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Characterization of Inflammation During Chronic Bronchopulmonary *Pseudomonas aeruginosa* Infection in Resistant and Susceptible Inbred Mouse Strains

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June 1998

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A Thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of Master of Science

Abstract

Studies in cystic fibrosis patients have suggested that the balance between pro- and anti-inflammatory cytokines may be important in controlling infection and tissue destruction during bronchopulmonary *Pseudomonas aeruginosa* (PA) infection. Here, the inflammatory response was characterized in a murine model of bronchopulmonary PA infection in resistant BALB/c and susceptible C57BL/6 mice following intratracheal inoculation with 10^5 CFU of mucoid PA entrapped in agar beads. On day 7 post infection, BALB/c mice had significantly less bacteria and significantly more IL-10 in the lungs as compared to C57BL/6 mice while the levels of TNF-α and IFN-γ levels were comparable. Bronchoalveolar lavage revealed that the significantly lower lung inflammatory response in BALB/c mice was composed of 45% PMN, 30% lymphocytes and 25% macrophages while that of C57BL/6 mice was composed of 62% PMN, 8% lymphocytes, and 30% macrophages. Alveolar macrophages from C57BL/6 mice spontaneously produced significantly higher NO levels while cells from BALB/c mice produced significantly more TNF-α, either spontaneously or following stimulation with LPS or heat-killed PA. There was no difference in IL-10 production. Lung PMN from either strain produced undetectable levels of NO and high levels of TNF-α. PMN from BALB/c mice, however, produced significantly higher levels of IL-10. The severity of infection in both strains of mice was exacerbated by administration of the anti-IL-10 mAb JES5-2A5. IL-10 neutralization resulted in an increased inflammatory response,

increased bacterial counts, and increased TNF-α levels in both mouse strains. The increased inflammatory response in resistant BALB/c mice was due to a significant influx of macrophages whereas in susceptible C57BL/6 mice a significant influx of PMN and decreased numbers of macrophages were observed. Taken together, these results suggest that an intricate network of cells and cytokines regulates the pulmonary inflammatory response to PA and that neutralizing IL-10 can exacerbate infection and inflammation irrespective of the genetic background of the host.

Résumé

Diverses études portant sur des patients atteints de fibrose kystique ont suggéré qu'un équilibre entre les cytokines pro et anti-inflammatoires serait important afin de contrôler l'infection et la destruction des tissus pulmonaires par *Pseudomonas aeruginosa* (PA). La réponse inflammatoire a été caractérisé chez le modèle murin suite à une infection par PA des voies broncho-pulmonaires de souris résistantes BALB/C et susceptibles C57BL/6 à l'aide d'une inoculation intratrachéale de 10^5 UFP de PA mucoïde emprisonné dans des billes d'agar. Les souris BALB/C avaient de façon significative moins de bactéries et plus de IL-10 dans les poumons 7 jours post-infection lorsque comparées aux souris C57BL/6 alors que les niveaux de TNF-α et d'IFN-γ étaient semblables. Des lavages broncho-alvéolaires ont également montré que la réponse inflammatoire significativement inférieure chez les souris BALB/C était composée à 45% de PMN, 30% de lymphocytes et 25% de macrophages alors que celle des souris C57BL/6 était composée à 62% de PMN, 8% de lymphocytes et 30% de macrophages. Par ailleurs, les macrophages alvéolaires des souris C57BL/6 produisaient de façon spontanée des niveaux de NO significativement plus élevés alors que les cellules des souris BALB/C produisaient plus de TNF-α de façon spontanée ou suivant une stimulation de PA inactivé par la chaleur ou avec du LPS. Il n'y avait pas de différence dans la production de IL-10 par ces macrophages. Les PMN pulmonaires des deux espèces murines ont produit des hauts niveaux de TNF-α mais n'ont pas produit de NO.

Cependant, les PMN des souris BALB/C ont produits des niveaux significativement plus élevés de IL-10. Chez les deux espèces murines, la sévérité de l'infection était exacerbée par une administration de l'anticorps monoclonal JES5-2A5 anti-IL-10. La neutralisation de IL-10 a amené une augmentation de la réponse inflammatoire, une augmentation du décompte bactérien ainsi qu'une augmentation des niveaux de TNF-α chez les deux espèces murines. L'augmentation de la réponse inflammatoire chez les souris résistantes BALB/C était due à un apport significatif de macrophages alors que chez les souris C57BL/6, un apport significatif de PMN et une diminution du nombre de macrophages était observée. La combinaison de ces résultats suggère qu'un réseau de cellules et de cytokines règle la réponse inflammatoire pulmonaire à PA et que la neutralisation de IL-10 peut exacerber l'infection et l'inflammation indépendamment du bagage génétique de l'hôte.

Acknowledgements

I am indebted to my supervisor Dr. Mary M. Stevenson for all her support and guidance. I could not have dreamed of a more wonderful introduction to immunology. She has given me the freedom to figure things out on my own and at the same time managed to keep me on course. Indeed, she exemplifies the virtues of perseverance and patience.

I am most grateful to Mifong Tam, not only for her technical wizardry, but also for her advice and opinions regarding both science and non-science matters. Her encouragement and optimism have meant the world to me.

I wish to thank all the members of Dr. Stevenson's laboratory, past and present, for putting up with me: Kusum, Mohan, Hakeem, Juliette, Catherine, Julie, and Zhong. In particular, I am grateful to Kusum for her help during my experiments and for our many discussions. Hakeem and Mohan, I am thankful for all their discussions and the advice they both have shared with me.

I wish to thank Dr. Danuta Radzioch for her critical review of this thesis and for her continued encouragement. Her insight and enthusiasm have been invaluable to me.

I am thankful for the guidance of Dr. Emil Skamene, Director of the McGill Centre for the Study of Host Resistance.

Special thanks to Claude Lachance for the French translation of the abstract.

I would like to acknowledge the financial support of the Canadian Cystic Fibrosis Foundation and FCAR-FRSQ.

Last, but by no means least, I would like to thank my parents, Steve and Glorianne, and my brother, Andrew. Through my ups and downs they have always been there and never flinched. I consider myself very fortunate to have their support and love.

Preface

The research work presented in this thesis will be compiled into a manuscript and submitted for publication as follows:

Stotland, P. K., Tam, M. F., Sapru, K., and M. M. Stevenson. Neutralization of IL-10 exacerbates chronic bronchopulmonary *Pseudomonas aeruginosa* infection in resistant and susceptible inbred mouse strains. *Manuscript in preparation*.

The candidate performed all the research work under the guidance of Dr. Mary M. Stevenson. The candidate was responsible for the infection of the mice, isolation of the airway cells, preparation of homogenates, and performed all ELISAs. Mifong Tam provided technical guidance in the preparation of JES5-2A5, during infection of the mice, and cell isolation and counting. Dr. Kusum Sapru assisted with the isolation and counting of the airway cells.

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List of Abbreviations

a.a.	amino acid
Ab	antibody
AMP	adenosine monophosphate
ANOVA	analysis of variance
ASF	airway surface fluid
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
cAMP	cyclic AMP
cDNA	complementary DNA
CFTR	cystic fibrosis transmembrane conductance regulator gene (human)
Cftr	cystic fibrosis transmembrane conductance regulator gene (murine)
CFTR	cystic fibrosis transmembrane conductance regulator protein
CFU	colony-forming unit
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
HBSS	Hank's balanced salt medium
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid

HKPA	heat-killed *Pseudomonas aeruginosa*
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IgG	immunoglobulin gamma
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
LPS	lipopolysaccharide
mAb	monoclonal antibody
MIP	macrophage inflammatory protein
mRNA	messenger RNA
NO	nitric oxide
PA	*Pseudomonas aeruginosa*
PBS	phosphate-buffered saline
PMN	polymorphonuclear neutrophils
RNA	ribonucleic acid
SEM	standard error of mean
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha

Chapter 1: Introduction

1.1. Literature Review

1.1.1. Introduction

Cystic fibrosis (CF), an autosomal recessive disease, is the most common, lethal, inherited disease in Caucasian populations. The disease is characterized by thick, mucous, secretions from epithelia resulting in abnormal sweat chloride, pulmonary disease, and pancreatic disease. Within affected airways, recurrent infection by an opportunistic Gram-negative bacteria, *Pseudomonas aeruginosa* (PA), leads to lung disease and eventual respiratory failure. Other mainfestations of disease include male infertility, malabsorption of gut contents due to pancreatic insufficiency, and intestinal blockage (meconium ileus) (Boat et al., 1989).

The major cause of morbidity and mortality among CF patients is chronic and recurrent lung infection with PA (FitzSimmons, 1993; Kerem, 1997). The pathology in the airways, induced by PA infection, is based on local inflammation and immune response(s) leading to inadequate clearance of bacteria which progressively damage the structural and functional integrity of the lungs (Koch and Hoiby, 1993; Cripps et al., 1995). It is widely recognized that an exaggerated inflammatory process in response to PA infection is the hallmark of lung disease in CF patients (Berger, 1991). Indeed, some infants with CF appear to have an inflammatory response in the airways even without any detectable bacterial infection (Khan et al., 1995).

1.1.2. Overview of Cystic Fibrosis

CF was identified in 1936 by Fanconi and further characterized 2 years later by Andersen (Andersen, 1938). Although the pathophysiology of the pancreas was the first manifestation of the disease to be described, today it is recognized that CF is the most common, lethal, autosomal recessive disorder affecting Caucasian populations (Wood et al., 1976; Davis et al., 1996; Hilman, 1997) wherein the incidence is approximately 1 in 2500 live births (Kane, 1988; Dodge et al., 1997). CF has a significant impact within Canada; in the Saguenay Lac-St. Jean region of Quebec the incidence can be as great as 1 in 891 live births (Corey and Farewell, 1996; Debraekeleer et al., 1997).

In most cases, the diagnosis of CF is considered when one or more features (shown in Table 1) are present and then confirmed by a finding of more than 60 mmol/L chloride in the sweat (Rosenstein and Zeitlin, 1998). Furthermore, most patients have chronic sinopulmonary disease, almost all postpubertal patients have obstructive azoospermia, and approximately 85-90% of patients have exocrine pancreatic insufficiency (Rosenstein and Zeitlin, 1998).

1.1.3. Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene and Protein

By the early 1980's, the physiologic abnormalities in maintaining homeostatic ion transport across epithelial-lined organs in CF patients was attributed to the failure of cAMP regulated chloride transport (Knowles et al., 1983; Quinton, 1986). In 1989, the

Table 1. Phenotypic Features Consistent with Diagnosis of CF

Chronic Sinopulmonary Disease

- Persistent colonization/infection with typical CF pathogens, including: *S. aureus*, non-typeable *H. influenzae*, mucoid and non-mucoid *P. aeruginosa*
- Chronic cough and sputum production
- Persistent chest radiograph abnormalities (i.e., bronchiectasis, atelectasis, infiltrates, hyperinflation)
- Airway obstruction manifested by wheezing and trapping
- Nasal polyps; radiographic or computed tomography abnormalities of paranasal sinuses
- Digital clubbing

Gastrointestinal and Nutritional Abnormalities

- Intestinal: meconium ileus; distal intestinal obstruction syndrome; rectal prolapse
- Pancreatic: pancreatic insufficiency; recurrent pancreatitis
- Hepatic: chronic hepatic disease manifested by clinical or histological evidence of focal billiary cirrhosis or multilobular cirrhosis
- Nutritional: failure to thrive (protein-calorie malnutrition); hypoproteinaemia and oedema; complications secondary to fat-soluble deficiency

Salt Loss Syndromes

- Acute salt depletion
- Chronic metabolic alkalosis

Male Urogenital Abnormalities

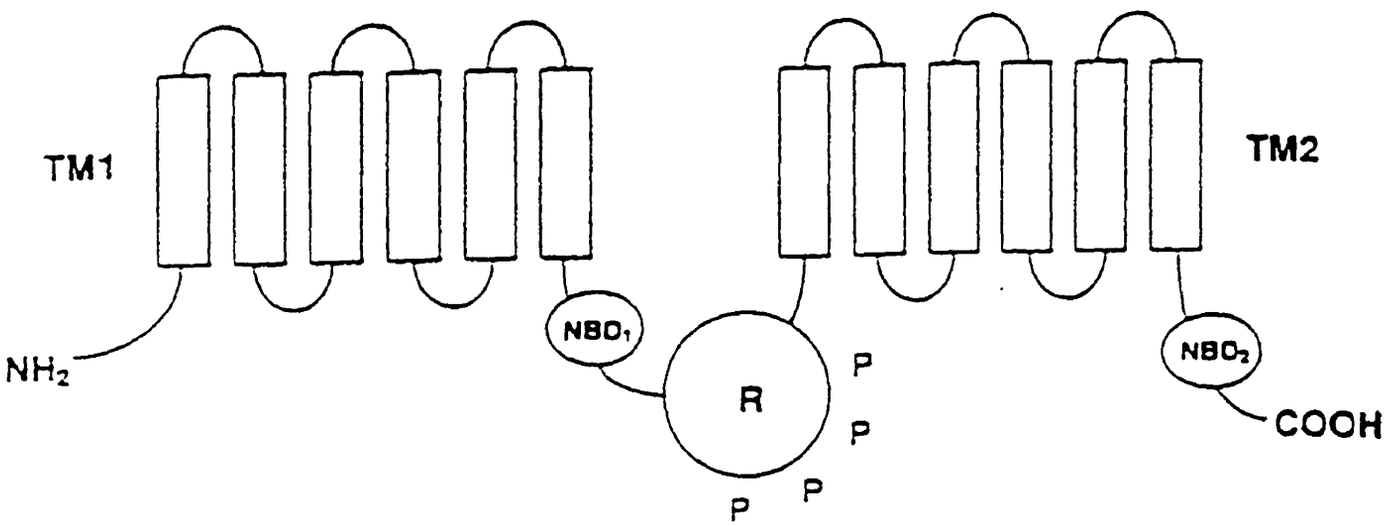
- Obstructive azoospermia

CF gene, named *CFTR*, was identified (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) by both linkage analysis (Tsui et al., 1985; Foroud, 1997; Wolff, 1997) and chromosome jumping (Collins, 1992). The CF gene was determined to reside in the region of chromosome 7q31.1 encompassing about 250 kb of genomic DNA and consisting of 27 exons. Expression of the 6.5 kb *CFTR* mRNA has been detected in the pancreas, nasal polyp, lung, colon, sweat gland, and liver – basically all epithelial tissues affected by CF – and encodes for a protein of 1480 amino acids, known as the cystic fibrosis transmembrane conductance regulator (CFTR).

The CFTR protein consists of 2 transmembrane domains with 6 transmembrane segments each and 2 nucleotide binding domains joined by a regulatory domain that has multiple phosphorylation sites for protein kinases A and C (see Figure 1) (Kerem et al., 1989; Riordan et al., 1989; Bear et al., 1992). Found in the apical membrane of epithelial cells, the CFTR protein functions as a low conductance chloride channel which is regulated by cAMP (Tsui, 1995). Indeed, mutations in the *CFTR* gene result in decreased epithelial chloride permeability and loss of cAMP mediated chloride secretion (Ackerman and Clapham, 1997; Wine, 1997). In addition to regulating chloride flux, the CFTR protein also appears to be involved in the regulation of other ion channels such as the outward rectifying chloride channel (ORCC) (Egan et al., 1992; Schwiebert et al., 1994) and the epithelial sodium channel (EnaC) (Stutts et al., 1995).

Sequences from CF cDNA libraries were found to contain a 3-bp deletion that corresponds to the loss of a phenylalanine at residue 508 (termed the ΔF508 mutation) of

Figure 1. Structure of CFTR predicted by the amino acid sequence. Two membrane-spanning domains (TM1 and TM2) with six transmembrane segments each, two nucleotide binding domains (NBD1 and NBD2), and an intracellular hydrophilic R domain that contains consensus phosphorylation sequences for PKA and PKC are illustrated. (Adapted from Davis et al., 1996).


TM1
TM2
NH2
NBD1
NBD2
R
P
P
P
P
COOH

the CFTR protein (Kerem et al., 1990; Zielenski and Tsui, 1995; Lenaerts, 1997). Subsequent analysis of CF and non-CF chromosomes has shown that the ΔF508 mutation occurs in approximately 70% of CF patients (Kerem et al., 1989) but over 650 other mutations have since been identified (Rosenstein and Zeitlin, 1998).

1.1.4. *CFTR*: Relationship to Disease Manifestations?

Following the identification of the *CFTR* gene, an intense effort was made to find an association between *CFTR* mutations and the severity of CF disease. Kerem et al. (1990) reported that patients who were homozygous for the ΔF508 mutation were diagnosed with CF at an earlier age and had a greater frequency of pancreatic insufficiency such that this condition was present in 99 % of the homozygous patients, 72 % of the heterozygous patients, and only 36 % of the patients with other genotypes. Although another group reported similar findings, a correlation between genotype and pancreatic status does not appear to be absolute (Borgo et al., 1993).

On the other hand, a correlation between CF genotype and the severity of lung disease has yet to be established. CF patients carrying the same *CFTR* mutations display a remarkable heterogeneity in the severity of lung disease (Santis et al., 1990; Hamosh and Corey, 1993), thereby suggesting the presence of either modifier genes exclusive of the *CFTR* locus (Rozmahel et al., 1996; Kent et al., 1997) or environmental factors (Davis et al., 1996). These putative modifier gene(s) may be responsible for initiating or modulating the host response to PA airway colonization. Thus, there is considerable

interest among CF researchers to elucidate the proposed existence and/or function of these genes.

1.1.5. *CFTR*: Link to PA Lung Disease?

The link between mutations at the *CFTR* locus and bronchopulmonary infection with PA remains unresolved. A number of models have been established and are described below.

One model proposes that CF patients have impaired cAMP mediated CFTR chloride secretion across the epithelial membrane of the respiratory tract. This view, then, supports the notion that epithelial ion transport promotes effective mucocilliary clearance by regulating both the volume and ionic composition of the airway surface fluid (ASF). Thus, with ASF concentrations of Cl^- being lower, increased absorption of Na^+ occurs, which in turn causes desiccation of mucus as water follows Na^+ across the epithelium. The net result of this altered ion transport is a reduced depth of ASF, impeded cilial action, and a viscous environment that predisposes airways to infection (Knowles et al., 1997). Indeed, according to this model, desiccated secretions may lead to trapping of bacteria in the lung with concomitant reduction in mucociliary clearance, and this failure of clearance may allow bacterial infections to become established (Davis et al., 1996). Another model proposes that if Na^+ were not accompanied by water, the depth of the ASF would remain unchanged; however, the ion concentrations would be lowered.

Subsequently, the reduced ion concentration in ASF would impede the killing potential of PMN (Mitzgerd et al., 1995).

Although the logistics of recovering tracheal ASF from CF patients has proven to be a difficult task (the ASF is only about 10 to 15 μm in depth), attempts to characterize the ASF from CF patients have been reported (Gilljam et al., 1989; Joris and Quinton, 1992; Joris et al., 1993; Smith et al., 1996; Goldman et al., 1997). In contrast to the idea that defects in the CFTR protein inhibit Cl^- secretion, these studies have suggested that an excessive salt concentration is apparent in the airways of CF patients. This model hinges upon the idea that airway epithelia regulate the ionic composition but not the volume of the ASF. Thus, in a similar manner to that of the sweat duct, the airway epithelium would appear to have a reduced ability to reabsorb salt. This excessive salt concentration is proposed to have a deleterious effect on the biological activity of a class of anti-microbial agents known as β-defensins (Smith et al., 1996; Goldman et al., 1997).

There are conflicting reports as to whether the salt concentrations in ASF of CF patients is indeed elevated. Knowles et al. (1997) reported no detectable differences in the ASF osmolarity between CF patients and normal counterparts. Moreover, the concentrations of ions found by these investigators in the ASF exceed the values consistent with β-defensin functions, thereby implicating the breakdown in CF lung defense to be factors other than salt-sensitive defensin-like molecules. In the most recent study, Hull et al. (1998) compared the ion concentrations in ASF from CF infants with various magnitudes of airway inflammation. They reported no increase in Cl^-

concentrations in tracheal ASF of CF patients compared to healthy controls. Moreover, they determined that in infants with CF, who do not have observable pulmonary inflammation, the tracheal ASF Cl^- levels are actually decreased, thus, refuting the hypothesis that increased ASF salt concentrations are important in the initial pathogenesis of CF lung disease.

In addition to regulating ion transport, it has been suggested that the CFTR can also regulate the expression of surface molecules on epithelial cells. Indeed, it has been reported that asialoGM1 was expressed on 12% of nasal polyp epithelial cells recovered from CF patients whereas its expression was detected on only 2.9% of cells from normal patients (Saiman and Prince, 1993). Thus, the increased expression of surface molecules, such as asialoGM1, could mediate increased PA attachment (Saiman et al., 1992). These results form the basis of the proposed mechanism implicating PA pili as the key player in binding (Tang et al., 1995).

Another possibility that may explain susceptibility to PA lung infection concerns the expression of CFTR in the epithelial cells from the CF lung. Pier et al. (1996a) hypothesized that the binding and internalization of respiratory pathogens by epithelial cells followed by desquamation could be an important mechanism for clearing bacteria from the airways. In support of this hypothesis, these investigators observed that cultured human airway epithelial cells expressing CFTR with the ΔF508 mutation were defective in the uptake of PA compared to cells expressing wild-type CFTR. Thus, they concluded that CFTR itself was a factor contributing to host defense against PA. Moreover, they

identified the LPS-core oligosaccharide as the bacterial ligand that binds to CFTR (Pier et al., 1996b). Subsequent experiments from the same group elucidated that CFTR is a cellular receptor that is responsible for the binding, endocytosing, and clearing of PA from the normal lung (Pier et al., 1997). However, it is unlikely that CFTR is the initial cellular receptor for PA binding because the normal level of membrane CFTR appears to be too low for effective initial binding.

1.1.6. CF Lung Disease: Initial PA infection

The intermediate steps connecting abnormal Cl^- transport and PA colonization in the airways remain unresolved. Nonetheless, chronic pulmonary disease ensues and its relentless course is the major cause of the morbidity and mortality associated with CF. Much effort has been directed to understanding why CF patients develop chronic PA lung infection and why their seemingly uncompromised immune systems are largely ineffective against this bacterium.

Respiratory viral infections, such as those caused by respiratory syncytial virus, have been hypothesized as initial triggering events leading to PA lung infection in CF patients (Ramphal et al., 1980; Marks, 1984; Hoiby and Koch, 1990). Virus replication and subsequent host responses cause damage to the respiratory mucosa, thereby, facilitating bacterial adherence (Zach, 1990). It is thought that the initial episodes of bacterial infection in the lower respiratory tract occur with organisms such as *Staphylococcus aureus* and *Hemophilus influenzae* during infancy (Wood et al., 1976;

Boat et al., 1989; Khan et al., 1995). Following *S. aureus* binding to fibronectin, an inflammatory response is evoked and, in turn, an increase in free elastase levels is observed in the sputum of CF patients. The increased level of elastase, released from infiltrating PMN, has marked proteolytic activity on fibronectin, thus, causing its cleavage. As infection proceeds and becomes established, clearance of these organisms becomes increasingly ineffective due to the mucous production within the airways coupled with the effects of an intense inflammatory response predominated by PMN. Infiltrating PMN release an array of oxidants, phagocytic enzymes, cytokines, and chemokines that have deleterious effects on delicate lung tissue (Sibille and Reynolds, 1990). Furthermore, the damage caused to fibronectin by both the bacteria and host response exposes binding sites on the surface of epithelial cells, thus, facilitating the adherence of PA (Woods et al., 1980). Recurrent infection subsequent to initial triggering events occurs and establishes a microenvironment that predisposes the airways to chronic PA colonization by mediating further injury to the respiratory mucosa (Warner, 1992).

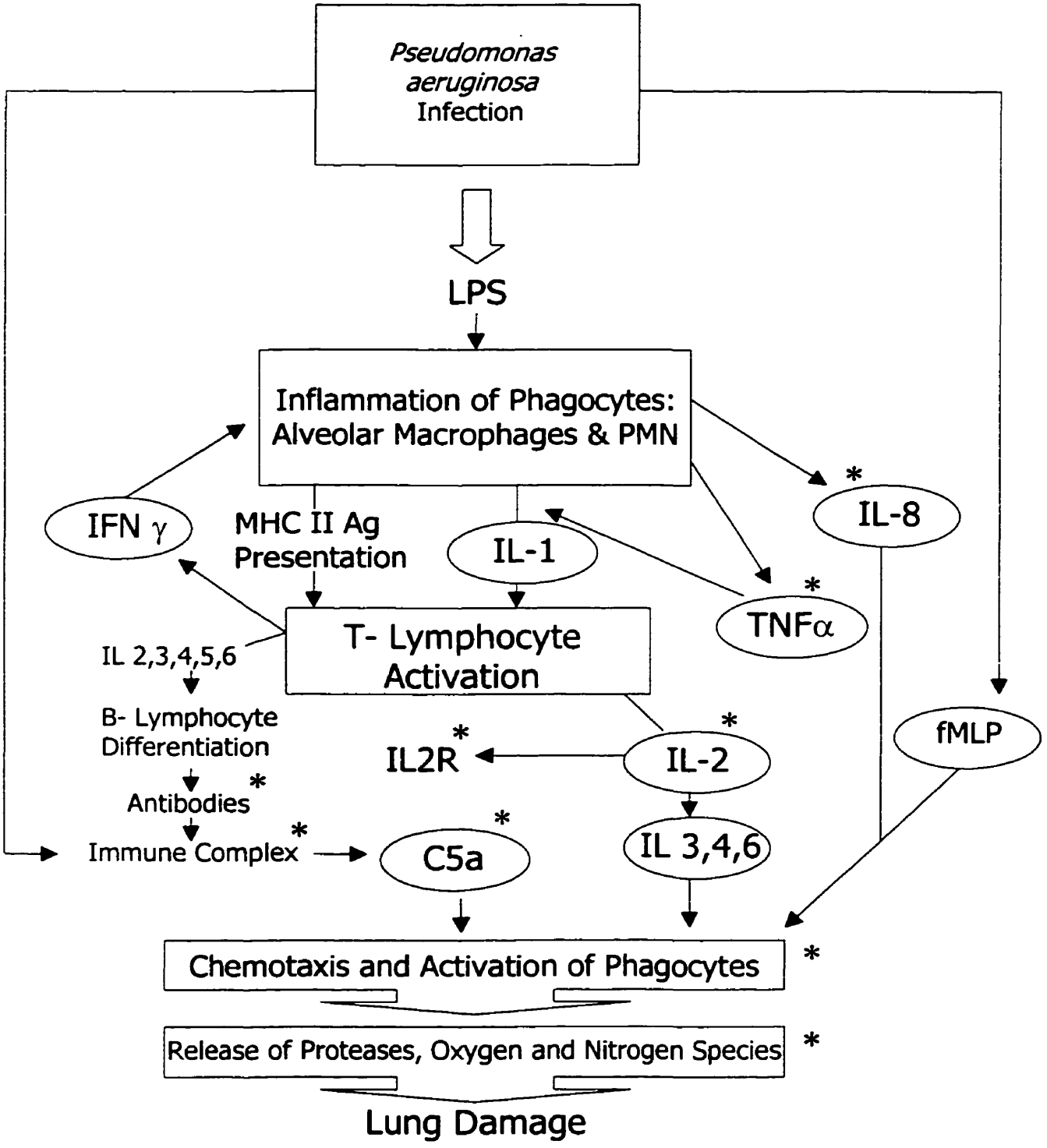
1.1.7. CF Lung Disease: Acute Inflammation

It remains unclear whether PA is required to incite a host inflammatory response or if inflammation precedes infection, thus, causing damage to the airways and, thereby, increasing the susceptibility of the lung to subsequent infection (Armstrong et al., 1995; Konstan and Berger, 1997). In order to investigate the latter possibility, Khan et al. (1995) characterized the inflammatory state of the airways of infants with CF. They

determined that infants as young as 4 weeks of age, with no observable infection (either bacterial, viral or fungal), had a significantly heightened inflammatory response as reflected by elevated numbers of PMN, and elevated levels of elastase/alpha$_1$-antiprotease inhibitor complexes and IL-8 in their BALF. Furthermore, these observations support the hypothesis that there are indeed genes exclusive of the *CFTR* that modulate host responses in CF.

Regardless of the sequence of initiating events, early in life, the ensuing host responses are inefficient to stop the inevitable course of persistent and recurrent PA infection and the resulting extensive tissue damage. Thus, before, during, or after initial PA infection, a vigorous acute inflammatory response predominated by PMN develops (Konstan and Berger, 1993; Danel et al., 1996). The infiltrating cells have substantial harmful effects to lung tissue, namely, by their ability to release elastase (Tosi et al., 1990; Zach, 1990; Meyer et al., 1991; Berger et al., 1994) and by the increased amount DNA released from dying PMN (Barton et al., 1976; Kirchner et al., 1996). Released elastase is capable of cleaving receptors on the surface of human T cells (Doring et al., 1988) and destroying key opsonins such as IgG (Doring et al., 1986; Berger et al., 1989), and complement receptors such as CR1 (Tosi et al., 1990). As well, elastase stimulates the release of neutrophil chemoattractants (Nakamura et al., 1992), and promotes hypertrophy and hyperplasia of the mucus-secreting apparatus (Sommerhoff et al., 1990), thus, causing damage to vulnerable lung tissue (Figure 2) (Berger, 1991). In addition to elastase, myriads of soluble factors (cytokines, chemokines, oxidants, and

Figure 2. Host response to bronchopulmonary *Pseudomonas aeruginosa* infection. * indicates areas of response which may be greatly exacerbated in CF. (Adapted from Berger, 1991 and Warner 1991).


Pseudomonas aeruginosa Infection
LPS
Inflammation of Phagocytes: Alveolar Macrophages & PMN
IFN γ
MHC II Ag Presentation
IL-1
IL-8
TNFα
T- Lymphocyte Activation
IL 2,3,4,5,6
B- Lymphocyte Differentiation
Antibodies
Immune Complex
IL2R
IL-2
fMLP
C5a
IL 3,4,6
Chemotaxis and Activation of Phagocytes
Release of Proteases, Oxygen and Nitrogen Species
Lung Damage

chemoattractants) are released and serve to establish a full-fledged inflammatory response (Table 2).

1.1.8. CF Lung Disease: Humoral Response

In addition to inflammation, the role of an antibody-driven response to PA infection has been documented. Sustained chronic PA infection may manifest a type III hypersensitivity reaction with immune complexes which, via complement, ultimately leads to the release of lysosomal enzymes and reactive metabolites from infiltrating PMN and, in time, from macrophages as well (Hoiby and Koch, 1990; Hoiby et al., 1990; Zach, 1990; Warner, 1992). Although in some bacterial infections opsonising antibodies may be protective, CF patients usually have high serum levels of anti-PA immunoglobulins, in particular non-opsonic IgG_2, which correlate with a poor prognosis (Doring and Hoiby, 1983; Meluleni et al., 1995). In addition, studies have shown that low immunoglobulin levels in CF children correlate with less severe lung disease (Turner et al., 1978; Matthews et al., 1980).

1.1.9. CF Lung Disease: Chronic Inflammation

Despite the fact that the acute inflammatory response may serve as a first line of defense in some bacterial infections, this response fails to control PA infection in the CF lung. At some point between the initial infection and inflammation, or shortly thereafter, PA acquires a mucoid phenotype (Zach, 1990; Warner, 1992). A considerable production

Table 2. Major Products Released by PMN

Cytokines/ Chemokines

- IL-1β
- IL-1ra
- IL-8
- TNF-α
- TGF-β
- MIP-1α
- IL-3
- GM-CSF
- IFN-α

Biologically active lipids

- Platelet-activating factor (PAF)
- Leukotriene B_4 (LTB_4)
- Prostaglandin E_2 (PGE_2)

Oxygen metabolites

- O_2, H_2O_2, $OH\cdot$

Proteins

- Fibronectin
- Heat shock proteins
- CR1
- CR3
- FcR
- Lactoferrin
- Defensins

Enzymes

- Elastase: serine and metalloenzyme
- Acid hydrolases
- Myeloperoxidase
- Lysozyme
- Neutral serine proteases
- Plasminogen activator
- Collagenase
- Gelatinase
- Heparanase

of alginate, an extracellular polysaccharide, forms a biofilm around the bacteria, thereby, encapsulating microcolonies of PA in the lungs (Meluleni et al., 1995). Bacteria are protected against the host's defense mechanisms by the production of alginate and the concomitant aggregation into microcolonies (Hoiby et al., 1990). As well, the release of toxins and proteases from PA may be important in preventing the host from mounting a protective local immune response (Hoiby and Koch, 1990). The development of the mucoid phenotype may help to explain the shift between acute and chronic inflammation within the CF lung.

The mucoid biofilm not only impedes opsonising antibody, and PMN and macrophage phagocytosis, but also contributes to a persistent stimuli to activate inflammatory cells and antigen presentation cells (Doring, 1994). Continuous induction of inflammatory processes mediates a vicious circle of cellular recruitment to sites of infection (Berger, 1991). Indeed, the shift to a chronic inflammatory state is highlighted by continuous PMN infiltration to the lung. As inflammation perpetuates, other cell types, including macrophages and lymphocytes, are recruited to attack the bacteria.

Attempts have been made to characterize the sustained inflammatory state of the CF lung by analysis of fluids obtained by both nasal and bronchoalveolar lavage. It is clear that a severe local inflammatory response is present in CF patients with mild lung disease who do not regularly produce sputum and do not exhibit clinically apparent exacerbation (Konstan et al., 1994). Bronchoalveolar lavage fluid (BALF) obtained from the airways of CF patients have significantly higher numbers of PMN (Wilmott et al.,

1990; Berger, 1991; Konstan and Berger, 1993; Meyer and Zimmerman, 1993; Konstan et al., 1994; Danel et al., 1996) and increased concentrations of elastase (Meyer et al., 1991; Konstan et al., 1994) as compared to healthy control subjects. High levels of IL-8 have been reported to play an important role in mediating PMN recruitment to the lungs (Dean et al., 1993; Armstrong et al., 1997; Noah et al., 1997), as well as the eicosanoid lipid mediator, leukotriene B_4 (LTB_4) (Konstan et al., 1993). Indeed, Richman-Eisenstat et al. (1993) demonstrated that treatment with a monoclonal antibody against the PMN chemokine, IL-8, significantly inhibited the chemotactic activity in CF patients' sputum, but not in induced sputum recovered from healthy subjects. It has been proposed that the high concentrations of IL-8 found within the CF lung negatively regulate neutrophils by downregulating their IL-8 receptor expression (Dai et al., 1994). Various sources of IL-8 in the CF lung have been reported and include epithelial cells (Bonfield et al., 1995), alveolar macrophages (Dean et al., 1993; Khan et al., 1995) and PMN themselves (Lloyd and Oppenheim, 1992; Inoue et al., 1994).

Other pro-inflammatory cytokines have been implicated in progression of inflammation during PA infection. Significantly elevated levels of IL-1 (Wilmott et al., 1990; Bonfield et al., 1995), IL-1β (Wilmott et al., 1990), and TNF-α (Kronborg et al., 1993; Bonfield et al., 1995) have been found within the BALF from CF patients. In addition, IL-6 has been reported to be modestly elevated in infected CF patients compared with healthy control subjects (Bonfield et al., 1995; Noah et al., 1997).

The process of eradicating PA from the lung represents a complex network of effector cells and cytokines. Taken together, the observations made from BALF studies suggest that airway damage likely results from the sum of acute and chronic infection and inflammation (Konstan et al., 1994; Cantin, 1995). The regulatory mechanisms involved in controlling this process are fine-tuned; either an excess production of pro- or anti-inflammatory cytokines by airway cells may compromise the efficiency of the inflammatory response. The anti-inflammatory cytokine, IL-10, has been proposed to play a key role in maintaining the inflammatory homeostasis in CF airways.

Bonfield et al. (1995) reported that BALF from CF patients contained significantly less IL-10 than did that of healthy control subjects. Interestingly, intracellular fluorescent staining revealed that alveolar macrophages from CF patients appeared to be a source of IL-10 whereas alveolar macrophages from healthy control patients did not appear to be producing IL-10. In a subsequent study by the same group, it was determined that bronchial epithelial cells from healthy airways are capable of producing IL-10 and this production is defective in chronically infected CF lungs (Bonfield et al., 1995). Thus, in addition to alveolar macrophages, the bronchial epithelium is implicated to play an important role in regulating the local immune response in the CF lung as demonstrated by the constitutive production of IL-10, which in turn may suppress macrophage activity, antigen presentation and neutrophil phagocytosis (Moore et al., 1993). CD4+ T cells from CF patients have also been shown to secrete significantly less IL-10 upon polyclonal activation in comparison to similarly

treated cells obtained from healthy control patients (Moss et al., 1996). Given the fact that high levels of pro-inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α) and low levels of anti-inflammatory IL-10 are observed in the lungs of CF patients, it becomes apparent that IL-10 may play a key role in modulating inflammatory homeostasis in the lung. Further, it is plausible that sufficient IL-10 production is needed to deter the onset of lung tissue damage ensuing from the over zealous inflammation in response to PA infection (Melvin Berger, personal communication).

1.1.10. Interleukin-10

IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF) based on its ability to downregulate the production of IFN-γ, IL-2, and TNF-β from T cells, macrophages and monocytes, and PMN (Moore et al., 1993). Although IL-10 was originally identified as an important mediator of T_H2 driven immune responses (Howard and O' Garra, 1992), it is now evident that IL-10 can also be secreted from T_H1 cells and activated macrophages (Abbas et al., 1996).

The human IL-10 gene is located on chromosome 1 and its expression is tightly regulated as very little constitutive expression of IL-10 is observed (de Vries, 1995). IL-10 is capable of downregulating its own mRNA production in an autocrine manner in human monocytes (de Waal Malefyt et al., 1991) and in alveolar macrophages stimulated with LPS (Toossi et al., 1996). Human interleukin-10 is composed of 160 a.a. and its molecular weight is 18.5 kDa. In order to exert a biological action and interact with its

110 kDa receptor, IL-10 must form a homodimer. IL-10 is produced by CD4+, T_H0, T_H1, B lymphocytes, mast cells, eosinophils, monocytes, macrophages and keratinocytes (Moore et al., 1993; de Vries, 1995; Lalani et al., 1997).

IL-10 has a diverse array of actions, which are dependent on the cell type, target, and surrounding immune stimulus. In murine models, IL-10 has been shown generally to inhibit T_H1 but not T_H2 cell growth by downregulating IL-12 production from antigen presenting cells (Muraille and Leo, 1998). Moreover, IL-10 is generally regarded as a potent anti-inflammatory cytokine capable of eliciting inhibitory activity on T cells (Fiorentino et al., 1991) and on macrophage and APC functions (Moore et al., 1993). IL-10 has been shown to reduce the production of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and granulocyte-macrophage colony stimulating factor (GM-CSF). IL-10 has also been shown to downregulate expression of class II histocompatibility molecules as well as costimulatory molecules for T cell activation including CD54, CD80, and CD86, and to inhibit the oxidative burst of phagocytes, and their production of nitric oxide (de Waal Malefyt et al., 1991; Fiorentino et al., 1991; Oswald et al., 1992; Ding et al., 1993; Willems et al., 1994; Chang et al., 1995). At the same time, IL-10 has been shown to upregulate the anti-inflammatory IL-1 receptor antagonist (Yssel et al., 1992; Gerard et al., 1993; Howard et al., 1993; Moore et al., 1993; Cassatella et al., 1994). Of particular relevance to CF airway inflammation, IL-10 has been reported to have potent inhibitory effects on IL-8

and TNF-α mRNA expression by PMN (Cassatella et al., 1993; Gerard et al., 1993; Howard et al., 1993; Kasama et al., 1994).

1.1.11. Role of IL-10 in Animal Models of Infection

IL-10 has been proposed to a play an important role in various infections. *In vivo*, treatment with IL-10 has been shown to protect mice from LPS-induced shock in response to the over-production of IL-1, IL-6, and TNF-α associated with endotoxemia (Gerard et al., 1993; Howard et al., 1993). Similarly, mice treated with anti-IL-10 polyclonal antibody before LPS administration resulted in a substantial increase in serum TNF-α and MIP-2 levels and significant increased LPS-induced mortality (Standiford et al., 1995). In contrast, IL-10 production during some infections, including *Streptococcus pneumonia* (van der Poll et al., 1996), *Listeria monoctyogenes* (Kelly and Bancroft, 1996), and *Trypanosoma cruzi* (Reed et al., 1994) can be detrimental to the outcome (van der Poll et al., 1996). Indeed, anti-IL-10 treatment induces the clearance of *Mycobacterium avium intracellulare* in mice (Bermudez and Champsi, 1993) and augments survival in a murine model of *Klebsiella pneumonia* infection (Greenberger et al., 1995).

The importance IL-10 in these models of infection can be attributed to the capability of this cytokine to maintain an appropriate balance between pro- and anti-inflammatory cytokines (Standiford et al., 1996a; Standiford et al., 1996b; Walley et al., 1996). When IL-10 administration results in protection to the host, it is generally thought

that this is due to the downregulation of septic-shock inducing cytokines, such as TNF-α and IL-1. Conversely, when IL-10 administration is detrimental to the host, it is proposed that the downregulation of important inflammatory cytokines prevents the generation of an appropriate inflammatory response necessary for the clearance of invading pathogens.

1.1.12. Animal Models of Chronic Lung Infection with PA

In order to study the inflammatory processes induced by chronic PA lung infection and to develop immunomodulatory measures, it is useful to employ animal models. The first experimental model of chronic PA infection was developed by Cash et al. (1979) in normal rats infected intratracheally with an inoculum of bacteria embedded in agar beads. Histological examination revealed that infection and subsequent host responses closely resembled CF lung disease; the initial bacteria load was not cleared but rather proliferated, and bacteria was still present 35 days after infection. Other groups also utilized the rat model of chronic PA lung infection using agar beads (Cochrane et al., 1988; Martin et al., 1988; Konstan et al., 1990; Iwata and Sato, 1991; Hart et al., 1993; Johansen, 1996; Johansen et al., 1996; Sato et al., 1997; Song et al., 1997) or seaweed alginate (Pedersen et al., 1990) to entrap bacteria. In addition to rats, other models of chronic PA lung infection have been developed in guinea pigs, cats, hamsters and rhesus monkeys (Johansen, 1996).

The agar bead model of chronic PA lung infection has also been developed in mice (Sordelli et al., 1992; Wilmott and Fiedler, 1994; Johansen, 1996; Feldman et al.,

1998). Starke et al. (1987) developed a similar agar bead model of infection with another bacteria, which increasingly causes infection in CF patients, namely, *Burkholderia cepacia*. The studies conducted by these groups, and others, have established the validity of the agar bead model of chronic PA lung infection.

Murine models of various infectious diseases have proven useful to gain an understanding of disease pathogenesis. Indeed, inbred strains of mice offer a significant advantage over other animals in terms of genetic analysis. As successfully demonstrated for the *Nramp1* gene, which controls host resistance to infection with a diverse spectrum of intracellular microorganisms, murine models have proven useful for determining the chromosomal location, physical mapping and cloning of a candidate gene (Vidal et al., 1993; Cellier et al., 1994; Blackwell et al., 1995). In a similar manner, researchers propose to utilize mouse strains identified as either genetically resistant or susceptible to chronic bronchopulmonary PA infection in order to identify the gene(s) other than the *Cftr* gene that are responsible for regulating the host response to chronic PA infection.

Previous reports employing the agar bead model of chronic PA lung infection with doses ranging from $1x10^4$ to $1x10^6$ CFU PA established that the C57BL/6 and A/J strains are more susceptible to PA lung infection compared to BALB/c mice (Gosselin et al., 1995; Morissette et al., 1995; Stevenson et al., 1995; Morissette et al., 1996). Furthermore, the DBA/2 strain of mice was deemed to be most susceptible among strains tested to PA lung infection as the majority of these mice succumb by 3 days post infection. (Morissette et al., 1995; Morissette et al., 1996). Infecting mice intratracheally

with a single dose of 10^5 CFU PA-impregnated beads, Stevenson et al. (1995) demonstrated the establishment of a prolonged, severe endobronchial infection lasting more than 4 weeks in susceptible C57BL/6 mice whereas resistant BALB/c mice cleared the infection in a significantly shorter time. Excessive, predominantly neutrophilic inflammation with concomitant tissue damage was apparent in the lungs of susceptible C57BL/6 mice whereas chronic, granulomatous inflammation with minor tissue damage was observed in the lungs of resistant BALB/c mice (Stevenson et al., 1995; Sapru et al., 1998; Tam et al., 1998).

Intrinsic differences in immune responses to PA lung infection may explain the inherent resistance and susceptibility of BALB/c and C57BL/6 strains of mice, respectively. Lung interstitial T cells from susceptible C57BL/6 mice had significantly reduced *in vitro* responses to mitogen and specific antigen compared to cells from resistant BALB/c mice, which did not have this defect (Stevenson et al., 1995). Similar to CF patients, susceptible C57BL/6 mice had pronounced serum levels of PA-specific antibodies (Stevenson et al., 1995). Gosselin et al. (1995) reported that early, high levels of TNF-α mRNA expression and TNF-α protein secretion in mice infected with PA were associated with host resistance. In addition, the authors also suggested that more efficient NO production in the first 24 hours post infection could exert a protective role in PA lung infection (Gosselin et al., 1995).

1.1.13. Role of IL-10 in Mouse Models of PA Infection

As in CF patients, IL-10 has been implicated to play a role in chronic PA lung infection in mice. Using a model of PA pneumonia, Sawa et al. (1997) determined that IL-10 production was increased in the lungs of BALB/c mice infected with cytotoxic strains of PA. It should be noted that this group employed a model of acute, non-persistent PA infection that incurred significant mortality rather than a model that incurred chronic pulmonary infection. Another group reported increased pathology in IL-10 gene knockout mice on a C57BL/6 background relative to wild-type C57BL/6 mice in a model which characterized the effects of repeated respiratory exposure to PA (Yu et al., 1998). This group utilized a model of repeated respiratory exposure to PA and did not report the bacterial burden within the lungs following infection. Thus, it is unlikely that this method induced a chronic bronchopulmonary infection.

1.1.14. Studies with CF Mice

The first evidence of the successful generation of a murine model for CF came from researchers at the University of North Carolina whose papers were published in 1992 (Clarke et al., 1992; Snouwaert et al., 1992). The *Cftr* locus was targeted and interrupted in a series of murine embryonic stem cell lines and then introduced into early mouse embyros. The resulting mice were used to generate heterozygotes, and these were bred to produce homozygous offspring with two copies of the defective gene, termed knockout mice. (This first knockout mice strain lacking the functional CFTR was

designated *Cftr*^{m1unc}). The breeding of these mice occurred in an expected mendelian ratio and there were no reproductive abnormalities (Collins and Wilson, 1992). However, these mice failed to develop any spontaneous lung pathology, even in adulthood, despite the onset of severe bowel disease, which consequently led to intestinal obstruction, perforation, fatal peritonitis, and death usually before 40 days (Collins and Wilson, 1992). Subsequent studies revealed that housing under sterile conditions and the use of sterile diets could prevent intestinal obstruction (Eckman et al., 1995; Kent et al., 1996). Other groups have also generated CF mice (Dorin et al., 1992; O'Neal et al., 1993; Ratcliff et al., 1993). In addition, models carrying the ΔF508 (Colledge et al., 1995; van Doorninck et al., 1995) and G551D (Delaney et al., 1996) mutations at the *Cftr* locus have been developed.

Rozmahel et al. (1996) using another strain of knockout mice, termed *Cftr*^{m1HSC}/*Cftr*^{m1HSC}, demonstrated a difference in the severity of intestinal disease between CF knockout mice on different genetic backgrounds. The authors suggested that modifier genes could influence the response in CF, which either directly or indirectly could partially correct the abnormalities of Na^+ and Cl^- epithelial ion transport. These include a non-*Cftr* Cl^- conductance gene, the activity of which may compensate, in part, for the lack of intestinal CFTR.

Kent et al. (1997) hypothesized from these findings that the mixed genetic background of the original *Cftr*^{m1unc} strain might influence the development of lung pathology. That is, there are likely to be alternative Cl^- channels produced (exclusive of

the CFTR) within the airways of mice that can compensate for the mutated CFTR and, thus, maintain lung homeostasis. Moreover, having alternative Cl^- channels available could play an important role in modulating the salt concentration of the ASF, as it has been shown that high salt concentrations can inactivate anti-bacterial molecules such as β-defensin-1, lysozyme and lactoferrin, as described above. In order to investigate this possibility, heterozygous mice from the original outbred *Cftr*^{m1unc} colony were backcrossed onto the C57BL/6 background for 18 generations to ensure 100% homozygocity for C57BL/6 alleles as determined by microsatellite typing (Matouk et al., 1996). The novel congenic strain was designated C57BL/6 *Cftr*^{m1unc}/*Cftr*^{m1unc} and these mice were found to develop features consistent with small airway disease, although no infectious pathogens common to CF were apparent in the airways (Kent et al., 1997).

Infection studies have been carried out in CF knockout mice. Snouwaert et al. (1995) reported that *Cftr*^{m1unc} (-/-) mice did not differ in their ability to clear single or repetitive intranasal infection with *S. aureus* compared to *Cftr*^{m1unc} (+/-). Using another congenic strain, *Cftr*^{m1HGU}, Davidson et al. (1995) reported higher bacterial loads and more pronounced disease in the lungs of *Cftr*^{m1HGU} (-/-) mice compared to *Cftr*^{m1HGU} (+/+) littermate controls after repeated aerosol exposure to *S. aureus* and *B. cepacia*. It should be noted that neither group studied lung infection with PA, the most common and distinctive pathogen in the CF lung. Recently, however, two groups reported findings using the agar bead model to induce chronic PA lung infection in CF mice (van Heeckeren et al., 1997; Gosselin et al., 1998).

van Heeckeren et al. (1997) used mice homozygous for the S489X mutation of the *Cftr* gene and observed that mortality of the CF mice was significantly greater than that of normal animals 10 days after intratracheal infection with PA. This group did not observe any significant difference in the bacterial burden between the CF and wild-type littermates whereas significantly higher concentrations of inflammatory mediators were found in the BAL of CF mice compared to wild-type littermates 3 days post infection. It should be noted that this group employed a unilateral method of infection in which only the right side lung of was instilled with bacteria. Furthermore, neither the genetic background nor the homozygocity of the mice used in the studies were indicated. In contrast, Gosselin et al. (1998) used C57BL/6-*Cftr*^{unc}/*Cftr*^{unc} (also referred to as C57BL/6-*Cftr*^{m1unc}/*Cftr*^{m1unc}) mice backcrossed 10 to 12 times with C57BL/6 mice to ensure 100% homozygocity for C57BL/6 alleles and reported significantly higher mortality and bacterial burden in the lungs of *Cftr* gene knockout (-/-) mice compared to littermate control (+/+) mice 6 days following bi-lateral intratracheal infection with 10^5 PA.

1.2. Objectives of the Proposed Study

The on-going goal of our laboratory is to characterize the host immune response to chronic bronchopulmonary PA infection. In order to do so, we (Stevenson et al., 1995; Sapru et al., 1998; Tam et al., 1998) and others (Gosselin et al., 1995; Morissette et al., 1995) have previously established that inbred mouse strains differ in their resistance and susceptibility to PA infection. Using genetically resistant BALB/c and susceptible C57BL/6 mice, we have developed a model of chronic lung PA infection by infecting mice intratracheally with $1\text{-}2x10^5$ CFU of mucoid PA entrapped in agar beads. This method of infection results in a sustained, local inflammatory response in the lungs of both strains of mice. However, resistant BALB/c were found to have a significantly reduced bacterial load within their lungs compared to susceptible C57BL/6 mice (Stevenson et al., 1995). This difference was apparent by day 7 post infection and could be consistently observed until 35 days post infection. Chronic, granulomatous inflammation was observed in the lungs of the resistant BALB/c mice and this appeared to correlate with protection as only minor tissue damage was observed (Sapru et al., 1998; Tam et al., 1998). In contrast, acute, predominantly neutrophilic inflammation with extensive tissue damage was apparent in the lungs of susceptible C57BL/6 mice (Sapru et al., 1998; Tam et al., 1998).

The purpose of my research was to further examine this marked difference in inflammation between the strains and determine the role of IL-10 during chronic

bronchopulmonary PA infection in resistant BALB/c and susceptible C57BL/6 mice. Based on previous observations (Sapru et al., 1998), we chose to examine the differences in the lung inflammatory response on day 7 days post PA infection, as this was the time when significant differences in various disease parameters could be readily observed. First, we examined the quantitative difference in the total and differential cell numbers recruited to the lung. Next, we determined the levels of various pro- and anti-inflammatory cytokines from the entire lung tissue, and in *in vitro* cultures of macrophages and PMN obtained from the lungs by lavage.

The second objective, elucidating the role of IL-10 during chronic bronchopulmonary PA infection, required the administration of neutralizing IL-10 mAb before and during infection. Anti-IL-10 treated groups of mice were compared to control rat IgG treated mice for differences in bacterial burden and levels of pro-inflammatory cytokines. Furthermore, the effect of anti-IL-10 treatment on cell recruitment to the lungs was also examined.

Our data, using non-CF mice, demonstrate inherent differences in the host response to PA lung infection, thus, supporting the proposition that genes outside the *Cftr* locus influence the host immune response to lung PA infection. Furthermore, we have shown that IL-10 depletion exacerbates PA lung infection and inflammation irrespective of the genetic background of the host.

Chapter 2: Materials and Methods

2.1. Mice

Female age-matched BALB/c and C57BL/6 mice, 8-10 weeks old, purchased from Charles River (St. Constant, QC) were used in all experiments according to guidelines and regulations of the Canadian Council on Animal Care. Food and water were provided *ad libitum*.

2.2. Anti-Murine IL-10 mAb and Neutralization Protocol

Anti-IL-10 monoclonal antibody (mAb)-containing ascites fluid was collected from BALB/c mice inoculated i.p. 7 days previously with 10^7 JES5-2A5 hybridoma cells (American Type Culture Collection; Rockville, MD). Neutralizing antibodies were purified by ammonium sulfate precipitation and concentration measured. Antibody concentration was determined both by ELISA and spectrophotometrically against known quantities of rat IgG. For IL-10 neutralization experiments, randomly designated animals were treated with anti-murine IL-10 mAb JES5-2A5 or rat IgG (Sigma, St. Louis, MO) as control Ab (Howard et al., 1993; Sawa et al., 1997). Mice were administered 200 μg of JES5-2A5 or rat IgG i.p. on days –2, 0, 2, and 4 throughout the course of PA infection.

2.3. *Pseudomonas aeruginosa*

PA, strain 508, was a kind gift from Dr. Jacqueline Lagacé (Université de Montreal, QC). This strain has a mucoid appearance when grown on blood agar and was originally isolated from the sputum of a CF patient at Ste-Justine Hospital, Montreal, QC.

2.4. Inoculum

PA entrapped in agar beads was prepared according to a modification of previously described methods (Starke et al., 1987; Stevenson et al., 1995). Briefly, log phase bacteria grown in 4% proteose peptone (Difco, Detroit, MI) were concentrated 10-fold and 1 ml was added to 9 ml of 1.5% trypticase soy agar pre-warmed to 50°C. This mixture was added to 150 ml heavy mineral oil at 50°C and stirred rapidly with a magnetic stirring bar for 6 min at 22°C, followed by cooling with continuous stirring for 10 min more. The oil-agar mixture was centrifuged at 15,000 x g for 20 min to sediment the beads. The oil was removed and the beads were subsequently washed 3 times in PBS at 400 x g for 10 min at 22°C. The size of the beads was verified microscopically and only those preparations containing beads predominantly 100-150 μm in diameter were used as inoculum. The number of bacteria was estimated by homogenizing the bacteria-bead suspension using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY) and plating 10-fold serial dilutions on trypticase soy agar (BBL, Becton Dickinson & Co., Cockeysville, MD). The plates were incubated overnight at 37°C and the number

of CFU counted. The inoculum for infection was prepared by diluting the bead suspension with PBS to $4x10^6$ CFU/ml.

2.5. Intratracheal Infection

Mice were anesthetized with a combination of ketamine (15 mg/ml) and xylazine (2 mg/ml) administered intramuscularly at a dose of 0.2 ml. Following a transverse cervical incision, the trachea was exposed and intubated with a sterile, flexible 22 g cannula attached to a 1.0 ml syringe. An inoculum of 50 µl, containing approximately 1-$2x10^5$ CFU, was implanted via the cannula into the lung. After inoculation, all incisions were closed by suture in an aseptic manner. None of the animals developed wound infection and healing occurred in 2-3 days.

2.6. Lung Homogenates

Lungs, harvested from infected mice, were homogenized for 60 sec at high speed (homogenizer PT10135 Brinkmann Instruments Co., Mississauga, ON) in 5 ml of PBS. Serial 10-fold dilutions of lung homogenates were plated on petri dishes containing TSA. The number of CFUs per lung was counted after overnight incubation at 37°C. For cytokine determinations, lung homogenates were centrifuged at 1500 x g at 4°C for 10 min. Samples were chilled on ice until the supernatants were removed and stored at -20°C until assayed for cytokine concentrations.

2.7. Bronchoalveolar Lavage

Mice were sacrificed by CO_2 overdose, exsanguinated by cutting the vena cava, and the circulation was flushed by slow intracardiac infusion of cation-free Hank's balanced salt solution (HBSS; GIBCO-BRL, Burlington, ON). The trachea was cannulated with a 22-gauge I.V. catheter placement unit (Critikon, Inc., Tampa, FL) connected to two 10 ml syringes via a 3-way stopcock with rotating collar. The lungs were lavaged with a total volume of 10 ml cation-free HBSS in 1.0 ml aliquots. Total cell numbers and types of inflammatory cells in the BALF from individual mice were determined by standard procedures. Briefly, total cells, diluted in Turk's fluid, were counted using a hemocytometer. Percent viability was determined by trypan blue exclusion and was greater than 90%. Differential counts were determined on Cytospin (Shandon Lipshaw, Pittsburgh, PA) preparations stained with Diff-Quick stain (Baxter Health Corporation, McGaw Park, IL). Individual BALF samples were pooled, pelleted by centrifugation at 350 x g for 10 min, and treated with lysis buffer (cold NH_4CL, 0.17 M) to remove the erythrocytes. The cells were washed and resuspended to $2x10^6$ macrophages/ml in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum (Hy-Clone, Logan, UT), 25 mM HEPES buffer (GIBCO-BRL) and 0.12% gentamycin (Schering, Pointe Claire, QC). Aliquots of 100 µl of the cell suspensions were plated in triplicate in 96-well flat bottom plates (Falcon Microtest II, Fisher Scientific, Lincoln Park, NJ). Macrophage monolayers were prepared by incubating plates for 2 hours at 37°C in a 5% CO_2 atmosphere. Non-adherent cells,

containing PMN, were removed by washing plates 3 times with warm HBSS and collected in sterile tubes. Total and differential cell counts were performed on recovered, non-adherent cells. Cells were resuspended to a concentration of $2x10^6$ PMN/ml, and aliquoted in flat-bottom plates, as described above. Macrophages and PMN were stimulated overnight for 20 hours by adding 0.1 μg *Escherichia coli* 0127:B8 lipopolysaccharide (LPS; Difco) or $2x10^6$ CFU heat-killed PA (HKPA) in complete medium. Unstimulated cultures were incubated with medium alone as controls. Supernatants were collected, immediately assayed for NO production and then frozen at -20°C until assayed for cytokines by ELISA.

2.8. NO Production

Levels of NO produced by bronchoalveolar macrophages and PMN following overnight stimulation *in vitro* were determined using Griess reagent. Briefly, 50 μl aliquots of each cell-free supernatant were added to an equal volume of Griess reagent (a freshly prepared 1:1 mixture of 1% sulfanilamide (Sigma Chemical Co., St. Louis, MO) in 2.5% H_3PO_4 and 0.1% *N*(-1-napthyl)-ethylenediamine dihydrochloride (Sigma) in distilled water) and the A_{550} was measured after 5 minutes using an ELISA reader. NO concentrations in μM were calculated using 10 mM $NaNO_2$ as a standard.

2.9. ELISAs

Cytokines were measured by two-site Ab sandwich ELISAs specific for TNF-α, IFN-γ, IL-10 or IL-12, as previously described (Sher and Coffman, 1992; Stevenson et al., 1992a; Stevenson et al., 1992b). Briefly, 96-well polyvinvylchloride microtitre Immunolon II plates (Dynatech, Chantilly, VA) were coated (50μl/well) with cytokine-specific primary mAb diluted in PBS. The primary antibodies used were as follows: hamster anti-murine TNF-α (Genyme, Boston, MA); murine anti-rat IFN-γ DB.1 (Bio-Source International, Camarillo, CA) which cross reacts with murine IFN-γ as previously described (van der Meide et al., 1986); rat anti-murine IL-10 JES5-2A5 (ATCC); and a mixture of hamster and rat anti-murine IL-12 (p35/p70) (PharMingen, San Diego, CA). After incubating overnight at 4°C, plates were washed 3 times with wash buffer (PBS and 0.1% Tween 20) and wells were blocked for 1 hr at room temperature with blocking buffer (PBS, 0.1% Tween 20, and 1% BSA). Cytokines standards used were as follows: recombinant murine TNF-α (Genzyme); recombinant murine IFN-γ (Genzyme); recombinant murine IL-10 (a kind gift provided by Dr. LuAnn Thompson-Snipes, Montreal General Hospital Research Institute, Montreal, QC); and recombinant IL-12 (a kind gift provided by Dr. S. Wolf, Genetics Institute, Cambridge, MA). Standards and samples were diluted in blocking buffer, added to plates (50μl/well), and incubated at room temperature for 1 – 2 hr, except for IL-12 which was incubated overnight at 4°C. After incubation, plates were washed 3 times with wash buffer and secondary antibodies, diluted in blocking buffer, were added (50μl/well) as follows: polyclonal rabbit anti-

murine TNF-α antiserum prepared and standardized in our laboratory (Stevenson et al., 1992a); polyclonal rabbit anti-murine IFN-γ antiserum prepared in our laboratory by standard methods (Stevenson et al., 1992b); biotin-conjugated rat anti-murine IL-10 mAB, SXC-1 (PharMingen); biotin-conjugated rat anti-murine IL-12 mAb, C15.6 prepared in our laboratory. After 1-2 hr incubation at room temperature, plates were washed 3 times with wash buffer. Horseradish peroxidase-conjugated to anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) or streptavidin (Bio-Rad) diluted in blocking buffer was added (50μl/well) and incubated for 1 hr at room temperature. After washing, ABTS substrate (Boehringer, Mannhein, Germany) was added (100 μl/well) and colour developed at room temperature. After 10 – 15 min, the colour reaction was read at 492 nm using a standard ELISA reader.

2.10. Statistics

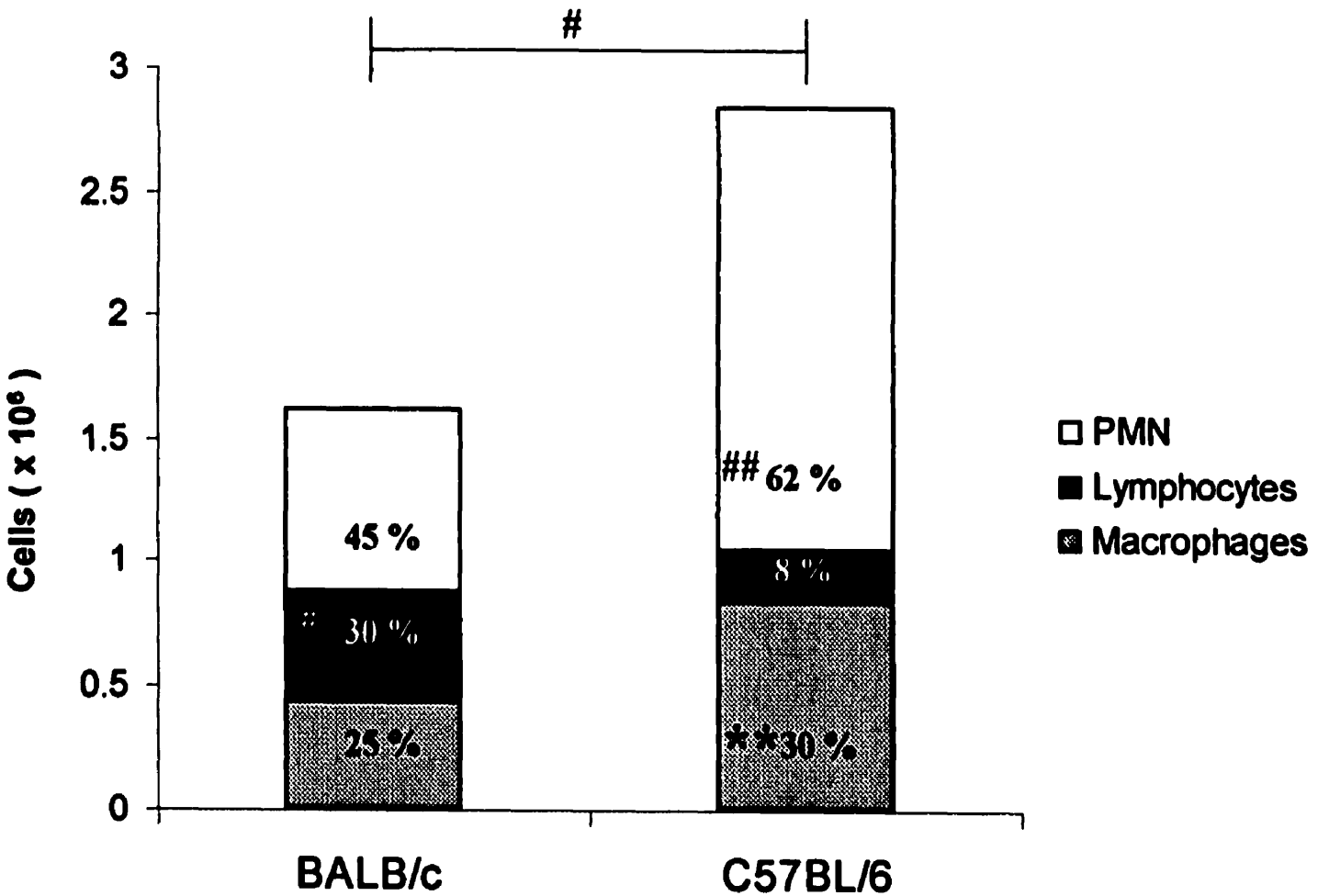
Data are presented as the mean ± SEM of replicate experiments as indicated. Statistical differences between means of controls and various treatment groups were evaluated using a nonparametric Student's t-test and ANOVA as indicated carried out with GraphPad InStat (version 3.00; GraphPad Software, San Diego, CA). Significance was set at a two-tailed p value of ≤ 0.05.

Chapter 3: Results

3.1. Cellular Composition after Intratracheal PA Infection

Initial experiments were performed to characterize the cellular response induced by intratracheal instillation of PA-impregnated agar beads. As shown in Figure 3, a pronounced inflammatory response was apparent by 7 days post infection in both mouse strains. Examination of BALF revealed significantly greater total cell numbers (2.85 ± 0.32 vs. $1.62 \pm 0.17 \times 10^6$; $p<0.005$), PMN (1.79 ± 0.24 vs. $0.74 \pm 0.10 \times 10^6$; $p<0.001$), and alveolar macrophages (0.83 ± 0.11 vs. $0.43 \pm 0.07 \times 10^6$; $p<0.005$), respectively, from susceptible C57BL/6 mice compared to resistant BALB/c mice. This coincided with significantly fewer lymphocytes (0.45 ± 0.05 vs. $0.22 \pm 0.04 \times 10^6$; $p<0.005$, respectively) in the BALF from susceptible C57BL/6 mice compared to resistant BALB/c mice. BALF obtained from uninfected, control mice of both resistant and susceptible strains revealed no significant differences in either the total or differential cell numbers (data not shown) with alveolar macrophages accounting for 99% of the cells recovered by lavage.

Figure 3. Number and type of cells harvested from the lungs of resistant BALB/c and susceptible C57BL/6 mice following PA infection. Mice were infected intratracheally with 1-2x10^5 CFU of PA-impregnated beads and cells were harvested by bronchoalveolar lavage 7 days later from 15-18 mice per strain. Contents of airways were flushed out with 10 ml cold cation-free HBSS. The total numbers of PMN (white bars), lymphocytes (black bars) and alveolar macrophages (grey bars) were calculated on the basis of differential cell counts performed on Diff-Quick cytospin preparations. Significance is indicated by ** ($p<0.01$), # ($p<0.005$), or ## ($p<0.001$) when comparing either total cell number or number of a specific cell type between strains.


#
3
2.5
2
1.5
1
0.5
0
Cells (x 10⁶)
45 %
30 %
25 %
62 %
8 %
**30 %
PMN
Lymphocytes
Macrophages
BALB/c
C57BL/6

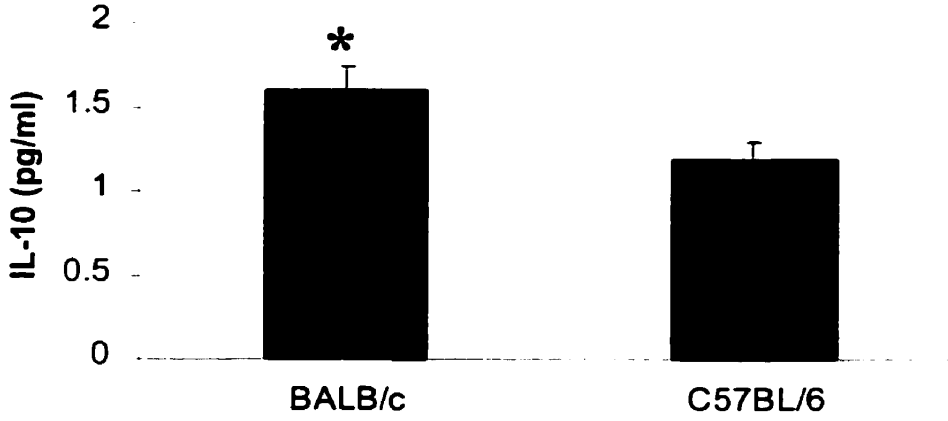
3.2. Lung Levels of Cytokines after PA Infection

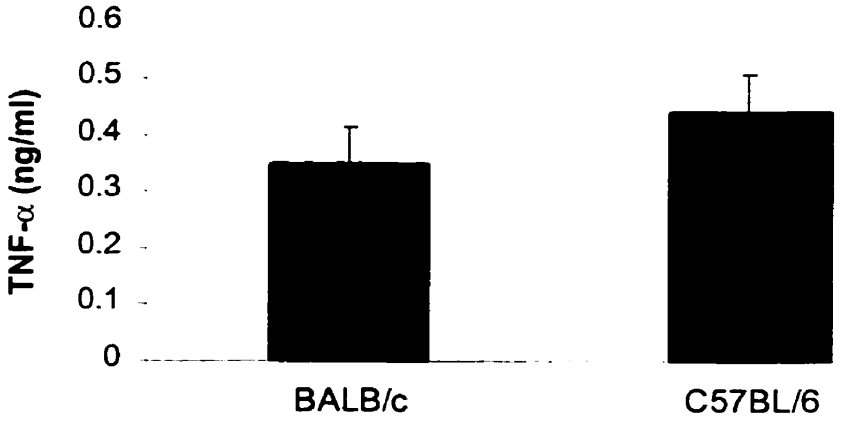
Having determined that susceptible C57BL/6 mice recruit a greater number of cells into the airways during PA infection, we next wanted to characterize the production of important cytokines during lung inflammation. As shown in Figure 4, the concentration of IL-10 apparent in the lung homogenates of resistant BALB/c mice was significantly greater ($p<0.05$) than the concentration measured in susceptible C57BL/6 mice. The levels of TNF-α in the lungs were marginally higher in susceptible C57BL/6 mice, however, this difference was not significant. Both strains of mice produced comparable levels of IFN-γ in their lungs. At day 7 post infection, IL-12 production was undetectable in the lungs of both resistant BALB/c and susceptible C57BL/6 mice (data not shown).

3.3. *In vitro* Cytokine Production from Inflammatory Cells

We next performed experiments to characterize the *in vitro* production of important cytokines by inflammatory cells following PA infection. As shown in Figure 5, the production of IL-10 by alveolar macrophages was comparable between the strains of mice. Macrophages recovered from the airways of resistant BALB/c mice, however, produced significantly higher levels of TNF-α both spontaneously ($p<0.001$) and following stimulation with LPS ($p<0.001$) or HKPA ($p<0.001$) compared to macrophages from C57BL/6 mice. In contrast, macrophages from susceptible C57BL/6 mice produced significantly higher NO levels both spontaneously ($p<0.001$) and following stimulation

Figure 4. *In vivo* production in the lung of IL-10, TNF-α, and IFN-γ from resistant BALB/c and susceptible C57BL/6 mice 7 days after intratracheal infection with 1-2x10^5 CFU of PA-impregnated beads. Lungs were removed aseptically and homogenized in sterile PBS. ELISAs were performed to assay cytokine levels in supernatants from lung homogenates. Values represent mean ± SEM of 5-16 mice per strain. Significance is indicated by * ($p < 0.05$) and represents differences between strains.


2
1.5
1
0.5
0
IL-10 (pg/ml)
*
BALB/c
C57BL/6


0.6
0.5
0.4
0.3
0.2
0.1
0
TNF-α (ng/ml)
BALB/c
C57BL/6

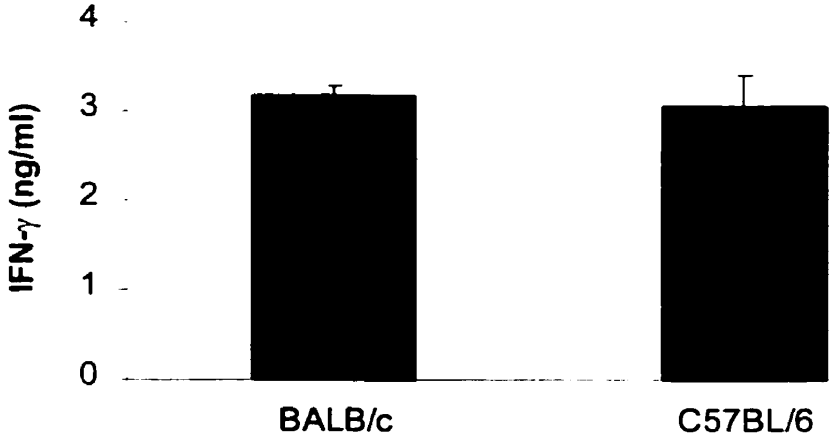
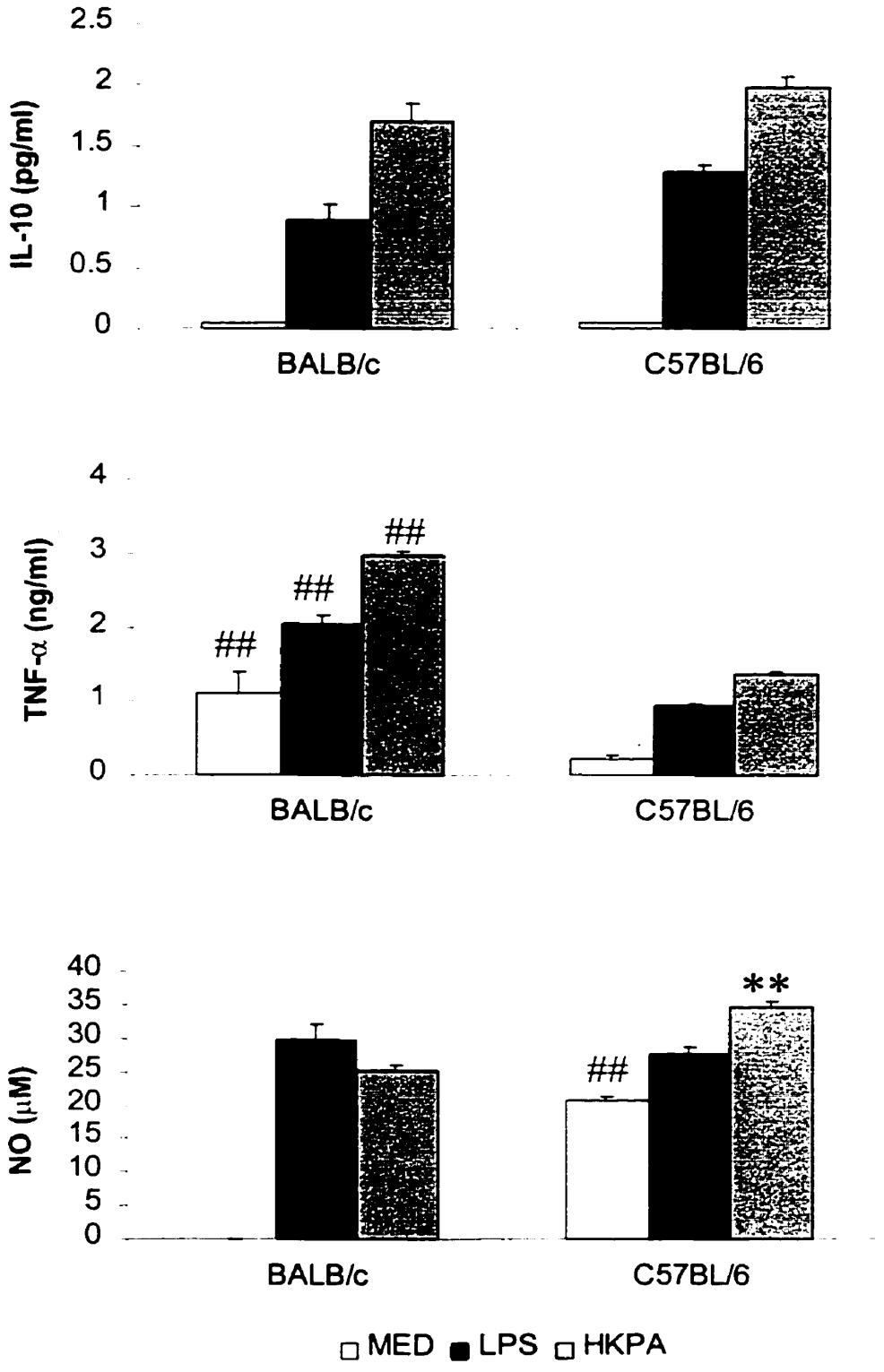

4
3
2
1
0
IFN-γ (ng/ml)
BALB/c
C57BL/6

Figure 5. *In vitro* production of IL-10, TNF-α, and NO by alveolar macrophages harvested by bronchoalveolar lavage from resistant BALB/c and susceptible C57BL/6 mice 7 days after intratracheal infection with 1-2x10^5 CFU of PA-impregnated beads. Cells were harvested from 5-8 mice per strain, pooled and macrophage monolayers prepared. Macrophages were incubated with medium (MED), 0.1 μg *E. coli* LPS (LPS) or 2x10^6 CFU heat-killed PA (HKPA). TNF-α and IL-10 concentrations in supernatants were measured by ELISA and NO concentrations were measured using Greiss reagent. Data are presented as mean ± SEM and are representative of an experiment repeated 3 times. Significance is indicated by **($p<0.01$) or ##($p<0.001$) when comparing differences between strains.


IL-10 (pg/ml)
2.5
2
1.5
1
0.5
0
BALB/c
C57BL/6
TNF-α (ng/ml)
4
3
2
1
0
##
##
##
BALB/c
C57BL/6
NO (μM)
40
35
30
25
20
15
10
5
0
##
**
BALB/c
C57BL/6
MED
LPS
HKPA

with HKPA ($p<0.01$). The production of IL-12 was not detected from macrophages of either strain (data not shown).

The *in vitro* production of cytokines by PMN recovered from the airways of PA infected mice was also examined (Figure 6). Lung PMN from resistant BALB/c mice produced significantly more IL-10 compared to susceptible C57BL/6 mice both spontaneously ($p<0.05$) and following stimulation with LPS ($p<0.05$) or HKPA ($p<0.01$). Lung PMN from both strains produced comparable levels of TNF-α after stimulation with either LPS or HKPA whereas NO production was undetectable in the culture supernatants. *In vitro* production of IL-12 from lung PMN harvested from either mouse strain was not detected (data not shown).

3.4. Effects of Anti-IL-10 Treatment on the Course of PA Infection

To determine if the anti-inflammatory properties of IL-10 play a significant role during chronic bronchopulmonary PA infection, experiments were performed to assess the biological effects of systemic IL-10 neutralization during PA infection. First, we wanted to determine if neutralization of IL-10 would have an effect on the outcome of infection in either resistant BALB/c or susceptible C57BL/6 mice. Groups of resistant BALB/c and susceptible C57BL/6 mice were treated intraperitoneally with 4 doses of neutralizing anti-IL-10 (200 μg/dose) or isotype matched rat IgG (200 μg/dose). As shown in Figure 7, treatment of animals with anti-IL-10 resulted in a significant increase ($p<0.05$) in the bacterial burden in the lungs of both resistant BALB/c and susceptible

Figure 6. *In vitro* production of IL-10, TNF-α, and NO by PMN harvested by bronchoalveolar lavage from resistant BALB/c and susceptible C57BL/6 mice 7 days after intratracheal infection with 1-2x10^5 CFU of PA-impregnated beads. Cells were harvested from 5-8 mice per strain, pooled, and non-adherent cells recovered. PMN were incubated with medium (MED), 0.1 μg *E. coli* LPS (LPS) or 2x10^6 CFU heat-killed PA (HKPA). TNF-α and IL-10 concentrations in supernatants were measured by ELISA and NO concentrations were measured using Greiss reagent. Data are presented as mean ± SEM and are representative of an experiment repeated 3 times. Significance is indicated by *($p<0.05$) or **($p<0.01$) when comparing differences between strains.

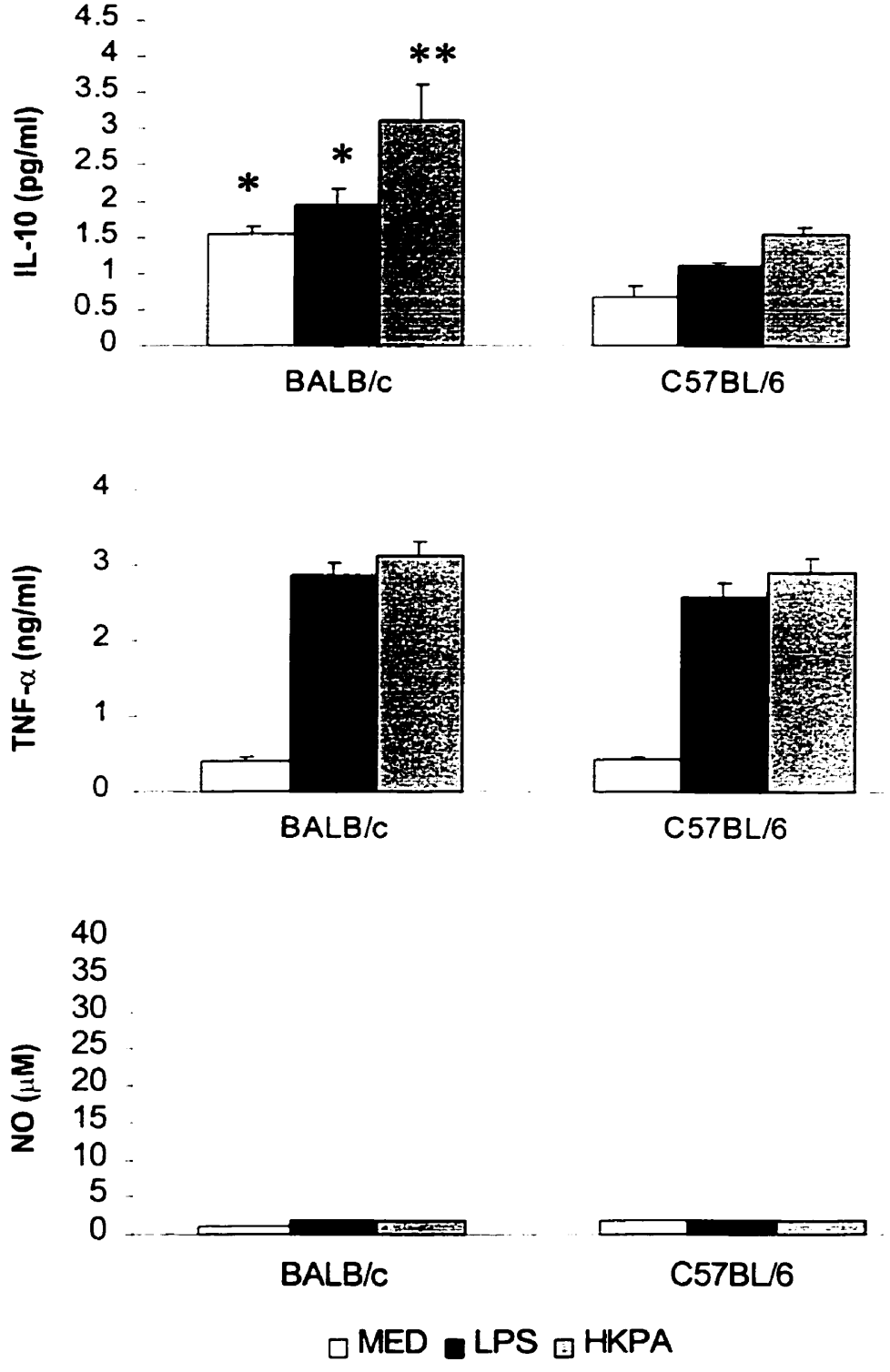
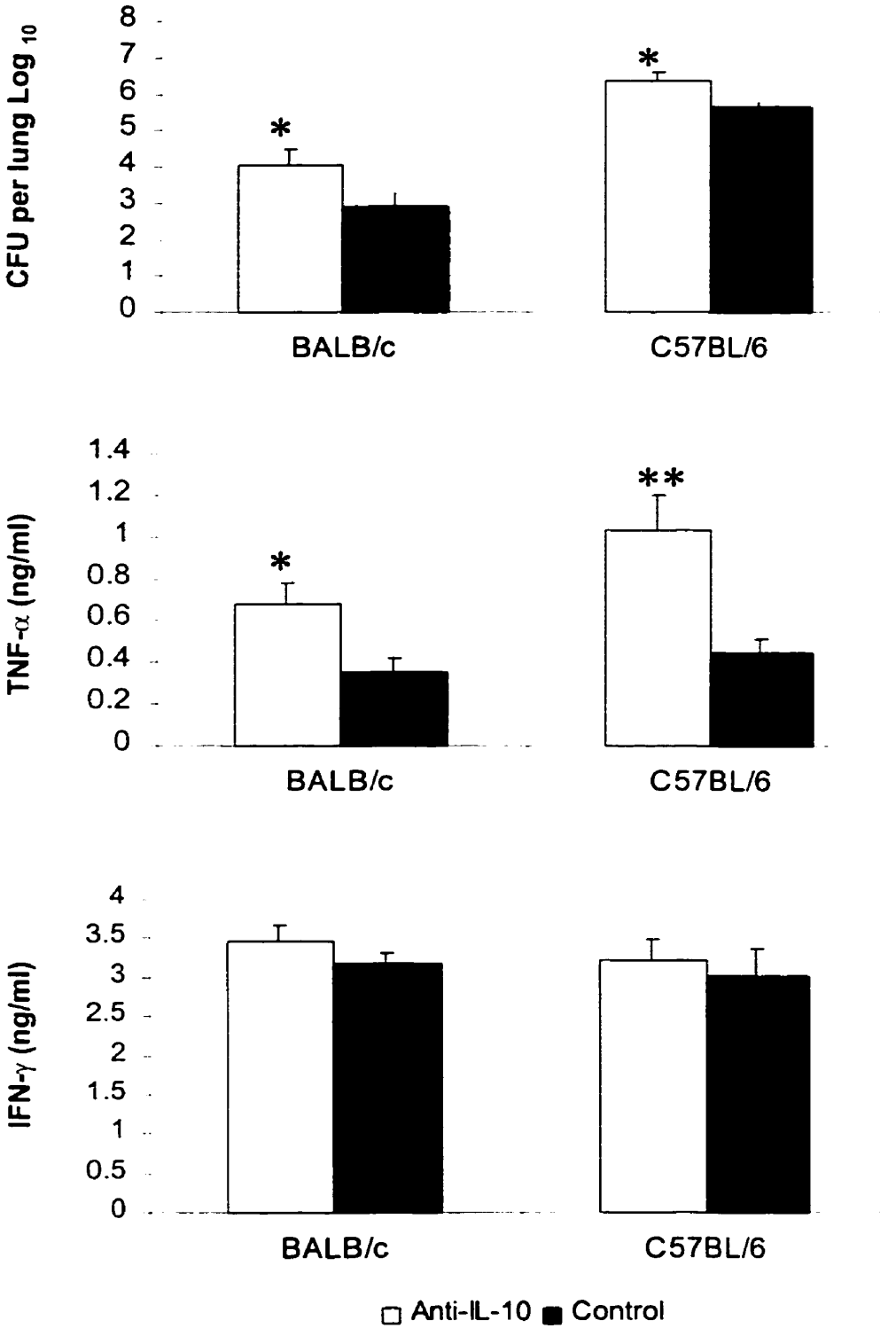

IL-10 (pg/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
**
BALB/c
C57BL/6
TNF-α (ng/ml)
4
3
2
1
0
BALB/c
C57BL/6
NO (μM)
40
35
30
25
20
15
10
5
0
BALB/c
C57BL/6
MED
LPS
HKPA

Figure 7. Effects of anti-IL-10 treatment on PA CFU, and concentrations of TNF-α and IFN-γ in the lungs of resistant BALB/c and susceptible C57BL/6 mice 7 days after intratracheal infection with 1-2x10^5 CFU of PA-impregnated beads. Lung homogenates were sequentially diluted and plated on agar plates for quantitative assessment of bacterial counts. Supernatants from lung homogenates were assayed for TNF-α and IFN-γ concentrations by cytokine-specific ELISAs. Values represent mean ± SEM from 15-18 mice per group. Significance is indicated by *($p< 0.05$) or **($p<0.001$) and represents differences between anti-IL-10 treated and control groups.

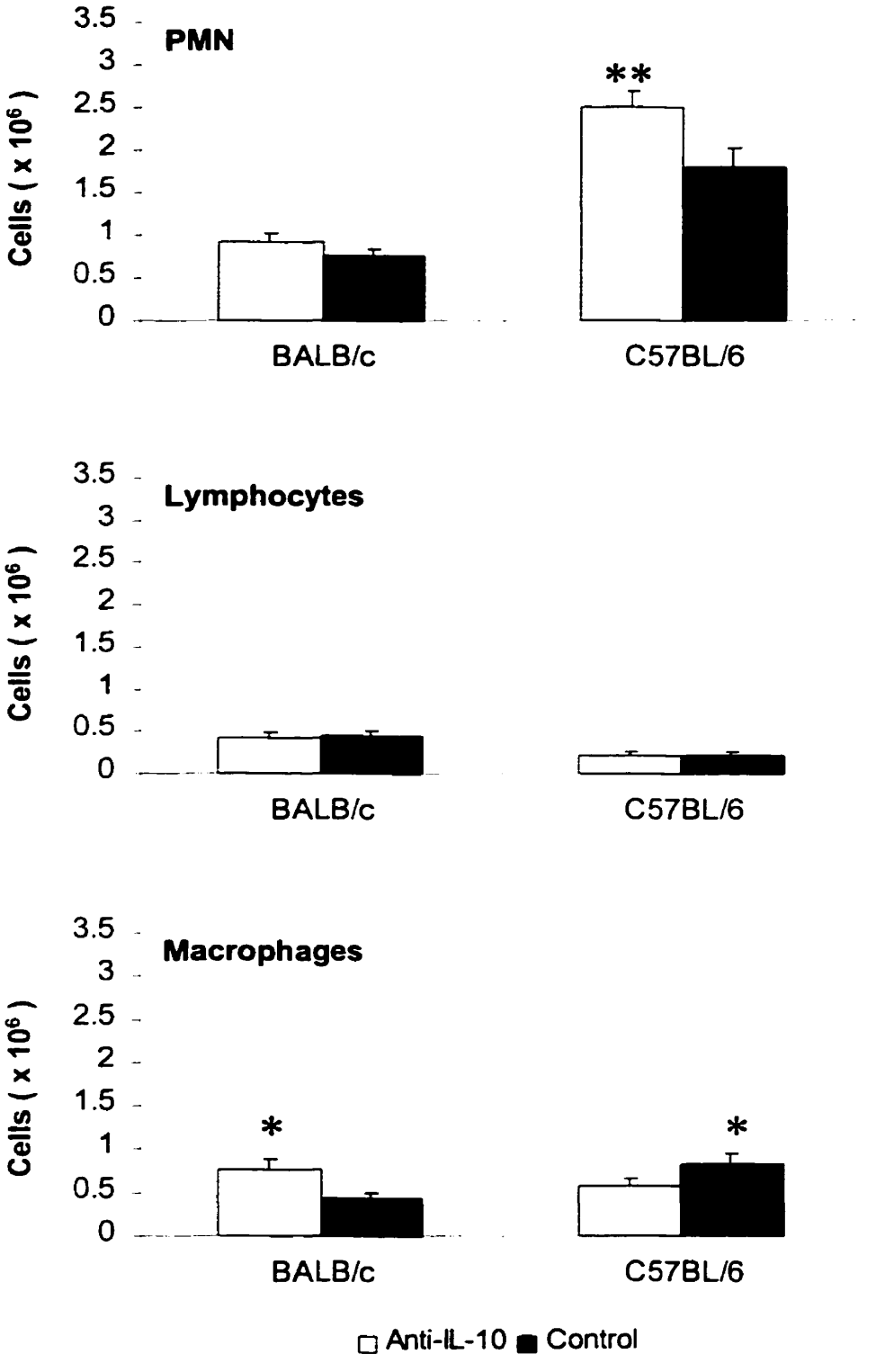

CFU per lung Log 10
8
7
6
5
4
3
2
1
0
*
*
BALB/c
C57BL/6
TNF-α (ng/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
BALB/c
C57BL/6
IFN-γ (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
BALB/c
C57BL/6
Anti-IL-10
Control

C57BL/6 mice. Interestingly, the increased bacterial proliferation was associated with significantly higher concentrations of TNF-α in the lungs of both BALB/c ($p<0.05$) and susceptible C57BL/6 ($p<0.01$) mice. No significant differences in the levels of lung IFN-γ were observed for either mouse strain after anti-IL-10 treatment. Levels of pro-inflammatory cytokine IL-12 were undetectable in the lungs of either mouse strain following neutralization of IL-10 (data not shown).

3.5. Effect of IL-10 Neutralization on Cell Recruitment to the Lungs

In order to determine if IL-10 neutralization altered the composition of the cells at the site of infection, the cell composition of BALF was also analyzed. As shown in Figure 8, neutralization of IL-10 induced a significant influx of macrophages ($p<0.05$) into the airways of resistant BALB/c mice but had no detectable effect on the number of PMN recruited to the lung. In contrast, following anti-IL-10 treatment, analysis of BALF from susceptible C57BL/6 mice revealed a significant influx of PMN ($p<0.01$) and a significant decrease ($p<0.05$) in the numbers of macrophages recovered from the airways. No significant differences in the number of lymphocytes were observed following systemic depletion of IL-10 in either mouse strain.

Figure 8. Number and type of cells harvested from the lungs of anti-IL-10 treated resistant BALB/c and susceptible C57BL/6 mice 7 days after intratracheal infection with 1-2x10^5 CFU of PA-impregnated beads. Contents of airways from 15-18 mice per strain were flushed with 10 ml cold cation-free HBSS. Total cell viability was determined using trypan blue and differential cell counts were performed by Diff-Quick cytospin preparations. Values represent mean ± SEM of 13-19 mice per group. Significance is indicated by *($p< 0.05$) or **($p<0.001$) and represents differences between anti-IL-10 treated and control groups.


PMN
Cells (x 10⁶)
3.5
3
2.5
2
1.5
1
0.5
0
**
BALB/c
C57BL/6
Lymphocytes
Cells (x 10⁶)
BALB/c
C57BL/6
Macrophages
Cells (x 10⁶)
*
*
BALB/c
C57BL/6
□ Anti-IL-10 ■ Control

Chapter 4: Discussion

Chronic bronchopulmonary PA infection is the major cause of morbidity and mortality among CF patients (FitzSimmons, 1993; Kerem, 1997). In response to infection, a pronounced recruitment of inflammatory cells to the airways with concomitant release of pro- and anti-inflammatory cytokines from both the inflammatory and resident cells is apparent (Bonfield et al., 1995; Davis et al., 1996). The excessive inflammation induced by chronic PA infection leads to a state of recurrent infection and inflammation ultimately leading to lung tissue damage (Konstan and Berger, 1993). In order to study the inflammatory response evoked by chronic PA infection and devise immunomodulatory approaches, we (Stevenson et al., 1995; Sapru et al., 1998; Tam et al., 1998) and others (Gosselin et al., 1995) have developed a model of chronic bronchopulmonary infection using PA-impregnated agar beads surgically introduced into the airways of genetically resistant BALB/c and susceptible C57BL/6 mice. Following intratracheal infection with 1-2x10^5 CFU of mucoid PA enmeshed in agar beads, resistant BALB/c were found to have a significantly reduced bacterial load within their lungs compared to susceptible C57BL/6 mice (Stevenson et al., 1995). This difference was apparent by day 7 post infection and could be consistently observed until 35 days post infection. Chronic, granulomatous inflammation was observed in the lungs of the resistant BALB/c mice which appeared to correlate with protection as only minor tissue damage was observed (Sapru et al., 1998; Tam et al., 1998). In contrast, acute,

predominantly neutrophilic inflammation with extensive tissue damage was apparent in the lungs of susceptible C57BL/6 mice (Sapru et al., 1998; Tam et al., 1998). In the present study, we have extended our characterization of this model of chronic bronchopulmonary PA infection by determining the production of important pro- and anti-inflammatory cytokines from the total lung homogenate and inflammatory cells, as well as the effects of IL-10 neutralization in resistant and susceptible strains of mice 7 days after intratracheal instillation with 1-2x10^5 CFU of PA-impregnated agar beads.

Our results indicate that it is not only important to evoke inflammatory cells to the sites of infection, but also that it is important that the cell types are present in the appropriate number and ratio (Figure 3). Based on observations in CF patients (Berger, 1991) and the results presented here using susceptible C57BL/6 mice, it is clear that a higher number of PMN is associated with chronic PA infection and tissue damage. The overabundance of PMN may be inefficient in eradicating bacteria by phagocytosis while concomitantly releasing an array of enzymes, proteases, oxidative species and cytokines which damage surrounding lung tissue (Sibille and Reynolds, 1990). As indicated by the findings from the lungs of resistant BALB/c mice, a more evenly balanced inflammatory response, composed of similar numbers of PMN and macrophages, may be better able to control bacterial proliferation and minimize tissue damage.

Alveolar macrophages have been postulated to play an important role in orchestrating the lung inflammatory response (Buret et al., 1994). Depletion of alveolar macrophages in a rat model of PA infection caused a substantial decrease in PMN

recruitment and TNF-α, CINC/gro, and MIP-2 production in the lung (Hashimoto et al., 1996). In contrast, in a murine model of *Klebsiella pneumonia*, depletion of alveolar macrophages resulted in an increase in PMN recruitment to the lungs (Broug-Holub et al., 1997). Although the exact mechanism(s) as to how alveolar macrophages influence the recruitment and activation of PMN remains unclear, it is most likely mediated through the release of various chemotactic cytokines. Future studies in our laboratory are aimed at elucidating the role of neutrophilic chemokines, such as MIP-2, during chronic PA infection.

In order to gain a better understanding of the inflammatory status of the mice, we determined the total lung concentrations of important pro- and anti-inflammatory cytokines 7 days after intratracheal infection with $1\text{-}2x10^5$ CFU of PA. We observed that genetically resistant BALB/c mice produced significantly more IL-10 in their lungs compared to susceptible C57BL/6 mice. Moreover, PMN harvested from the lungs of resistant BALB/c mice produced significantly more IL-10 compared to PMN from the lungs of susceptible C57BL/6 mice whereas alveolar macrophages from both mouse strains produced similar levels. Previous studies have indicated that IL-10 may play a role in controlling the over-zealous inflammation observed within the CF lung (Konstan and Berger, 1993; Konstan et al., 1994; Bonfield et al., 1995a; Bonfield et al., 1995b; Konstan and Berger, 1997). These studies suggest a beneficial role for IL-10 during chronic PA infection in maintaining homeostasis by modulating the inflammatory response to environmental irritants (Melvin Berger, personal communication).

IL-10 has been shown to have a beneficial effect in numerous rodent models of disease, including: acute visceral ischemia in mice (Hess et al., 1997), an IgG immune complex (BSA-anti-BSA) model of acute lung injury in rats (Mulligan et al., 1997), a murine model of *Staphylococcal enterotoxin* B (Hasko et al., 1998), and a murine model of endotoxemia (Gerard et al., 1993; Howard et al., 1993; Standiford et al., 1995). In contrast, IL-10 has also been shown to play a deleterious role during *Klebsiella pneumoniae* (Greenberger et al., 1995) and *Streptococcus pneumoniae* (van der Poll et al., 1996) infections. The importance IL-10 in these infections may be due to the ability of this cytokine to maintain an appropriate balance between pro- and anti-inflammatory cytokines (Standiford et al., 1996a; Standiford et al., 1996b; Walley et al., 1996). Thus, given the significantly greater production of IL-10 in the lungs of resistant BALB/c mice (Figure 4), IL-10 may be important in controlling PA lung infection and limiting inflammation-induced lung injury in this mouse strain.

IL-10 has also been shown to be a specific chemotactic factor for CD8+ T (Gesser et al., 1997) cells while it suppresses the ability of CD4+ T cells to migrate in response to IL-8 and RANTES (Tan et al., 1995). Thus, the higher numbers of lymphocytes apparent in the lungs of resistant BALB/c mice (Figure 3) may be composed of a greater number of CD8+ T cells. Although it would be simple to conclude that the higher production of IL-10 found within the BALB/c lungs could account for the increased number of lymphocytes, our results from the IL-10 neutralization experiments do not support this

possibility (Figure 6). Future experiments are planned to determine the exact phenotypes of the lymphocyte populations in the BALF and their role during PA lung infection.

In order to evaluate the cell source of the cytokines in the lung, we examined the *in vitro* production of cytokines from alveolar macrophages and PMN of both mouse strains. Consistent with our previous findings, alveolar macrophages from resistant BALB/c mice produced significantly higher levels of TNF-α and significantly lower levels of NO compared with cells from susceptible C57BL/6 mice 7 days following intratracheal infection with PA (Sapru et al., 1998). PMN from both strains produced similarly high levels of TNF-α and undetectable levels of NO. As mentioned above, IL-10 production by PMN harvested from resistant BALB/c mice was significantly higher compared to those harvested from susceptible C57BL/6 mice.

High levels of TNF-α production may contribute to protection as well as pathogenesis (Beutler, 1995). In particular, high levels of TNF-α produced early in response to PA infection have been associated with protection in BALB/c mice (Gosselin et al., 1995). Possible sources of TNF-α in the lung are alveolar macrophages, interstitial macrophages (Franke-Ullmann et al., 1996; Broug-Holub et al., 1997;), epithelial cells (Zhang and Phan, 1996), or, as we have shown, PMN themselves. TNF-α is chemotactic cytokine responsible for PMN recruitment (Laichalk et al., 1996; Takashima et al., 1997). However, the higher TNF-α production from alveolar macrophages recovered from resistant BALB/c mice did not correlate with a dramatic recruitment in the number of PMN. Thus, it is unlikely that TNF-α exerts a protective effect through the recruitment of

inflammatory cells. Furthermore, it is possible that other chemokines, such as MIP-2, could play a more prominent role in PMN recruitment to the airways.

The production of cytokines by PMN from both mouse strains underscores the concept that this cell type plays an important role in the afferent arm of the immune response (Lloyd and Oppenheim, 1992; Cassatella, 1995). In addition to the cytokines we examined, PMN can also secrete an array of chemokines including MIP-1α (Kasama et al., 1993) and MIP-1β (Kasama et al., 1994), which are potent chemoattractants for monocytes and T cells. Thus, the production of IL-10, along with other chemokines and cytokines, by PMN at the site of infection may play a crucial role in the outcome of infection by switching the inflammatory response from an acute, predominantly PMN response to a chronic response composed of PMN and macrophages.

To fully appreciate the dynamics of the lung inflammatory response to PA, data from the lung cellular composition (Figure 3) must be analyzed together with the production of cytokines from total lung homogenates (Figure 4) and phagocytes (Figure 5 and Figure 6). Taken together, these data suggest that the massive number of PMN in the lungs of susceptible C57BL/6 mice, which are capable of producing both TNF-α and IL-10, are not effective in lowering the bacterial burden but instead incite damage to surrounding lung tissue. In addition to the damage mediated by TNF-α release, the sustained high levels of NO produced by alveolar macrophages during chronic PA infection may also contribute to lung damage rather than protection (Beckman et al., 1990; Nathan, 1997; Sapru et al., 1998). Conversely, the lung inflammatory response to

PA in resistant BALB/c mice, which is composed of a more balanced ratio of macrophages and PMN, appears to be better able to reduce the bacterial burden, control inflammation, and limit tissue damage. Alveolar macrophages and PMN from resistant BALB/c mice may contribute to protection through the high production of TNF-α and IL-10, respectively.

Finally, in order to better understand the *in vivo* role of IL-10 during chronic bronchopulmonary PA infection, we administered neutralizing anti-IL-10 antibody to both PA-infected resistant and susceptible mice and evaluated the effect of the treatment on the bacterial load, lung cytokine levels and cell recruitment to the airways. Irrespective of the host's genetic background, neutralization of IL-10 exacerbated the course of PA infection leading to significantly higher bacterial burden in both strains together with elevated levels of TNF-α in the lung (Figure 7).

Taken together, our results suggest that increased TNF-α does not contribute to the clearance of PA from chronically infected lungs. In support of our findings, it has been previously reported that TNF-α on its own does not inhibit proliferation of PA *in vitro* (Buret et al., 1994). On the other hand, TNF-α has been proposed to play an indirect protective role during lung PA infection by enhancing the phagocytic activity of PMN (Buret et al., 1994) or by modulating the release of NO from macrophages (Gosselin et al., 1995) *in vivo*. Thus, it is likely that the timing and production of this septic shock-inducing cytokine play a pivotal role in the balance between protection and pathogenesis. Too high a concentration of TNF-α, as is the case following IL-10 neutralization, fails to

reduce bacterial proliferation but still incites an inflammatory response leading to tissue damage.

The elevated levels of TNF-α observed in our experiments following anti-IL-10 treatment may be responsible for the increase in PMN observed in the lungs of susceptible C57BL/6 mice (Ming et al., 1987; Kolls et al., 1993; van Furth et al., 1994) although no effect on the BALF cell composition was noted in anti-TNF-α treated BALB/c mice infected with PA (Gosselin et al., 1995). It is possible that anti-IL-10 treatment caused the upregulation of other PMN chemoattractants, including MIP-2 (Greenberger et al., 1995; Standiford et al., 1995), the proposed murine equivalent of IL-8 (Schmal et al., 1996). Furthermore, we observed a differential effect of IL-10 neutralization on the recruitment of macrophages when comparing the lung inflammatory response of resistant and susceptible strains of mice (Figure 8). This difference could be attributed to the modulation of secondary chemoattractants regulated by IL-10, such as MIP-1α and MIP-1β (Kasama et al., 1995). Studies are underway to elucidate the identity of these factors and their regulation within the PA infected murine lung.

Our results add to the findings of previous studies describing the role of IL-10 during lung PA infection in mice. In a model of acute lung infection with various non-cytotoxic and cytotoxic strains of PA, Sawa et al. (1997) determined that IL-10 administration required the concomitant presence of IFN-γ in order to exert a protective effect when BALB/c mice were infected intratracheally with cytotoxic strains of PA. In addition, our results imply that IL-10 may play a protective role during chronic PA lung

infection, although at this point we can only speculate on the mechanism. Further studies will be required to determine the exact mechanism of IL-10 during chronic PA lung infection. In contrast to our findings of elevated bacterial proliferation after anti-IL-10 treatment, Yu and et al. (1998) showed that IL-10-deficient knockout mice on the PA susceptible C57BL/6 background cleared PA infection significantly better than wild-type mice. It should be noted that the initial CFU deposition in the lungs was higher than the dose we used ($7.1x10^6$ CFU vs. $1\text{-}2x10^5$ CFU) and their method of infection was by means of repeated aerosol challenge, thus representing an acute model of infection. Even more perplexing were the observations of reduced levels of TNF-α and MIP-2 found in the lungs of the IL-10-deficient knockout mice relative to the wild-type littermate control mice. The authors suggest that this may be due to the faster clearance of PA from the lungs of the IL-10-deficient knockout mice whereas the residual bacterial burden in the lungs of the C57BL/6 mice continues to elicit more TNF-α and MIP-2. Taken together with our results, it is evident that lung PA infection presents a situation in constant flux involving the initiating inflammatory stimulus, the bacterial burden, and the evolving cytokine milieu in the lung. Indeed, the course of lung PA infection represents a dynamic interaction of pathogen, immunocompetent cells, and soluble mediators that may differ depending if the infection is acute or chronic. It will be necessary to fully characterize this complex lung network in order to develop potential immunomodulatory measures capable of ameliorating the devastating course of inflammation apparent in PA lung infection.

In summary, PA resistant BALB/c mice were found to have significantly higher IL-10 concentrations in their lungs compared to susceptible C57BL/6 mice 7 days after the intratracheal instillation of $1\text{-}2x10^5$ CFU of PA-impregnated agar beads. This correlated with a significantly lower number of total inflammatory cells recruited to the lungs of resistant BALB/c mice comprised of similar numbers of PMN and macrophages. In contrast, a pronounced influx of inflammatory cells, primarily composed of PMN, was observed in the lungs of susceptible C57BL/6 mice. Alveolar macrophages from resistant BALB/c mice produced significantly more TNF-α and significantly less NO compared to cells harvested from susceptible C57BL/6 mice. PMN from both strains produced high levels of TNF-α whereas IL-10 production was significantly greater from cells harvested from resistant BALB/c mice. Finally, neutralization of IL-10 exacerbated PA infection and inflammation irrespective of the genetic background of the host. We have shown here that IL-10 plays a role in chronic lung PA infection; however, the kinetics and amount of its production probably mediate a crucial effect in determining its protective role. Future studies are required to delineate these parameters in the hope of designing IL-10-based therapy as a strategy to fight CF.

Chapter 5: Bibliography

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