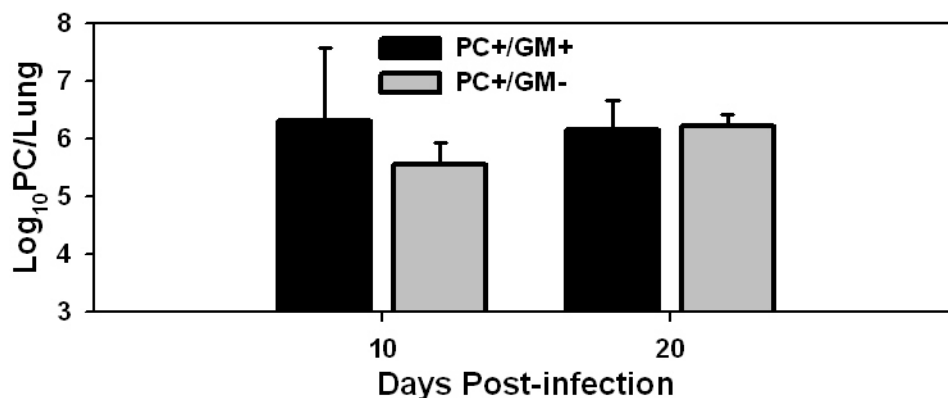


Figure 5.17 rmGM-CSF administered i.p. did not reduce PC lung burden in pup mice. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 0.25µg/g i.p. rmGM-CSF or sterile, deionized water twice daily for 7 days beginning day 7 post-infection. Whole lungs were collected on days 10 and 20 post-infection, processed into single cell suspensions. Aliquots were spun onto glass slides, stained with Diff-Quick[®] and enumerated microscopically. Data are representative of the mean ± SD of four to five mice per group and 2 separate experiments.

4. rmGM-CSF administered i.n. improves Pneumocystis clearance

The next rmGM-CSF experiment included seven C57BL/6 x DBA/2J (B6D2F1/J) pup mice, which were infected with PC at 72 hours old. On day 7 post-infection 3 of the 7 mice were treated with i.n. rmGM-CSF daily (5ng/g) for 5 days (254-256); the 4 control mice received i.n. PBS on the same dosing schedule. On day 18 post-infection, BALF and lungs were collected and processed for flow cytometry and microscopic analysis as previously described. Mice treated with rmGM-CSF had a reduced PC burden without a concomitant increase in the number of LMs expressing activation markers (Figure 5.18a). All cells were first gated on large nonlymphocytes and CD11c. None of the markers tested were increased in the treatment group compared to the control group, including CD11b, MHC class II, dectin-1, and mannose receptor. Figure 5.18b depicts the PC burden in the lungs of both the rmGM-CSF-treated and the control mice. Although both groups of mice have a relatively large PC burden and no statistically significant differences were found, the rmGM-CSF-treated group had fewer overall PC organisms in their lungs compared to the control group

(Figure 5.18b). This data suggests that rmGM-CSF is capable of reducing PC burden without increasing the number of LMs expressing an activated phenotype. Such a pattern would be desirable in an infant whose developing lungs are vulnerable to damage from excessive inflammatory responses. It is possible that the reduced PC lung burden observed in the rmGM-CSF-treated mice resulted in the decreased number of macrophages expressing activation markers compared to the untreated group.

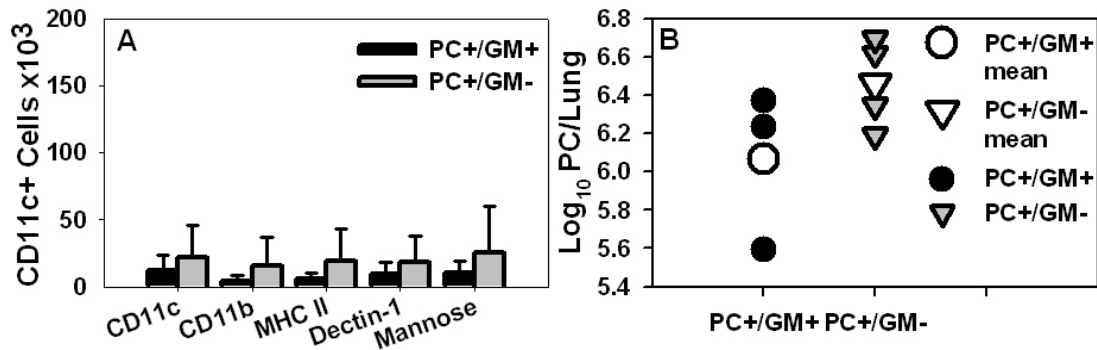


Figure 5.18 rmGM-CSF administered i.n. reduced PC burden in pup mice. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 5ng/g i.n. rmGM-CSF or PBS daily for 5 days beginning day 7 post-infection. Lavage fluid and whole lungs were collected on day 18 post-infection and processed into single cell suspensions. (A) Cells were stained with antibodies specific for CD11c, CD11b, dectin-1, MHC class II, and mannose receptor and examined using flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter and were gated on CD11c+ cells. Data are representative of three to four mice per group. (B) Aliquots of lung digest were spun onto glass slides, stained with Diff-Quick[®] and enumerated microscopically. White symbols represent the mean log₁₀PC/Lung.

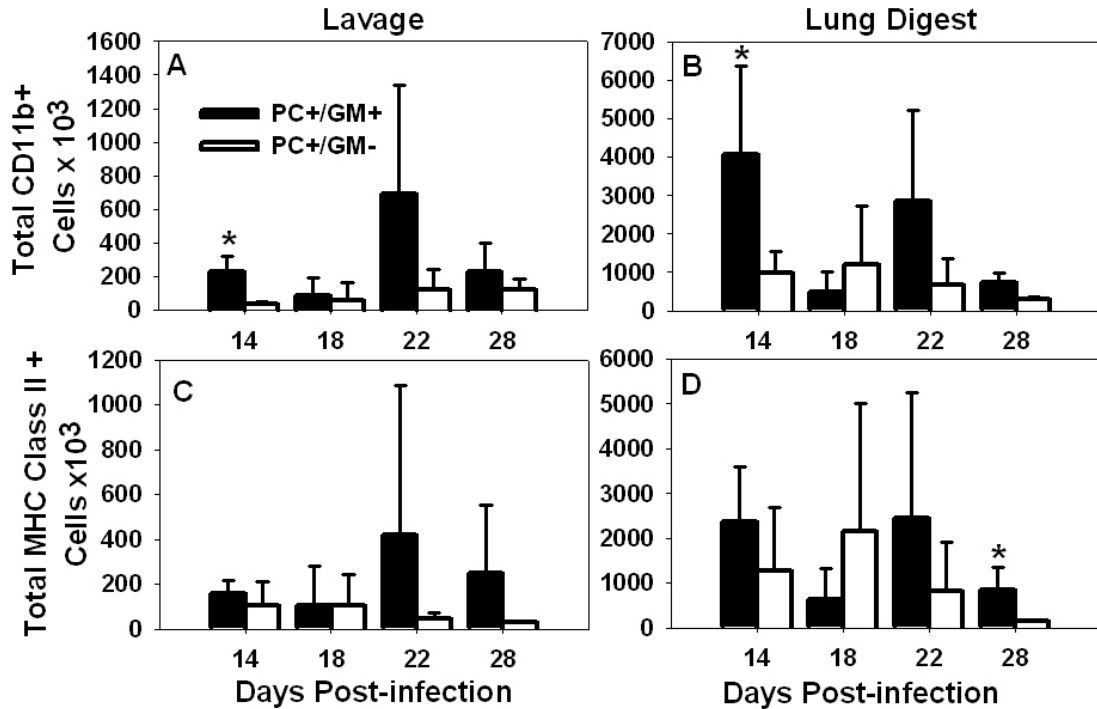


Figure 5.19 rmGM-CSF administered i.n. increased the number of LMs expressing activation markers in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 5ng/g i.n. rmGM-CSF or PBS daily for 7days beginning day 7 post-infection. Lavage fluid and whole lungs were collected on days 14, 18, 22, and 28 post-infection and processed into single cell suspensions. Cells were stained with antibodies specific for CD11c, (A-B) CD11b, and (C-D) MHC class II and examined using flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter and were gated on CD11c+ cells. Data are representative of the mean \pm SD of three to four mice per group. * $p < 0.05$ compared to respective PC+/GM- groups.

Based on this data, a second rmGM-CSF experiment was designed to include multiple timepoints. As previously described, mice were infected with PC at 72 hours old, and treatment with 5ng/g rmGM-CSF i.n. was begun 7 days post-infection and continued for 7 days. In addition to looking at LMs, flow cytometry was used to compare differences in T cell infiltration and activation in both the lungs and lymph nodes in the treatment group compared to the control group. At day 14 post-infection there was a significant increase in LMs (CD11c+/CD11b+ non-lymphocytes) in both the BALF and the lung tissue in the rmGM-CSF-treated mice compared to the untreated mice (Figure 5.19a and b);

suggesting an increase in LM infiltration and/or activation of macrophages or granulocytes. In both tissues this difference was lost by day 18 post-infection. While these cells increased again in both tissues by day 22 post-infection in the treatment group compared to the untreated group, the difference was not found to be statistically significant. The number of nonlymphocytes expressing MHC class II was also assessed in both the BALF and the lung tissues. As can be seen in figures 5.19c and d, there was a lot of intra-group variability, thus the only significant difference noted in the number of cells expressing MHC class II was at day 28 post-infection in the lung tissue (Figure 5.19d). Taken together, this data suggests that rmGM-CSF may both increase the infiltration of AMs into the alveolar spaces as well as increase the number of infiltrated and tissue macrophages expressing activation markers in the lungs of PC-infected pups.

With an increase in the infiltration and activation of LMs in the BALF as well as the lung tissue, one would expect an increase in the infiltration and activation of T lymphocytes within the same time frame. Based on this premise, flow cytometry was used to evaluate the presence of both CD4+ and CD8+ T cells with a high expression of CD44 and a low expression of CD62L (an indication of T lymphocyte activation). As expected, the infiltration and activation of both CD4+ and CD8+ T cells followed a very similar pattern to that of the AM and tissue macrophages (Figure 5.20). Pups treated with rmGM-CSF had an increase in activated CD4+ and CD8+ T cells in the BALF and the lung tissue. This increase occurred earlier at day 14 post-infection in the lung tissue compared to day 22 post-infection in the BALF, likely reflecting the time it takes for lymphocytes to extravasate across the lung tissues to reach the alveolar spaces. (Figure 5.20a-d). Overall, no statistically significant differences between the pups receiving rmGM-CSF and the pups receiving vehicle only were found. The TBLNs were analyzed for activated T lymphocytes to determine if rmGM-CSF treatment improved the time to specific antigen response in the regional lymph node, presumably through expedited or improved TBLN homing and/or antigen presentation. Treatment with rmGM-CSF did not improve lymphocyte activation time in the TBLN compared to the untreated group (Figure 5.21a-b).

Lastly, figure 5.21c depicts the PC lung burden in each mouse from both the treatment and control groups at each timepoint tested. While the lung burden of PC organisms at day 14 post-infection was the same in both groups, a divergence is obvious at day 18 post-infection whereby the rmGM-CSF-treated group has a lower burden compared to the control group (NS). This divergence is lost, however by day 22 post-infection and overall, no statistically significant differences in PC burden were observed between the two groups (Figure 5.21c).

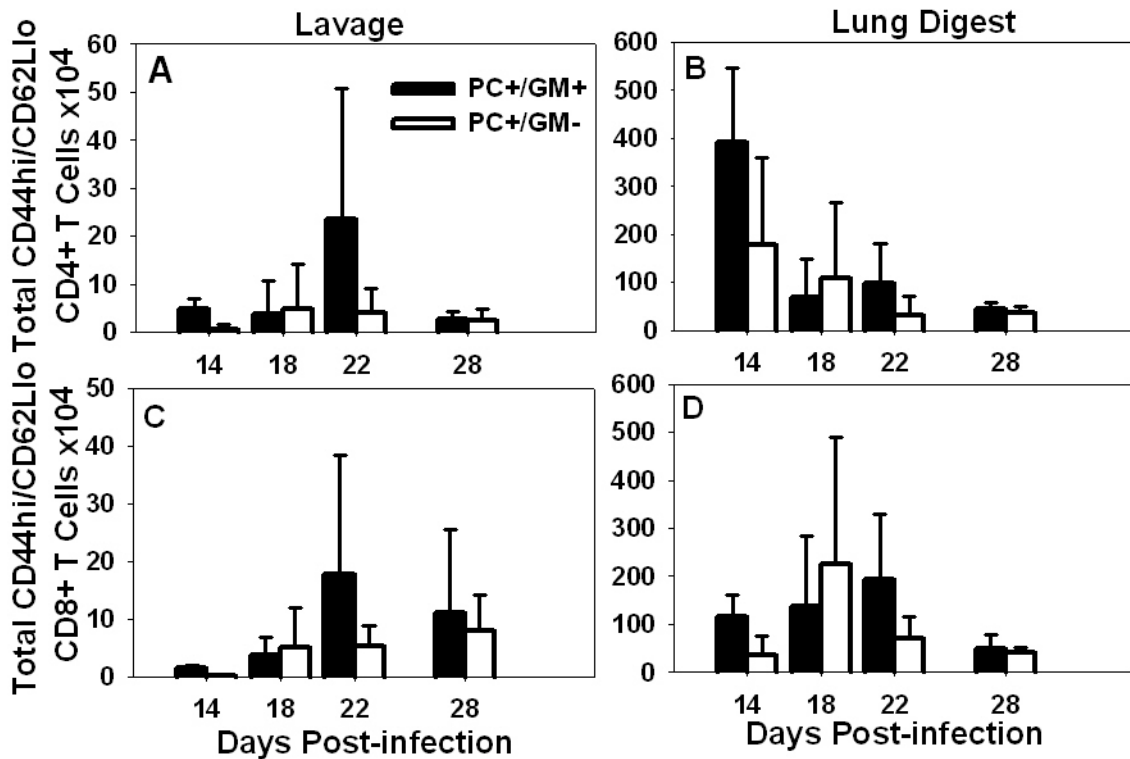


Figure 5.20 rmGM-CSF administered i.n. did not significantly increase the number of activated T cells in PC-infected pup lungs. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 5ng/g i.n. rmGM-CSF or PBS daily for 7days beginning day 7 post-infection. Lavage fluid and whole lungs were collected on days 14, 18, 22, and 28 post-infection and processed into single cell suspensions. Cells were stained with antibodies specific for CD4 (A-B), CD8 (C-D), CD44, and CD62L and examined using flow cytometry. Cells were gated for small, non-granular cells by using forward and side light scatter. Data are representative of the mean \pm SD of three to four mice per group.

Taken together, the data describing this rmGM-CSF *in vivo* experiment demonstrates that mice infected with PC as neonates have a more robust LM response to the organism following treatment with rmGM-CSF compared to untreated mice. The increase in activated LMs at day 14 post-infection in the rmGM-CSF group occurred approximately 7 days prior to the reduced PC burden seen at day 18 in comparison to the control group. Overall, rmGM-CSF increased the activation of LMs in PC-infected pups. While the reduction in PC burden did not reach statistical significance, it is clear that rmGM-CSF bolstered the overall immune response to PC infection in pup mice.

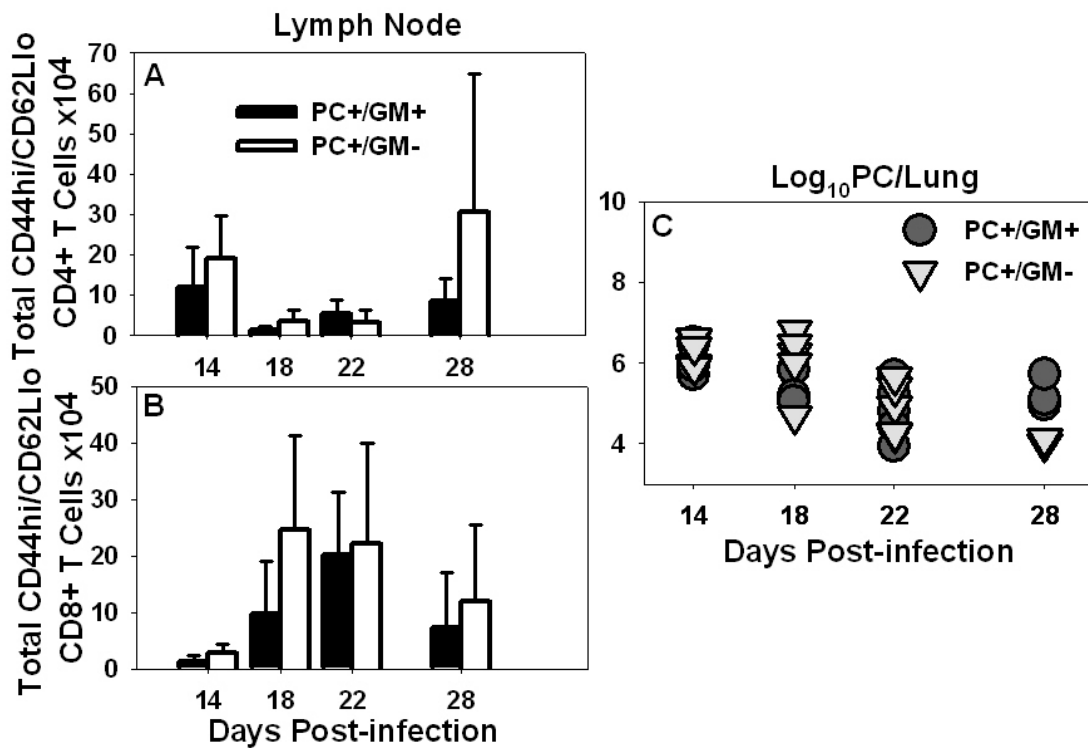


Figure 5.21 rmGM-CSF administered i.n. did not significantly reduce PC burden in pup mice. Mice were infected with PC as neonates (24 to 72 h after birth) and then treated with 5ng/g i.n. rmGM-CSF or PBS daily for 7days beginning day 7 post-infection. TBLNs and whole lungs were collected on days 14, 18, 22, and 28 post-infection and processed into single cell suspensions. Cells were stained with antibodies specific for CD4 (A), CD8 (B), CD44, and CD62L and examined using flow cytometry. Cells were gated for small, non-granular cells by using forward and side light scatter. (C) Aliquots of lung digest were spun onto glass slides, stained with Diff-Quick[®] and enumerated microscopically. Data are representative of the mean \pm SD of three to four mice per group.

This data differs from the first i.n. rmGM-CSF experiment which demonstrated a reduction in PC burden without a concomitant increase in LM expressing activation markers (Figure 5.18). Two explanations can be offered when comparing the results of these two experiments. First, is that the second experiment continued rmGM-CSF therapy for 7 days, unlike the first experiment in which treatment was extended for only 5 days. The extra 2 days of therapy may have contributed to the increased number of LMs expressing activation markers. Second, as previously stated, the reduced PC burden seen in the first i.n. rmGM-CSF experiment was likely driven by the reduced PC burden. The second experiment also had a reduction in PC burden at day 18 post-infection which correlated with a lower number of LMs expressing activation markers. This second experiment, however, looked at days further out from infection and as will be described in the next two figures, demonstrated somewhat of a rebound in PC burden with a concomitant increase in activation markers. Overall this data suggests that rmGM-CSF treatment alone is not sufficient to treat PC infection.

D. Recombinant mouse granulocyte macrophage-colony stimulating factor plus Trimethoprim/sulfamethoxazole

Although the standard of care treatment for PC pneumonia is TMP/SMX, the use of combination therapy for infectious diseases is a common, well-accepted practice depending on the severity of the infection, its location, and the virulence of the infecting organism (257). Considering the use of combination therapy together with the rationale of using immunomodulatory agents to fight infectious diseases, we hypothesized that the use of GM-CSF and TMP/SMX together could improve PC clearance in pup mice. Experiments were designed to include four groups of pups; all were infected with PC between 24 and 72 hours old. The infection was allowed to grow for 7 days and become established before beginning one of the following treatment regimens: 40mg/kg TMP/ 200mg/kg SMX orally twice daily for 14 days, 5ng/g rmGM-CSF i.n daily for 7 days, 40mg/kg TMP/ 200mg/kg SMX orally twice daily x 14 days + 5ng/g rmGM-CSF i.n daily x 7 days, or vehicle only i.n. once daily x 7 days.

1. Pneumocystis burden

As demonstrated in Figure 5.22, the groups treated with TMP/SMX alone and TMP/SMX plus rmGM-CSF had significantly lower PC burdens by day 22 post-infection compared to control mice. By day 29 post-infection the PC burden in the combination group remained low. However, the burden in the TMP/SMX group seems to rebound slightly upon discontinuation of the drug. This observation suggests that in the combination group, the TMP/SMX was responsible for the brunt of the work in reducing PC, but the addition of rmGM-CSF enabled the host's immune system to maintain that reduction more efficiently after the TMP/SMX was removed.

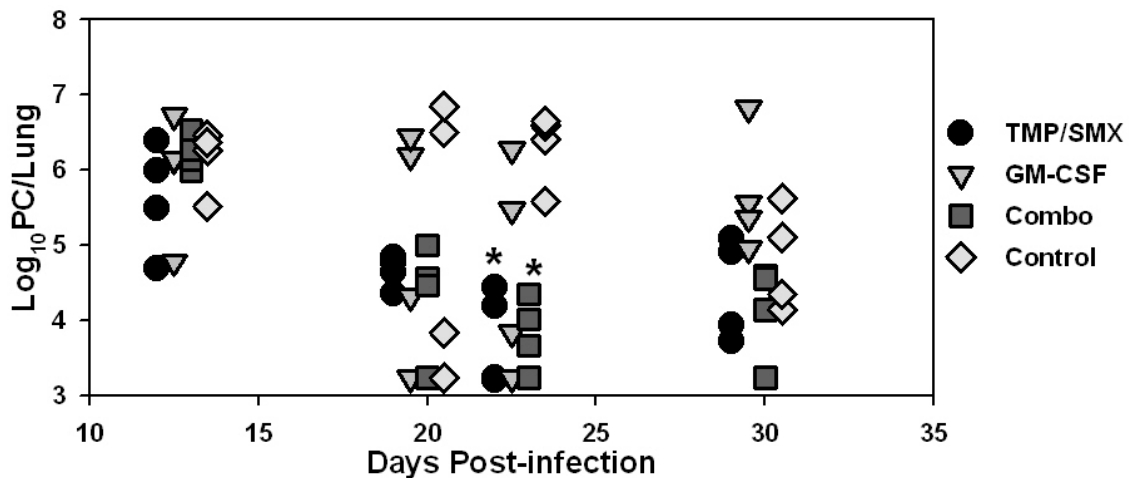


Figure 5.22 TMP/SMX and the combo groups significantly reduced PC burden in pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l normal saline (NS) daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs were collected on days 12, 19, 22, and 29 post-infection, processed into single-cell suspensions. Aliquots were spun onto glass slides, stained with Diff-Quik[®], and enumerated microscopically. Data represent four to five mice per group and are representative of 3 separate experiments. * $p < 0.05$ mean \pm SD compared to the control group at the indicated time point.

2. rmGM-CSF levels in the lung

To determine if the rmGM-CSF sufficiently made it to the lungs via the i.n. route of administration, an ELISA was performed on the BALF collected after each timepoint. Since the rmGM-CSF was exogenously administered for 7 days beginning 7 days after the pups were infected with PC, the last day of rmGM-CSF dosing was day 14 post-infection. Thus, the results observed in the GM-CSF ELISA were somewhat predictable in that there was a significantly elevated level of GM-CSF on day 12 post-infection in the rmGM-CSF-treatment group which was not observed at the later time points (Figure 5.23). Unexpectedly, however, there was less GM-CSF in the rmGM-CSF/TMP/SMX (combination) group than in the untreated controls. By day 19 post-infection, the exogenous administration of rmGM-CSF had ceased and no groups were producing significant levels of the cytokine endogenously. On days 22 and 29 post-infection the TMP/SMX and rmGM-CSF single-treatment groups were producing elevated levels of GM-CSF compared to the control group, but not enough to reach statistical significance (Figure 5.23).

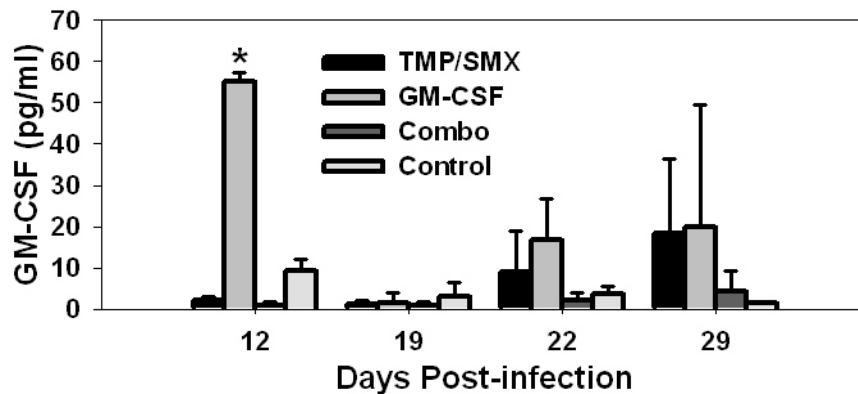


Figure 5.23 GM-CSF levels were significantly elevated in GM-CSF-treated pup lungs. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Lungs were lavaged on days 12, 19, 22, and 29 post-infection and GM-CSF levels were determined by ELISA. * p <0.05 compared to all other groups at day 12 post-infection. Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments.

3. Lung macrophage activation markers reflect rmGM-CSF treatment and Pneumocystis burden

Similar to previous experiments, flow cytometry was used to evaluate the level of LM and lymphocyte infiltration and activation within the lungs of mice from each of the groups outlined above. As depicted in figure 5.24, the infiltration and activation of pup LMs in the BALF were somewhat variable. A look at non-lymphocytes expressing CD11c in figure 5.24a shows us that there is a slight surge of macrophage infiltration into the alveolar spaces on day 12 post-infection in the rmGM-CSF-treated group compared to all other groups, although the differences are not statistically significant. On day 19 post-infection, all groups demonstrated similar numbers of CD11c+ cells and then interestingly, on day 22 post-infection, there was significantly lower numbers of CD11c-expressing cells in the TMP/SMX group compared to the untreated group. At day 29 post-infection the combination group had significantly fewer CD11c+ expressing cells compared to the untreated group, whereas numbers in the TMP/SMX group had rebounded back up to the level of the untreated group. It is important to consider the timeframe of rmGM-CSF and TMP/SMX treatments when analyzing this data. To relate fluctuations in the surface marker expression to the treatment period of each drug, a timeline is provided in figure 5.24b. One can see, by comparing the expression of CD11c+ cells to the timeline that the greatest numbers occurred in the rmGM-CSF-treated group 5 days into the treatment period; the subsequent drop in cells expressing CD11c occurring at day 19 post-infection may reflect the discontinuation of the drug at day 14 post-infection. Overall, this data suggests that rmGM-CSF may have been driving the infiltration of CD11c+ cells early on and upon discontinuation of the drug, the number of CD11c+ expressing cells returned to baseline. Furthermore, in the group receiving TMP/SMX alone, the number of CD11c+ cells appeared to be driven by PC burden (Figure 5.22), which was decreased by the activity of TMP/SMX. This decreased number of CD11c-expressing cells was not seen in the combination group most likely, due to the promotion of CD11c+ cell infiltration imparted by the rmGM-CSF. Thus the

overall reduction in CD11c+ cells in the combination group by day 29 post-infection likely represents a reduction in PC burden (compare to figure 5.22)

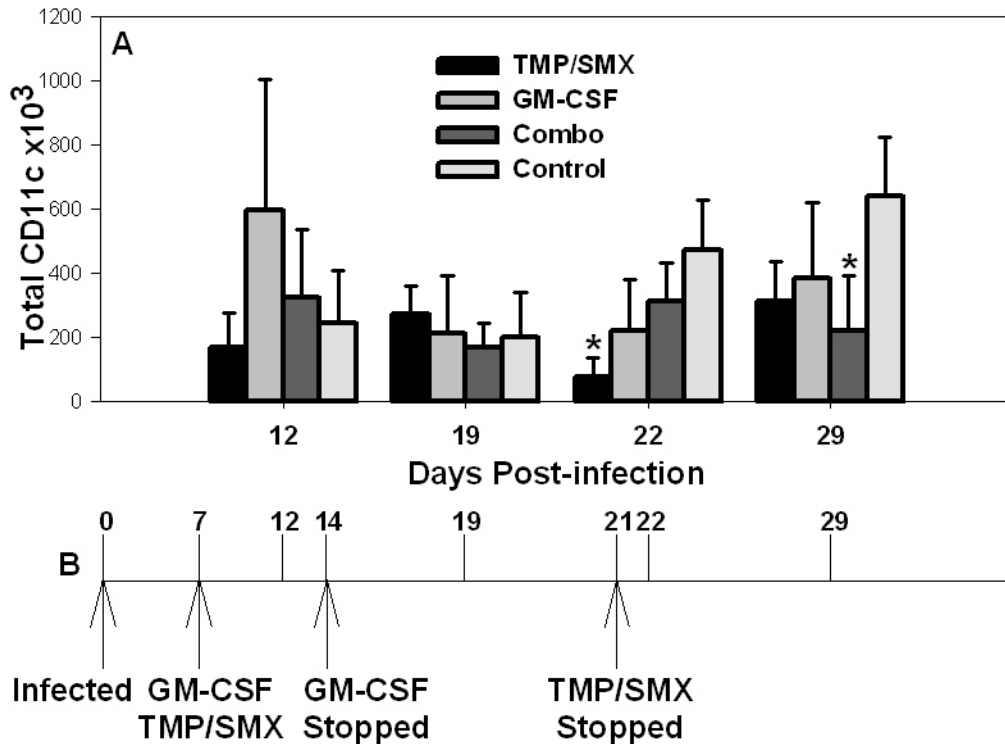


Figure 5.24 GM-CSF drove an increase in infiltrating AMs in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l normal saline (NS) daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7 days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7 days (Combo), or 10 μ l NS daily for 7 days (Control). Lungs were lavaged on days 12, 19, 22, and 29 post-infection and cells were stained with antibodies specific for CD11c and analyzed by flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter. (A) Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. * p <0.05 compared to respective control groups at the indicated time points. (B) Timeline depicts the start and discontinuation of treatments.

The other markers of macrophage activation tested showed very similar trends to the number of cells expressing CD11c. The number of cells dually expressing CD11c and CD11b appeared to correlate with PC burden (Figures 5.25a and 5.22). This is most apparent when looking at day 12 compared to day 22 at which time the number of CD11c/CD11b+ cells are significantly less in the

groups receiving TMP/SMX compared to the control group. Treatment with rmGM-CSF alone is not capable of significantly reducing PC burden. However, at day 12 post-infection the groups receiving rmGM-CSF had more cells expressing CD11c and CD11b (Figure 5.25a). Cells dually expressing CD11c and dectin-1 or MHC class II had very similar patterns. While still driven by PC burden, at day 22 post-infection the combination group had more dual positive cells compared to the TMP/SMX group despite the drop in PC burden (Figures 5.22 and 5.25b and d). Unlike the other markers described here, the cells co-expressing CD11c and mannose receptor did not appear to correlate with PC burden (Figure 5.25c). Furthermore, these cells did not reflect treatment with rmGM-CSF by increasing in number at day 12 post-infection.

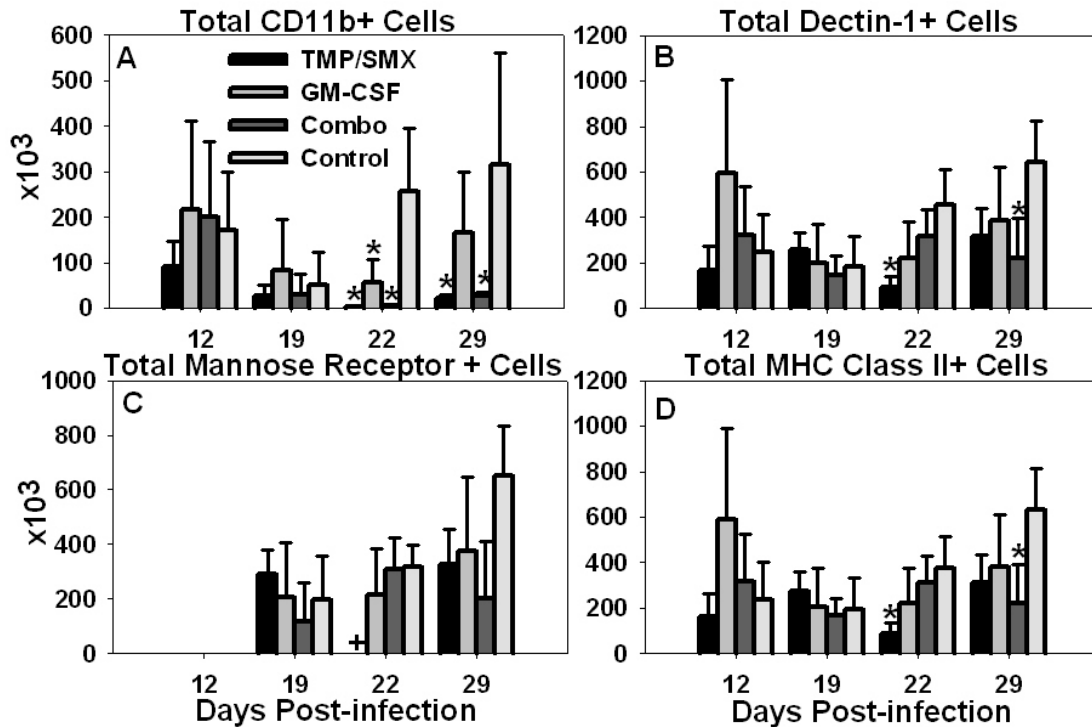


Figure 5.25 Groups receiving TMP/SMX had reduced numbers of LMs expressing activation markers. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Lungs were lavaged on days 12, 19, 22, and 29 post-infection, and cells were stained with antibodies specific for CD11c, CD11b (A), Dectin-1 (B), Mannose receptor (C), and MHC class II molecules (D) and analyzed by flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter and were gated on CD11c+ cells. Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. *Total number of cells expressing the marker indicated is significantly greater than the control group at the indicated time point, $p < 0.05$. +Total number of cells expressing the marker indicated is significantly greater than all other groups at the indicated time point, $p < 0.05$.

The same markers were analyzed in the lung digest in order to capture both resident macrophages as well as cells that were actively translocating into the alveolar spaces. In this case, a similar pattern was observed among all the receptors, including mannose receptor (Figures 5.26 and 5.27). Early on, cell activation markers were driven by treatment with rmGM-CSF. Upon discontinuation of rmGM-CSF, these markers began to more closely correlate

with PC burden with TMP/SMX groups having fewer cells expressing CD11c, CD11b, dectin-1, MHC class II, or mannose receptor. By day 29 post-infection, a significant rebound in cells expressing these markers occurs, likely due to the discontinuation of TMP/SMX on day 21 post-infection. Together, these data suggest that rmGM-CSF is capable of driving an increase in LM activation; however it did not demonstrate an improvement in PC burden beyond that produced by TMP/SMX alone. By looking at day 29 post-infection, one can see that the PC burden in the combo group remained relatively low compared to the TMP/SMX group; this is reflected in the lower number of cells expressing activation markers in both the BALF and the lung digest (Figures 5.22-5.27).

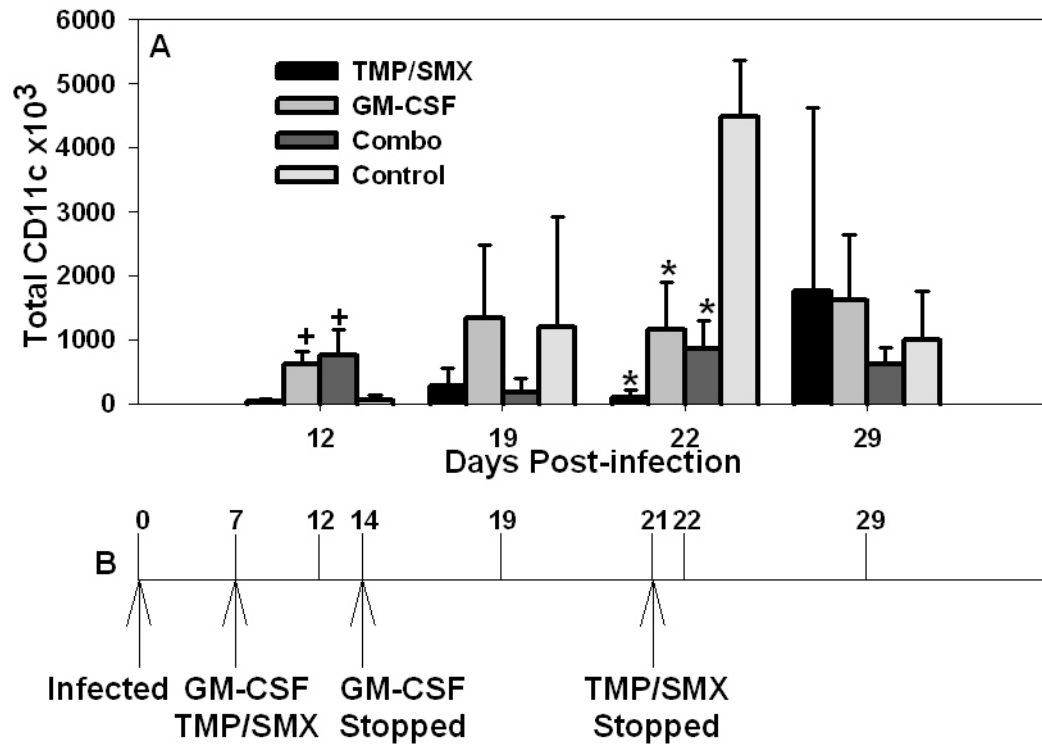


Figure 5.26 GM-CSF drove an increase and TMP/SMX drove a decrease in infiltrating AMs in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs were collected on days 12, 19, 22, and 29 post-infection and processed into a single-cell suspension. Cells were stained with antibodies specific for CD11c and analyzed by flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter. (A) Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. ⁺ p <0.05 compared to TMP/SMX and control groups; ^{*} p <0.05 compared to control group. (B) Timeline depicts the start and discontinuation of treatments.

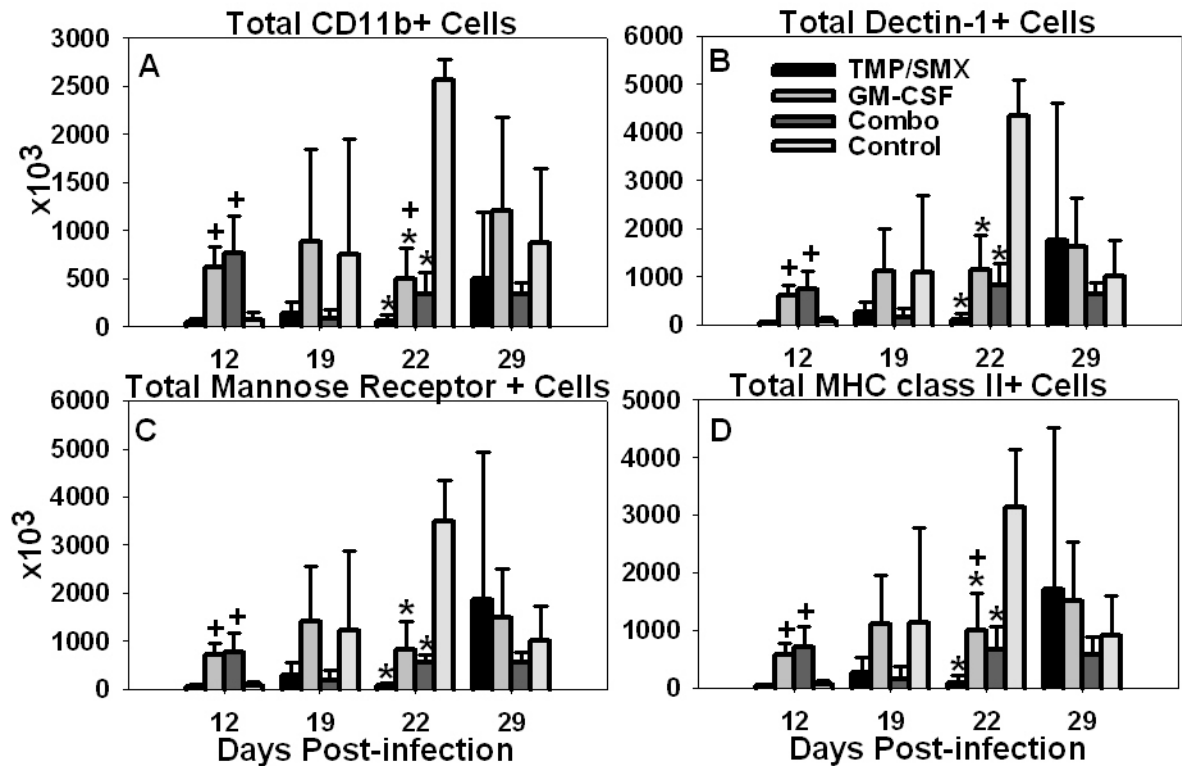


Figure 5.27 GM-CSF drove an increase and TMP/SMX drove a decrease in infiltrating and activated AMs in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7 days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7 days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs were collected on days 12, 19, 22, and 29 post-infection and processed into a single-cell suspension. Cells were stained with antibodies specific for CD11c, CD11b (A), Dectin-1 (B), Mannose receptor (C), and MHC class II molecules (D) and analyzed by flow cytometry. Cells were gated for large non-lymphoid cells by using forward and side light scatter and were gated on CD11c+ cells. Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. + p <0.05 compared to TMP/SMX and control groups; * p <0.05 compared to control group.

4. rmGM-CSF increases cytokine production in mouse lungs

Another method by which the activation level of LMs can be assessed is through their production of cytokines; therefore, levels within the BALF were analyzed for each of the four groups at each timepoint tested. Treatment with rmGM-CSF \pm TMP/SMX appeared to stimulate the production of TNF α and IL-

12p70 as well as MCP-1 early on but did not stimulate IL-6 production (Figure 5.28a-d). rmGM-CSF alone was important early in the experiment (days 12 and 19) for driving the production of $\text{TNF}\alpha$ and MCP-1. These two cytokines may have contributed to the increase in activated resident macrophages in the lung digest observed at day 22 post-infection and the subsequent decrease in PC burden during this same time (Figures 5.22, 5.26-27).

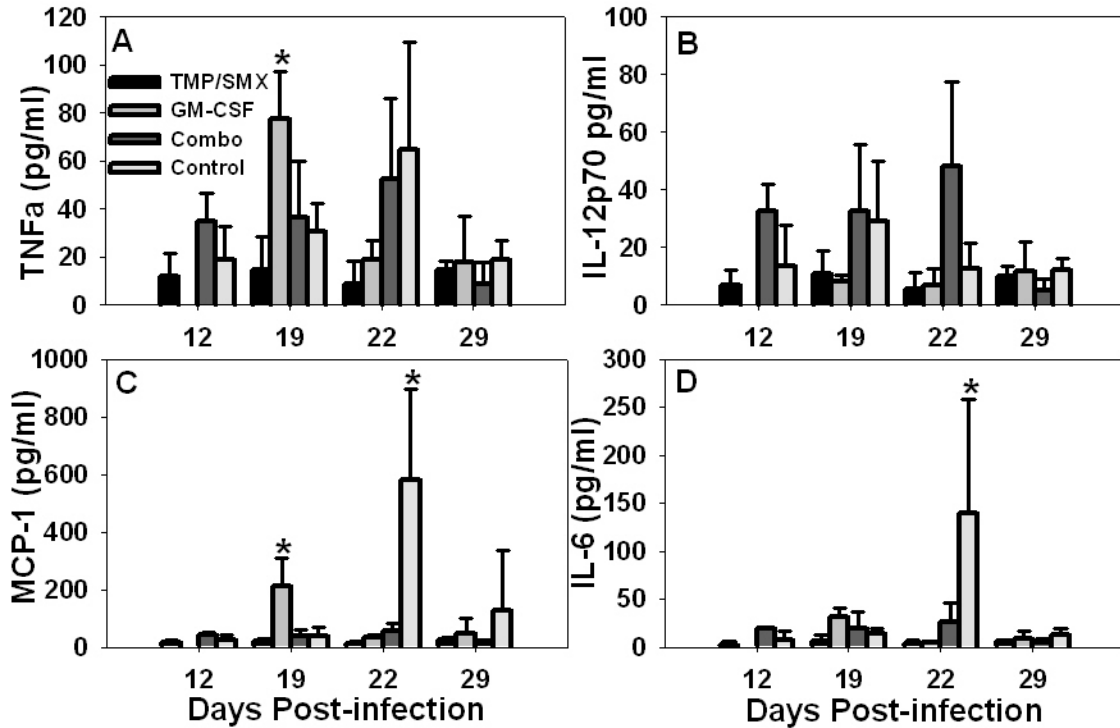


Figure 5.28 GM-CSF drove early $\text{TNF}\alpha$ and MCP-1 production in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 7 $\mu\text{l/g}$ TMP/SMX orally twice daily for 14 days + 10 μl NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 7 $\mu\text{l/g}$ TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μl NS daily for 7 days (Control). Lungs were lavaged on days 12, 19, 22, and 29 post-infection and analyzed for cytokine production using a BD CBA[®] kit specific for (A) $\text{TNF}\alpha$, (B) IL-12p70, (C) MCP-1, and IL-6 (D). Data represent the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. (A) * $p < 0.05$ compared to TMP/SMX and control groups; (C-D) * $p < 0.05$ compared to all other groups at the indicated time points.

5. Lymphocyte infiltration was driven by rmGM-CSF and Pneumocystis burden

In addition to looking at the LM response secondary to rmGM-CSF ± TMP/SMX, the infiltration and activation of T lymphocytes into the lungs and lymph nodes was also analyzed. The influx of activated cells by day 12 post-infection likely reflects rmGM-CSF treatment, whereas the decrease in these cells by day 19 most likely reflects the discontinuation of rmGM-CSF along with the decrease in PC burden in the mice receiving TMP/SMX (Figure 5.22 and 5.29a and c). Once again, at day 29 post-infection there is a considerable rebound in the number of activated lymphocytes infiltrating into the lungs in the TMP/SMX group alone compared to the combo group (Figure 5.29a and c). The presence of activated T cells in the lymph node reflect the ability of TMP/SMX to reduce PC burden as the groups receiving TMP/SMX required less overall T cell activation (Figure 5.29b and d).

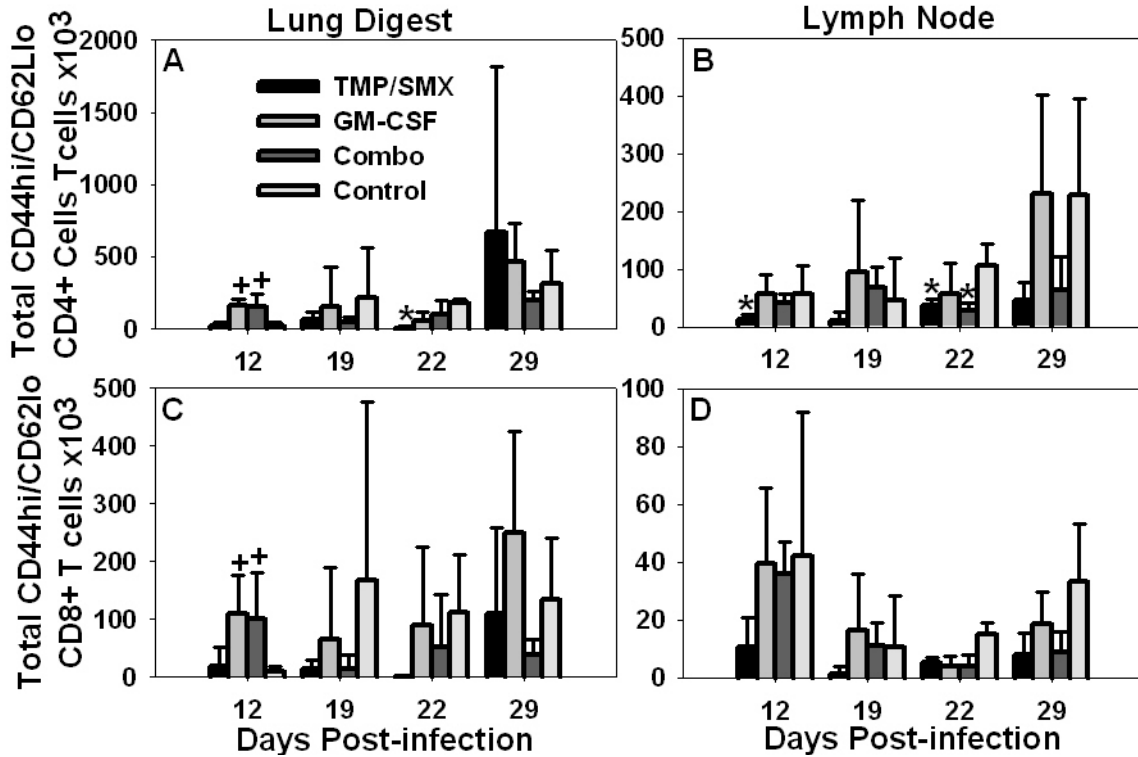


Figure 5.29 GM-CSF drove an increase and TMP/SMX drove a decrease in infiltrating AMs in PC-infected pup mice. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 40 mg/kg TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 5ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 40 mg/kg TMP/SMX orally twice daily for 14 days and 5ng/g i.n. rmGM-CSF daily for 7 days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs and TBLNs were collected on days 12, 19, 22, and 29 post-infection and processed into single-cell suspensions. Cells were stained with antibodies specific for CD4 (A-B), CD8 (C-D), CD44, and CD62L and analyzed by flow cytometry. Cells were gated for small non-granular cells by using forward and side light scatter. Data represent of the mean \pm SD of four to five mice per group and are representative of 3 separate experiments. + p <0.05 compared to TMP/SMX and control groups; * p <0.05 compared to control group.

6. High dose rmGM-CSF reduced Pneumocystis burden in the combination group

Based on the initial TMP/SMX + rmGM-CSF experiment, a final rmGM-CSF experiment was designed to evaluate the role of rmGM-CSF at a higher dose. The experimental design was the same as described above except that the rmGM-CSF dose was 50ng/g body weight instead of 5ng/g. Two time points were analyzed at days 17 and 22 post-infection. As in the previous experiment, PC burden was reduced in the TMP/SMX and rmGM-CSF plus TMP/SMX groups. The use of a higher dose of rmGM-CSF, however, reduced PC burden significantly compared to the TMP/SMX group alone at day 22 post-infection (Figure 5.30)

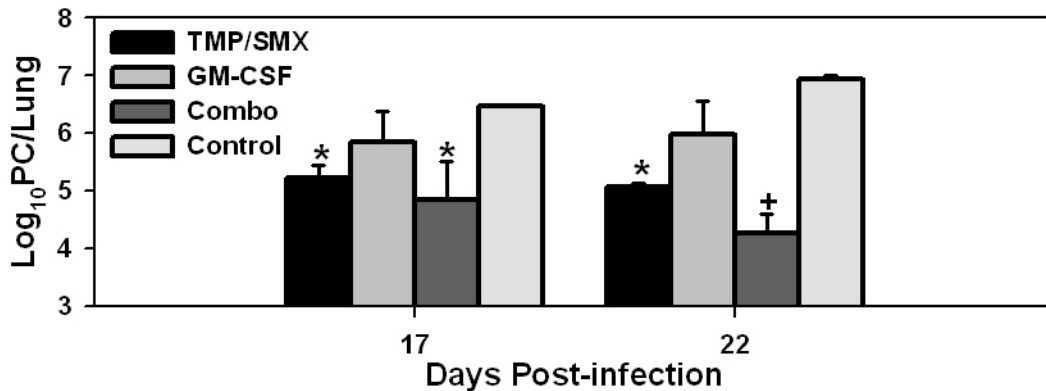


Figure 5.30 PC burden was significantly reduced in the combination group compared to TMP/SMX alone. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 7µl/g TMP/SMX orally twice daily for 14 days + 10 µl NS daily for 7 days (TMP/SMX), 50ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 7 µl/g TMP/SMX orally twice daily for 14 days and 50ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 µl NS daily for 7 days (Control). Whole lungs were collected on days 17 and 22 post-infection and were processed into single-cell suspensions. Aliquots were spun onto glass slides, stained with Diff-Quick[®], and enumerated microscopically. **p*<0.05 compared to control groups at the indicated time points. †*p*<0.05 compared to TMP/SMX and control groups at the indicated time point. Data represent the mean ± SD of four to five mice per group.

7. Lung macrophage activation markers with high dose rmGM-CSF

The same macrophage activation markers were evaluated as previously described, except that instead of looking at mannose receptor, TLR2 and TLR4 were included instead. TLR2 has recently been shown to be a receptor for PC antigens and TLR4 stimulation, although not previously shown to interact with PC, is a known inducer of $\text{TNF}\alpha$ (Figure 5.31) (44, 246-249). The increased dose of rmGM-CSF did not alter the number of cells expressing CD11c, CD11b, dectin-1, or MHC class II compared to the lower treatment dose (data not shown). The number of cells expressing TLR2 in the lung digest at day 22 resembles the pattern seen with LM activation markers during the initial rmGM-CSF/TMP/SMX experiment in which the cells followed the burden of PC (Figure 5.31d). No additional trends in cells expressing TLR2 or TLR4 appeared to correspond with mGM-CSF treatment or PC burden (Figure 5.31 a-c).

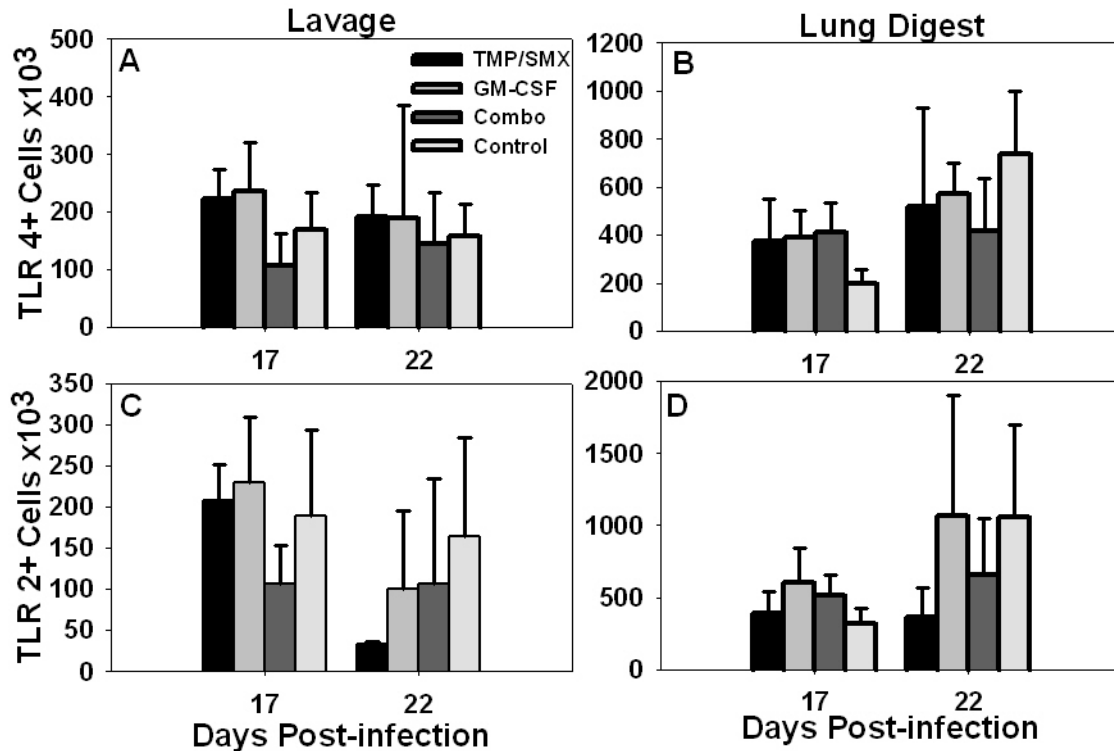


Figure 5.31 TMP/SMX drove a reduction in the number of cells expressing TLR2. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 7 μ l/g TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 50ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 7 μ l/g TMP/SMX orally twice daily for 14 days and 50ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs and lung lavaged was collected on days 17 and 22 post-infection and were processed into single-cell suspensions. Cells were stained with antibodies specific for CD11c, TLR4 (A-B), and TLR2 (C-D). Cells were gated for large non-lymphocytes using forward and side scatter and were gated on CD11c+ cells. Data represent the mean \pm SD of four to five mice per group.

The infiltration of activated CD4+ and CD8+ T cells in the lung digest appeared to be primarily driven by rmGM-CSF treatment with cell numbers being greatest at day 17 post-infection in the groups receiving rmGM-CSF (Figure 5.32 a-b). The levels of TNF α , IL-12p70, MCP-1, and IL-6 produced in the lung subsequent treatment with 50ng/g of rmGM-CSF were comparable to those produced following the lower dose of rmGM-CSF (data not shown). Taken together, these data suggest that the combination of TMP/SMX plus rmGM-CSF is more efficient at reducing PC burden and keeping the burden low compared to

TMP/SMX alone in pup PC-infected pup mice. The addition of rmGM-CSF to the treatment of this opportunistic infection appears to cooperate with TMP/SMX by recruiting and activating macrophages within the lung.

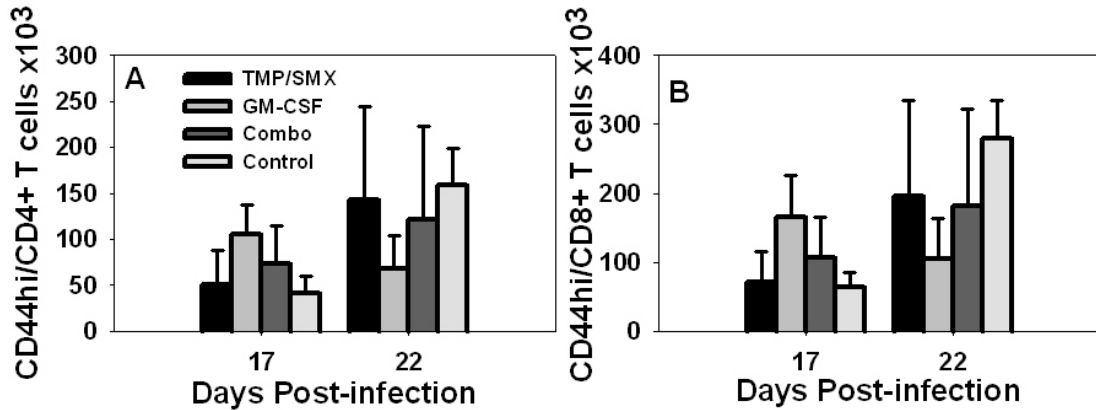


Figure 5.32 GM-CSF drove an early increase and late reduction in T cell infiltration into PC-infected pup lungs. Mice were infected with PC as neonates (24 to 72 h after birth). Beginning on day 7 post-infection, mice were treated with either 7 μ l/g TMP/SMX orally twice daily for 14 days + 10 μ l NS daily for 7 days (TMP/SMX), 50ng/g i.n. rmGM-CSF daily for 7days (GM-CSF), 7 μ l/g TMP/SMX orally twice daily for 14 days and 50ng/g i.n. rmGM-CSF daily for 7days (Combo), or 10 μ l NS daily for 7 days (Control). Whole lungs were collected on days 17 and 22 post-infection and were processed into single-cell suspensions. Cells were stained with antibodies specific for CD44, CD4 (A), and CD8 (B). Cells were gated for small non-granulocytes using forward and side scatter. Data represent the mean \pm SD of four to five mice per group.

CHAPTER 6: Human neonatal lung macrophages have a poor activation profile compared to adults

Infants, predominantly those that are immunocompromised, appear to be particularly susceptible to primary PC infection. It is believed that the higher incidence of PC as well as other pulmonary infections among infants is likely due to an immature immune system as the neonatal lung environment is deficient immunologically in preterm as well as term infants (7, 8). Decreased phagocytic capacity of monocytes and macrophages in newborns may contribute to this increased risk of infection from inhaled pathogens (7, 8). To evaluate the response of human neonatal versus adult macrophages against exogenous stimuli and extend the findings derived from our animal studies, a series of experiments were performed using human-derived macrophages.

1. LPS stimulates less cytokine production in neonatal compared to adult lung macrophages

Our studies in mouse models have taught us that pup LMs are less efficient than adult LMs in mounting a response against PC, as well as other stimuli such as LPS. To determine if human LMs respond similarly to exogenous stimuli, *in vitro* experiments were performed to evaluate the ability of neonatal LMs to produce cytokine compared to adult LMs. Peripheral blood was collected from healthy adult volunteers. The monocytes were isolated, cultured, and allowed to differentiate into macrophages. At the same time, suctioned sputum samples from intubated neonates were collected and the macrophages were isolated. The adult peripheral blood monocyte-derived macrophages and the isolated neonatal LMs were aliquoted into 96-well tissue culture plates and stimulated with LPS for 24 hours to determine if neonatal and adult cells would produce similar levels of cytokine. Cytokine production among the adult macrophages was significantly greater than that produced by neonatal LMs (Figure 6.1). The production of $\text{TNF}\alpha$, IL-10, IL-6, and IL-1 was significantly greater among adult macrophages compared to neonatal LMs following 24 hours

of LPS stimulation (Figure 6.1a-d). Interestingly, IL-8 production was equivalent between adult and neonatal cells (Figure 6.1e). This data suggests that neonatal cells do not produce cytokine as efficiently as adult cells despite exposure to a strong stimulus such as LPS. The discordance between IL-8 production and the other cytokines analyzed may indicate that alternate receptors are present which recognizing LPS on neonatal LMs resulting in adequate IL-8 production while other cytokines are reduced compared to adult cells. It is possible that the neonatal LPS receptors which ultimately lead to the production of $\text{TNF}\alpha$, IL-10, IL-6, and IL-1 are deficient in either their expression or function, whereas the LPS receptor responsible for IL-8 production is fully expressed and functional compared to that of an adult.

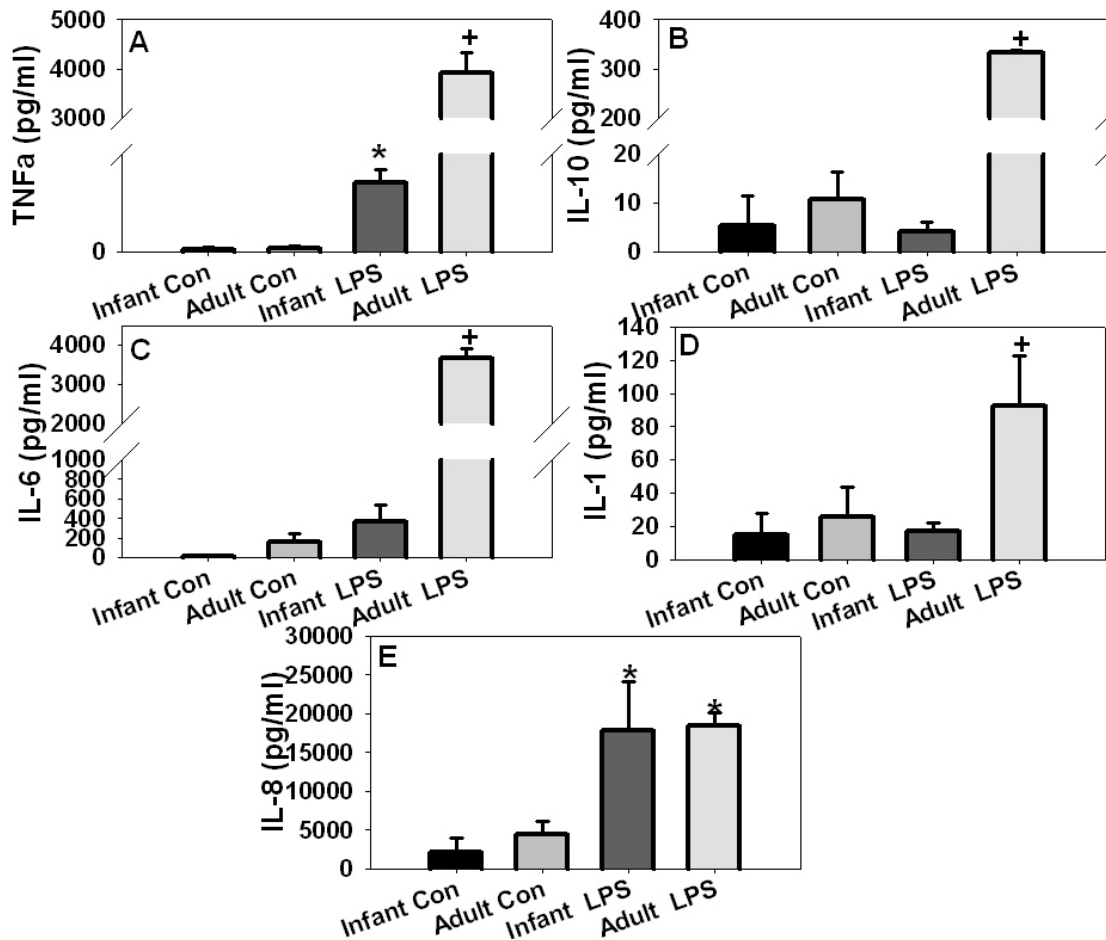


Figure 6.1 Human adult macrophages produced more cytokine than neonatal LMs stimulated with LPS. Adult peripheral blood monocyte-derived macrophages and neonatal LMs were isolated and cultured in a 96-well tissue culture plate at 2×10^5 cells per well in 200 μ l of human-specific culture media. After 24 hours, media was replaced with fresh media containing 100ng/ml of LPS (LPS) or media alone (Con). BD CBA[®] kit was used to determine cytokine concentrations after 24 hours of stimulation. * $p < 0.05$ compared to respective neonate or adult control groups. + $p < 0.05$ compared to neonatal LPS and adult control groups. Data represent the mean \pm SD of three to four wells per group and are representative of 3 separate experiments.

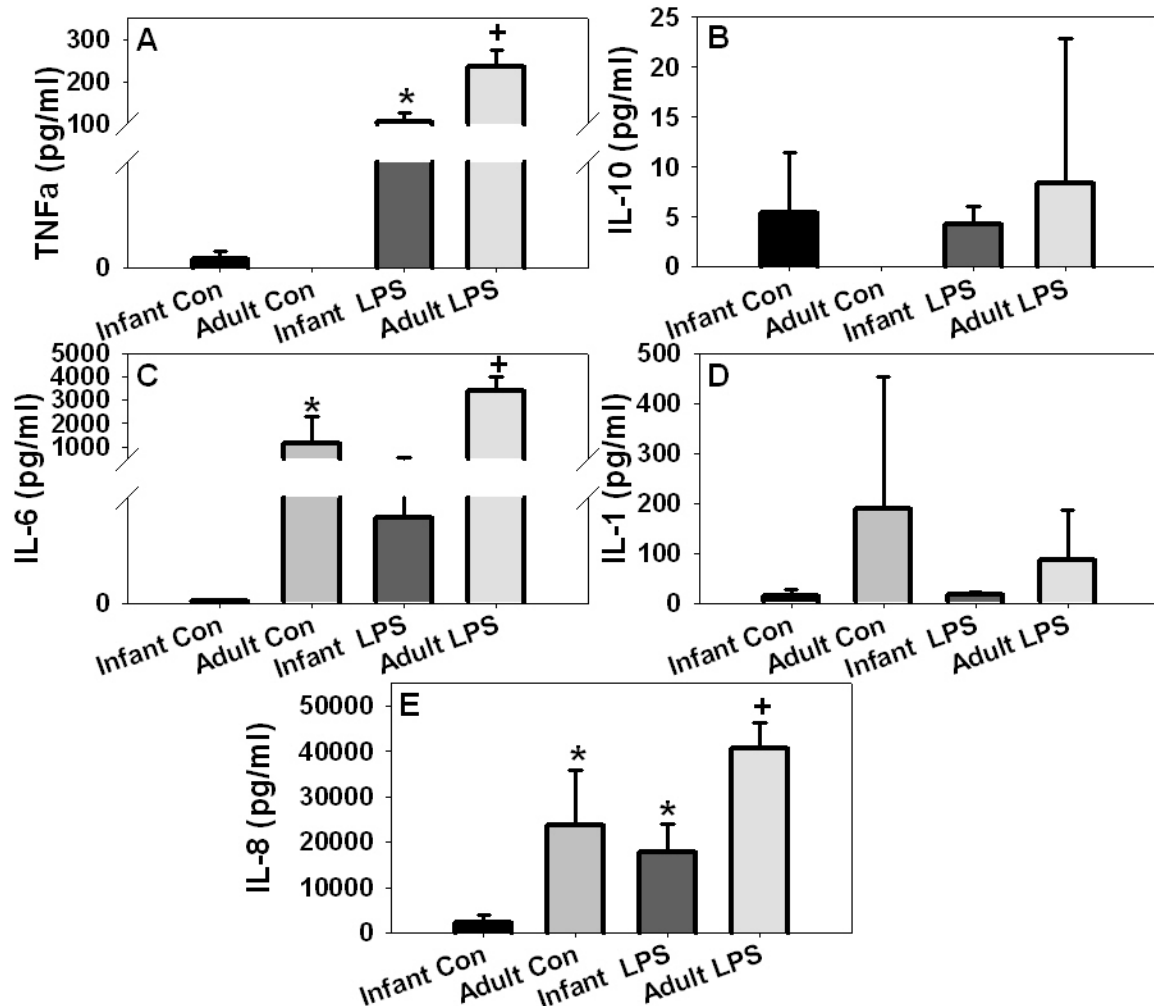


Figure 6.2 Human adult LMs produced more cytokine than neonatal LMs stimulated with LPS. Adult and neonatal LMs were isolated and cultured in a 96-well tissue culture plate at 2×10^5 cells per well in 200 μ l of human-specific culture media. After 24 hours, media was replaced with fresh media containing 100ng/ml of LPS (LPS) or media alone (Con). BD CBA[®] kit was used to determine cytokine concentrations after 24 hours of stimulation. * $p < 0.05$ compared to respective neonate or adult control groups. + $p < 0.05$ compared to neonatal LPS and adult control groups. Data represent the mean \pm SD of three to four wells per group and are representative of 3 separate experiments.

The expression of surface molecules found on macrophages can vary depending on the tissues in which they reside. It is possible; therefore, that cytokine production may also vary among tissue-specific macrophages. Thus, this experiment was repeated using adult macrophages isolated from bronchiolar lavage samples and neonatal suctioned sputum samples. As in the previous

experiment, the production of $\text{TNF}\alpha$ and IL-6 were significantly greater among the adult cells stimulated with LPS compared to the neonatal samples (Figure 6.2a and c). While IL-1 and IL-10 maintained similar patterns, there were no significant differences between the two groups (Figure 6.2b and d). The production of IL-8 was significantly greater in adult compared to the neonatal LMs in both the stimulated and unstimulated cells, demonstrating that adult LMs constitutively produce IL-8 in the absence of exogenous stimuli (Figure 6.2e). Furthermore, this data suggests that PRRs on human adult LMs vary compared to adult peripheral blood monocyte-derived macrophages. Overall, these data show that human neonatal lung macrophages are less responsive to LPS than adult lung macrophages. This finding may indicate a deficient expression of the LPS receptor, TLR4, or either of its two co-molecules, CD14 or MD-2. Alternatively, human neonatal LMs may be deficient in their intracellular signaling pathway downstream from TLR4.

2. Neonatal lung macrophages respond similarly to adult lung macrophages when stimulated with Zymosan

To determine if human neonatal LMs were capable of responding to an antigenic stimuli that targeted PRRs different than those targeted by LPS, zymosan was used. Zymosan is known to bind to dectin-1, mannose receptor, and TLR2, receptors which are also thought to ligate PC antigenic components. These experiments were performed using macrophages from both human neonatal and adult sputum specimens. Culture supernatants were collected after 12 and 24 hours of stimulation with zymosan or media alone. At both 12 and 24 hours post-stimulation, $\text{TNF}\alpha$, IL-6, and IL-10 production was significantly increased among neonatal and adult LMs compared to their control cells (Figure 6.3a-f). The levels of cytokine produced by neonatal versus adult LMs varied depending on the cytokine. $\text{TNF}\alpha$ and IL-8 production was greater among adult LMs (Figure 6.3a-b, g-h), however IL-6 and IL-10 was greater among neonatal LMs (Figure 6.3c-f). No differences in IL-1 production were observed between groups (data not shown). The higher IL-10 levels seen in neonates compared to

adults following zymosan stimulation coincide with what we saw in mice and may be part of a protective mechanism specific for developing neonatal lung macrophages. This zymosan data is in contrast to stimulation with LPS in which adult LMs produced significantly greater levels of all cytokines tested (Figure 6.2). The variable response to zymosan suggests that neonatal LMs are more responsive to stimuli that targets both dectin-1 and TLR2.

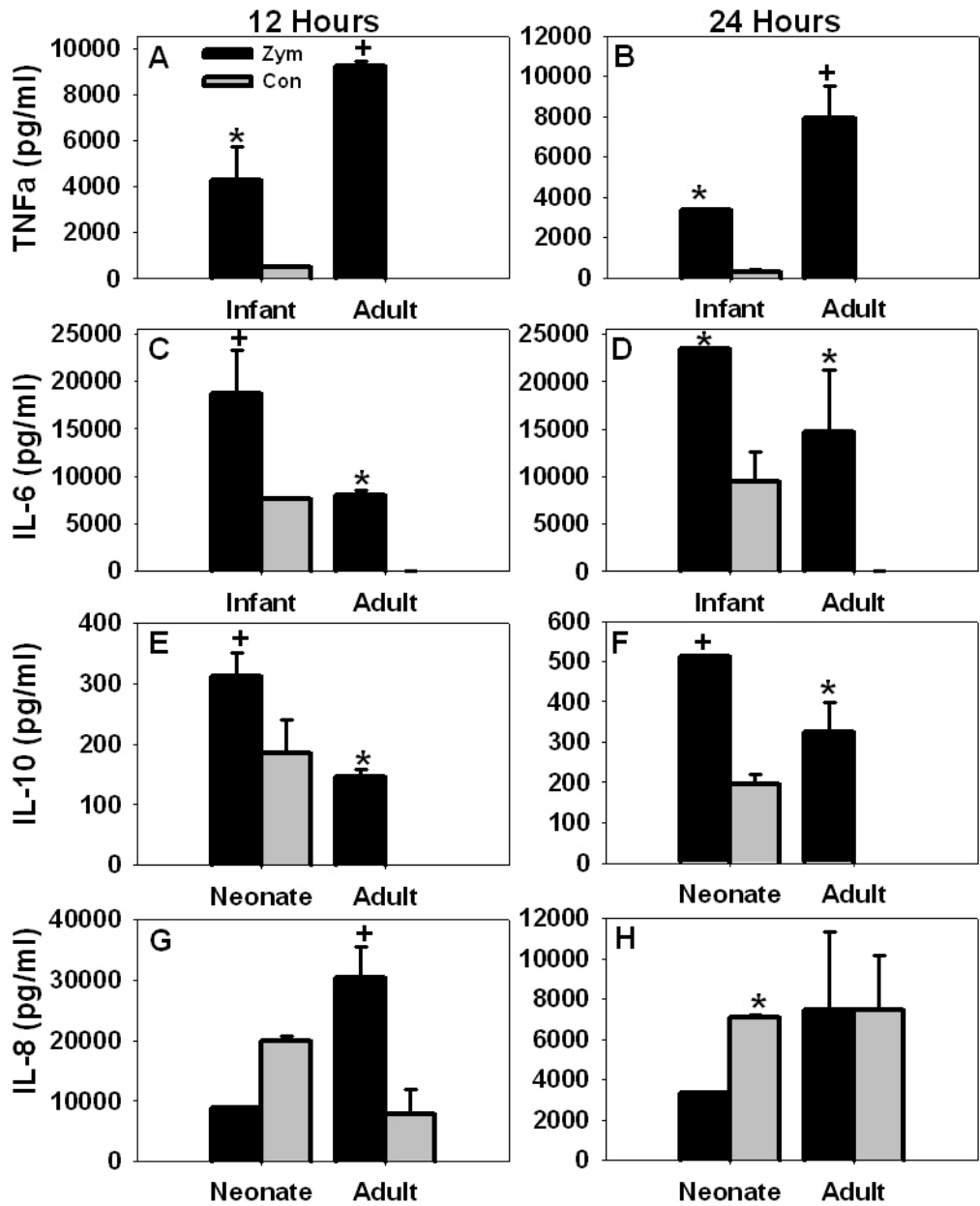


Figure 6.3 Zymosan stimulated comparable levels of cytokine production in neonatal LMs compared to adult LMs. Adult and neonatal LMs were isolated and cultured in a 96-well tissue culture plate at 2×10^5 cells per well in 200 μ l of human-specific culture media. After 24 hours, media was replaced with fresh media containing 250 μ g/ml of zymosan (zym) or media alone (Con). BD CBA[®] kit was used to determine cytokine concentrations after 12 and 24 hours of stimulation. The first column represents data collected after 12 hours of stimulation and the second column represents data collected after 24 hours of stimulation. * $p < 0.05$ compared to respective neonate or adult control groups. + $p < 0.05$ compared to neonatal LPS and adult control groups. Data represent the mean \pm SD of three to four wells per group and one to two neonate or adult pooled samples per well and are representative of 3 separate experiments.

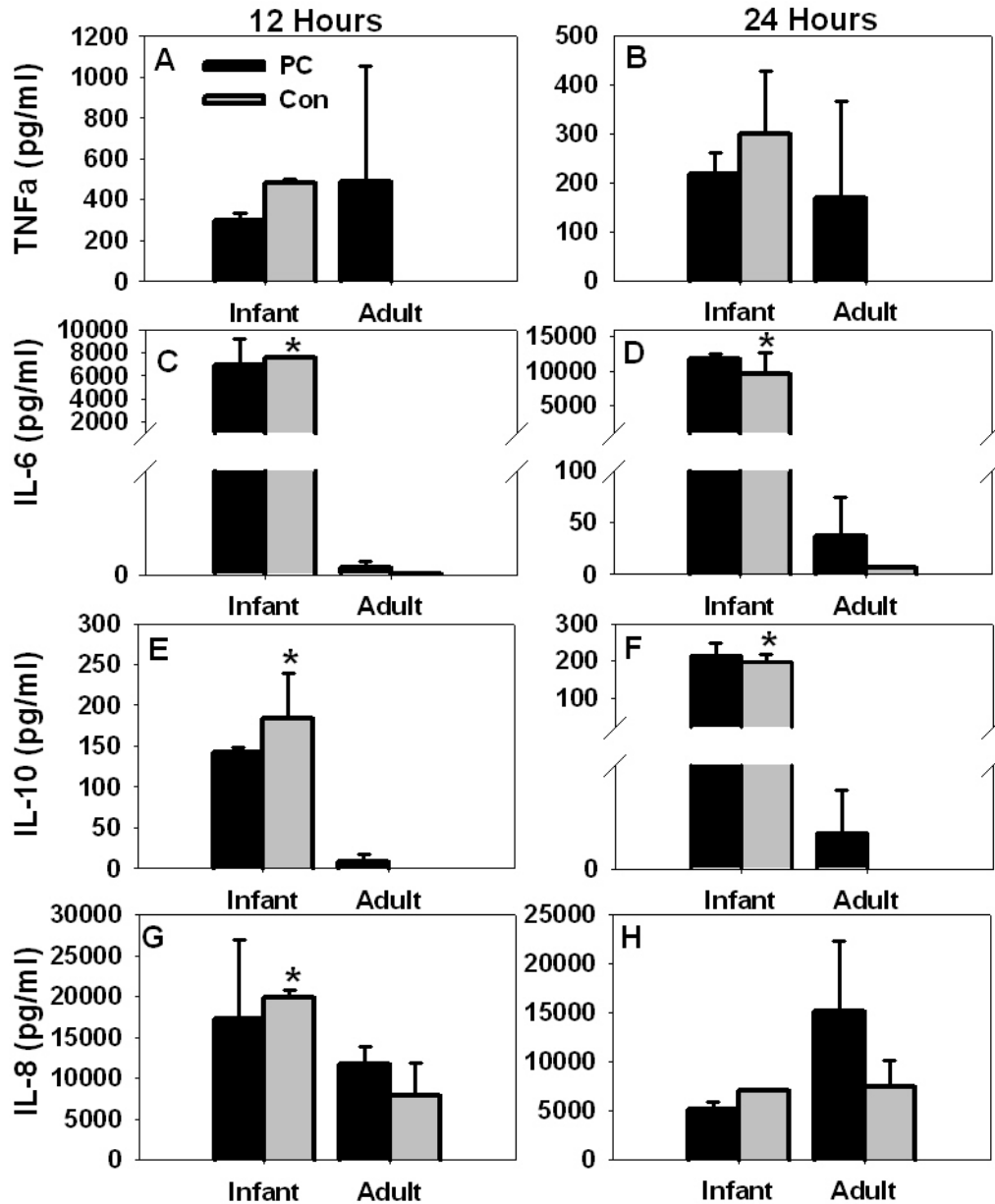


Figure 6.4 PC did not stimulate cytokine production in neonatal LMs. Adult and neonatal LMs were isolated and cultured in a 96-well tissue culture plate at 2×10^5 cells per well in 200 μ l of human-specific culture media. After 24 hours, media was replaced with fresh media containing PC at a concentration of 50 PC organisms to every 1 macrophage (1:50) or media alone (Con). BD CBA[®] kit was used to determine cytokine concentrations after 12 and 24 hours of stimulation. The first column represents data collected after 12 hours of stimulation and the second column represents data collected after 24 hours of stimulation. * $p < 0.05$ compared to respective PC-stimulated adult LMs. Data represent the mean \pm SD of three to four wells per group and one to two neonate or adult pooled samples per well and are representative of 3 separate experiments.

3. Pneumocystis did not stimulate neonatal lung macrophage cytokine production above background levels

We have demonstrated that human neonatal LMs respond poorly to LPS compared to adult LMs, but respond similarly to zymosan. Since PC contains some of the same antigen motifs as zymosan, namely beta-glucan and mannose, we next stimulated with PC to determine if cytokine production was similar to that seen with zymosan. Overall, cytokine production from neonatal LMs surpassed that of adult LMs in both the PC-stimulated and control cells (Figure 6.4a-h). However, PC did not provide a strong enough stimulus for the neonatal LMs to overcome their own background levels of cytokine production. Adult cells stimulated with exogenous PC responded with increases in TNF α , IL-6, IL-10, and IL-8 (Figure 6.4a-h) production relative to their control cells; however these differences did not reach statistical significance. The high background level of cytokines produced by the neonatal LMs may be due to the mechanical ventilation that these neonates were receiving at the time the lavage samples were retrieved and make this data somewhat difficult to interpret. Overall, however, these data demonstrate that human adult cells are capable of stimulation with PC, but neonatal cells are not.

4. rhGM-CSF did not increase cytokine production in Pneumocystis-stimulated neonatal lung macrophages

GM-CSF has been shown to enhance the clearance and improve outcomes of many different pulmonary infections (24), (25), (26, 27), (28), including PC (29), presumably through its ability to activate macrophages for enhanced activity against invading pathogens (23) (24). Based on this premise, we hypothesized that the addition of rhGM-CSF to PC-stimulated neonatal LMs would enhance the cellular response and increase cytokine production. To test this hypothesis, we repeated the experiments described above comparing human neonatal and adult LMs with the addition of rhGM-CSF to PC-stimulated cells. Overall, the addition of rhGM-CSF did not increase the level of cytokine production among neonatal or adult LMs (Figure 6.5a-h). Interestingly, after 24

hours of stimulation rhGM-CSF alone, neonatal cells appeared to produce more $\text{TNF}\alpha$ than PC alone or the combination of rhGM-CSF plus PC suggesting that PC may cause some cytokine inhibition in neonatal cells (Figure 6.5b). Conversely, the combination of rhGM-CSF plus PC in adult cells induced slightly more $\text{TNF}\alpha$ compared to PC alone. Overall, these data suggest that the addition of rhGM-CSF to PC does not stimulate an increase in cytokine production among human neonatal LMs compared to PC alone.

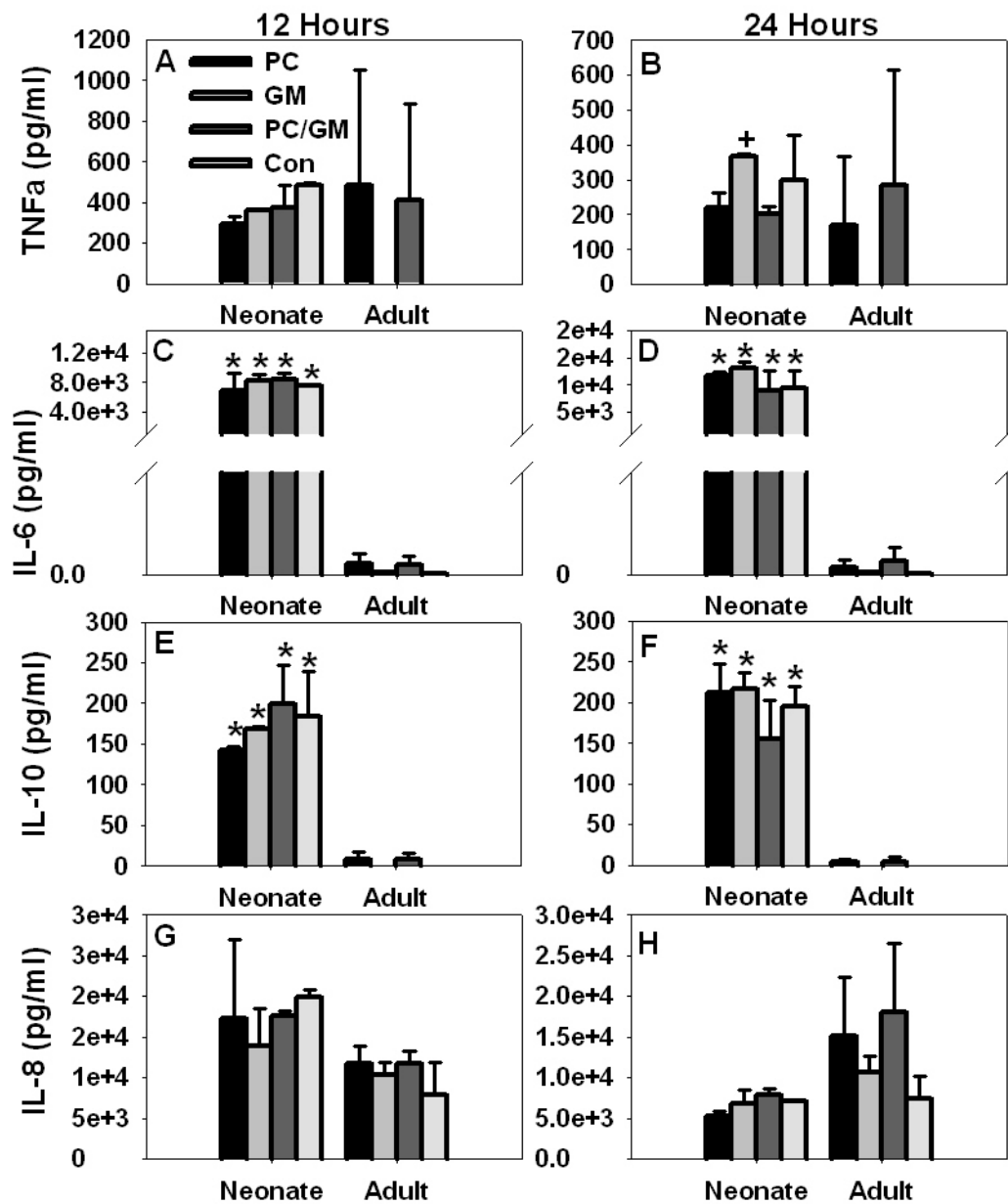


Figure 6.5 rhGM-CSF increase TNF α production in neonatal LMs after 24 hours. Adult and neonatal LMs were isolated and cultured in a 96-well tissue culture plate at 2×10^5 cells per well in 200 μ l of human-specific culture media. After 24 hours, media was replaced with fresh media containing 1:50 of PC (PC), 100ng/ml or rhGM-CSF (GM), 1:50 of PC plus 100ng/ml of rhGM-CSF (PC/GM) or media alone (Con). BD CBA[®] kit was used to determine cytokine concentrations after 12 and 24 hours of stimulation. The first column represents data collected after 12 hours of stimulation and the second column represents data collected after 24 hours of stimulation. * $p < 0.05$ compared to respective adult LM groups. + $p < 0.05$ compared to neonatal PC and PC/GM and adult GM groups. Data represent the mean \pm SD of three to four wells per group and one to two neonate or adult pooled samples per well and are representative of 3 separate experiments.

5. rhGM-CSF improves neonatal lung macrophage phagocytosis of Pneumocystis

We have previously shown that rmGM-CSF reduces PC burden in pup lungs without an associated increase in LM cytokine production (Figure 5.18). We hypothesized then, that human neonatal LMs were capable of improved phagocytosis, despite the lack of cytokine production, through exogenous rhGM-CSF stimulation. Adult and neonatal LMs were co-cultured with live PC organisms at a ratio of 10 PC to every 1 LM (1:10) with or without rhGM-CSF for 25 minutes at 37°C. Following several washes, an aliquot from each group was spun onto glass slides and stained with Diff-Quick[®]. Phagocytosis was quantified using oil-immersion microscopy (1000x) by examining at least 200 cells and counting the number of internalized PC organisms in each one. A representative slide can be seen in Figure 6.6. The amount of phagocytosis was calculated according to the following formula: phagocytic index (PI) = (percent macrophages containing at least 1 organism) x (mean number of PC per positive cell)(235-237). Treatment of the neonatal LMs co-cultured with PC increased their PI to the level of adult LMs (Figure 6.7). Only a minimal increase in the PI was observed with the adult rhGM-CSF-treated LMs, likely due to the already high level of phagocytosis occurring within these cells. Overall, these data suggest that treatment of neonatal LMs with rhGM-CSF is capable of stimulating phagocytosis without a concurrent increase in cytokine production.

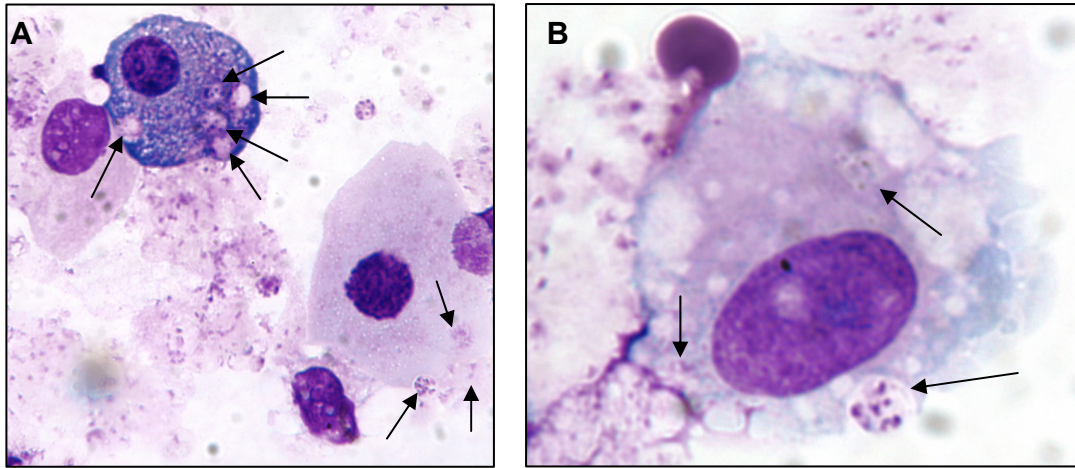


Figure 6.6 Human LMs are capable of phagocytosing murine-specific PC. Human LMs were co-cultured with *Pneumocystis carinii* f. sp. *muris* at a ratio of 1 macrophages:10 PC organisms for 25 minutes. Aliquots were spun onto glass slides and PI was determined: $PI = (\text{percent macrophages containing at least 1 organism}) \times (\text{mean number of PC per positive cell})$; 200 cells per slide were counted. Figure is representative of one slide for each human neonate (A) and adult (B) lavage sample collected; 1000x objective. Arrows indicate phagocytosed PC organisms.

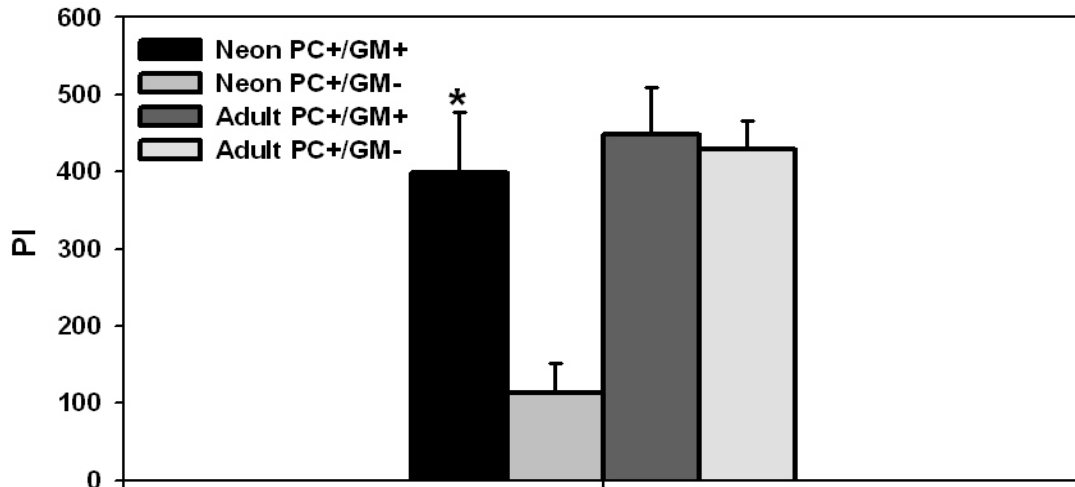


Figure 6.7 rhGM-CSF improves human neonatal LM phagocytosis of PC organisms. Adult and neonatal LMs were co-cultured with live PC organisms (1:10) with or without rhGM-CSF for 25 minutes at 37°C. Aliquots from each group were spun onto glass slides and stained with Diff-Quick®. Phagocytosis was quantified using oil-immersion microscopy (1000x) by examining at least 200 cells and counting the number of internalized PC organisms in each one. The phagocytic index (PI) was calculated according to the following formula: $PI = (\text{percent macrophages containing at least 1 organism}) \times (\text{mean number of PC per positive cell})$. * $p < 0.05$ compared to the Neon PC+/GM- group.

CHAPTER 7: Discussion

A. Results Summary

The goal of this research was to determine if stimulation of LMs from neonates with exogenous stimuli would improve activation of these cells and expedite the clearance of PC. We began our investigations using a mouse model and an *in vitro* culture system. The number of cells expressing many surface molecules known to be upregulated during infection, including CD11c, CD11b, MHC class II, CD40, CD80, CCR2, and CCR5 were all shown to be delayed in pup compared to adult LMs infected with PC. The infiltration of T lymphocytes into the lungs of PC-infected pups as well as cytokine production was also shown to be delayed compared to adults.

We have demonstrated that IFN γ alone is not sufficient to expedite clearance of PC organisms in pup mice. We proceeded to show that pup LMs are capable of responding to the exogenous stimuli, LPS and are as efficient as adult LMs at responding to zymosan. Furthermore, we demonstrated that pup mice treated with aerosolized HKEC were able to clear PC infection faster than untreated mice. This increased clearance correlated with CD4 and CD8 T cell infiltration, LM activation and LM cytokine production. Using this HKEC model we also reiterated the importance of the macrophage-T-cell interactions, but demonstrated that in the absence of T cells, pup LMs can be stimulated exogenously to improve phagocytosis.

We next explored the effect of GM-CSF on pup LMs and the clearance of PC. We employed the use of GM-CSF specifically in these experiments due to its commercial availability and because it has been proven safe and effective in the immunocompromised patient population at improving macrophage function (23, 24) as well as improving outcomes in pulmonary infections (24-28), including PC (29). *In vitro* stimulation of PC-exposed pup LMs with rmGM-CSF resulted in increased cytokine production relative to their PC-exposed, untreated cells; for TNF α , this increase was even greater than PC-exposed, rmGM-CSF-treated

adult LMs. We transitioned our GM-CSF experiments *in vivo* first with i.p administration of rmGM-CSF followed by i.n. administration at medium and high doses. Overall, treatment with rmGM-CSF resulted in increased pup LM activation and T cell infiltration as well as a slight reduction in PC burden.

Combining the increasing data supporting the use of combination therapy with the rationale of using immunomodulatory agents in infectious diseases we next explored the use of TMP/SMX together with GM-CSF for the treatment of neonatal PC infection in mice. We discovered that co-treatment with rmGM-CSF and TMP/SMX significantly reduced PC burden in pup lungs. We determined that rmGM-CSF acted early to increase LM activation, cytokine production, and T cell infiltration but that the subsequent reduction in PC burden led to a reduction in LM activation, cytokine production, and T cell infiltration. We further demonstrated that pup mice receiving combination therapy maintained a lower PC burden after therapy was stopped unlike the mice receiving TMP/SMX alone which had a rebound in PC burden following discontinuation of TMP/SMX.

Lastly, we extended our investigations to the human population by collecting macrophages from human adult and neonatal lung aspirates. We discovered that neonatal LMs were capable of exogenous stimulation with LPS but their cytokine production was not to the extent that adult LMs achieved. Stimulation with zymosan, however, a fungal cell wall preparation that binds to different PRRs than LPS, resulted in cytokine production in neonatal LMs equivalent to those in adult LMs and in some instances, even greater. Alternatively, incubation with PC stimulated adult but not neonatal LM cytokine production; this data was complicated by a high baseline cytokine production among neonatal LMs. The addition of rhGM-CSF did nothing to improve the cytokine profile in PC-infected neonatal LMs, but once again was complicated by high baseline levels of cytokine production in the neonatal cells. Treatment of neonatal LMs co-cultured with PC organisms led to an increase in the phagocytic index reaching the level of adult LMs. Overall, we learned that treatment of human neonatal LMs with rhGM-CSF improves the phagocytic capacity against PC organisms without a concomitant increase in cytokine production.

B. Lung Macrophage Activation is Delayed in Pneumocystis-infected Pup Compared to Adult Mice

1. Activation markers

The finding that adult LMs express activation markers such as CD11b, MHC class II, CD40, and CD80, approximately 2 weeks earlier than pup LMs suggests that there is also at least a 2 week delay in the function that these molecules provide. CD11b is the α -subunit of CD11b/CD18 (Mac-1) belonging to the β 2 subfamily of integrins. Functionally, Mac-1 promotes endothelium adhesion, phagocytosis, and oxidative burst (258). Its expression on macrophages is increased during states of activation making it a useful monitoring tool for assessing activation status (258, 259). Throughout this research we define alveolar and lung macrophages as being CD11c+. We further describe the activated form of these cells in pups as being CD11c+/CD11b+. CD11b is a β -glucan receptor that is capable of recognizing the β -glucan component of fungal cell walls, including PC. Its upregulation during PC infection may thus, contribute to clearance of the organism. We acknowledge that a small population of these cells is likely dendritic cells, however, we are confident that the vast majority are macrophages and the changes observed following immunomodulatory therapy represents a change in macrophage rather than dendritic cell phenotype. Tissue dendritic cells sample the alveolar spaces for antigen by protruding their dendrites into these spaces; they generally do not reside in the alveolar spaces. This is in contrast to AMs which reside in the alveolar spaces and act as a first line of defense against inhaled pathogens. Based on this information, we have assumed that CD11c+ cells derived from lung lavaged fluid are almost entirely AMs. Although dendritic cells do reside in the lung tissue, the concentration of lung macrophages far outways that of dendritic cells (260). Subsequently, it is assumed that the majority of CD11c+ cells derived from the lung tissue are macrophages. Thus, phenotypic differences observed in tissue-derived CD11c+ cells following immunomodulation represent changes in macrophages rather than dendritic cells.

Fc γ R is an important mediator of phagocytosis of IgG-coated antigens (261). Its role in PC infection has not been previously described. We have shown here that an increase in the number of LMs expressing Fc γ R is delayed approximately 2 weeks compared to adult LMs. MHC class II, CD40, and CD80 are all involved with the macrophage:T cell interaction. The delay in the expression of these molecules likely contributed to the delayed T cell activation and may further lead to a delay in initiation of the adaptive immune response. Wright and colleagues have demonstrated the importance of MCP-1 and MIP-1 in the focal targeting of inflammatory cell recruitment to sites of PC infection (262). Here we have demonstrated that there is a delay in the number of cells expressing the receptors for these chemokines (CCR2 and CCR5). Taken together, the delayed expression of these functional activation markers on LMs likely all contribute to the delayed PC clearance observed in pup mice compared to adults.

When comparing pup and adult markers of activation, it is important to consider the total number of cells as this number is used to determine the total number of cells expressing the marker of interest. In most experiments, percent change from control was used to account for large differences in total cell numbers; however, in some experiments total cell numbers were used. Figure 7.1 demonstrates the differences observed in total cell numbers derived from lavaged fluid in pups versus adults over time.

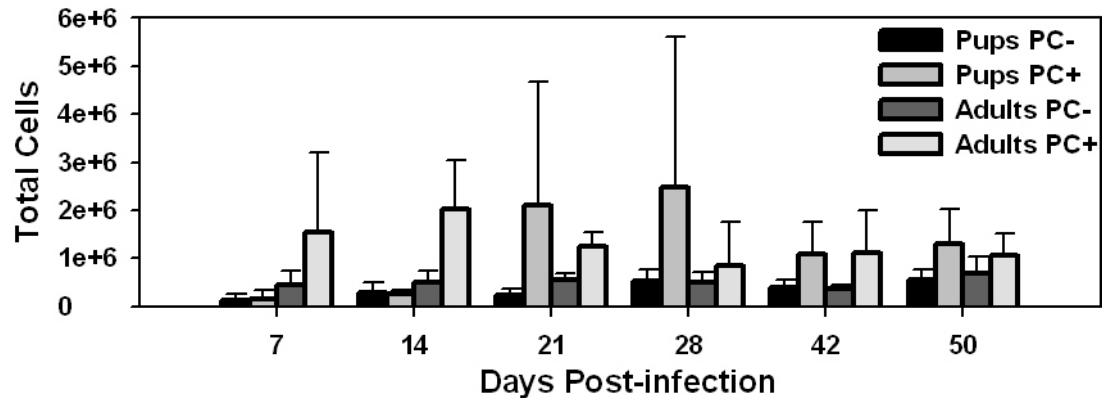


Figure 7.1 Total cells in pup versus adult Lungs. The total number of cells enumerated in the lavaged fluid taken from pups and adults trends toward being greater in PC-infected adults compared to pups early post-infection. No statistically significant differences were observed overall. Pup and adult mice were lavaged at the indicated time points and cells were counted using a hemocytometer. Data represent the means \pm SD for four to five mice per group.

2. T lymphocyte infiltration

The infiltration of CD4 and CD8 T cells into PC-infected pup lungs is delayed at least 2 weeks compared to adults. This data supports what has previously been shown in our lab (9, 10, 234). For both pups and adults, the infiltration of T cells precedes the onset of PC clearance by about 1 week. One reason for this delayed T cell infiltration could be due to a failure of neonatal lung endothelial cells to up-regulate adhesion molecule pairs allowing for lymphocyte extravasation into the lung. It has previously been shown in neonatal lungs that ICAM-1 and VCAM-1 fail to up-regulate in response to PC infection (73). Without this up-regulation, their integrin counterparts (LFA-1 and VLA-4, respectively) expressed on lymphocytes fail to bind and no extravasation occurs. Alternatively, we have also shown that there is a delay in the upregulation of pup LMs expressing MHC class II molecules following PC infection (Figure 3.5). Without the presentation of antigen in the context of these MHC class II molecules, T cells fail to recognize antigen and thus their activation would be delayed. Although macrophages are considered to be professional antigen presenting cells, their primary function is phagocytic killing and cytokine production during pathogen invasion. Dendritic cells (DCs) are the primary antigen presenting cells

in the lungs therefore; we must consider that the delayed T cell infiltration may be related to deficient antigen presentation function in neonatal DCs. Our lab has previously demonstrated that there is a delay in DCs expressing MHC class II molecules in PC-infected pup mice compared to adults (239). Thus deficient antigen presentation in both LMs and DCs likely contributes to the delayed infiltration of activated T cells into PC-infected pup lungs.

3. Cytokine production

The developing neonatal lung environment is a complex network of immune stimulating and inhibiting mechanisms. They work to protect the lung from pathogenic invasion while simultaneously trying to prevent damage caused by host inflammation. Whenever a single cell is removed from this regulatory environment, caution must be taken in interpreting the results, as they do not account for the effects of the immune environment from which they came. This is true for our *in vitro* culture stimulation experiments in which pup or adult LMs are stimulated *ex vivo*. There are also benefits to this *in vitro* culture system. We were able to identify cytokine production specific to the pup or adult LM being stimulated and did not have to worry about possible unknown antigenic stimulation as would have been the case in an *in vivo* system.

In evaluating cytokine production from pup and adult LMs, we varied time and antigenic stimuli. We determined that in culture, pup LM TNF α and IL-6 production following PC stimulation is both delayed and lower than that of adult LMs. The poor response seen in the pup cells may be multifactorial. It has been proposed that TLR2 mediates the LM response to PC in mice (44) and that TLR2 co-localizes with dectin-1 to initiate macrophages' pro-inflammatory response (263). We have shown that the expression of both TLR2 and dectin-1 are present in equal amounts on both pup and adult LMs, however it is possible that the co-localization of these molecules are impaired on pup cells following stimulation with PC organisms. The timing and extent of release for individual cytokines is guided by factors other than TLR2 and dectin-1 expression. Tachado and colleagues demonstrated that co-expression of mannose receptor (MR) and

TLR2 are required for PC-mediated IL-8 release (79). Although we have also demonstrated that MR is equally expressed on both pup and adult LMs, it is possible that other TLR signaling intermediates are involved with this delayed cytokine response observed in pup compared to adult LMs stimulated with PC. TLR2 ligand recognition involves cooperation with TLR1 or TLR6, which confer discrimination among different microbial components, specifically, triacyl and diacyl lipopeptides respectively (248, 264, 265). It is possible that there is a delayed or impaired expression of either TLR1 or TLR6 on pup LMs. In addition to potential differences in PRRs, there may also be differences in signaling intermediates or transcription factors involved in TLR-mediated cytokine activation. We have shown that NF κ B nuclear translocation was minimal, but similar in both pup and adult LMs stimulated with PC. It is possible however, that pup LMs may generate cytokine production through activation of an alternate transcription factor such as activation protein-1 (AP-1), NF-IL6, or a member of the IRF family of transcription factors (248). Further research to determine if pup LMs stimulated with PC initiate a MyD-88-independent pathway or transcription factors other than NF κ B may help explain the differential cytokine production between pup and adult LMs.

MCP-1 production was produced very early following PC stimulation in both pup and adult LMs with no difference in concentrations observed. Although TNF α , IL-6, and MCP-1 are all known gene products of the transcription factor NF κ B (266), it is unknown whether NF κ B plays a significant role in the differential production of NF κ B-dependent cytokines or in coordinating the production of these cytokines. Furthermore, it is thought that both the timing and the relative amounts of NF κ B-dependent cytokines produced following stimulation are likely controlled by the interaction of NF κ B with other transcription factors as well as NF κ B-independent factors (266). Thus, it appears that MCP-1 production is controlled by factors other than those involved in TNF α and IL-6 production in PC-stimulated mice.

C. Exogenous Immunomodulation can Improve Lung Macrophage Function and Pneumocystis Clearance in Pup Mice

1. Inteferon gamma

It has previously been demonstrated that IFN γ and TNF α are important for the clearance of PC and that the delayed clearance of PC in pup mice correspond with a delay in the up-regulation of these two cytokines (9, 10, 234). Furthermore, it has been shown that SCID mice made to specifically overexpress IFN γ in the lungs were able to stimulate clearance of the organism (243, 244). It is curious, then, why treatment of neonatal mice with i.n. IFN γ demonstrated no increased activation, cellular infiltration, or PC clearance. The most likely explanation is that the addition of just one of the cytokines, IFN γ or TNF α alone, does not increase susceptibility to PC infection, but rather both cytokines together are important and necessary for clearance of the organism (267). IFN γ acts on macrophages by sensitizing them to a second stimulatory signal. This second signal is often TNF α , thus without also increasing the amount of TNF α the microbicidal effect of IFN γ was likely lost. Other stimulatory signals, such as certain pathogenic organisms, are also able to provide this second signal. We have shown here that PC is unable to provide this second stimulatory signal and that the combination of IFN γ plus PC is insufficient for macrophage activation. This finding further demonstrates the inability of neonatal LMs to respond to PC organisms despite the added stimulus of IFN γ .

2. LPS/Heat-killed E. coli

My initial *in vitro* cytokine data demonstrated that LPS significantly increases cytokine production in pup macrophages compared to either control or PC-stimulated cells, but overall, adult cells produced more cytokines than pup cells following stimulation with LPS and PC. In general, PC appears to be a relatively weak stimulus for both pup and adult LMs. There are many variables involved in the type and level of cytokines produced following antigenic stimulation in LMs, including the offending pathogen and the lung environment

itself. By isolating AMs from pups and adults and stimulating them in culture in a controlled environment we were able to eliminate the effects of the surrounding lung environment. Differences in cytokine production between pup and adult LMs, therefore, are most likely the result of differences in receptor expression or intracellular signaling. There is growing evidence suggesting that dectin-1 (β -glucan receptor) is an important receptor for the clearance of PC (61, 65, 268). The β -glucans are important cell wall components of fungi and yeast cell walls (269), including PC (65). A recent study done in dectin-1 knock-out mice demonstrated that dectin-1 is critical for the production of reactive oxygen species (ROS) in macrophages but is not necessary for normal T cell responses (268). Furthermore, this study demonstrated that dectin-1 was required for PC clearance in immunocompromised mice. Since β -glucans are found on PC organisms (44, 65), we hypothesized that pup mice did not express dectin-1 as well as adults. We discovered, however that dectin-1 was highly expressed on both pup and adult mice, ruling out poor dectin-1 expression as the possible cause of poor PC clearance in pup mice. It is possible that although dectin-1 expression is adequate, its function or downstream signaling may be deficient resulting in a poor cytokine response following the recognition of β -glucan particules.

The toll-like family of receptors is known to be important in mediating the immune response to invading pathogens (248). Recently, TLR2 has been shown to contribute to the innate immune response to PC (44, 61). Furthermore, TLR2 has been shown to collaborate with dectin-1 in mediating the macrophage response to β -glucan (44, 61, 263), which is a major component of the PC cell wall. This collaboration has been shown to stimulate macrophages to increase the production of pro-inflammatory cytokines (60, 61). We demonstrated that pup mice express TLR2 at the same level as adult mice, suggesting that the delayed PC clearance in pup mice may be due to a delayed expression of TLR1 or TLR6 or a inefficient co-localization with TLR2. The decreased expression of TLR4 in pup versus adult mice, however, may contribute to the reduced cytokine production in pup mice stimulated with LPS. To initiate the intracellular signaling

cascade, TLR4 must first form a complex with LPS-bound CD14 and MD-2 (66, 270, 271). Therefore, a deficiency in expression of CD14 or MD-2 or impaired co-localization could also cause a reduction in cytokine production following LPS stimulation.

Recently, Sadeghi and colleagues demonstrated similar findings in human newborns compared to adults (272). They showed that TLR4 expression and cytokine secretion following LPS stimulation increases with gestational age, but TLR2 levels did not differ between age groups. Alternatively, they found that MyD88 levels were lower and p38 and ERK1/2 was impaired in newborns compared to adults following stimulation with TLR-specific ligands. They also showed that IL-1 β , IL-6, and IL-8 cytokine production was significantly reduced ($P < 0.001$) compared to adults. MyD88 together with p38 and ERK represent 2 parallel intracellular signaling cascades that are initiated upon TLR ligation and ultimately cytokine production (273, 274). Impairment in both of these signaling systems would significantly contribute to the impaired cytokine production observed in pups/neonates compared to adults.

One transcription factor ultimately stimulated to generate many pro-inflammatory cytokine genes is NF κ B. In our mouse model we showed that NF κ B translocation in PC-stimulated adult mice was significantly greater than unstimulated adult mice but that PC stimulation did not effect NF κ B nuclear translocation in pup mice. Alternatively, LPS-stimulation significantly increased the nuclear translocation of NF κ B in adult cells compared to both the adult control cells as well as LPS-stimulated pup cells. Data previously generated in our lab (unpublished; Brown and Qureshi) demonstrates that PC-stimulation induces significantly more NF κ B nuclear translocation compared to pup cells in mice (Figure 7.1). Although my data demonstrated an increase in NF κ B nuclear translocation in PC-stimulated adult LMs, my data did not demonstrate a significant increase over PC-stimulated pup LMs. The difference between these two pieces of data can best be interpreted by understanding the differences between how each of these studies was conducted. The experiment performed by Brown and Qureshi use whole lungs compared to my experiments which use

specifically LMs. It is possible then, that NFκB expression in the nucleus of PC-stimulated adult LMs may be less pronounced than in other cells found in the lung tissue such as bronchial epithelial cells. Additionally, the earliest time point analyzed by Brown and Qureshi was at 24 hours in contrast to my experiment which analyzed NFκB translocation after 1 hour of PC-stimulation. The longer exposure time coupled with the inclusion of other lung cell types, such as lung epithelial cells, may contribute to the discordant findings in my *in vitro* data compared to Brown and Qureshi's *in vivo* NFκB data. It is also possible that cells removed from their lung environment respond to stimuli differently than they would otherwise. It is possible that LM cytokine production occurs via an alternative pathway (ex. MyD-88-independent) or via alternative transcription factors (ex AP-1) when removed from its natural lung environment and thus differences in NFκB were not detected *in vitro*.

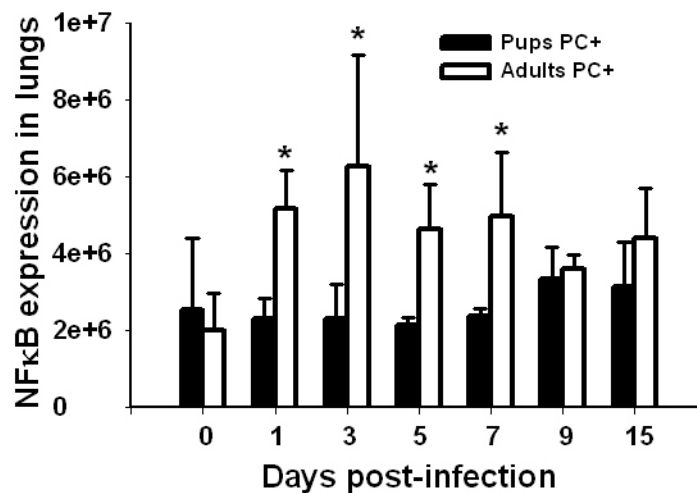


Figure 7.2 PC-infected adults express significantly more NFκB compared to PC-infected pup mice. (Unpublished data; Brown and Qureshi) Pup mice were infected with 1×10^6 PC at 24-72 hours old and adult mice were infected with 1×10^7 PC as adults. Whole lungs were collected at the indicated days post-infection and nuclear extracts were isolated. Electic mobility shift assays (EMSA) were performed to identify NFκB in the nuclear extracts. Data represent the mean \pm SD for 3-5 mice per group per time point. * $P < 0.05$ compared to the pup groups at the same time points.

In vitro stimulation with zymosan, a ligand for TLR2 and dectin-1 (65, 248), generated a high level of pro-inflammatory cytokine production in both pup and adult LMs. This suggests that given the appropriate stimulation, pup LMs are capable of producing cytokines at the level of adult cells and that PC specifically, does not stimulate pup cells adequately. The finding that TLR2 and dectin-1 expression is similar on both pup and adult LMs supports this finding.

To evaluate the effects of LPS *in vivo*, we aerosolized pup mice with HKEC, a method that has been shown to improve PC clearance in CD4 T cell-depleted adult mice (238). We demonstrated that HKEC improves PC clearance in pup mice. Moreover, we demonstrated that HKEC also influenced the PC-infected pup lung environment by increasing immune cell infiltration, LM activation, and cytokine production. While $\text{TNF}\alpha$, $\text{IFN}\gamma$, and MCP-1 were most influenced by the combination of PC infection and HKEC treatment, IL-6 was primarily driven by HKEC. The differences in cytokine production between this HKEC *in vivo* data (Figure 5.10) and the cytokine data generated *in vitro* following stimulation with LPS (Figure 5.3) underscores the influence of factors other than NF κ B in controlling the amount of cytokine produced, including the lung environment and antigenic stimulus itself. The importance of T cells in clearing PC infection has previously been established in the literature (241, 242). Taking this a step further, we demonstrated that T cells are required for efficient phagocytosis of PC organisms by LM. This interaction between T cells and LMs is likely driven by $\text{IFN}\gamma$, a cytokine produced by T cells and required for LM activation (50). Macrophages require two signals to become activated; the first is $\text{IFN}\gamma$. The second signal varies, but can be provided by invading pathogens (ex. LPS), $\text{TNF}\alpha$ (autocrine activity), but also by CD40L expressed on T cells (50). Thus, both signals required for macrophage activation can be provided entirely by T cells, reiterating the importance of the cognate interactions between LMs and T cells during infection.

Overall, it is clear that pup LMs can be stimulated through exogenous stimulation to expedite the clearance of PC organisms and that the best results occur in the presence of functional CD4 T cells. The mechanism behind this

appears to be primarily through LM activation, which is enhanced through the cognate interactions with infiltrating T cells.

3. Granulocyte macrophage colony-stimulating factor

The gamut of infectious diseases has shifted over the past several years to include those occurring predominantly in immunocompromised individuals. Limited efficacy within the current antimicrobial armament in the immunocompromised host combined with increasing resistance patterns has led to an urgent need for new therapies against these infectious diseases. It has been well documented that this crisis in antimicrobial therapy stems predominantly from antibiotic overuse, misuse, and a limited number of antimicrobial agents in the pipeline (275). However, another important factor that limits the utility of antimicrobial-based therapy is the decreased efficacy of these agents in immunocompromised individuals versus to those with intact immune systems (257). The era of HIV and chemotherapy has taught us a lot about the relationship between host immunity and microbial pathogenesis. Pathogens that are thought to be trivial in individuals with an intact immune system become life-threatening in those that are immunocompromised. This underscores the crucial bond between host immunity and pathogenesis and provides a powerful rationale for fighting infectious diseases with immunomodulatory agents (257).

In the absence of GM-CSF, mice are more susceptible to PC organisms (81), most likely due to impaired LM phagocytosis (81, 276) and reduced cytokine production (81, 277, 278). In adults, treatment with GM-CSF has been shown to enhance the clearance and improve outcomes in PC infection (29) as well as many other pulmonary infections (24-28). Based on the rationale for fighting infectious diseases with immunomodulatory agents and the aforementioned GM-CSF literature, we hypothesized that GM-CSF would improve PC clearance and LM function in pup mice. By isolating pup and adult AMs and culturing them *in vitro* with or without rmGM-CSF and PC we were able to elucidate that alterations in cytokine production were secondary to AM stimulation rather than other immune cells located within the lung environment.

The increased production of $\text{TNF}\alpha$, MCP-1, and IL-6, but not IL-10 following treatment with rmGM-CSF suggests that it functions primarily to activate LMs regardless of the presence or absence of PC; although cytokine production is greater in the presence of the organism. We proceeded to administer rmGM-CSF to pup mice *in vivo* to evaluate the response while considering the effect of the lung environment. Varying doses using i.p. and i.n. administrations, we determined that treatment of PC-infected pup mice with rmGM-CSF increases LM activation and T cell infiltration. While PC burden was reduced in the groups receiving rmGM-CSF, there were no statistically significant differences compared to the control groups.

Similar to the previously described studies in which treatment with GM-CSF demonstrated an increase in cytokine production (24, 29), our studies demonstrated that rmGM-CSF is also able to stimulate cytokine production in pup LMs. Unlike these same studies which describe an improvement in the clearance of Group B streptococcus (24), *Histoplasma capsulatum* (26, 27), and *Pneumocystis carinii* (29) in adult mice, our research demonstrates that PC-infected pup mice failed to achieve a significant reduction in PC burden. There are several factors which must be considered, however, when interpreting these findings. First, Mandujano et al. and Deepe and Gibbons demonstrated a reduced microbial burden in CD4-depleted adult mice treated with rmGM-CSF. Second, LeVine and colleagues demonstrated that rmGM-CSF did not improve LM phagocytic uptake but did improve the production of ROS. Taken together these previously published findings suggest that rmGM-CSF works primarily on LMs to increase the amount of ROS, but not phagocytosis. There is conflicting evidence in the literature regarding the phagocytic abilities of pup versus adult macrophages (279-281). Nakano et al. observed that thioglycollate-elicited macrophage from newborn mice phagocytose better than those from adult mice (279, 280). Starobinas et al. was unable to reproduce the findings published by Nakano and furthermore demonstrated that the phagocytic index of a 5 day-old mouse is significantly less than that of an adult mouse (281). Previous research performed in our lab demonstrated that pup mice are less efficient than adult

mice at phagocytosing fluorescent beads and migrating with them to the draining lymph nodes (234). If pup LMs are immature and innately deficient in their phagocytic capacity, then rmGM-CSF may be unable to improve phagocytic killing. The slight reduction in PC burden observed in rmGM-CSF-treated PC-infected pup mice may be generated from the stimulation of cytokine production and the subsequent T cell infiltration, but without adequate phagocytic function, the increased ROS production imparted by rmGM-CSF in adult LMs would be rendered useless in pup LMs.

The functional activation of pup LMs may vary depending on the offending organism. Using a rabbit model, Bellanti and colleagues demonstrated that the phagocytic ability of the LM is well developed in the early post-natal period with regard to *Staphylococcus aureus* and *Escherichia coli* but the microbicidal activity does not fully develop until after the first postnatal month of life (282). The postulated explanation for these conflicting observations is that while the microbicidal mechanisms of the LM may be fully developed at birth, it may be inhibited at this time by large quantities of phagocytosed surfactant-related material (SRM) present in these cells during the early postnatal period (283). This large phagocytic uptake has been found to correlate with a reduction in observable lysosomes (283) and depletion of cellular energy reserves (284), effects of which may interfere with normal microbicidal activity (282). Based on this literature, two theories could possibly explain our data demonstrating a failure of rmGM-CSF to significantly reduce PC burden but seemingly increase LM activation. The first is that rmGM-CSF could not overcome the effects of SRM present in the pup LMs and the subsequent reduction in lysosomes and depleted energy reserves. The second theory is that pup LMs do not recognize or efficiently bind PC organisms compared to other invading organisms resulting in decreased phagocytosis that can not be overcome by rmGM-CSF.

The failure of rmGM-CSF to elicit a significant PC reduction could be related to other immature functions associated with the neonatal lung, such as poor T cell infiltration. This hypothesis is less likely, however, as we have shown that treatment with rmGM-CSF improves T cell infiltration into the lungs of PC-

infected pup mice. Thus, our rmGM-CSF data supports the literature which demonstrates a phagocytic deficiency among infant mouse LMs to phagocytose PC organisms or that these LMs are overwhelmed by SRM rendering them much less responsive to rmGM-CSF. It is also possible that the use of a different dosing strategy may generate a more functional activated LM in pup mice. We used several different dosing strategies in our GM-CSF experiments. We began with i.p. administration of 0.25 μ g per gram of body weight twice daily for 5 days. This regimen was based on a work previously done in our lab in which rmGM-CSF was used for the expansion of DCs (239). Other authors have shown improved microbial clearance from the lungs of mice using doses ranging from 0.05 μ g – 0.25 μ g/g/day i.p. (26, 29). While these authors treated anywhere from 7 to 21 days, they saw reduction in microbial burdens within a week of starting GM-CSF therapy. Based on the short half-life (2 hours) of GM-CSF, we initially chose to administer the dose in 2 equally divided doses. While we demonstrated an increase in LM activation and T cell infiltration, we did not see a reduction in PC burden. In order to initiate a specific pulmonary immune response and potentially improve PC clearance, we used the inhaled route of administration in subsequent pup experiments (252-254, 256). Once again we demonstrated an increase in LM activation and macrophage and T cell infiltration, but no reduction in PC burden. For each experiment we waited 7 days following PC inoculation before beginning GM-CSF treatment to allow time for the organisms to establish an infection. Many of the other authors showing reductions in microbial burdens began treatment on day 1 post-infection or even prior to infection (24, 26, 29). To more closely mimic a clinical timeframe from infection to treatment, we chose to wait seven days before initiating GM-CSF treatment. However, this may be one reason why we were unable to demonstrate a reduction in PC burden. Alternatively, it is possible that our pup mice were metabolizing the drug at a much faster rate than adult mice. Thus despite using a higher dose/gram of body weight compared to that used in adult mice, we were unable to demonstrate a reduction in PC burden. It is possible that longer treatment duration or higher

dose could improve PC clearance; this may be an area to explore in future research.

4. Granulocyte macrophage colony-stimulating factor/Trimethoprim-Sulfamethoxazole

Based on our findings in PC-infected pup mice treated with rmGM-CSF along with recent reports supporting the use of combination therapy for infections in immunocompromised individuals (257), we took our research forward by next looking at the use of rmGM-CSF together with the standard of care treatment for PC, TMP/SMX. While not always considered a part of the immunocompromised population, neonates have been shown to be highly susceptible to pulmonary infections due to the immaturity of the developing lungs (7, 8). Opportunistic pathogens, including PC, which are often thought of as markers of immunosuppression, can cause infection in neonatal lungs (102, 115). While the standard of care treatment for PC pneumonia is TMP/SMX, the use of combination therapy for infectious diseases is a common, well-accepted practice depending on the severity of infection, its location, and the virulence of the infecting organism (257). Taking the use of combination therapy together with the rationale of using immunomodulatory agents to fight infectious diseases, we hypothesized that the use of GM-CSF and TMP/SMX together could improve PC clearance in pup mice.

The results generated from the use of combination therapy with GM-CSF and TMP/SMX appeared to volley between the stimulating effects of GM-CSF and the control of PC burden by TMP/SMX. LM activation markers, cytokine production and T cell infiltration all appeared to increase early on in the experiments during active treatment with rmGM-CSF, although the addition of TMP/SMX blunted these effects in the combination group. After rmGM-CSF was discontinued, the reduction in LM stimulation appeared to be influenced by the decrease in PC burden in the groups receiving TMP/SMX. Two important findings were generated from these data. The first is that combination therapy with TMP/SMX plus 50ng/g of i.n. rmGM-CSF reduces PC burden significantly

more than TMP/SMX alone in PC-infected pup mice. The second is that control of the PC infection is maintained better in the combination group compared to the TMP/SMX group upon discontinuation of TMP/SMX. This second finding suggests that the addition of rmGM-CSF boosts the immune system to help TMP/SMX fight the infection and continues this enhanced effect even after TMP/SMX is withdrawn.

Using an adult mouse model, Deepe and Gibbons demonstrated that i.p. administration of GM-CSF together with a suboptimal dose of amphotericin B reduced *Histoplasma capsulatum* CFU and improved survival compared to optimal doses of amphotericin B alone (26). It is possible that the dose of TMP/SMX could be reduced or the length of therapy shortened when given in combination with GM-CSF. It is also possible that GM-CSF may be able to improve the activity of alternative drugs for PC that are less effective than TMP/SMX when administered alone, such as dapsone (221).

The need for more treatment options for PC infection is undeniable. While the resistance patterns of PC organisms are difficult to define due to its inability to be cultured, many studies have linked mutations in the DHPS gene with chronic TMP/SMX prophylaxis (18, 210-215). A link between the DHPS polymorphism and TMP/SMX treatment failures have also been identified (212, 217). This DHPS polymorphism is thought to be brought about by selective evolutionary pressure from SMX (209); further overuse and misuse of TMP/SMX will likely lead to increases in treatment failures against PC. Adverse reactions to TMP/SMX are common, particularly in the immunocompromised population, the population which most frequently uses this particular antibiotic. Fifteen percent of children develop substantial adverse reactions to TMP/SMX (16), ranging from rash to interstitial nephritis (13-15) and hyperbilirubinemia (199-201).

This is the first study to demonstrate an improved treatment option for fighting PC infection in neonates since pentamidine was replaced with TMP/SMX in 1974 (190-192) and was proven safe for children in 1977 (193, 194). The use of GM-CSF has been proven safe in both immunocompetent as well as immunocompromised children (285), making it an attractive option for

combination therapy in order to reduce the dose or treatment duration of TMP/SMX or possibly even dapsone or caspofungin; further studies are needed to assess these options.

D. Human Neonatal Lung Macrophage Activation is Delayed

Within the United States, PC infection remains primarily a disease of the immunocompromised host, but in underdeveloped countries PC pneumonitis is still a major cause of morbidity and mortality, particularly in young children (1). Infants appear to be particularly susceptible to primary PC infection, likely due to an immature immune system and a lung environment that protects against damaging inflammation early after birth (5). Fortunately the United States has remained somewhat sheltered from this ever-present infection. Despite this, PCP is still one of the deadliest and most prominent infections among the immune deficient population throughout the world. Research which furthers our understanding of this organism and new drugs designed to target this organism remains an extremely important task. Through our research using a mouse model and murine cell lines, we have learned a lot about the neonatal immune response to PC infection. We have also demonstrated that by combining GM-CSF with TMP/SMX we can clear PC infection from neonatal lungs and maintain this reduction after discontinuation of therapy. While some inferences can be made in human infants based on the research we have done in mice, it is important to determine if human LMs respond similarly to mouse LMs following stimulation with PC, LPS, zymosan and GM-CSF. Considering that our research has clinical implications the work we have done using human LMs will strengthen our overall findings and perhaps lead to clinical trials implementing the use of GM-CSF in populations vulnerable to PC infection as well as other opportunistic infections.

1. LPS

Our initial experiments were performed using adult peripheral blood monocyte-derived macrophages compared to neonatal LMs isolated from bronchiolar suctioned sputum. Subsequently we performed the same experiments using both adult and neonatal LMs isolated from bronchiolar suctioned sputum. The differences in cytokine production observed in macrophages derived from different areas, peripheral blood monocyte versus lung, emphasizes the distinctive roles that macrophages play based on their tissue specificity and the requirements for that particular organ. Adult macrophages, regardless of source, produced more cytokines than neonatal LMs following stimulation with LPS. Lung derived adult macrophages produced significantly less TNF α than peripheral blood monocyte-derived macrophages (233 pg/ml vs. 3910 pg/ml; $P < 0.05$). This is likely because a large amount of TNF α produced locally in the lung would initiate a huge inflammatory response. Such a response would be detrimental in the lung environment where the delicate balance between defending against invading pathogens and preventing a damaging host response is crucial. Overall, the cytokine response of human neonatal and adult LMs to LPS resembles that of pup and adult LMs in mice. After 24 hours of LPS stimulation neonatal LMs produce significantly less TNF α and IL-6 compared to adult LMs. This similarity between human and mouse data would suggest that, as with mice, there may be a delayed or reduced expression of TLR4, CD14, MD-2, or an impaired co-localization of these molecules.

2. Zymosan

As with LPS, stimulation of human LMs with zymosan produced similar trends in cytokine production as those observed in mice with the exception of TNF α . Pup mouse LMs produced more TNF α at both 12 and 24 hours post-stimulation, although these differences were not statistically significant. Conversely, human adult cells produced significantly more TNF α than neonatal cells after 12 and 24 hours of zymosan stimulation and thus more closely resemble the stimulation response seen with LPS. The production of IL-6 and IL-

10 more closely followed the patterns observed in mouse cells stimulated with zymosan; human neonatal cells produced significantly more of these cytokines compared to adults. In mice we demonstrated that TLR2 and dectin-1 expression levels were similar on both pup and adults, which was supported by the finding that $\text{TNF}\alpha$ and IL-6 production was also similar between pup and adults. The discordance in $\text{TNF}\alpha$ and IL-6 production observed in human neonatal and adult cells following zymosan stimulation is unlikely due to differences in TLR2 expression. We have shown in mice that TLR2 expression is similar for pup and adult LMs. Furthermore, if TLR2 expression on neonatal LMs was low compared to adult cells, it is likely that $\text{TNF}\alpha$ and IL-6 production in neonatal LMs would also be low compared to adults. Since this is not the case, it is more likely that a downstream signaling component is responsible for the difference observed in these two cytokines between neonatal and adult LMs. As was observed in mouse LMs, zymosan-stimulated cells produced significantly more cytokine than LPS in both neonates and adults. This observation suggests that signaling through TLR2 produces a more pronounced cytokine response than TLR4-dependent signaling.

3. Pneumocystis

The failure of PC to stimulate cytokine production above baseline in neonatal macrophages coincides with our pup mouse data. It is possible that if we had incubated the neonatal cells with PC for 48 hours we may have detected a difference, as we did with $\text{TNF}\alpha$ in our pup mouse data. Adult human and adult mouse LMs both responded to PC with an increase in cytokine production above that of their respective control cells, although these differences were not statistically different in the human cells. The differential response of neonatal/pups compared to adults to PC may be due to immature intracellular signaling in the neonatal/pup cells. We have shown that there is no difference in the expression of TLR2 or dectin-1 on mouse LMs. Additionally there are no differences in the number of cells expressing these surface receptors. We have also shown that there is no difference in the expression of mannose receptor on pup versus adult mouse macrophages (data not shown). Mannose receptor is

another receptor thought to bind pathogen-associated mannose structures found on bacteria, yeast, and fungi (56). While some studies have suggested that MR is important in the recognition and clearance of PC (51, 52), other studies found that MR-/- mice were no more susceptible PC than their wild-type counterparts (57) (58).

Another factor which must be considered pertains to the PC organism itself. We have previously described that these organisms are species specific and cannot be grown in culture. In our lab, our PC source is kept in a SCID mouse colony and is therefore, mouse-specific (*Pneumocystis carinii* f. sp. *muris*). Ideally, we would have liked to use human-specific PC (*Pneumocystis carinii* f. sp. *hominis*); however, since this organism cannot be grown in culture, the *hominis* strain of the organism is very difficult to acquire. There has been a lot of debate over the classification and nomenclature of PC organisms since its discovery in 1909. It is now clear that many different species make up the genus *Pneumocystis*. Extensive research of this organism has taught us that almost every mammalian species harbors at least one species of PC, which can thrive only in its specific mammalian host and which are genetically distinct from one another (286, 287). Within the rat, for example, there are at least five known PC species (288). Comparisons of gene sequences among different PC species using gene trees and bootstrap statistics have provided us with some idea of the similarities between specific sequences responsible for proteins such as superoxide dismutase (SOD), DHPS, and DHFR (289, 290). Unfortunately, it is still unclear what makes a species specific to a mammalian host. Research published by Gigliotti and Hughes has demonstrated that a single monoclonal antibody (MAb) directed at the gp120 surface glycoprotein found on PC organisms (Mab 5E12) was able to recognize PC from rat, rabbit, ferret, and humans (291). This is an important finding considering that gp120 has been implicated in binding of PC to type I pneumocytes and macrophages (292, 293) as this would suggest that PC species specific to one mammalian host is capable of binding to the pneumocytes of another. Overall, this published data together with the increased cytokine production we observed within the human adult LMs

suggests that human cells are capable of recognizing mouse-specific PC. As such, this data demonstrates that human neonatal LMs, as with pup mouse LMs, fail to respond to PC organisms compared to their adult counterparts.

Interestingly, the baseline level of cytokine production in neonatal cells was much higher than the baseline production in adult cells. Since no demographic or disease state information was collected on individual human subjects it is difficult to provide an absolute explanation for this finding. It is possible, however, that this observation can be attributed to the fact that each of the neonatal subjects were receiving ventilation support, unlike the adult subjects. This type of pulmonary stress may have stimulated the LMs leading to an increase in cytokine production.

Unlike what was observed in mouse cells treated with rmGM-CSF, human LMs co-cultured with PC did not demonstrate an increase in cytokine production when stimulated with rhGM-CSF. Despite this, rhGM-CSF significantly improved phagocytosis in neonatal LMs co-cultured with PC. There are a couple of possibilities which could explain this discordance between cytokine production and phagocytosis. The first is a possible timing issue. The phagocytosis index was determined after 25 minutes of incubation with PC and GM-CSF. Alternatively, aliquots for cytokine analysis were taken at 12 and 24 hours post-stimulation. It is possible that GM-CSF produces an immediate early cytokine response that was missed by collecting aliquots after 12 hours. The second possible explanation for this discordance is that GM-CSF improved LM phagocytosis without stimulating cytokine release. Deepe and Gibbons demonstrated that there was no increase in cytokine production in mice infected with *Histoplasma capsulatum* following GM-CSF treatment (26). An improvement in phagocytosis without a concomitant increase in cytokine production may be favorable depending on the type of organism and the extent of infection. As described in the introduction, PC pathogenesis can be divided into two categories; PC-mediated and immune-mediated lung injury. Although PC can cause direct damage to the host lung by killing pneumocytes (147, 150) and producing lung-damaging enzymes (151-157), it is possible that the majority of

damage comes from immune-mediated lung injury. In the clinical setting, the presentation of PCP is highly dependent on the immune status of the patient, suggesting that the host immune response to PCP is contributing to its pathogenesis by initiating immune-mediated lung injury. The initial observation of PCP in cancer patients often occurs once immunosuppressive steroid therapy is being tapered (163) (164); in bone marrow transplant recipients it typically occurs after engraftment and immune function restoration (165) (166); and in AIDS patients it generally occurs following rapid recovery of CD4 T lymphocytes secondary to initiating highly active antiretroviral therapy (HAART) (167, 168). Additionally, it has been well documented that lung injury during moderate to severe PCP is minimized with the administration of adjunctive steroid therapy, reiterating the detrimental effects of the host immune response to PC in the infected lung (169) (170). If the majority of lung damage is immune-mediated, it would be favorable for LMs to ingest and kill the organisms without mounting a more fulminate immune response.

Overall, the significant improvement in neonatal LM phagocytosis initiated by GM-CSF is very promising. The failure of GM-CSF to increase adult LM phagocytosis above baseline is likely because the baseline level of phagocytosis among the adult LMs was already maximized. Based on these data, populations vulnerable to pulmonary infection from PC and other pulmonary-specific pathogens could benefit from the addition of GM-CSF to the standard antimicrobial therapy.

E. Conclusion

Neonatal lung research is difficult due to the size and delicate nature of the still developing lung and homeostatic complexities. Balance between protecting the neonatal lung from invading pathogens and an overwhelming immune response is critical. When using immunomodulatory agents to treat pulmonary infections, one must consider this delicate balance and make sure not to tip the scale heavily in one direction over the other. The need for more treatment options to accommodate the growing population of immune-deficient adults and children is obviated by increasing antimicrobial resistance patterns coupled with fewer new antimicrobials in the pipeline (294). GM-CSF has been used clinically for many years in both immune-competent and incompetent individuals. Its safety in children and efficacy in stimulating LMs have been proven (23, 24, 285). Combination therapy has the benefit of often being able to reduce doses or durations of therapy to minimize toxicity (257). It is known that term and pre-term infants are more susceptible to pulmonary infection and often have a more fulminate course of the disease (7, 8, 282). Since LMs are often the first line of defense against inhaled pathogens we hypothesized that neonatal LM function was impaired or delayed. We have shown, in pup mice that LMs are at least partially responsible for this increased susceptibility due to a delay in activation and cytokine production. We demonstrated in both mouse and humans that functionally impaired neonatal LMs can be exogenously stimulated to expedite activation and cytokine production and improve PC clearance. We have demonstrated in human neonatal LMs that GM-CSF improves phagocytosis of PC organisms without increasing cytokine production. GM-CSF was shown to increase cytokine production in pup mouse LMs; however the failure to produce cytokines in human neonatal LMs may be favorable for an organism which imparts much of its lung damage through initiation of the innate and adaptive immune systems. Taking together these mouse and human data, we believe that combination therapy with GM-CSF and TMP/SMX is a viable treatment option for

PC infections among immunocompromised populations, including the neonatal population.

F. Future Directions

There are many avenues left to explore in this area of research; one such area involves looking at surfactant proteins. Surfactant proteins A and D (SP-A and SP-D) are large hydrophilic glycoproteins which participate in innate lung immunity through interactions with a variety of organisms, including PC (295, 296). SP-A and SP-D have both been shown to bind PC (297-300) as well as interact with and modulate the function of macrophages (301-303). Specifically, SP-A has been shown to increase phagocytosis while SP-D increases the production of ROS during PC infection (295, 296, 303, 304). The importance of these surfactant proteins for clearance of PC infection has been demonstrated in both animal models and in humans. Linke and colleagues demonstrated delayed PC clearance in a SP-A^{-/-} mouse model (298), while Hughes and colleagues showed the efficacy of exogenous surfactant replacement using a PC-infected rat model (305). In humans a limited number of publications have demonstrated that surfactant treatment in PC-infected infants improves gas exchange and overall oxygenation (306-309). However, none of these studies address the effect of surfactant on LM function and PC clearance. Combining the fact that surfactant treatment has already been used clinically to treat PC-infected infants with the published evidence that surfactant improves LM function against PC organisms, it would be reasonable to extend our research to determine if surfactant can expedite PC clearance and improve outcomes in PC-infected infants.

Although there are several immunomodulatory agents commercially available, including GM-CSF, they are not all optimized for delivery directly to the lung. Historically, clinicians have used the available i.v. or s.c. formulations, however these are designed to be delivered to the lung. The lungs are distinctly different from the venous environment and thus a formulation and delivery system that are specifically designed for the lung environment may help improve

the overall affect of GM-CSF. Furthermore, a delivery system that is designed to instill the greatest amount of drug to the lung would also help optimized therapy and waste as little drug as possible. Thus, efforts to optimize dose, delivery, and formulation of GM-CSF as well as other immune modulating agents is an important future objective with the overall goal of improving outcomes and minimizing toxicity.

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Publications

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