NOTE TO USERS

TLR SIGNALING PATHWAYS ARE INVOLVED IN THE DEVELOPMENT OF THE INNATE IMMUNE RESPONSE TO *PSEUDOMONAS AERUGINOSA* INDUCED LUNG INFECTION

by

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DEDICATION

This work is dedicated to my daughter, Jenavieve. I hope that it inspires her to face adversity; stand up for what she believes in and finish her work.

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ABSTRACT

Toll-like receptors (TLR) induce distinct patterns of innate immune responses through myeloid differentiation factor 88 (MyD88) dependent and/or independent pathways, depending on the nature of the pathogen. *Pseudomonas aeruginosa* is a cause of serious lung infection in immunocompromised individuals and cystic fibrosis patients. The role of TLR pathways in *P. aeruginosa* induced lung infection were examined *in vivo* in this study. MyD88 deficient mice demonstrated a delayed clearance of *P. aeruginosa* from the lung and a delay in neutrophil recruitment to the airways. These observations were associated with a delay in the production of inflammatory mediators that affect neutrophil recruitment, including macrophage inflammatory protein 2 (CXCL2/MIP-2), tumor necrosis factor (TNF), and interleukin (IL)-1β in the airways of MyD88 deficient mice. Similarly, in the lung of MyD88 deficient mice nuclear factor κB (NFκB) activation was inhibited following *P. aeruginosa* infection. Innate immune responses to *P. aeruginosa* lung infection in TLR2 deficient and TLR4 mutant mice were partially inhibited compared with the responses of respective control mice. Thus, the full development of host responses to *P. aeruginosa* lung infection involves a MyD88 dependent and a MyD88 independent mechanism. Toll-IL-1 receptor domain containing adapter inducing IFNβ (TRIF) is an adaptor molecule that mediates a distinct TLR signaling pathway. The role of TRIF in *P. aeruginosa* infection was also investigated. Following *P. aeruginosa* infection, TRIF deficient mice showed a complete inhibition of CCL5/RANTES production, severe impairment in TNF and CXCL1/KC production, but normal CXCL2/MIP-2 and IL-1β production in the lung. This was associated with a delayed recruitment of neutrophils into the airways. These results demonstrate that TRIF mediates a distinct cytokine/chemokine profile in response to *P. aeruginosa* infection. Importantly, TRIF deficient mice had a delayed clearance of *P. aeruginosa* from the lung when compared with wild type mice. Thus, TRIF is an adaptor molecule that is required for the development of the innate immune response to *P. aeruginosa* infection. These results indicate that the MyD88 and TRIF pathways are essential for the development of innate immune responses to *P. aeruginosa* infection, leading to the clearance of this bacterium.

LIST OF ABBREVIATIONS USED

AP-1	Activating protein-1
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ELISA	Enzyme linked immunosorbant assay
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesufonic acid
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of κB
IKK	IκB kinases
IL	interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISRE	IFN-stimulated response element
ITAM	Immunoreceptor tyrosine activation motif
ITIM	Immunoreceptor tyrosine inhibition motif
CXCL1/KC	Keratinocyte chemoattractant
kDa	kiloDalton
KO	Knock out
LBP	LPS-binding protein
LPS	lipopolysaccharide/endotoxin
LRR	leucine-rich repeats
MAL	MyD88 adaptor-like protein
min	minute(s)
MIP	Macrophage inflammatory protein
MPO	myeloperoxidase
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor κB
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PGN	peptidoglycan
PKR	dsRNA-dependent protein kinase
PMN	polymorphonuclear leukocyte/neutrophil
PRR	pattern recognition receptor
CCL5/RANTES	Regulated upon activation normal T cell expressed and secreted
RT	reverse transcription
SEM	standard error of the mean
SD	standard deviation
TAB	TAK1-binding proteins
TAK	TGF-β-activated kinase
TBK	TANK-binding kinase
TICAM-1/2	Toll-interleukin 1 receptor domain-containing adaptor molecule
TIR	Toll-interleukin-1 receptor
TIRAP	Toll-interleukin-1 receptor associated protein
TIRP	TIR-containing protein
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine substrate
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factor
TRAM	TRIF-related adapter protein
TRIF	TIR-domain-containing adapter-inducing IFNβ
TTBS	Tris-buffered saline + Tween20

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Frank Deford, the father of Alex a little girl who had CF wrote "Once *Pseudomonas* begins to march through the lungs, it multiplies with impunity and sweeps everything in its path. For any cystic fibrosis patient *Pseudomonas* is the harbinger of death" (1). I want to recognize and acknowledge the pain suffered by parents who have children with CF. May this research continue.

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CHAPTER 1. Introduction

1.1 Immune Response: An Overview

The immune system's function is to protect the host from infection. Inflammation results from the initiation of the immune response. The five cardinal signs of inflammation are redness, swelling, heat, pain and loss of function (3-5). Normal host defense responses include both the innate and adaptive immune response. The first line of defense is the innate immune response which occurs within minutes, after invasion of the host and is activated to the same extent regardless of how many times the infectious agent is encountered. The acquired (adaptive) immune response is slow to develop and improves with repeated exposure to a given infectious agent.

The innate immune response involves structural defenses, cells that release inflammatory mediators (basophils, mast cells, and eosinophils), phagocytic cells (neutrophils, monocytes, and macrophages), and natural killer cells. The molecular components of innate responses include complement, acute-phase proteins, and cytokines (6, 7). The role of the innate immune system includes initial defense against and clearance of microorganisms, as well as activation of the adaptive immune system. The innate immune response was previously thought to be relatively nonspecific, but increasing evidence describes the complexity and specificity of the innate immune system (8). To activate the acquired immune response, antigen presenting cells (APC), such as macrophages and dendritic cells, display antigen to lymphocytes leading to the activation and proliferation of helper T cells. T cells help B cells secrete antibodies that are specific for antigen and eliminate extracellular microorganisms. T cells can eradicate intracellular microbes by activating macrophages and by killing virally infected cells. Therefore, a combination of the innate and acquired immune responses are usually responsible for eliminating microbes (6).

1.2 Innate Immunity (Primarily in the Context of Gram-Negative Bacterial Lung Infections)

Microorganisms gain access to the internal environment of the host by damaging innate immune barriers such as the skin and mucous membranes. The lung serves as a

major interface between the host and the external environment and thus is a major portal of entry for microbes. In the lung, the innate immune responses are primarily responsible for protecting the host from inhaled microbes (9, 10). The lung is protected by nonspecific factors, the humoral response and a response by innate immune cells .

1.2.1 Brief Overview of Structural and Humoral Components of the Innate Immune Response

The nonspecific structural component of the innate immune response that protect the host against microbial infection include the ciliary beat, the cough reflex and mucus clearance. As a microbe comes into the host past the nonspecific defenses, it is confronted with natural antibodies and complement which are components of the humoral response important in the innate immune response to microbes (11, 12).

1.2.2 Cells of the Innate Immune System

Pulmonary innate immunity is also mediated by cells of the airway such as alveolar epithelial cells (13, 14), alveolar macrophages (15-18) and resident mast cells (19-22) which recognize microbes and are capable of producing inflammatory mediators such as leukotriene B4 (23), interleukin-1 (IL-1), tumor necrosis factor (TNF) (18) and chemokines including CXCL8/IL-8, macrophage inflammatory protein 2 (CXCL2/MIP-2) and keratinocyte-derived chemokine (CXCL1/KC) (24-30). The secretion of these inflammatory mediators leads to the increased microbicidal activity of phagocytes at the site of infection, recruitment of additional cells that can aid in the killing of microbes (Figure 1.1), and can act on the bone marrow to increase hematopoiesis, the central nervous system to induce fever, and the liver to produce acute phase proteins, as reviewed by Kapetanovic (12).

1.2.2.1 Phagocytic Cells at the Site of Infection (Macrophage)

Macrophages at the site of infection are able to recognize microbes and will phagocytose and kill the pathogen by a process which is mediated by complement (31), Fc receptors (32) and carbohydrate recognition receptors such as the mannose receptor (33). The role of Toll-like receptors (TLRs) in activating phagocytosis is not yet clear. Blander *et al.* (34) have suggested that phagocytosis of bacteria by macrophages is

impaired when there are deficiencies in TLR signaling. However, Yates *et al.* (35) did not find any impact on phagocytosis as a result of TLR signaling in a model of phagocytosis induced by coated beads. More recently, in a review, Blander *et al.* (36) suggest that the role of TLR in phagocytosis may be overshadowed by other mechanisms of phagocytosis. They hypothesize that beads coated with IgG or mannose induce maximal phagocytosis and differences may not be observed in the presence or absence of TLRs (36). Overall, the role of TLRs in inducing phagocytosis is not clear.

The recruitment of neutrophils from the pulmonary vasculature into the alveolar space is particularly important because neutrophils are more effective in phagocytosing and eliminating virulent microbes than macrophages (8, 9, 37, 38). Macrophages can become better at killing microbes by obtaining secreted signals from Natural Killer (NK) cells. Interleukin (IL)-12 produced by macrophages activates NK cells to produce interferon (IFN)-γ (39). The IFN-γ in turn activates the macrophage to increase its microbicidal activity (40, 41).

1.2.2.2 Natural Killer (NK) Cells

NK cells are important in defense against pathogens (42). NK cells are activated by directly interacting with ligands on target cells and by cytokines such as IL-12 (43), produced by macrophages and dendritic cells. The activation of NK cells leads to the lysis of target cells by the exocytosis of perforin and granzymes (44) and the production of cytokines such as IFN-γ (45). IFN-γ is important for activating phagocytes to increase their microbicidal activity (40, 41). NK cells may kill bacteria directly or kill bacterial infected cells by antibody dependent cell mediated cytotoxicity (ADCC). There is currently very little or no evidence in the literature indicating that NK cells can directly kill bacteria. Infected cells or bacteria alone that are opsonized with IgG may interact with the Fc region of the antibody with FcR on the NK cell (46). The activation of a NK cell is determined by the balance of inhibitory and activating signals it receives when it interacts with other cells. In the context of bacterial infection, NK cells are important in controlling the infection by producing IFN-γ (47) and by killing infected cells that have increased levels of activating receptors (48) or a decreased level of inhibitory receptors on their surface. NK cells express TLRs and respond to LPS stimulation by activating

nuclear factor-κB (NFκB). Stimulation of NK cells with a combination of IL-12 from activated macrophages or dendritic cells, and bacterial peptidoglycan enhanced the production of IFN-γ and several chemokines (49). Mice given IL-12, intranasally, before infection with *Streptococcus pneumoniae* had increased survival and enhanced bacterial clearance that was dependent on the expression of IFN-γ (50). These authors suggest that exogenous IL-12 activates NK cells, leading to IFN-γ production which induces macrophages to secrete TNF. This then leads to the expression of CXCL1/KC, increased neutrophil recruitment and susequent bacterial clearance (50). Therefore, NK cells may be involved in bacterial clearance by lysing cells with an intracellular bacterial infection, possibly lysing bacteria through ADCC, and interacting with microbes via TLRs to produce cytokines and chemokines to activate macrophages and recruit cells (42).

1.2.3 Complement

Complement is a vital part of the innate immune response and is critical for host defense (11). An important function of complement is to fight microbial infection. Complement split products opsonize bacteria for phagocytic recognition and the terminal membrane attack complex (MAC) can form in the membrane of the microbe leading to lysis. The products of complement, especially C5a and C3a, are important in local inflammatory responses that increase vascular permeability, vasodilation and smooth muscle contraction (51-53). There are regulatory mechanisms in place that allow the deposition of complement on the surface of microbes and inhibit the deposition of complement on host cells (11). The activation of complement and the three complement pathways have been extensively reviewed in the literature (11, 12, 54). The pathways will be briefly discussed here.

1.2.3.1 The Classical Complement Pathway

The classical complement pathway is initiated by the binding of C1q to two Fc regions on IgG or IgM that is bound to the surface of a microbe (Figure 1.2) (55). This initiates activation of protease activity in the C1r subunit which cleaves C1s into active protease (56). C1s then cleaves C4 into C4a and C4b (57, 58). C4b binds the surface of the target close to C1 and C4a is released and is an anaphylatoxin (59). C2 binds C4b

and C1s cleaves C2 into C2a and C2b (60) making C4b2a which is a C3 convertase (61-63). The C3 convertase converts C3 into C3b and C3a (64). C3b binds C4b2a to make C4b2a3b which is a C5 convertase (65). C5 convertase converts C5 into C5a and C5b. A cascade of events occur that lead to assembly of the MAC which is made up of C5b, C6, C7, C8, C9 forming a pore or hole in the membrane of the target, lysing it (66-69).

1.2.3.2 The Lectin Pathway

The lectin pathway involves the binding of mannose binding lectin (MBL) or ficolins (70, 71) to a microbe (Figure 1.2). MBL binds mannose on microorganisms and is similar to C1q in structure (72). Ficolin is similar in structure to C1q and MBL and also binds carbohydrate regions on microbes. When MBL binds its target, MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2) act to cleave C4 and then C2 (73, 74), generating a C3 convertase. The pathway then follows the classical pathway for activation of complement.

1.2.3.3 The Alternative Pathway

C3 is hydrolyzed spontaneously at low levels into C3b and C3a (Figure 1.2) (75). C3b can bind to microbe membranes (76) due to a decrease in the sialic acid which enables C3b to stick (77, 78). Factor B binds C3b and target membranes and Factor D is able to convert Factor B into Bb and Ba (79), generating C3bBb which is a C3 convertase. Properdin is needed to stabilize this C3 convertase (80-82). The C3 convertase converts C3 into C3a and C3b, generating C3bBb3b which is a C5 convertase (83). The C5 convertase acts to convert C5 into C5b and C5a leading to the formation of the MAC (66, 84).

1.2.3.4 Anaphylatoxins

Anaphylatoxins such as C3a (85), C4a (59) and C5a (86) are generated during the activation of complement. Anaphylatoxins can bind receptors on mast cells and basophils to induce degranulation and the release of mediators such as histamine (87, 88), resulting in an increase in vascular permeability (51) and smooth muscle contraction (52). C3a and C5a also recruit monocytes and neutrophils to the site of inflammation (89, 90).

1.2.3.5 Complement Receptors (CR)

The anaphylatoxins released during complement activation bind receptors and mediate inflammatory functions such as chemoattraction of leukocytes, histamine release from mast cells and the release of lysosomal enzymes from leukocytes, as reviewed by Gasque (11). The receptor for C3a is called the C3aR and the receptor for C5a is C5aR/CD88. An additional receptor has been identified to interact with C5a, C3a and C4a and is called C5L2 (91, 92). These receptors are 7 transmembrane spanning G protein coupled receptors that regulate the immune and inflammatory response after stimulation with anaphylatoxins. It was originally thought that C5L2 was a non-signaling receptor; however, recently evidence suggests that C5L2 plays a role in signaling for C5a and C3a (93). C5L2 may also play a role in controlling pro-inflammatory and anti-inflammatory responses and LPS signaling.

There are several different complement receptors such as CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) that interact with products of the complement pathway that are involved in promoting phagocytosis (11). CR1 is found on myeloid cells and interacts with complement components like C3b, C4b (94), C1q (95) and MBL (96). CR3 is found on the surface of neutrophils, monocytes and NK cells and interacts with ligands such as iC3b (31), LPS (97) and ICAM-1 (98-100) to promote phagocytosis, LPS clearance and cell adhesion, respectively. CR4 is present on dendritic cells and macrophages and interacts with iC3b (101), LPS (102) and ICAM-1 (103), promoting similar functions as CR3.

C1qRp (CD93) was thought to bind the complement components C1q and MBL; however, evidence suggests that this receptor does not bind directly to C1q. Experiments using cells expressing C1qRp did not reveal enhanced binding in comparison with control

cells(104), and a chimera made up of a recombinant C1qRp-Fc did not interact with immobilized C1q (104). Therefore, C1qRp may not be a receptor for C1q. Instead, C1qRp may contribute to the removal of apoptotic cells (105). C1q and MBL mediate the phagocytosis of apoptotic cells by interacting with calreticulin (also known as cC1qR) and CD91 on macrophages (106).

The α2β1 integrin has been identified as a receptor for the complement protein C1q and the collectins MBL and SP-A. The interaction of C1q and pathogen immune complexes with α2β1 has been shown to play a role in mast cell activation and cytokine production (107). In the past, studies using antibodies to α2β1 indicated that this integrin may not play a role in C1q interaction with platelets because the antibodies only moderately inhibited the adhesion of platelets to C1q (108). However, reinterpreting this data may suggest that there are multiple C1q receptors on platelets (107). Since α2β1 has been shown to mediate cytokine production in mast cells, it may act as a co-receptor with other innate immune receptors, such as TLRs involved in regulating cytokine production (107).

1.2.3.6 Complement and the LPS Receptor

Complement components bind the appropriate receptors on cells to activate the immune response. Connections can be made between complement and other innate immune receptors in mediating and amplifying an immune response. One recent study (109) indicates that activation of complement and CD14 are required for CD11b up-regulation, phagocytosis and oxidative burst of granulocytes and monocytes after stimulation with Gram-negative bacteria. Inhibiting the opsonization of C3 and the formation of C5a blocked phagocytosis of a Gram-negative bacteria and oxidative burst in granulocytes. C5a was found to be important in up-regulating CD11b on granulocytes and monocytes. Using antibodies to CD14 and C5a completely abrogated the up-regulation of CD11b in granulocytes and monocytes. (109). These findings are interesting because it highlights the interactions between different components of the innate immune response, particularly complement and LPS recognition. There is a recent report indicating that CR3 and TLR4, are required for the phagocytosis of a Gram-negative bacterium and the activation of the nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase, respectively (110). Therefore, the innate immune response may work in a sequential manner dependant on several components.

1.3 Recognition of PAMPs

The ultimate action of the innate immune system is the recognition of microbes for destruction and removal from the host. The recognition of conserved sequences on microbes known as pathogen-associated molecular patterns (PAMPs) initiates the innate immune response. PAMPs are produced only by microbial pathogens, and not by their hosts. They are invariant structures shared by entire classes of microbes and are essential for the survival or pathogenicity of the microorganism (111). PAMPs are recognized by pattern-recognition receptors (PRRs) on epithelial cells and leukocytes. The specificity of PRRs allows the host to defend against pathogens but to "ignore" nonpathogenic particles that come in contact with the pulmonary epithelium (8). The recognition of microbes by PRR induces signals in the cell causing activated transcription factors to go to the nucleus and regulate transcription of genes important in the innate immune response. The transcription of cytokines, chemokines and adhesion molecules is enhanced. Cytokines and chemokines act locally in an autocrine or paracrine manner, binding to their respective receptors to enhance the innate immune defense and recruit neutrophils to the site of infection for bacterial clearance (112). Some PRR include collectins, scavenger receptors and toll-like receptors (TLRs). Of these PRR, collectins will be mentioned briefly and TLRs will be discussed in detail. These PRR have emerged as fundamental components of the innate immune response and provide a link between the innate and adaptive immune response (7).

1.3.1 Collectins

Collectins are a family of Ca^{2+}-dependent lectin proteins that bind carbohydrate ligands. Collectins, including MBL, surfactant protein-A (SP-A) and SP-D are able to recognize pattern associated molecular patterns on microbes. The tertiary structure of collectins is similar to that of C1q; however, C1q is not itself a lectin. Some domains that are vital for collectin function include a disulfide bond at the N-terminus, a collagen like domain, a coiled-coil neck domain and a carbohydrate recognition domain (CRD) (113).

1.3.1.1 Interactions Between Collectins and Components of the LPS Receptor

Collectins alter the interactions of LPS with the LPS receptor complex. Interactions between CD14 and collectins seem to be important for the innate immune response. MBL (114), SP-A and SP-D (115) interact with CD14, which is part of the LPS receptor.

MBL and CD14

The interaction between MBL and CD14 is not inhibited by excess mannose, therefore the lectin binding region is likely separate from the CD14 interaction region (114). Perhaps the interaction of MBL with CD14 provides a way to ensure the host recognizes microbial infection.

SP-A, CD14 and TLR4

Pulmonary surfactant is a mixture of proteins and lipids produced by the epithelium that are present in the alveolar spaces produced by the epithelium and are important in reducing surface tension during expiration (116). One of the most abundant proteins in the surfactant is SP-A. The interaction of SP-A with CD14 regulates the host response to LPS. SP-A interacts with CD14 via its neck domain and with LPS through the CRD. SP-A can interact with CD14 and rough LPS (LPS lacking the O antigen) to augment the host response; however, the interaction of SP-A with CD14 inhibits the host response to smooth LPS (117). Using TLR4 mutant mice, it has been shown that SP-A-induces activation of the NFκB signaling pathway and up-regulation of cytokines depends on a functional TLR4 (118). More recently, evidence suggests that SP-A interacts directly with TLR4 and the co-factor MD-2 and prevents smooth LPS signaling (119). Therefore, interactions between collectins such as SP-A and the LPS receptor complex are important for regulating an innate immune response.

SP-D, CD14 and TLR4

SP-D interacts with CD14 via its CRD and inhibits binding of CD14 to smooth and rough LPS. The collectin, SP-D, interacts with CD14 in a way to down-regulate responses to LPS (115). Recently, SP-D has been shown to interact with TLR4 via its

CRD and antibodies to SP-D inhibited this observed interaction (120). Therefore, the collectin SP-D is involved in regulating LPS signaling.

1.4 Toll-Like Receptor (TLR)

1.4.1 Identification and Structure

TLR were originally discovered in *Drosophilia melanogaster.* The Toll gene was identified as a requirement for antimicrobial resistance (121, 122). In 1997, a human homologue of *Drosophilia* Toll was identified to signal the activation of adaptive immunity (123). The *Drosophilia* Toll signals via mechanisms that are similar to the IL-1R signaling pathways, which is important in vertebrates for many immune and inflammatory responses (123). TLRs are transmembrane proteins; the extracellular domains are comprised largely of leucine rich repeats which are responsible for the recognition of microbes (124), while the intracellular Toll/IL-1 receptor (TIR) domain are homologous with IL-1R and are essential for initiating intracellular signaling. TLRs transduce signals via intracellular components shared with the IL-1R signaling machinery leading to the activation of NFκB to produce inflammatory mediators (125, 126).

1.4.2 Specificity of TLRs

TLRs function as pattern recognition receptors that recognize conserved motifs on microbes (127), and are important in initiating immune responses. The fact that there are several TLRs indicates that the innate immune system is more selective than it was originally deemed. Each TLR binds or interacts specifically with a particular microbe component (Table 1.1). These receptors are required to keep microbes in check while the acquired immune response develops and also may be able to initiate and direct the acquired immune response (128). It is likely that the acquired immune responses would be delayed if it was not for the presence and proper function of TLRs and their signaling pathways. The role that these receptors play in the innate immune response is critical in sites that interact directly with the external environment, such as the airways (9, 129).

Currently, eleven different TLRs have been identified in mammalian species (130-135). TLR1 – 9 are conserved between mouse and human. TLR10 is presumed to

be functional in the human but not in the mouse (136). TLR11 is functional in mice but not in humans (135). TLR2 is activated by peptidoglycans, lipopeptides and lipoteichoic acid, which are major components of the Gram-positive cell wall (131, 137-139). TLR2 can heterodimerize with TLR1 or TLR6, increasing the repertoire of ligands TLR2 can bind (138, 140). TLR3 binds viral double stranded RNA (141). TLR4 interacts with LPS the main component of the Gram-negative cell wall (126, 142). Motility is an important virulence factor for bacteria and bacterial flagella bind TLR5 (143). TLR7 and TLR8 recognize single-stranded RNA viruses (144, 145). TLR9 binds bacterial DNA (133). Uropathogenic bacteria are recognized by TLR11 but the component is not yet known (135). TLRs have a distinct localization pattern, where TLR1, 2, 4, 5 and 6 are present on the surface of the cell and TLR3, 9 and possibly TLR7 and 8 are present on intracellular compartments such as phagosomes and endosomes; however, surface TLRs are also likely to be present intracellularly as well (146, 147), reviewed in (148, 149). These receptors are required to keep microbes in check while the acquired immune response develops.

The LPS receptor complex

TLR4 is responsible for the signaling of LPS (126, 142). LPS binds to LPS-binding protein (LBP), an acute phase protein that circulates in the blood stream. The LPS/LBP complex then binds to CD14 on the cell surface. The complex of CD14-LPS/LBP interacts with MD-2 that is found in association with TLR4, resulting in activation of downstream signaling (150-152). Evidence suggests that CD14 is required for MyD88 dependent TLR4 signaling of smooth LPS but not rough LPS (153). Similarly, TRIF mediated TLR4 signaling by rough LPS does require CD14 (154).

TABLE 1.1 TLRs and Corresponding Ligands

TLR	Ligands	Microbial sources
TLR1/2	triacyl lipopeptides	bacteria and mycobacteria (138)
TLR2	lipoteichoic acid	Gram-positive bacteria (139)
	peptidoglycan	Gram-positive bacteria (131)
	zymosan	Yeast (155)
TLR2/6	diacyl lipopeptides	Gram-positive bacteria (137, 138)
		viruses, poly(I:C) (141)
TLR3	double-stranded RNA	Gram-negative bacteria (126, 142)
TLR4	LPS	Gram-positive and –negative flagellated bacteria (143)
TLR5	flagellin	viruses (144, 145)
		viruses (145)
		bacteria (133)
TLR7	single-stranded RNA	
TLR8	single-stranded RNA	
TLR9	CpG unmethylated DNA	

Adapted from (7)

1.4.3 Signaling

The cytoplasmic protein, MyD88 (Figure 1.3), has been identified as an important adapter molecule that is recruited to TIR domains of TLRs and plays a critical role in the IL-1R/TLR signaling cascade (156-158). Most TLRs are able to signal through the adaptor molecule MyD88 (159, 160). However, there have been several other adaptor molecules identified that induce TLR signals, including TIR associated protein (TIRAP/Mal) (161), TIR domain containing adaptor inducing IFNβ (TRIF/TICAM-1) (162, 163), TRIF relate adaptor molecule (TRAM/TIRP/TICAM-2) (164) and sterile α-armadillo motif containing protein (SARM) (165). Of interest, TIRAP/Mal is involved in the MyD88 dependent signaling pathway for TLR2 and TLR4 (161, 166), whereas TRIF/TICAM-1 is important for TLR3 and TLR4 signaling, and TRAM/TIRP/TICAM-2 is involved in the MyD88 independent signaling pathway through TLR4 (162, 167).

1.4.3.1 Myeloid Differentiation Factor 88 (MyD88)

MyD88 is a cytoplasmic protein that has been identified as an adapter molecule that is a critical component of the IL-1 receptor and TLR signaling cascade (157, 158). The full length nucleotide sequence of mouse MyD88 was determined in 1990. It was isolated as an early transcript in the differentiation of myeloid cells after induction by IL-6 (168), and it was also postulated to be involved in macrophage differentiation (169). The human homolog of MyD88 was characterized in 1996 (170). The N-terminal region MyD88 contains a death domain (DD), while the C-terminal region in MyD88 is similar to the cytoplasmic domains of IL-1R and TLRs called the TIR domain (171, 172). These domains dictate what signaling molecules interact with MyD88. A mutated form of MyD88, lacking the intermediate domain between the DD and the TIR domain, inhibits the IL-1R/TLR signaling (157). MyD88 deficient cells are unable to produce cytokines in response to LPS (173). Most TLRs can signal through MyD88 (159, 160), therefore MyD88 deficient mice have been used as a mouse model for impaired TLR function.

1.4.3.2 MyD88 Dependent Signaling

Activation of TLR1, 2, 4, 5, 6, 7, 8 and 9 involves the recruitment of MyD88 following ligand binding (Figure 1.3). The MyD88 dependent signaling cascade through

TLR and TLR4 also requires the adaptor molecule TIRAP/Mal (166). This leads to the recruitment and activation of the serine/threonine kinases IL-1R associated kinase 1 (IRAK1) (174, 175) and IRAK4 (176). Four members of the IRAK family have been found, including IRAK1, IRAK2, IRAK4, and IRAK-M. IRAK2 (177) and IRAK-M (178) are negative regulators of TLR signaling. IRAK1 and IRAK4 have intrinsic serine/threonine protein kinase activities (179). IRAK4 is important in NFκB activation and the up-regulation of several inflammatory cytokines after stimulation with TLR ligands (Figure 1.3). IRAK4 mutations have been reported in patients with recurrent infections particularly *Streptococcus pneumoniae* and poor production of inflammatory mediators (180). Similarly, IRAK4 deficient mice have impaired cytokine responses and NFκB activation in response to IL-1β, LPS and ligands for TLR2, TLR3 and TLR9 (181).

After stimulation, IRAK4 and IRAK1 are phosphorylated and then dissociate from MyD88, resulting in the activation of tumor necrosis factor receptor associated factor 6 (TRAF6) (182). TRAF6 activates transforming growth factor-β activated protein kinase 1 (TAK1) (183). TAK1 activates the IκB kinase (IKK) complex leading to NFκB activation and translocation to the nucleus (183, 184) as well as MAP kinase activation which leads to activator protein (AP)-1 activation and nuclear translocation (184) (Figure 1.3).

NFκB is present in the cytoplasm in an inactive form in unstimulated cells because of its association with inhibitor of κB (IκB). After TLR ligand stimulation a cascade of events occurs that leads to the phosphorylation of IκB, which then becomes ubiquitinated and subsequently degraded (179, 185). Degradation of IκB releases NFκB, allowing NFκB to translocate to the nucleus where it induces the expression of specific genes (186). There are five mammalian NFκB proteins identified, including NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), RelA/p65, RelB, and c-Rel (187). The prototypical NFκB is thought to be a heterodimer of p65 and p50, which is present in most cells (179). NFκB regulates the inducible gene expression of many cytokines, chemokines, adhesion molecules, acute phase proteins, and antimicrobial

peptides (187, 188). The release of these mediators at the site of infection induces the recruitment of immune cells to clear the microbe.

1.4.3.3 TIR Domain-Containing Adapter Inducing IFNβ (TRIF) Dependent Signaling

The adaptor molecule TRIF was identified by Yamamoto *et al.* by performing a database screening for the TIR domain (162). The transcript for TRIF is ubiquitously expressed in human tissues (162), and cells expressing TRIF were able to activate the IFNβ promoter and the NFκB promoter (162). The same study found that the N-terminal region of TRIF was important for IFNβ promoter activation, whereas both N-terminal and C-terminal portions of TRIF were involved in the NFκB dependent promoter activation (162). A dominant negative form of TRIF hindered TLR3 mediated signaling, indicating the involvement of TRIF in TLR3 signaling (Figure 1.3). The dominant negative form of TRIF also inhibited the activation of NFκB after stimulation with ligands of TLR2, TLR4 and TLR7, which suggests TRIF is involved in these signaling pathways in addition to MyD88 (162). The development of TRIF deficient mice has allowed further examination of the importance of TRIF in cells after stimulation with TLR ligands.

Macrophages from TRIF deficient mice stimulated with poly(I:C) exhibited an impairment in IFNβ, CCL5/RANTES, CXCL10 and CCL2 mRNA expression as compared to wild type controls (163). This finding suggests that TRIF is important in the TLR3 signaling pathway.

In order to examine the role of TRIF in TLR4 signaling, TRIF deficient cells were examined after stimulation with LPS. TRIF deficient embryonic fibroblasts stimulated with LPS showed a significant reduction in the production of CXCL10, CCL5/RANTES and CCL2 mRNAs, while TRIF deficient peritoneal macrophages stimulated with LPS had decreased production of TNF, IL-6 and IL-12 (163). TRAM is an adaptor molecule that has been identified to link TRIF to TLR4 (164). These data show that TRIF is involved in TLR4 mediated signaling (Figure 1.3).

The stimulation of TLR3 and TLR4 results in TRIF dependent activation of the transcription factor interferon regulatory factor 3 (IRF3). This was identified because the dimerization of IRF3 after stimulation with poly(I:C) or LPS was not observed in TRIF

deficient cells (163, 173). However, it has been shown that TLR3 induces a stronger activation of IRF3 than TLR4 signaling, which correlates with higher levels of IFNβ production (189). These studies demonstrate that TRIF plays a role in the MyD88 independent activation of IRF3 in response to TLR3 and TLR4 signaling.

There is evidence that other transcription factors such as NFκB are involved in the TRIF dependent signaling pathway. MyD88 independent signaling after TLR4 stimulation also results in the late phase activation of NFκB through TLR4 signaling (160, 190).

1.4.3.4 IRF

The expression of the type I interferons (IFNα and IFNβ) is controlled by the IRF transcription factors. The two important transcription factors for the type I interferons are IRF3 (191) and IRF7 (192). IRF3 is constitutively expressed whereas IRF7 expression is weak in unstimulated cells but can be induced by stimulation with LPS (167, 189, 193), type I IFN (194) or viral infection (195). IRF3 can be activated by viral components (196) and LPS (173). The activation of IRF3 involves the phosphorylation of IRF3, which leads to its dimerization and nuclear translocation (197). IRF3 binds DNA elements at the IFNβ promoter by interacting with co-activators to promote gene transcription (198), reviewed in (179, 199).

IRF7 is a secondary response gene and its up-regulation requires protein synthesis after treatment with LPS (189). The production of IRF7 is induced in many cells following autocrine or paracrine type I IFN signaling (192). However, IRF7 is present in the cytoplasm of plasmacytoid DC at basal levels (200), which is likely accounts for the high IFN production by these cells. Phosphorylation and dimerization of IRF7 leads to its nuclear translocation and the up-regulation of IFNα (192).

Recent information suggests that the transcription factor IRF5 is activated downstream of both of the adaptor molecules MyD88 and TRIF (201). It has been reported that IRF5 interacts with and is activated by MyD88 and TRAF6 (202). IRF5 is able to increase the transcription of pro-inflammatory cytokine genes (202).

1.4.3.5 Important Molecules in TRIF Dependent Activation of IRF

TRAF family member associated NFκB activator (TANK) binding kinase (TBK1) and IκB kinase ε (IKKε also known as inducible IKK (IKKi)) are the main kinases responsible for activating IRF3 and IRF7 after viral infection (203). The role of these kinases in TLR signaling has been examined recently. Treatment of macrophages with LPS increased the activity of TBK1 15 min after stimulation. The activity of IKKε was induced 8 h after stimulation with LPS, indicating that there may be a delayed role for IKKε after LPS stimulation. Using a RNA interference (RNAi) technique, it was shown that down-regulating TBK1 and IKKε lead to a decrease in the mRNA levels of IFNβ and IRF7 after stimulation with LPS, suggesting that TBK1 and IKKε play a role in LPS induced type I IFN production (193). Similarly, Matsui *et al.* (204) demonstrated signaling by TBK1 but not IKKε at 2 and 4 h after LPS stimulation. In another study, TBK1 deficient fibroblasts were deficient in IFN-stimulated response element (ISRE) binding and activation of the IFNβ promoter after stimulation with LPS and dsRNA (205). A role for IKKi in IRF3 activation after LPS stimulation was not identified at 2 h (205). TBK1 deficient macrophages stimulated with LPS had an abolished IFN response (206). Therefore, an essential role for TBK1 in activation of IRF3 after LPS stimulation and a lessor role for IKKε at later time points.

TRIF may directly interact with kinases that phosphorylate IRF3/7, or there may be molecules that bridge them together. It has been shown that TBK1 interacts directly with TRIF (207), but recent evidence indicates that TRAF3 can also associate with TRIF and TBK1 and IKKε. TRAF3 may be an important link between TRIF and the kinases that are needed for the activation of IRF (208).

To add to the complexity of the signaling pathways, it has been shown that the TRIF mediated IRF3 activation may be required for the activation of NFκB. Specifically, the IRF3 dependent protein synthesis of TNF is required for subsequent NFκB activation. TNF signals via the TNF receptor in an autocrine positive feedback loop activating the late phase NFκB (190).

1.4.3.6 TRIF Activation of NFκB

There are at least two signaling molecules downstream of TRIF (Figure 1.3) that can link TRIF to the activation of NFκB. RIP1 has been shown to be involved in the TRIF dependent activation of IκB kinases and ultimately NFκB (209, 210). TRIF also interacts with TRAF6 to activate IκB kinases and then NFκB. A mutant TRIF that cannot bind TRAF6 is incapable of activating NFκB (207). Sato *et al.* (207) show that TRAF6 and TBK1 bind and interact with the N-terminus of TRIF in yeast and mammalian cells. The TRAF6 binding motif in TRIF is important for the activation of NFκB but not the IFNβ promoter (207). The TRIF to RIP1 and TRIF to TRAF6 pathways may converge at the IKK complex for optimal activation of NFκB (179). Therefore, activation of NFκB may be the result of direct signaling pathways; however, there is a suggestion that protein synthesis may be involved.

The MyD88 independent pathway may involve protein synthesis to activate NFκB. Covert *et al.* (190) show that LPS-stimulated mouse embryonic fibroblasts deficient in MyD88 increased transcription of genes such as chemokine CXCL10, CXCL2/MIP-2 and TNF. However, the pretreatment of MyD88 deficient cells with soluble TNF receptor blocked the activation of NFκB after LPS stimulation. Since the TNF promoter has several potential IRF binding sites, Covert *et al.* (190) hypothesized that IRF3 might be responsible for increasing the transcription of TNF. The depletion of endogenous IRF3 protein expression in MyD88 deficient mouse embryonic fibroblasts impaired the activation of NFκB. Thus, they concluded that the LPS induced TRIF dependent signaling pathway activates TNF production through IRF3. The secreted TNF binds its receptors on the cell leading to NFκB activation (190).

These studies show that there are multiple, overlapping ways the host can activate NFκB to induce the innate immune response. This is most likely important to increase the likelihood of an early immune response and quick bacterial clearance.

1.4.3.7 IRFs in MyD88 Dependent Signaling

The TLR signaling pathways are not as straight forward as initially thought; there is cross-talk between the MyD88 dependent and TRIF dependent signaling pathways.

The IRF transcription factors are not only involved downstream of the TRIF signaling pathway but are also involved in the MyD88 dependent signaling pathway. IRF7 interacts with MyD88, but not IRF3, after stimulation with a TLR9 ligand to induce IFN production. Confocal microscopy indicates that MyD88 and IRF7 colocalize in the cytoplasm and MyD88 is required for the nuclear translocation of IRF7 after TLR9 stimulation. IRAK1 and IRAK4, which are kinases downstream of MyD88, have been implicated in the phosphorylation of IRF7 (211).

1.5 Negative Regulators of TLR Signaling

SIGIRR and ST2

Single immunoglobulin IL-1R-related molecule (SIGIRR) is a receptor that contains a TIR domain. SIGIRR has been shown to inhibit IL-1R and TLR signaling when over expressed *in vitro* (212), and intraperitoneal injection of LPS resulted in a more potent inflammatory response in SIGIRR deficient mice, which lead to rapid mortality in these animals (212). It was reported that SIGIRR interacted specifically with the IL-1R, TLR4 and TRAF6 (212). ST2 is another negative regulator of TLR signaling. ST2 deficient macrophages expressed increase levels of cytokines after LPS stimulation. Specifically, ST2 interacts with and inhibits MyD88 and Mal/TIRAP but not TRIF or IRAK (213). SIGIRR and ST2 inhibit TLR signaling by sequestering MyD88 through TIR domain interactions (212).

IRAK-M

IRAK-M is involved in the negative regulation of TLR signaling (178). IRAK-M deficient macrophages showed an increase in cytokine expression after stimulation with TLR agonists and whole bacteria (178). IRAK-M interacts with IRAK1/4, which may prevent the dissociation of IRAK1/4 from MyD88 to block TLR signaling (178). IRAK-M is induced by TLR signaling and functions during the second or continuous exposure to stimulation.

Suppressor of cytokine signaling (SOCS)

SOCS is another negative regulator of TLR signaling (214). SOCS deficient mice die as a result of excessive inflammation (215). Recent evidence suggests that SOCS is involved in down regulating TLR signaling by the degradation of TIRAP/Mal (216).

Tollip

Another adaptor protein named Toll-interacting protein (Tollip) was first found to associate with the TIR domain of the IL-1R after stimulation with IL-1β (217). Tollip also associates with TLR2 and TLR4, inhibiting TLR signaling by suppressing the kinase activity of IRAK. Tollip may benefit the host by limiting the production of pro-inflammatory mediators during inflammation and infection (218).

NADPH oxidase

TLR signaling may also be negatively regulated by NADPH oxidase. NADPH oxidase has an essential role in the intracellular killing of engulfed microbes. It has been suggested that TRAF4 is produced after TLR signaling and interacts with $p47^{phox}$, IRAK1, TRAF6 and TRIF, thereby inhibiting TLR signaling (219). This demonstrates an important mechanism for negative feedback control of TLR signaling.

IRF4

IRF4 is involved in the negative regulation of TLR signaling. IRF4 competes for binding with IRF5 because increasing the expression level of IRF4 inhibited the interaction of IRF5 but not IRF7 with MyD88. The function of IRF4 as an important negative regulator of TLR signaling is highlighted by the evidence that IRF4 deficient mice showed a more potent inflammatory response and succumbed more rapidly than wild type mice after stimulation with unmethylated CpG-oligodeoxynucleotides (220)

1.6 Control of the Cellular Response to Interaction with Microbes

It is not completely clear how the expression of genes is controlled (199). The expression of genes is controlled in a complex manner. There are promoter sequence specificity, signaling specificity and cell type specific regulation.

There is evidence that suggests stimulating cells with LPS can induce nucleosome remodeling around a specific promoter (221). Specific regions of DNA that are normally hidden by tight complexes with histones are exposed, increasing the accessibility of the promoter region to transcription factors, which increases specific gene transcription. Therefore, chromatin/nucleosome remodeling yields another mechanism for controlling the outcome of TLR signaling (199).

The signaling pathways that are activated as a result of several different host-microbe interactions leads to the activation of multiple transcription factors that are able to up-regulate the transcription of several different genes. Co-repressors and co-activators recruited to DNA bound transcription factors play an important role in regulating gene transcription. Microbes target specific cells which recognize patterns on the microbe to activate signaling pathways that ultimately activate the transcription factors NFκB, AP-1 and/or IRF3/7. These transcription factors, along with co-repressors and co-activators, lead to the transcription of a number of genes including cytokines. Secreted cytokines bind receptors and then act in an autocrine or paracrine fashion to further activate the host for microbe clearance (222).

Examining the regulation of the function of transcription factors has led to the discovery that transcription factors work together under certain stimuli to up-regulate gene transcription. For example, it has been shown that IRF3 can act as a cofactor with NFκB for the transcription of CXCL10 (223). The p65 subunit of NFκB interacts with IRF3 to induce transcription after LPS stimulation. NFκB is required for the activation of IRF3 by LPS, and, in the absence of the p65 subunit of NFκB, TLR4 actually fails to induce ISRE dependent gene expression (224). Since co-activators are important for transcriptional activation, the interaction of p65 and IRF3 at the ISRE may be mediated by the IRF3 co-activator cAMP-responsive element-binding protein-binding protein (CBP), which interacts with both IRF3 and p65 (198, 225). The interaction of p65 with IRF3 could have a synergistic effect or alter the conformation of IRF3 to activate

transcription (224). The fact that transcription factors can function together to activate gene transcription is important because it increases the complexity and specificity of the response to each microbe component.

1.7 Cytokines and Chemokines

1.7.1 Cytokines

Cytokines are small molecules, approximately 8 to 40 kDa in size, that are secreted from a variety of cells and that allow cells to communicate without direct contact. Cytokines are made in response to the recognition of microbes and are vital to the activation and function of the immune system. Most cytokines are often referred to as interleukins because they allow ease of communication between leukocytes; however, other cells including epithelial and endothelial cells can produce cytokines and respond to cytokines. Some important features of cytokines are that they can have pleiotropic effects and that multiple cytokines play redundant roles. A cytokine can act in an autocrine manner on the cell that produced it, in a paracrine manner on nearby cells and in an endocrine manner on cells in distant areas of the body. The expression of cytokine receptors is highly regulated. Cytokine receptors relay a signal into the cell to trigger a response. Many cytokine-receptor interactions are promiscuous, meaning the receptor may bind many ligands and ligands may bind several different receptors. When cytokines bind their receptor they can activate gene transcription of other cytokines, enhance expression of adhesion molecules and act in a negative feedback manner to inhibit their own effect. Cytokines help regulate development, haematopoiesis, tissue repair, inflammation, the innate and adaptive immune responses (227-231).

There are structural similarities between the cytokine receptors that allow them to be divided five major receptor families: the haematopoietic growth factor receptor family (type I cytokine receptors), the TNFR group, members of the Ig-supergene receptor family, the receptors for IFNs and IL-10 (type II cytokine receptors), and the 7-transmembrane spanning G-protein coupled chemokine receptors. Part of the explanation for the redundancy of cytokines is that many receptors in the same family use a common

signal transducing receptor component or share common intracellular signaling molecules (228, 230).

1.7.2 Chemokines

Chemokines are a special type of cytokine that are specifically involved in the recruitment of cells to sites of inflammation by chemotaxis. The chemokine family members are classified into four groups according to the position of conserved cysteine residues near the amino terminus of the amino acid sequence: C, CC, CXC and CX_3C. The chemokine receptors are G protein coupled receptors with 7 transmembrane domains (232-234). Chemokines are produced to recruit leukocytes to a specific tissue (235, 236), some other roles of chemokines that have been reviewed include involvement in development, survival, angiogenesis, homeostasis, increasing gene transcription and the activation of cells (237-239).

1.7.3 Some of the Important Cytokines and Chemokines in this Model

Some cytokines increase inflammation and are called pro-inflammatory while others reduce inflammation and are called anti-inflammatory. IL-1β and TNF are pro-inflammatory cytokines that produce fever, inflammation, tissue destruction and, in severe cases, shock and death (229). Some of the important cytokines in the innate immune responses that I have examined include TNF, IL-1β and the chemokines CXCL2/MIP-2, CXCL1/KC and CCL5/RANTES.

1.7.3.1 Tumor Necrosis Factor (TNF)

TNF is a 17 kDa cytokine that forms trimers of 51 kDa and is secreted mainly by monocytes and macrophages but can also be produced by mast cells and lymphocytes (18, 38). The receptors for TNF, TNFR1 and TNFR2, are expressed on most cell types (240). TNFR1 primarily binds soluble TNF and TNFR2 preferentially binds membrane bound TNF (241, 242). TNF is an endogenous pyrogen (243) that plays a role in cachexia (244). It can lead to the production of prostaglandins (245), cytokines and chemokines (24, 243, 246), and increase expression of adhesion molecules (247), and

MHC class I and class II (248). In addition, TNF can signal to induce apoptosis (240, 249).

TNF was originally identified by its necrosis inducing properties but it also plays an important role in the innate immune response to infection. TNF is produced early on during an infection and has been described as an "alarm" cytokine. Lipopolysaccharide (LPS) is a potent stimulator of TNF production (9, 16). Evidence suggests that TNF affects neutrophil recruitment directly (250) and indirectly by playing a role in the production of CXC chemokines such as CXCL8/IL-8 (18). TNF can induce leukocyte rolling and recruitment by activating endothelial cells to express adhesion molecules (251, 252).

Several studies indicate that efficient bacterial killing in the lung requires TNF signaling (253-255). However, the inhibition of TNF signaling has shown varied effects on the recruitment of neutrophils into the lungs dependent on the stimuli and timing (253-255). Signaling through the TNFR1 has been found to be vital for appropriate host defense to pathogens however the role of TNFR2 appears to be minimal (256, 257). Skerrett *et al.* (258) found that neutrophil recruitment was impaired TNFR1 deficient mice following challenge with LPS but not *P. aeruginosa.* Ulich *et al.* (259) reported that rats given soluble TNFR1 showed decreased neutrophil migration at 6h after LPS challenge but not at 4 or 12 h. Laichalk *et al.* (254) showed that inhibiting TNF diminished neutrophil migration 48 h after an infection but not 24 h. TNF signaling may therefore play a role in neutrophil migration at specific time frames, depending on the stimuli (241).

1.7.3.2 IL-1β

IL-1β is a 15 kDa pro-inflammatory cytokine that was originally described by its ability to induce fever (260). IL-1β induces the expression of cyclooxygenase 2 (261), cytokines, chemokines (246) and adhesion molecules (262). IL-1β has several similar functions as TNF; however, one important difference is that it does not induce apoptosis, discussed in the review by Dinarello (260). The precursor to IL-1β is found in the cytosol and in secretory vesicles and is cleaved by active caspase-1, an intracellular cysteine protease that processes the precursor to IL-1β into the mature form of IL-1β

which can be secreted (260, 263). IL-1β binds the type I IL-1 receptor (IL-1RI), which is a member of the TIR superfamily (264). IL-1β induces MyD88 dependent signaling (265) leading to nuclear factor κB (NFκB) (266) and AP-1 (267) activation to increase gene transcription of chemokines, cytokines, acute-phase proteins and cell adhesion molecules, reviewed in (268). IL-1β also binds IL-1RII, which is a decoy receptor. Both receptors for IL-1β are found in membrane bound and soluble forms (264). The soluble forms of the IL-1R prevent signaling of IL-1β and can essentially soak up available IL-1β. There is an IL-1 receptor antagonist (IL-1Ra) that also interferes with IL-1β signaling through preventing IL-1β from binding to its receptor by directly interacting with the IL-1R (260, 264, 269). IL-1β and TNF activate and are activated by NFκB generating a positive regulatory loop that may amplify and perpetuate local inflammatory responses (270).

Mice with a deficiency in TNFR1 and IL-1R have impaired neutrophil recruitment after bacterial challenge (271) that is more severe than in mice with deficiencies in TNFR1/TNFR2 (253) or IL-1R alone (271), suggesting that these mediators produce additive or synergistic effects. There was a significant decrease in the production of the neutrophil chemoattractant CXCL1/KC, but not CXCL2/MIP-2, in the TNFR1 and IL-1R doubly deficient mice, which suggests that CXCL1/KC is produced as a result of the TNFR and IL-1R signaling pathways and may in part be responsible for the decrease in neutrophil recruitment (271). These data suggest that the downstream signaling of these combined receptors is important for neutrophil recruitment after bacterial infection.

1.7.3.2 Neutrophil Chemoattractants (CXCL2/MIP-2 and CXCL1/KC)

The CXC chemokine, CXCL8/IL-8, is associated with increased neutrophil influx into the airspace in humans (30, 272, 273). In rodents, the two most important chemokines for neutrophil recruitment into the lung are CXCL1/KC (274, 275) and CXCL2/MIP-2 (275-277), which are both 8kDa in size. Interestingly, CXCL2/MIP-2 and CXCL1/KC production can be initiated by TNF (278). Blocking antibodies against CXCL1/KC or CXCL2/MIP-2 decreased neutrophil recruitment in lungs of rats after stimulation with LPS (279, 280), and in mice after pulmonary infection with *P.*

aeruginosa but did not have an effect on bacterial clearance or survival (281). Blocking the function of CXCL2/MIP-2 during an infection by *Klebsiella pneumoniae* inhibited the recruitment of neutrophils to the lungs of mice (282). Interestingly, transgenic expression of CXCL1/KC enhanced the clearance of *K. pneumoniae* from the lung (283).

CXCL1/KC and CXCL2/MIP-2 mediate neutrophil migration into the lung (284); however, in mice both chemokines act through the CXCR2 receptor (285). Blocking antibodies against CXCR2 led to a decrease in neutrophil recruitment, impaired bacterial clearance and an increase in the mortality of mice infected with *P. aeruginosa* (281). In a model of *P. aeruginosa*-induced corneal infection, CXCR2 deficient mice exhibited an impaired neutrophil recruitment and bacterial clearance (286). Similarly, after inhalation of LPS neutrophil recruitment was significantly impaired in CXCR2 deficient mice (287, 288). Therefore, the neutrophil chemoattractants CXCL1/KC and CXCL2/MIP-2 and their receptor, CXCR2, are important in neutrophil recruitment and bacterial clearance after infection.

1.7.3.4 Regulated Upon Activation Normal T Cell Expressed and Secreted (CCL5/RANTES)

CCL5/RANTES is an 8kDa (289) CC chemokine that plays a fundamental role in inflammatory processes (290, 291) and is a chemoattractant for T cells (292), monocytes (290, 292) and eosinophils (293). CCL5/RANTES is expressed by activated T cells (289), while fibroblasts (294) and epithelial cells (295, 296) can produce CCL5/RANTES many hours after stimulation with TNF. The differences in the induction of CCL5/RANTES transcription in different cell types suggests there may be distinct control mechanisms that regulate production of this chemokine (16).

Inflammatory cytokines can promote the production of CCL5/RANTES by activating NFκB. The binding sites for NFκB in the CCL5/RANTES promoter contribute to the transcription of CCL5/RANTES after stimulation with TNF and IL-1β (297). The NFκB region of the CCL5/RANTES promoter is important for activation of the CCL5/RANTES promoter in lung alveolar epithelial cells around 24 h after stimulation with TNF (296). However, the production of inflammatory mediators may not be required for the production of significant levels of CCL5/RANTES. Macrophages

stimulated with LPS induce the transcription of CCL5/RANTES as a direct result of signal transduction but the process does not require de novo protein synthesis, as determined by treatment with cycloheximide (189). Therefore, inflammatory mediators and direct signaling can induce CCL5/RANTES production.

The control of CCL5/RANTES production is complex and has been shown to be determined by the transcription factors NFκB and IRF3, depending on the stimulus. The signaling adaptor molecule TRIF is important in the production of CCL5/RANTES after stimulation with LPS and poly(I:C) (163). A dominant negative form of the transcription factor interferon regulatory factor 3 (IRF3) inhibits the virally induced expression of the CCL5/RANTES promoter, therefore demonstrating the involvement of IRF3 in the regulation of CCL5/RANTES gene expression (298). It is not completely clear to what degree LPS induced CCL5/RANTES production depends on NFκB and/or IRF3. In one study, it was shown that LPS induced production of CCL5/RANTES involved IRF3, since IRF3 deficient macrophages did not induce CCL5/RANTES expression after LPS stimulation (299). However, in another report the expression of CCL5/RANTES was up-regulated in IKKi and TBK1 deficient fibroblasts stimulated with LPS (205), suggesting that CCL5/RANTES may be up-regulated by TRIF but not through the TBK1 and IRF3 pathway. Jang *et al.* (300) show that LPS induced CCL5/RANTES production is dependent on NFκB and that TAK1 and NFκB inducing kinase (NIK) are responsible for activating NFκB. The differences observed in these studies may be a result of the cell types used. Perhaps the regulation of CCL5/RANTES production is variable, depending on cell type and stimulation. Therefore, further research need to be done to resolve the conflicting evidence regarding whether IRF3, NFκB or both are responsible for CCL5/RANTES production.

1.7.4 Relevance of Inflammatory Mediator Production

Inflammatory mediator production is important in alerting the host that there is an infection. The inflammatory mediators produced after cells local to the infection recognize a pathogen act to alert other cells and mobilize their innate immune responses (227, 229). Cytokines and chemokines are vital for the recruitment of neutrophils to the site of infection, and up-regulating their anti-microbial functions (301, 302).

1.8 Neutrophils

Neutrophils are short-lived phagocytes (303) that are rapidly recruited from the circulation to sites of inflammation and are essential to host defense against many microbial infections (15, 304). Neutrophils are derived from myeloid precursors in the bone marrow and released into the circulation as mature neutrophils (305). Host and/or microbe derived components recruit neutrophils to sites of infection and prime neutrophils to enhance microbicidal activity (304). Within 3 – 4 h after lung infection, significant numbers of neutrophils are recruited into the airways in animal models (16, 306-308). The importance of neutrophils in killing bacterial and fungal pathogens is evident in patients that lack neutrophils (neutropenic) (301, 302). Neutrophils can kill microbes by binding and ingesting microbes through the process of phagocytosis. The fusion of lysosomal granules with the phagosome and the production of reactive oxygen species are effective in killing most bacteria (304). Excessive neutrophil activation may damage tissues (303); therefore, regulation of the innate immune response is important for the effective resolution of infection and inflammation.

1.8.1 Neutrophil Recruitment

The active recruitment of neutrophils to the site of infection is fundamentally important for the innate immune response (304). Host factors produced that can recruit neutrophils (Figure 1.1) include CXCL8/IL-8 (30, 272, 273), C5a (89) and leukotriene (LT)B4 (309). Neutrophil recruitment is also mediated by bacterial components such as *N*-formylated peptides (fMLP) (310). These chemoattractants are also able to prime neutrophils for enhanced function, thereby facilitating host defense.

Selectins are glycoproteins that have extracellular lectin-like domains that interact with sialylated carbohydrate determinants and mucin-like glycoproteins (304, 311). In mammals, three structurally similar family members have been identified and the expression of each is cell type dependent; for example, E-selectin and P-selectin are present on the surface of cytokine activated endothelial cells (251), while P-selectin is found on activated platelets (312), and L-selectin is found on eutrophils and other leukocytes (313). Selectins mediate the initial tethering of circulating leukocytes with endothelial cells (314) (Figure 1.1). P-selectin is up-regulated to the surface of

endothelial cells from pre-formed intracellular granules (Weibel-Palade bodies) by stimulation with mediators such as C5a (89), LTB4 (315), CXCL8/IL-8 (315) and histamine (316). In contrast, E-selectin requires synthesis which is usually mediated by cytokine stimulation. Tethering and rolling can be initiated when E- and P-selectin interact with CD162 (P-selectin glycoprotein ligand-1) on neutrophils (304, 317).

Rolling allows neutrophils to scan the surface of the endothelium for the presence of chemokines (Figure 1.1). Resident cells at the site of infection produce TNF, IL-1β (27, 29, 30) and histamine (318), which induces the endothelium to secrete CXCL8/IL-8 (246, 319). CXCL8/IL-8 is held near the surface of the endothelium by glycoproteins (320), where it interacts with the chemokine receptors CXCR1 and CXCR on human neutrophils, resulting in the activation of integrins such as LFA-1 (CD11a/CD18) (98, 321). Conformational changes in LFA-1 lead to high affinity binding and increased avidity for its ligand ICAM-1, which leads to firm adhesion of the neutrophil to the endothelium (98-100). Other neutrophil chemoattractants can influence integrin binding and, therefore, firm adhesion of the neutrophil to the endothelium. LTB4 can induce an increase in integrin clustering and surface mobility (avidity), and fMLP evokes a large increase in the affinity of β2-integrins, as well as in avidity (322). After neutrophils are firmly attached to the endothelium, transmigration through the endothelium occurs, preferentially at tricellular corners (323), moving up the chemotactic gradient of chemokines made by various cell types (24-30), resulting in the accumulation of neutrophils at the site of infection.

The conventional model of neutrophil tethering, rolling, activation, firm adhesion and extravasation through the endothelium is appropriate for describing neutrophil migration into larger diameter pulmonary arterioles and venules, but not lung capillaries (324). Neutrophils must change their shape in order to squeeze through the narrow capillaries and the conventional model for neutrophil rolling is not necessary in this instance (325).

1.8.2 Neutrophil Function

1.8.2.1 Neutrophil Priming

Priming of a neutrophil has been defined as an enhancement of the neutrophil respiratory burst (326, 327) in response to an activating stimulus following exposure to an agent that does not induce a respiratory burst on its own (328). However, neutrophil priming can also result in enhanced neutrophil adhesion (329), phagocytosis (330), cytokine production (331), leukotriene synthesis (332), and degranulation (330) in response to a second stimulation. Some agonists that prime neutrophils include cytokines such as GM-CSF and TNF (332, 333), chemokines such as CXCL8/IL-8 (333), and lipid derived signaling molecules such as platelet-activating factor (PAF) (334) and LTB4 (327, 335). Microorganisms produce several agonists that can prime neutrophils; many of these components from microorganisms are also toll-like receptor (TLR) agonists such as LPS (326). The most important function of priming is the promotion of clearance of microbes (304).

1.8.2.2 Neutrophil Phagocytosis

Neutrophils are vital in the killing of microbes. Neutrophils bind to and engulf microbes by a process called phagocytosis. During phagocytosis, a pseudopod extends to engulf particulate matter including microbes and the phagosome closes. The phagosome fuses with lysosomes (336, 337), exposing microbes to cytotoxic granule contents. Neutrophil phagocytosis can be initiated by microbes opsonized with collectins, complement, antibody and possibly TLRs (12, 36). Neutrophils are more efficient phagocytes than alveolar macrophages and are able to effectively clear bacterial lung infections (338).

1.8.2.3 Overview of Neutrophil Involvement in Host Defense

Neutrophils recognize and engulf microbes by phagocytosis (339). The neutrophil's ability to kill microbes comes from pre-stored granule mediators that are released into the phagolysosome or into the extracellular environment. There are oxygen dependent and independent mechanisms of killing microbes (304, 340).

There are different types of granules in a neutrophil. The primary granules are also referred to as peroxidase positive or azurophilic granules (340) because of the high content of myeloperoxidase (337) and the affinity of these granules for the basic dye azure A (340). Other granules are peroxidase negative and have been divided into specific (secondary) and gelatinase (tertiary) granules. The specific granules have a high content of lactoferrin and the gelatinase granules contain high levels of gelatinase (341).

1.8.2.4 Oxidative Burst

Reactive oxygen species (ROS) are produced after stimulation and especially increased after phagocytosis (342). The NADPH oxidase is made of multiple components. The cytosolic components of NADPH oxidase include $p40^{PHOX}$ (343, 344), $p47^{PHOX}$ (345), $p67^{PHOX}$ (345) and small Rho-like GTPases (Rac1 (346) and Rac2 (347)). The components of NADPH oxidase that are on the membrane of peroxidase-negative granules are cytochrome b_{558} (composed of two subunits $p22^{PHOX}$ and $gp91^{PHOX}$ (348)) and Rap1A (349). NADPH oxidase is dormant in resting cells but it can be activated quickly by chemokines (350), Fc and complement receptor ligation with bacteria (351). Stimulation results in the phosphorylation of $p47^{PHOX}$ (352), which initiates assembly of the NADPH oxidase complex at the membrane and conversion of molecular oxygen to superoxide by transferring electrons from NADPH in the cytoplasm to phagosomal or extracellular oxygen (353-355). Superoxide is converted to hydrogen peroxide spontaneously or the reaction is catalyzed by superoxide dismutase (356, 357). The azurophilic granules of neutrophils contain myeloperoxidase (MPO) (358), which is an enzyme that catalyzes the reaction of hydrogen peroxide (H_2O_2) (359) and chloride (Cl^-) (360) to produce HOCl (361-363). HOCl can react with other molecules such as amines to produce toxic substances that are antimicrobial (358, 364, 365).

Support for the role of ROS in killing microbes is found in patients with chronic granulomatous disease (CGD) (366, 367). Humans with CGD are unable to generate ROS and are susceptible to bacterial and fungal infections, as reviewed in (355). Patients with a deficiency in MPO (368) do not succumb to bacterial infections (369) however patients that have other conditions such as diabetes mellitus have an increased susceptibility to infections with *Candida albicans* (370). Recent evidence (371), contrary

to previous results (372), indicate that MPO deficient neutrophils are impaired in the bacterial killing of *Staphylococcus aureus* (371). This suggests that oxidants are critical but the role of MPO in bacterial killing many not be essential.

1.8.2.5 Non-Oxidative Killing Mechanisms

Neutrophil granules also contain mediators involved in microbe killing that do not require oxygen. There are many antimicrobial substances in the granules of the neutrophil, a few of which will be discussed here such as antimicrobial peptides, lysozyme, and proteases (358).

Defensins

Defensins are small, cationic peptides that have conserved cysteine residues and are stored within the primary granules of neutrophils (373). Defensins associate with the cell walls of bacteria, fungi and enveloped viruses via electrostatic attractions disrupting the integrity of the membrane by creating pores in it (374).

Bactericidal/permeability-increasing protein (BPI)

BPI is another cationic antimicrobial peptide in the primary granules of neutrophils that interacts with negatively charged residues of LPS; specifically, BPI has a high affinity for the lipid A component of LPS (375). BPI is cytotoxic and disrupts the cell membrane by interacting with LPS (376), neutralizes the endotoxicity of LPS (377) and opsonizes Gram-negative bacteria to enhance phagocytosis (378). BPI is the closest relative to LBP and they both bind LPS; however, BPI binds LPS with a higher affinity that LBP (379). LBP enhances a pro-inflammatory response to LPS; however, BPI decreases the immune response to LPS. It has been suggested that BPI functions in a negative feedback loop which opposes LPS activation. The stronger affinity of BPI for LPS over LBP may play a role in the observed inhibition of the LBP induced pro-inflammatory response when BPI and LPS are present (379). A recent study shows that BPI is able to suppress the activation of the IFN-β and NFκB promoters by LPS/lipid A. This suggests that BPI released from neutrophils may act as a negative regulator after infection and may be able to play a role in controlling cytokine expression by inhibiting

TLR signaling through MyD88 and TRIF (380). The mechanism by which BPI works in this system is not clear. BPI may alter LPS in such a way that it is no longer able to interact with its receptor and initiate signaling.

Proteases

There are proteases in the primary granules of neutrophils that are microbicidal such as proteinase-3 (381), cathepsin G (382) and elastase (383). A controversial view concerning the importance of the production of ROS has been presented by Segal (384). He believes that proteases in the granules of neutrophils have a primary role in killing bacteria as neutrophils from mice deficient in both cathepsin G and elastase could not kill bacteria. His model suggests that the MPO disposes of H_2O_2 and does not play an important direct role in microbial killing. Segal proposes that superoxide is a nucleophile used to soak up free protons in the phagosome, which is important in raising the pH to levels favorable for efficient protease function. He claims that the function of the peroxidases and their products are to aid the function of the proteases (372). Recently evidence suggests that the method used to determine neutrophil microbial killing may provide inaccurate results. The method used to lyse neutrophils and determine viable bacteria has been called into question recently (371), therefore Segal's argument may not be valid. The mechanisms of microbe killing inside the neutrophil are complex and likely require a combination of mediators oxygen dependent and independent.

Lactoferrin

Lactoferrin is a glycoprotein found in specific granules that has broad antimicrobial effects on Gram-negative and Gram-positive bacteria. Early studies on lactoferrin indicated that iron sequestration was the primary reason for its antimicrobial activity, reviewed in (340, 385). However, a cationic peptide released from lactoferrin called lactoferricin has been shown to exhibit antimicrobial properties (386).

Lysozyme

Lysozyme is a cationic peptide that is found in all neutrophil granules (340, 387) and is produced by others cells including macrophages (388) and epithelial cells (389).

Lysozyme is cytotoxic for both Gram-negative and Gram-positive bacteria (340). Lysozyme harms the cell wall of Gram-negative bacteria by binding to LPS (390) and disrupting the membrane. Lysozyme functions to kill Gram-positive bacteria by hydrolyzing peptidoglycan polymers (391). Increasing the expression of lysozyme in the lungs of transgenic mice enhanced bacterial killing after infection with *P. aeruginosa* and group B *Streptococcus* (392). Mice that are deficient in lysozyme had a decreased ability to kill bacteria and reduced survival following lung infection with *Klebsiella pneumoniae* (393). After lung infection with *P. aeruginosa*, lysozyme deficient mice had an impaired bacterial clearance (394). Therefore, lysozyme is a vital component of the host defense in the lungs.

1.8.2.6 Production of Cytokines and Chemokines by Activated Neutrophils

Once recruited to the site of infection, neutrophils are able to actively participate in the host response to infection because they can interact with microbes and produce cytokines such as TNF, IL-1β, and CXCL2/MIP-2 (9, 306).

1.8.2.7 Neutrophil Apoptosis

There is an important balance between survival and apoptotic signals that define the lifespan of a neutrophil. Neutrophils are short-lived cells having an *in vivo* half-life of about 6 – 8 h (303, 395). Neutrophils undergo apoptosis by engagement of TNF and Fas receptors, stress stimuli and phagocytosis (396, 397). Cytokines and LPS delay the spontaneous apoptosis of the neutrophil (398). TLR activation has been shown to be important for neutrophil survival and may inhibit apoptosis of neutrophils (399). A combination of TLR signaling and activation signals from macrophages seem to be required for enhanced inhibition of apoptosis in neutrophils (400). This is important because apoptosis is inhibited during the early stages of inflammation when cytokines are produced. However, phagocytosis mediated by complement receptors or FcR, which is responsible for killing microbes, enhances apoptosis (396, 401). The β_2 integrin known as either Mac-1, CD11b/CD18 or CR3 is a complement binding receptor that is important for neutrophil activation, recruitment and phagocytosis (11). It has been shown that phagocytosis-induced cell death does not occur in CR3 deficient murine neutrophils.

Therefore, phagocytosis-induced cell death proceeds through a CR3 dependent pathway (402). Recent evidence indicates that a functional NADPH oxidase impairs the apoptosis of neutrophils through caspases. Therefore, the distinction between survival and apoptosis signals may involve a sequence of events after phagocytosis and killing of microbes in the phagosome (403). Between 3 – 6 h after phagocytosis there is an increase in transcription in apoptosis related genes (401). The phagocytosis of a microbe produces ROS, which induce lysosomal membrane permeabilization and evidence suggests that this process is involved in inducing apoptosis by cleaving the proapoptotic Bcl-2 protein Bid (404). Apoptotic neutrophils are phagocytosed by nearby macrophages (405). The apoptosis and subsequent removal of phagocytic neutrophils from a site of inflammation is pertinent to the resolution of inflammation (304).

1.9 The Importance of *Pseudomonas aeruginosa* Infections

The immune response is normally effective in eliminating *P. aeruginosa* from the host and promoting healing. However, cystic fibrosis patients suffer from chronic respiratory infections, indicating that the immune response is not effective in eradicating microbes from their lungs. It seems as though the inflammation present in the lungs of cystic fibrosis patients is damaging enough to harm and destroy the lung architecture but it is not effective enough to clear a *P. aeruginosa* infection (10).

1.9.1 *P. aeruginosa*

P. aeruginosa is a Gram-negative bacillus which is ubiquitous in the environment (406). It is an opportunistic pathogen that preferentially infects immunocompromised individuals. Life threatening infections can develop in burn (407), cancer (408), and neutropenic patients, and in the lungs of immunocompromised patients, especially patients with cystic fibrosis (CF) (409). Uncontrolled *P. aeruginosa* infections can develop into sepsis (410); however, CF patients rarely develop blood stream invasion by *P. aeruginosa*. The virulence factors that enable *P. aeruginosa* to infect mammals include cell associated and secreted virulence factors. The cell associated virulence factors are endotoxin (LPS) (411), pili (412), flagella (413) and alginate capsules (414). Secreted virulence factors include proteases (415), exoenzymes (416) and exotoxins

(417). *P. aeruginosa* have a type III secretion system, whereby, they can inject specific proteins directly into cells when they come into direct contact (418, 419).

Nonmucoid forms of *P. aeruginosa* colonize the airway and then convert to a mucoid form that that overproduce an extracellular polysaccharide alginate that promotes the generation of biofilms (418, 420). Mutations in the *mucA* gene (Figure 1.4) are predominantly responsible for the conversion of *P. aeruginosa* isolates to the mucoid form (421, 422). MucA is a negative regulator of *algU* (Figure 1.4), preventing the transcription of enzymes that are important in alginate biosynthesis (423). Evidence suggests that the mutations in *mucA* are introduced by the activity of the DNA polymerase IV and defective *mutS* (Figure 1.4), which is responsible for recognizing mismatched DNA for repair (422). There are several regulators of alginate synthesis (424). Three new positive regulators have recently been identified for alginate biosynthesis *mucE*, *mucP* and *algW* (Figure 1.4), which result in increased levels of *algU* and decreased *mucA* levels (425). The conversion of the non-mucoid form of *P. aeruginosa* to the mucoid form happens during times of stress and involves mutations in key regulatory elements.

1.9.2 *P. aeruginosa* Infection Model

There are several *P. aeruginosa* infection models. The agar bead model is representative of an infection with *P. aeruginosa* in a mature biofilm. An acute infection model may be more representative of the innate immune response after the initial infection with *P. aeruginosa* (426). Therefore, acute pneumonia models with *P. aeruginosa* are useful for studying pneumonias in non-CF patients and in studying the initial stages of *P. aeruginosa* colonization in the CF airway (427).

Although mice with a deficiency in cystic fibrosis transmembrane conductance regulator (CFTR) exist, they do not represent a good model of human disease because they do not spontaneously develop lung infections. There may also be a second chloride transporter besides CFTR that is able to function in these mice and compensate for a deficiency in CFTR (428). Therefore, a good mouse lung model for CF has been difficult to develop.

1.9.3 Cystic Fibrosis (CF)

The first description of cystic fibrosis was published in 1936 by Dr. Guido Fanconi (429, 430) and was further characterized by Dr. Dorothy Andersen in 1938 (431). CF is the most common fatal autosomal recessive genetic disorder affecting Caucasians (432, 433). It arises from mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which results in improper chloride transport (434-437). It is estimated that one in every 3,600 children born in Canada has CF (438). CF is characterized by abnormally thick mucus layers in the lumens of all secretory organs. The most problematic organs for these patients are the lungs and the gastrointestinal track. Although the exact mechanisms of the defects, are under debate, CF patients suffer from chronic sinopulmonary infection (431), abnormally high concentrations of electrolytes in sweat (439), as well as gastrointestinal, nutritional, and other abnormalities (433).

Patients with CF have several medical problems due to the CFTR gene mutation but currently the most problematic feature of the disease is chronic pulmonary infection leading to progressive lung destruction (431), primarily due to lung infection with *P. aeruginosa* (440-442). *P. aeruginosa* infection is the most important bacterial pathogen in lungs of CF patients (443). Currently, chronic infection with *P. aeruginosa* is responsible for lung function decline and the mortality of CF patients. Approximately, 80 to 95% of CF patients will die from respiratory failure due to chronic bacterial infection and airway inflammation (433, 440, 441, 444, 444, 445). CF has not always been a disease characterized by chronic pseudomonal lung infection. Before 1946, the reports of CF pseudomonal lung infections were low. At this time, Di Sant'Agnese and Andersen reported that infection with *Staphylococcus aureus* is more prevalent than *P. aeruginosa* (446). The importance of pseudomonal infection was first described by Garrard *et al.* in 1951 (447). The emergence of *P. aeruginosa* as an important pathogen in CF patients may be due to prolonged use of antibiotics (447). In 1991, a study by Abman *et al.* reported that *S. aureus* was the most frequent isolate found in young infants with CF but that *P. aeruginosa* was recovered more often after 2 years of age (448). It was found that the same strain of *P. aeruginosa* persisted in individual patients but

differed between patients, indicating that the initial colonizing strain persists in patients but undergoes phenotypic changes (448).

1.9.4 Understanding *P. aeruginosa* Infection in CF Patients

Patients with CF commonly suffer from *P. aeruginosa* lung infections (449). Since *P. aeruginosa* is successful in colonizing the CF host, it can be surmised that there may be deficiencies in the innate immune response of the CF patient. *P. aeruginosa* sets up a chronic infection in these patients by generating a biofilm that leads to persistent inflammation (426). It has been shown that patients with CF benefit from the use of a *P. aeruginosa* vaccine that reduces the proportion of CF patients developing chronic *P. aeruginosa* infection and increases the time to infection (450). The possibility of an effective vaccine may prove to be very promising for lengthening the lifespan of CF patients.

CF patients do not clear *P. aeruginosa* and suffer chronic lung infections. In the normal host, *P. aeruginosa* interacts with resident cells, which leads to the production of cytokines and chemokines that recruit effector cells. Initially, neutrophils are recruited that phagocytose *P. aeruginosa* and clear the infection. Bacterial clearance does not occur in CF patients, but the mechanisms that underlie the incompetence of the innate immune response in CF patients are unclear. There has not been classical immunodeficiency identified to account for the susceptibility of CF patients to lung infections; however, they have dehydrated mucus which impedes mucociliary clearance, and may play a role in impaired bacterial clearance (451, 452). In understanding the problem associated with effective bacterial clearance in CF patients, it is important to determine what the appropriate molecular mechanisms are in the innate immune response to *P. aeruginosa* in order to effectively evaluate and then test what may be wrong in CF patients.

A correlation has been found between MBL deficiency and *Pseudomonas* infections in cystic fibrosis patients, suggesting that MBL deficiency in CF patients may be associated with poor prognosis and early death (453). Another study found no correlation between MBL levels in the serum and clinical outcome in a group of CF patients. They found that the genotype frequency of MBL in CF patients was similar to

healthy controls (454). Therefore, further investigation into whether other components of the innate immune response are impaired is vital for improving treatments for CF patients.

1.9.5 The Role of the CFTR in *P. aeruginosa* Infection

The functional defect in CF patients is a defective CFTR, which is important for chloride transport (437). There are links between functional CFTR and proper immune responses. Although *P. aeruginosa* is generally thought to be an extracellular pathogen; evidence suggests that *P. aeruginosa* can be internalized into cells such as epithelial cells and survive (455). There are conflicting reports regarding the mechanisms of internalization of *P. aeruginosa.* Darling *et al.* (455) show that *P. aeruginosa* can internalize into epithelial cells with a mutant CFTR and survive for prolonged periods, while the internalization of *P. aeruginosa* is decreased by the presence of a functional CFTR, suggesting that functional CFTR can inhibit cellular invasion of *P. aeruginosa* (455). The report by Darling *et al.* (455) conflicts with reports by Pier *et al.* (456, 457)that indicate CFTR mediates the uptake and internalization of *P. aeruginosa* into epithelial cells. However, this group has subsequently found that epithelial cells without CFTR fail to activate the nuclear translocation of NFκB after LPS stimulation (458). and that functional CFTR is required for *P. aeruginosa* induced IL-1β secretion (14).

1.9.6 Impaired Neutrophil Recruitment and Antimicrobial Activity of Airway Surface Liquid in CF Patients

Neutrophil recruitment may be impaired in the early stages of *P. aeruginosa* infection in CF patients due to a decrease in neutrophil chemoattractant production (14). A decrease in the inflammatory response during the initial stages of infection may lead to the chronic colonization of *P. aeruginosa* in the CF lung (14, 455).

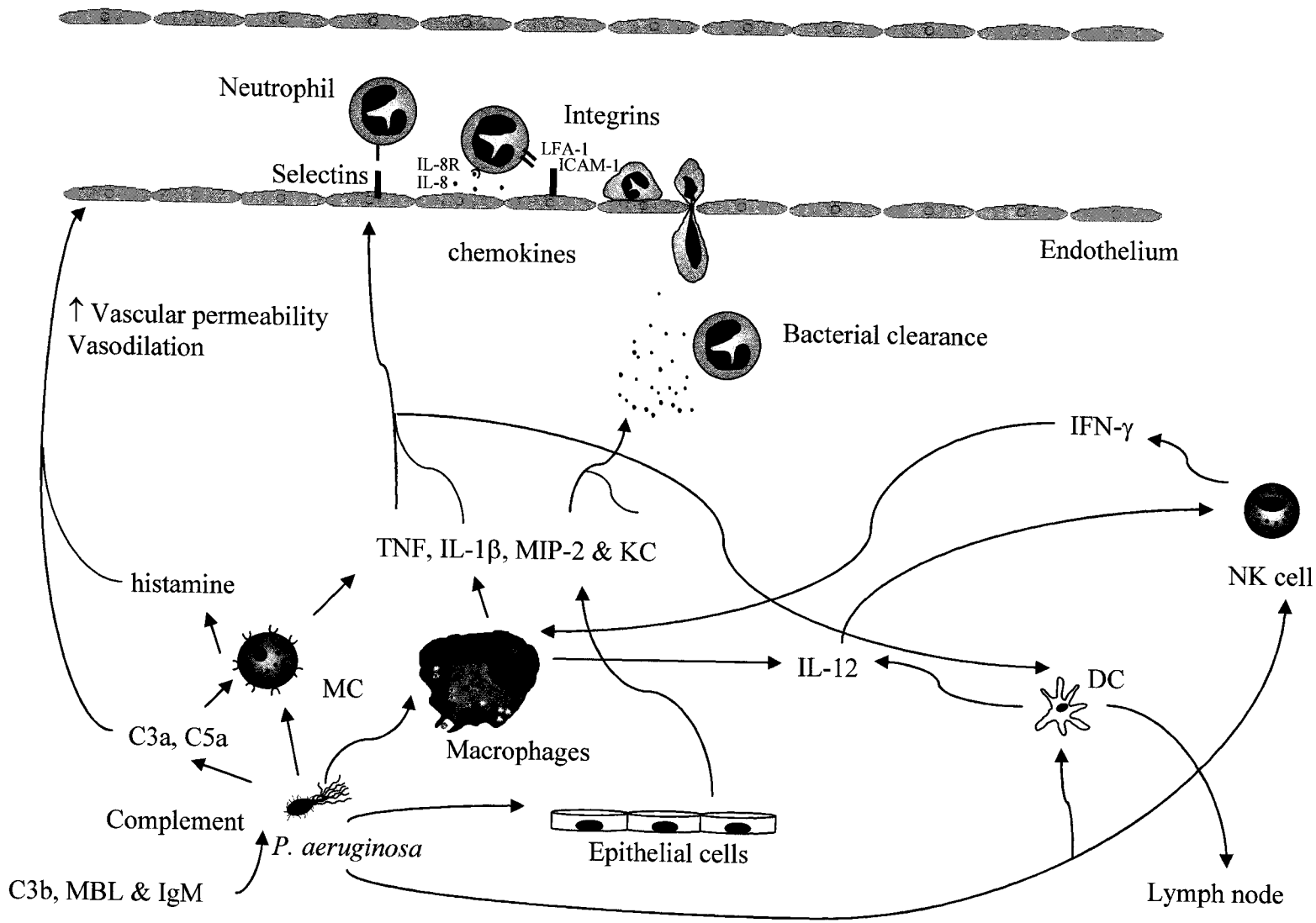
In addition, the airway surface liquid from CF patients has an impaired bactericidal activity when compared to the airway surface liquid of healthy individuals. The study by Moraes *et al.* (459) found that there was an impaired bacterial clearance in the presence of airway surface liquid from CF patients. This study found that the airway surface liquid of CF patients, which has a high ionic strength, does not impair neutrophil

function. They suggest that the airway surface liquid may have impaired direct antimicrobial effects on bacterial clearance.

1.10 Hypothesis

Neutrophils are directed from the circulation to sites of infection by cytokines and chemokines. Therefore, it was hypothesized that the cytokines released from resident cells, after recognition of *P. aeruginosa* ligands in the lung, attract neutrophils to the site of infection (Figure 1.1). Similar *in vivo* models using wild type mice show that the number of neutrophils increase at the site of *P. aeruginosa* infection (460-463) and after LPS inoculation (462). Neutrophils are able to actively phagocytose *P. aeruginosa*, therefore neutrophils actively participate in the bacterial clearance of *P. aeruginosa* (460, 461). I have set up a murine intranasal model of *P. aeruginosa* lung infection and developed a myeloperoxidase (MPO) assay to detect neutrophil recruitment in mice. I hypothesize that neutrophil recruitment and bacterial clearance will be hindered in animals without important components of the TLR signaling pathways, due to impaired production of vital cytokines and chemokines.

FIGURE 1.1 Major activating pathways contributing to the innate immune response to *P. aeruginosa* infection. As a pathogen comes into the airway, it interacts with resident cells such as epithelial cells, macrophages and mast cells, which recognize the pathogen and produce inflammatory mediators. Complement is activated, resulting in the production of C3a and C5a, which have direct effects on the endothelium to increase vascular permeability, vasodilation and neutrophil recruitment. Mast cells degranulate after interaction with C3a, C5a or the pathogen, releasing mediators such as histamine which is able to increase vascular permeability and vasodilation. The cytokines and chemokines produced after interaction with the pathogen increase the expression of adhesion molecules on the endothelium. Selectins mediate neutrophil rolling, while chemokines present on the endothelium activate rolling neutrophils to increase the affinity of integrins for their ligands. Integrins mediate firm adhesion of the neutrophil to the endothelium. Neutrophils extravasate through the endothelium up a chemoattractant gradient to the site of infection to facilitate bacterial clearance. Macrophages and dendritic cells (DC) produce IL-12 after recognition of a pathogen, which activates NK cells to produce IFNγ that acts back on the macrophage to increase its microbicidal activity. NK cells may also kill bacteria or bacterial infected cells directly. DCs move to the local draining lymph node to present antigen and initiate the adaptive immune response. Adapted from (11, 464).


Neutrophil
Integrins
Selectins
IL-8R
IL-8
LFA-1
ICAM-1
chemokines
Endothelium
↑ Vascular permeability
Vasodilation
Bacterial clearance
IFN-γ
NK cell
TNF, IL-1β, MIP-2 & KC
histamine
MC
Macrophages
IL-12
DC
C3a, C5a
Complement
P. aeruginosa
Epithelial cells
C3b, MBL & IgM
Lymph node

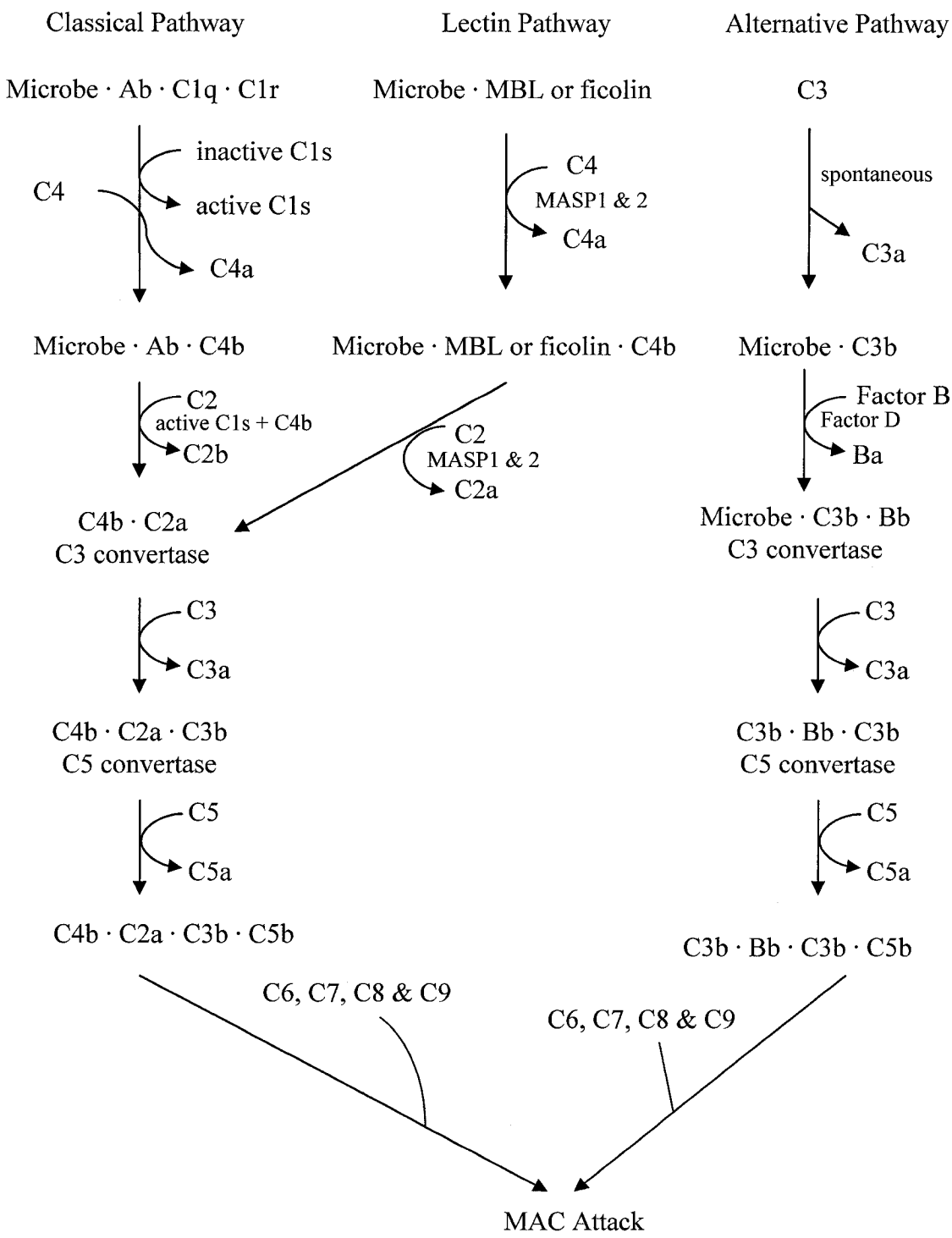
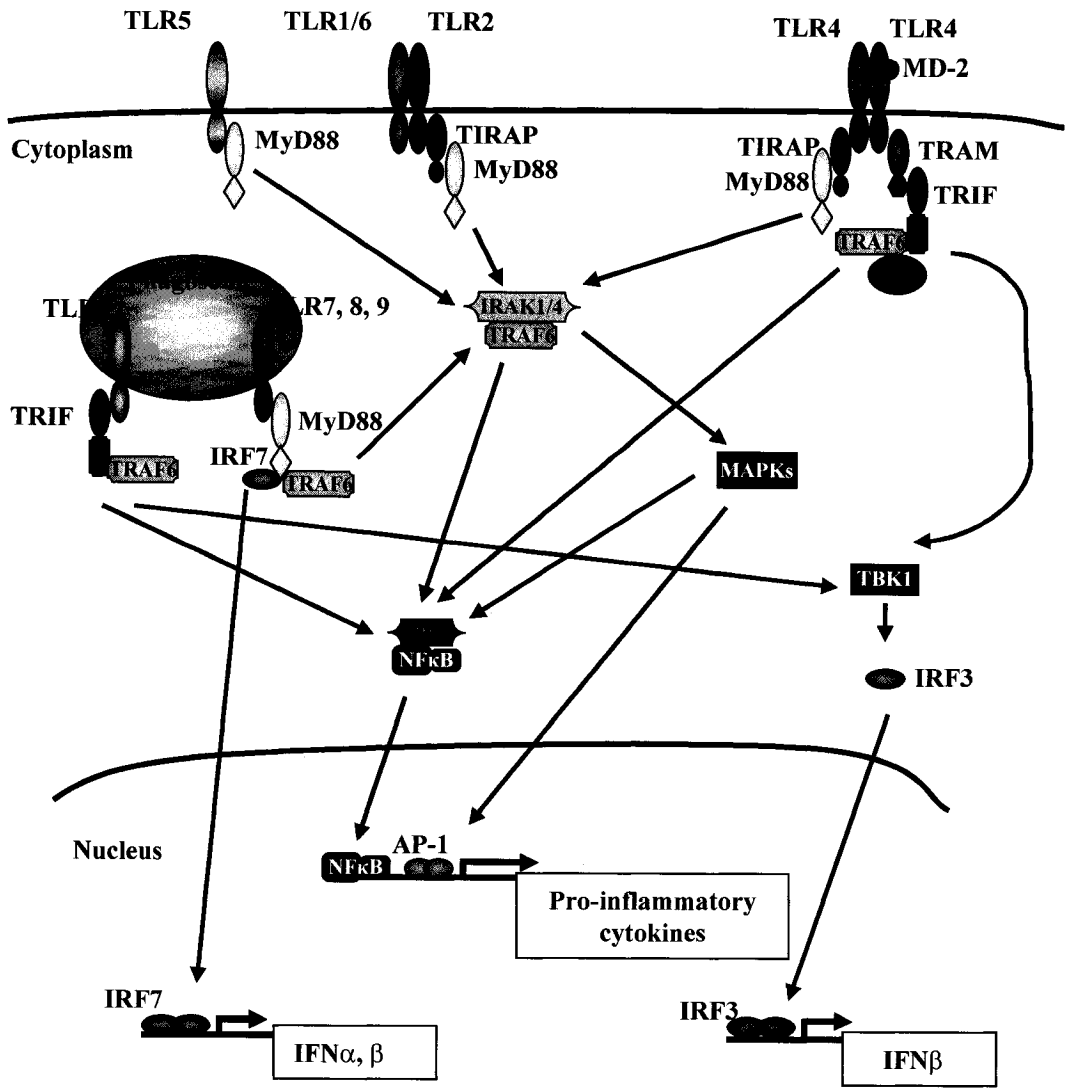


FIGURE 1.2 Activation of complement via the classical, lectin and alternative pathways.

FIGURE 1.3 TLR Signaling Pathway. TLRs can signal through MyD88 dependent and independent mechanisms. The MyD88 dependent signaling pathway is activated by TLRs that interact with the adaptor molecule MyD88, initiating a cascade of events that activates the translocation of NFκB to the nucleus, which induces the production of inflammatory mediators. Activation of TLR1, 2, 4, 5, 6, 7, 8 and 9 involves the recruitment of MyD88; however, TLR2 and TLR4 also require the adaptor molecule TIRAP/Mal. The serine/threonine kinases IL-1R associated kinase 1 (IRAK1) and IRAK4 become activated via phosphorylation and then dissociate from MyD88 to activate tumor necrosis factor receptor associated factor 6 (TRAF6). This leads to the activation of the IκB kinase (IKK) complex, upstream of NFκB and MAP kinases that initiate AP-1 activation. NFκB is present in the cytoplasm in an inactive form in unstimulated cells because of its association with inhibitor of κB (IκB). The phosphorylation of IκB via IKK leads to its ubiquitination and subsequent degradation. Degradation of IκB releases NFκB, allowing NFκB to translocate to the nucleus where it induces the expression of specific genes, including cytokines, chemokines, adhesion molecules, acute phase proteins, and antimicrobial peptides. The release of these mediators at the site of infection induces the recruitment of immune cells to clear the pathogen. An important MyD88 independent signaling pathway involves the adaptor molecule TRIF. TRIF may directly interact with kinases that phosphorylate IRF3/7 or there may be molecules that bridge them together. It has been shown that TBK1 interacts directly with TRIF but recent evidence indicates that TRAF3 can also associate with TRIF and TBK1 and IKKε. TRAF3 may be an important link between TRIF and the kinases that are needed for the activation of IRF. TRIF also leads to the activation of NFκB. There are at least two signaling molecules downstream of TRIF that can link TRIF to the activation of NFκB. RIP1 and TRAF6 have been shown to be involved in the TRIF dependent activation of IκB kinases and ultimately NFκB. Adapted from reviews by (148, 179, 193, 222, 226).


TLR5
TLR1/6
TLR2
TLR4
TLR4
MD-2
Cytoplasm
MyD88
TIRAP
MyD88
TIRAP
MyD88
TRAM
TRIF
TRAF6
TLR7, 8, 9
IRAK1/4
TRAF6
TRIF
MyD88
IRF7
TRAF6
TRAF6
MAPKs
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Pro-inflammatory cytokines
IRF7
IFNα, β
IRF3
IFNβ

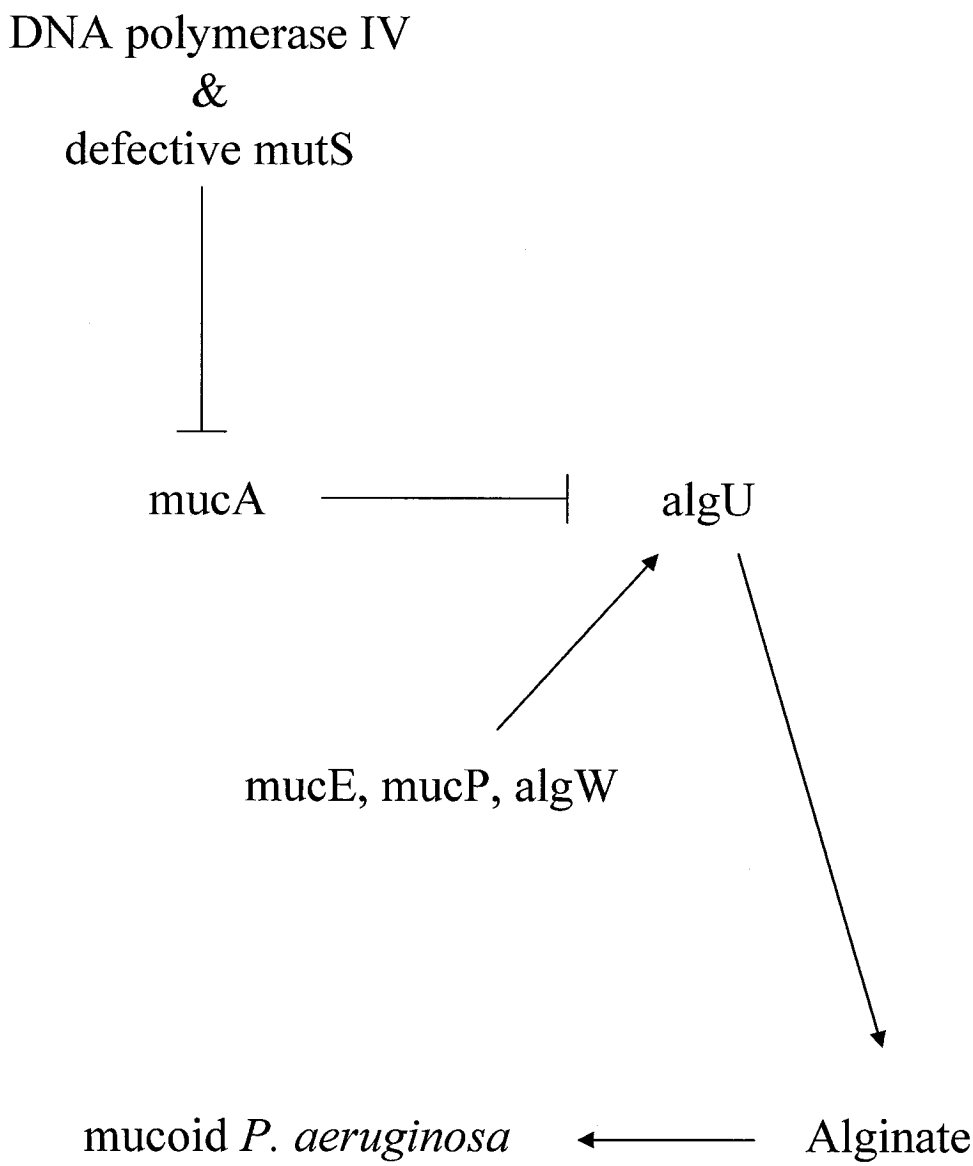


FIGURE 1.4 Regulation of alginate production in *P. aeruginosa.* Mucoid *P. aeruginosa* is most commonly due to a mutation in *mucA.* MucA is a negative regulator of *algU*, which is responsible for making alginate. Mutations in *mucA* are caused by DNA polymerase IV and defective *mutS*, which normally recognizes mismatched DNA. Some positive regulators of alginate are *mucE*, *mucP* and *algW*.

CHAPTER 2. Materials and Methods

2.1 Mice

MyD88 deficient (MyD88$^{-/-}$), TLR2 deficient (TLR2$^{-/-}$) and TRIF deficient (TRIF$^{-/-}$) mice were backcrossed eight times against the C57BL/6 strain (Osaka University, Osaka, Japan) (131, 163, 465). C57BL/6 mice were purchased from Charles River (Senneville, QC). MyD88 deficient mice were age and sex matched with C57BL/6 mice. C3H/HeJ mice (TLR4 mutant) and control, C3H/HeOuJ, (TLR4$^{+/+}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/10ScN mice having a deletion of *Tlr4* gene (TLR4$^{-/-}$) and C57BL/10ScSn (TLR4$^{+/+}$) were purchased from Jackson Laboratories (Bar Harbor, ME). The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

Genotyping was performed by extracting DNA from an ear sample made by an ear punch. DNA was extracted using the XNAT REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich Chemical CO., Missisauga, ON). The PCR was run according to the manufacturers protocol with the annealing temperature specific to the primers used for 35 cycles. The annealing temperature was 65°C for MyD88. A band present for primers a and b was indicative of a wild type gene while having a band present for the primers b and c was indicative of a knock out gene.

The primers for MyD88 were:

a) 5'-AGACAGGCTGAGTGCAAACTTGTGCTG-3'

b) 5'-AGCCTCTACACCCTTCTCTTCTCCACA-3'

c) 5'-ATCGCCTTCTATCGCCTTCTTGACGAG-3'

The primers for TRIF were:

a) 5'-CAAGATTGGACTTCACCTGGGTCCTTA-3'

b) 5'-CTGACACACTGTGTACTTACTAGGTGC-3'

c) 5'-CTAAAGCGCATGCTCCAGACTGCCTTG-3'

2.2 Infection Model

2.2.1 Preparation of *P. aeruginosa*

P. aeruginosa strain 8821 (a gift from Dr. A. Chakrabarty, University of Illinois, Chicago, IL) is a mucoid strain isolated from a cystic fibrosis patient (466). 10µl of *P. aeruginosa* was taken each week from frozen stock and plated onto a Luria Bertani agar plate (1% NaCl, 1% Tryptone peptone , 0.5% yeast extract, 1.5% Bactoagar). The plate was incubated overnight at 37°C. Approximately 18 – 24 h later a single colony was taken from the plate to inoculate 5 - 7 ml LB broth and shaken overnight at 200 rpm at 37°C. Approximately 18 h later, the culture was centrifuged 12 000 x g for 3 min at room temperature. The supernatant was discarded and the *P. aeruginosa* pellet was resuspended in an equal volume of 1x Dulbecco's phosphate buffer saline (PBS) (Invitrogen, Burlington, ON). The optical density was determined with a spectrophotometer at 620nm at a 1/10 dilution. The *P. aeruginosa* in PBS was centrifigued for 3 min at 12,000 x g. The supernatant was discarded and the pellet was resuspend in 1 OD/20 µl saline for infection with 1 x 10^9 *P. aeruginosa* or 1OD/ml for infection with 1 x 10^7 *P. aeruginosa.*

2.2.2 Lung Infection with *P. aeruginosa* and Collection of Lung and Bronchoalveolar Lavage Fluid

Mice were anesthetized with ketamine/xylazine and infected intranasally with 1 x 10^7 or 1 x 10^9 CFU of *P. aeruginosa* in 20µl of saline. After 4, 8, 24 or 48 h, mice were sacrificed by cardiac puncture, and PBS was infused into the heart to remove blood from the lungs. Bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lung three times with 1 ml of 1x PBS containing 100 µg/ml soybean trypsin inhibitor (Sigma-Aldrich Chemical CO., Missisauga, ON). Lung tissue was obtained for the detection of cytokines and myeloperoxidase (MPO) activity, for bacterial colony forming units (CFU) (left lobes), and for histology, RT-PCR, and EMSA (right lobes).

2.2.3 Lung Processing

Lung tissue was homogenized in 50 mM HEPES buffer (Sigma-Aldrich Chemical CO., Missisauga, ON), calculated by 4 µl/mg lung tissue, and containing 100 µg/ml soybean trypsin inhibitor. For counting bacterial CFU, 10 µl of the homogenate was plated on an agar dish and incubated for 24–48 h at 37 °C. The homogenate was centrifuged at 18,000 x g for 30 min at 4 °C. The supernatant was stored at -80 °C for later analysis of cytokines. The pellet was resuspended and homogenized in 0.5% cetyltrimethylammonium chloride (4 µl/mg lung tissue) and centrifuged as above. The clear extract was used for the MPO assay.

2.2.4 BALF Processing

BALF (10 µl) was plated on an agar dish and incubated for 24 h, and then bacterial CFU were counted. For the detection of cytokines and MPO activity, BALF was centrifuged at 300 x g for 5 min at 4 °C. Supernatants were used for cytokine analysis. The pellets were resuspended in 1ml of 0.15M NH_4Cl and spun as before to lyse red blood cells. The supernatants were discarded, and the pellets were resuspended in 250µl/mouse of 0.5% cetyltrimethylammonium chloride (Sigma-Aldrich Chemical CO., Missisauga, ON) and centrifuged, and the clear extracts were used for MPO assay.

2.3 MPO Assay

The MPO assay was used to determine the infiltration of neutrophils into the lungs of the mice. The protocol was adapted from (467). The BALF and lung extracts in duplicate (75µl) were mixed with equal volumes of the substrate. The substrate was made up of 3mM 3,3',5,5'-tetramethyl-benzidine dihydrochloride (Sigma-Aldrich Chemical CO., Missisauga, ON), 120µM Resorcinol (Sigma-Aldrich Chemical CO., Missisauga, ON), and 2.2mM H_2O_2. The reaction was timed for 2 min and was stopped by adding 150 µl of 2M H_2SO_4. The optical density was measured at 450nm using an ELISA plate reader.

2.4 Real-Time Quantitative RT-PCR

The extraction of RNA was performed using the Trizol/Qiagen RNeasy kit hybrid protocol. 1ml of Trizol (Life Technologies Gibco) was used to extract total RNA from lung samples (~0.100g) by homogenizing with a baked glass homogenizer. Once disrupted in Trizol the sample was stored at –80°C until needed. The homogenized samples were set out at room temperature for 5 min then 0.2ml of chloroform was added per 1ml of Trizol and tubes were shaken vigorously for 15 s and then incubated at room temperature for 3 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C. The mixture separated into a lower red, phenol-chloroform phase, an interphase and a clear upper aqueous phase. RNA was found in the clear aqueous phase. The aqueous phase was transferred to a new tube and the RNA was precipitated with 0.5ml isopropyl alcohol per 1ml Trizol. The samples were incubated at room temperature for 10 min. and centrifuged at12,000 x g for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 1ml of 75% ethanol. The sample was vortexed and then centrifuged at 7,500 x g for 5 min at 4°C. The supernatant was removed. The pellet was partially dried and then RNA pellet dissolved in 30µl RNase free water.

Total RNA, was measured by OD_{260} and was used in a 20µl reverse transcription reaction with SuperscriptII to make cDNA, according to the manufacturer's protocol, including an RT- control. Briefly, 2µg of total RNA, 1µl of oligo-dT and RNase free water up to a volume of 12µl was added to a 0.2ml PCR tube per sample. Samples were incubated on the PCR block at 70°C for 10 min then chilled on ice. Then 4µl of x5 RT buffer, 1µl of 0.1M DTT, 1µl of 10mM dNTP and 1µl RNase out was added per sample and incubated at 42°C for 2 min. 1µl of SuperScriptII (Invitrogen, Burlington, ON) was added per sample and incubated for 50 min at 42°C. The SuperScriptII was heat deactivated by incubating at 70°C for 10 min. The cDNA prepared in this way was diluted 5µl in 95µl molecular grade water, then 5µl of this dilution was used in each 20µl real time RT-PCR reaction.

Real-time quantitative RT-PCR was performed using a 7000 Sequence detector (PerkinElmer Life Sciences). The plate was set up with duplicate wells per sample for both the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the endogenous

reference and the gene of interest primer/probe. The standard curve was set up with serial dilutions using a 10-fold dilution series with 5 dilution points (1/4, 1/16, 1/64, 1/256, 1/1024) in a 20μl reaction. Controls used include an RT- control to test what amplification may be occurring due to genomic DNA, and a water control. Specific quantitative assays for CXCL2/MIP-2, TLR2, TLR4 and IRF7 were performed using Assays-on-Demand reagents containing 6-FAM dye-labeled TaqMan minor groove binder probes (PerkinElmer Life Sciences) according to the manufacturer's protocol (468). Data were analyzed using the relative standard curve method according to the manufacturer's protocol, and values from wild type untreated mice were used as the calibrator. Values were normalized by the endogenous reference and then expressed as the –fold increase relative to wild type untreated mice.

2.5 Cytokine Production

The concentrations of cytokines and chemokines in the lung and BALF were determined by ELISA using antibody pairs from R&D Systems (Minneapolis, MN) for IL-1β (cat # DY401), TNF (cat # DY410), CXCL2/MIP-2 (cat # DY452), IFNγ (cat # DY485), CXCL1/KC (cat # DY453), CCL5/RANTES (cat # DY478), IL-12 (cat # DY419), IFNα (cat # 42100-1) and IFNβ (cat # 42400-1). The optimal concentration of each antibody was specified by the manufacturer for each lot.

Plates were coated with antibody specific to the cytokine of interest in 6ml bicarbonate buffer (0.1 M $NaHCO_3$ / 0.5 M NaCl / dH_2O) by adding 50 μl/well of coating antibody solution. The plate was incubated overnight at 4 °C. Approximately 18 h later the plate was washed once with 0.01% Tween-20/PBS buffer and tapped dry on paper towels. 100 μl of blocking buffer (2% BSA/PBS) was added to each well and incubated at room temperature for 2 h. The plate was washed 3 times with 0.01 % Tween-20/PBS and tapped dry on paper towels. 50 μl of sample and standard were added to the appropriate wells. Standards were diluted in ELISA assay Buffer (0.2% BSA/0.05% Tween-20/PBS) and serial dilutions were made according to manufacturer's instructions. The plate was incubated at 4 °C overnight. The plate was washed four times with 0.01 % Tween-20/PBS buffer and patted dry. The plate was incubated for 1 h at room temperature with 50 μl/well of biotinylated antibody (made in ELISA assay buffer)

according to the manufacturer's instructions. The plate was washed four times with 0.01 % Tween-20/PBS buffer and patted dry. The plate was incubated for 30 min with 50 µl/well Streptavidin-Alkaline phosphatase (1.5 µl Strep-AP (GibcoBRL, cat # 19589-019) to 6 ml ELISA assay buffer per plate). The plate was washed five times with Tween-20/TBS buffer and pat dry. The plate was incubated for 1 h with 50 µl/well substrate (GibcoBRL, cat # 19589-019). The amplifier (GibcoBRL, cat # 19589-019) was added at 50 µl/well. The plate was watched closely, and the reaction was stopped using 50 µl 0.3 M H_2SO_4. The plates were read at 490 nm using an ELISA plate reader.

2.6 Electrophoretic Mobility Shift Assays

Nuclear protein extracts were obtained using a nuclear extract kit (ActiveMotif, Carlsbad, CA), according to the manufacturer's protocol. Briefly, the lung tissue was weighed and cut up with scissors and homogenized on ice using 3ml of 1x hypotonic buffer (supplemented with 3µl of 1M DTT and 3µl of detergent per 3ml of 1x hypotonic buffer) per gram of tissue. The sample was then incubated on ice for 15 min and centrifuged in a microcentrifuge tube for 10 min at 850 x g at 4°C. The supernatant was transferred to a pre-chilled microcentrifuge tube and saved for later to be pooled with the cytoplasmic fraction. Cells were resuspended gently in 500µl 1x hypotonic buffer by pippetting up and down several times and then incubated on ice for 15 min. At this time, 25µl of detergent was added per sample and each sample was vortexed for 10 s on high. The suspension was centrifuged for 30 s at 14,000 x g at 4°C. The supernatant (the cytoplasmic fraction) was pooled with the supernatant saved earlier and stored at -80°C. The pellet was resuspended in 50µl of complete lysis buffer (50µl DTT, 5µl protease inhibitor cocktail and 445µl lysis buffer) by pipetting up and down. The sample was vortexed on high for 10 s. The suspension was set on a rocking platform at 150 rpm for 30 min on ice. The suspension was vortexed on high for 30 s. The sample was centrifuged for 10 min at 14,000 x g at 4°C. The supernatant (nuclear fraction) was transferred to a pre-chilled microcentrifuge tube and stored at -80°C. Total protein concentrations were determined by comparison to a standard curve using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Missisauga, ON).

A consensus double-stranded NFκB oligonucleotide (Promega, Madison, WI) (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was used as a probe. The probe was labeled with (r-32p) ATP in a mixture (5 µl DNase/RNase free water, 2µl of 1.75pmol/µl NFκB oligo, 1µl of 10x T4 kinase buffer, 1µl of T4 kinase and 1µl of (r-32p)ATP) for 15min on the heating block at 37°C. The reaction was stopped with a mixture of 1µl of 0.5M ethylene diamine tetracetic acid (EDTA) and 89µl of TE buffer (10mM Tris-HCl at pH 8.0 and 1mM EDTA). The labeled probe was collected and separated from unlabeled probe by using a microspin G-25 sephadex column (GE Healthcare, Baie d'Urfé, QC).

Nuclear protein (10 µg) was added to 10 µl volume of binding reaction with 1 µg of poly(dI-dC) (GE Healthcare) and incubated at room temperature for 15 min. Double-stranded NFκB oligonucleotide labeled with ^{32}P, then added to each reaction mixture, incubated at room temperature for 30 min and separated by electrophoresis on a 6% polyacrylamide gel in 0.5 x Tris-boric acids–EDTA buffer for 1 h at 300V. Gels were vacuum-dried at 80°C for 2h and subjected to autoradiography.

2.7 Histology

Mouse lungs were fixed in 10% acetate buffered formalin overnight and then washed twice with 70% ethanol. The tissues then undergo dehydration, clearing and paraffin wax infiltration with the Autotechnicon tissue processor. The tissues were dehydrated using stages of 70% ethanol, 95% ethanol and then 100% ethanol. They were cleared using a mixture of equal parts 100% ethanol and xylene for 1 h and then xylene alone. Tissues were embedded with paraffin (Tissue Prep, Fisher Scientific, melting point 56 - 57°C) and put in blocks. Sections were made at 5µm using a Reichert-Jung rotary microtome. The cut sections were placed in a 45°C water bath and put on silinated slides. Slides dried overnight at 37°C. The slides were deparaffinized using xylene and hydrated using stages of ethanol starting at 100%, 95%, 70%, 50% then running tap water. Slides were stained with Harris hematoxylin for 5 min. The slides were rinsed with running tap water for 5 min. Differentiated with dips in acid alcohol and then rinsed with tap water. The slides were then immersed in Scott's solution to blue. The slide is counter stained with 0.5% Eosin with 0.5% $CaCl_2$ in distilled water for 5 s. The slide was

rinsed with tap water then placed in 95% ethanol for 30 s. The slide was dehydrated with 100% ethanol and then xylene. A coverslip was placed on top with cytoseal and left to dry. Slides were viewed by an Eclipse 600 Nikon light microscope.

2.8 Statistics

Data are presented as mean ± S.E. of the indicated number of experiments. Statistical significance was determined by assessing means with analysis of variance and the Tukey-Kramer multiple comparison test or by using an unpaired *t* test, as appropriate. Differences were considered significant at $p < 0.05$.

CHAPTER 3. The Development of Early Host Response to *Pseudomonas aeruginosa* Lung Infection is Critically Dependent on Myeloid Differentiation Factor 88 in Mice

Power M.R, Y. Peng, E. Maydanski, J.S. Marshall, and T.J. Lin. *J. Biol. Chem.* 2004; 279:49315-49322. (Addition and Correction in: *J. Biol. Chem.* 2005; 280:2395-6). (307)

3.1 Introduction

Toll-like receptors (TLRs) are a family of pattern recognition molecules that initiate intracellular signaling cascades on exposure to microbial molecules (469). There are at least 10 TLRs (TLR1–TLR10) that induce signal transduction through adaptor proteins. Five TLR-associated adaptor proteins have been described previously (470, 471), including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (MAL) (also known as TIRAP), Toll-interleukin-1 receptor domain-containing adaptor molecule-1 (TICAM-1) (also know as TRIF), TICAM-2 (470), and TRIF-related adaptor molecule (TRAM). The adaptor usage by different TLRs provides a molecular basis for the differences in gene expression patterns induced by distinct TLRs (471). Given that MyD88 transduces a core set of TLR-induced signals (469, 471), microbially induced immune responses can be divided broadly into the MyD88 dependent and MyD88 independent pathways.

The MyD88 dependent pathway is essential for the host defense against microbial infections in vivo from organisms such as *Staphylococcus aureus* (472) and *Toxoplasma gondii* (473). In contrast, resistance to *Mycobacterium tuberculosis* infection is effected largely through MyD88 independent pathways (474, 475). In acute polymicrobial peritonitis, the effective antibacterial immune response occurs in the absence of MyD88 (476). In other cases, both MyD88 dependent and independent mechanisms are involved. For example, *Listeria monocytogenes* activates an immune response through an ordered, sequential MyD88 independent and dependent fashion (477). Specific microbes utilize individual TLR and adaptor pathways to induce immune responses that are tailored to the given microbial infection (478).

Pseudomonas aeruginosa, an opportunistic Gram-negative bacillus, is the major pathogen in cystic fibrosis patients (432) and a common cause of nosocomial pneumonia

(479, 480). Two major features of *P. aeruginosa* lung infection are the recruitment of neutrophils and the production of various cytokines and chemokines in the local tissue (481). Neutrophils play an essential role in the clearance of *P. aeruginosa* from the lung (15, 482). Recruitment of neutrophils to the lung is largely induced by the production of inflammatory mediators in the airways (483). *P. aeruginosa*-induced mediator production in the airways is initiated by the interaction between *P. aeruginosa* and the host cell receptors (482). A major effort has been made to identify the molecules responsible for the initiation of *P. aeruginosa*-induced inflammation. Several cell surface molecules have been implicated in the direct interaction with *P. aeruginosa*, including the cystic fibrosis transmembrane conductance regulator (457, 458), complement receptor 3 (484), gangliotetraosylceramide (485), CD91 (486), and syndecan-1 (487). More recently, TLR2, -4, and -5 have been associated with *P. aeruginosa* infection (488-491). *In vivo*, decreased expression of TLR4 appears to correlate with impaired resistance to *P. aeruginosa* infection (489). *In vitro*, TLR2 and TLR5 are involved in *P. aeruginosa* flagella-induced activation of epithelial cells (488), and TLR2 and TLR4 are involved in monocyte and macrophage activation by a component of *P. aeruginosa* alginate (490). However, the contribution of the TLR-MyD88 pathway to the effective host response to *P. aeruginosa* lung infection remains a critical area of investigation.

Here we demonstrate that MyD88 deficient ($MyD88^{-/-}$) mice showed little or no neutrophil recruitment or production of the neutrophil attractants CXCL2/MIP-2, TNF, and IL-1β and had an impaired ability to clear *P. aeruginosa* from the lung, which vastly contrasted with the findings of robust CXCL2/MIP-2, TNF, and IL-1β production, neutrophil infiltration, and bacterial clearance demonstrated in wild type animals. These findings suggest an essential role for the MyD88 dependent pathway in the host defense against *P. aeruginosa* lung infection *in vivo*. The different immune responses seen in MyD88 deficient, TLR2 deficient, and TLR4 mutant mice suggest that the host defense against *P. aeruginosa* lung infection may involve multiple members of the MyD88 dependent TLR/IL-1 superfamily.

3.2 Results

3.2.1 Impaired Clearance of *P. aeruginosa* in MyD88 Deficient Mice

We used MyD88 deficient mice to examine the role of MyD88 in the host defense against *P. aeruginosa* lung infection *in vivo*. $MyD88^{+/+}$ and $MyD88^{-/-}$ mice were inoculated intranasally with 1 x 10^9 CFU of *P. aeruginosa* strain 8821. BALF and lung tissue were collected 24 h later for the detection of viable bacteria by counting CFU. $MyD88^{+/+}$ mice are able to clear *P. aeruginosa* (Figure 3.1). Significantly higher CFU counts could be seen in both the BALF and lung tissue from $MyD88^{-/-}$ mice (Figure 3.1), demonstrating that $MyD88^{-/-}$ mice have impaired bacterial clearance in comparison with $MyD88^{+/+}$ mice.

3.2.2 Impaired Neutrophil Recruitment to the Lung in $MyD88^{-/-}$ Mice

Given that neutrophils are essential for the clearance of *P. aeruginosa* during acute lung infection (482), we tested whether the impaired bacterial clearance in $MyD88^{-/-}$ mice was caused by defective neutrophil infiltration. $MyD88^{+/+}$ and $MyD88^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* strain 8821. After 4 or 8 h, the BALF and lung tissue were collected for measuring MPO activities. In $MyD88^{+/+}$ mice, infection with *P. aeruginosa* for 4 h induced an increased activity of MPO in the lung but not in the BALF, suggesting that at this time point neutrophils in circulation were recruited to the lung but had not yet reached the airways. After 8 h of infection, a significant increase in MPO activity was observed in both BALF and lung tissue of $MyD88^{+/+}$ mice (Figure 3.2*A* and *B*). Strikingly, little change in MPO activity was observed in either BALF or lung tissue after 4 or 8 h of *P. aeruginosa* lung infection in the $MyD88^{-/-}$ mice (Figure 3.2*A* and *B*), suggesting an impaired neutrophil recruitment to the lung in $MyD88^{-/-}$ mice. This finding is confirmed by histological examination of the lung. Lung tissue from $MyD88^{-/-}$ mice showed little neutrophil infiltration after 8 h of *P. aeruginosa* infection, compared with the prominent neutrophil recruitment in $MyD88^{+/+}$ mice (Figure 3.2*C* – *F*).

3.2.3 *P. aeruginosa*-Induced Production of CXCL2/MIP-2, CXCL1/KC, TNF, and IL-1β in MyD88$^{+/+}$ but not in MyD88$^{-/-}$ Mice

MyD88$^{-/-}$ neutrophils have normal migration ability *in vivo* because neutrophil recruitment to the septic focus has been shown to be normal in MyD88$^{-/-}$ mice during polymicrobial infection (476). Thus, we reasoned that the impaired neutrophil recruitment in the lungs of MyD88 deficient mice might be caused by a defect in the production of neutrophil chemoattractants. Accordingly, several cytokines and chemokines that are important for neutrophil migration *in vivo* were examined. These include the direct neutrophil chemoattractant CXCL2/MIP-2 (492, 493) and indirect attractants TNF and IL-1β (76). To examine CXCL2/MIP-2 mRNA expression by real-time RT-PCR, lung tissue from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice after 4 h of *P. aeruginosa* infection was used to isolate RNA. A significant increase of CXCL2/MIP-2 mRNA expression was seen in MyD88$^{+/+}$ mice. In contrast, there was little CXCL2/MIP-2 response in MyD88$^{-/-}$ mice (Figure 3.3*A*). To determine CXCL2/MIP-2 and CXCL1/KC production at the protein level, BALF and lung tissue homogenates from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice after 4 or 8 h of *P. aeruginosa* lung infection were used to determine CXCL2/MIP-2 and CXCL1/KC protein concentration by ELISA. *P. aeruginosa* infection induced a significant increase of CXCL2/MIP-2 in the BALF and lung tissue in MyD88$^{+/+}$ mice. In contrast, there was little CXCL2/MIP-2 or CXCL1/KC production in MyD88$^{-/-}$ mice (Figure 3.3).

Levels of TNF and IL-1β were also examined because of their essential roles in neutrophil recruitment to the lung *in vivo* (483). In MyD88$^{+/+}$ mice, *P. aeruginosa* lung infection induced an increased production of IL-1β in the lung tissue (Figure 3.4*B*) and TNF in the BALF (Figure 3.4*C*). In contrast, there was little response of IL-1β or TNF in lung tissue or BALF from MyD88$^{-/-}$ mice (Figure 3.4*B* and *C*). It is also noteworthy that in wild type mice, *P. aeruginosa*-induced IL-1β remained largely in the lung tissue (Figure 3.4*B*), whereas the majority of TNF was secreted in the BALF (Figure 3.4*C*).

Given that IFNγ and IL-12 have been implicated in *P. aeruginosa* lung infection (494, 495), these two cytokines were examined in both MyD88$^{+/+}$ and MyD88$^{-/-}$ mice. Mice were challenged for 4 or 8 h with an intranasal inoculation of *P. aeruginosa* (strain 8821, 1 x 10^7 CFU/mouse). The BALF and lung tissue homogenates were used to

determine the levels of production of IFNγ and IL-12 by ELISA. In contrast to the robust response of neutrophil attractants CXCL2/MIP-2, IL-1β, and TNF, there was little IFNγ or IL-12 response in either MyD88$^{+/+}$ and MyD88$^{-/-}$ mice after 4 or 8 h of *P. aeruginosa* lung infection (Figure 3.*5A-D*).

To examine the role of MyD88 in *P. aeruginosa*-induced NFκB activation, lung tissue homogenates from *P. aeruginosa*-infected (4 h) or untreated mice were used to isolate nuclear extracts for the determination of NFκB activation by electrophoretic mobility shift assay. *P. aeruginosa*-induced NFκB activation was seen in wild type mice (Figure 3.6). In contrast, *P. aeruginosa*-induced NFκB activation was markedly reduced in MyD88$^{-/-}$ mice. These data are consistent with the defect of *P. aeruginosa*-induced mediator production in MyD88$^{-/-}$ mice as shown in Figures 3.3 and 3.4.

3.2.4 Increased TLR2, but not TLR4, mRNA Expression in the Lung after *P. aeruginosa* Stimulation

MyD88 is important in TLR signaling, therefore the lack of a *P. aeruginosa*-induced response in MyD88$^{-/-}$ mice suggests that TLR may have a critical role in *P. aeruginosa*-induced host responses. To examine whether *P. aeruginosa* infection induces changes in TLR2 and TLR4 expression, lung tissue from *P. aeruginosa*-infected (4 h) MyD88$^{+/+}$ and MyD88$^{-/-}$ mice was used to determine mRNA levels by real-time RT-PCR. Results were expressed as the –fold increase relative to the level in wild type untreated mice by using the average value from these animals as a calibrator. As shown in Figure 3.7*A*, the basal levels of TLR2 expression in MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were similar. Interestingly, *P. aeruginosa* infection induced a greater increase in TLR2 expression in MyD88$^{+/+}$ mice (7.5-fold) than in MyD88$^{-/-}$ mice (1.7-fold) (Figure 3.7*A*), supporting the concept that TLR2 is involved in *P. aeruginosa* lung infection. However, little change in TLR4 level in the lung was found in MyD88$^{+/+}$ and MyD88$^{-/-}$ mice (Figure 3.7*B*).

To examine whether TLR4 plays a role in *P. aeruginosa*-induced up-regulation of TLR2 expression, TLR4 mutant mice were used. Lung tissue from saline-treated or *P. aeruginosa*-infected (4 h) mice was subjected to real-time RT-PCR analysis for TLR2 mRNA. Data are expressed as the –fold increase relative to the level in saline-treated mice. As shown in Figure 3.7*A P. aeruginosa* infection had little effect on TLR2

expression, suggesting an important role for TLR4 in *P. aeruginosa*-induced TLR2 expression.

3.2.5 *P. aeruginosa*-Induced Differential Responses in TLR2$^{-/-}$ and TLR4 Mutant Mice

To determine the specific contributions of TLR2 and TLR4 in the development of early immune responses in the lung following *P. aeruginosa* infection, TLR2$^{-/-}$ and TLR4 mutant mice as well as their corresponding control mice were infected intranasally with *P. aeruginosa* strain 8821 for 4 h. Lung homogenates were used to determine neutrophil infiltration (MPO) and cytokine production. TLR2$^{-/-}$ mice showed a partial decrease in neutrophil recruitment compared with wild type mice (Figure 3.8*A*). However, a significant *P. aeruginosa*-induced CXCL2/MIP-2 and IL-1β production was observed in TLR2$^{-/-}$ mice (Figure 3.8*B* – *D*), suggesting that TLR2 alone may not be a major component in mediating *P. aeruginosa*-induced responses. Similarly, TLR4 mutant mice showed *P. aeruginosa*-induced production of CXCL2/MIP-2, IL-1β, and TNF when compared with saline-treated mice (Figure 3.8*F* – *H*). Compared with TLR4$^{+/+}$ mice, TLR4 mutant mice demonstrated a significant decrease of neutrophil recruitment and CXCL2/MIP-2, IL-1β, and TNF production (Figure 3.8*E* – *H*).

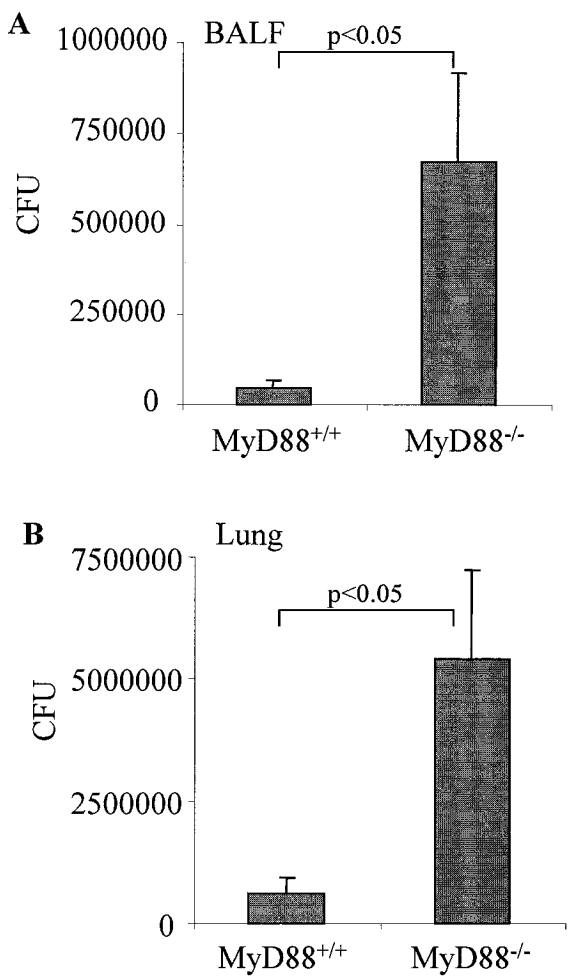
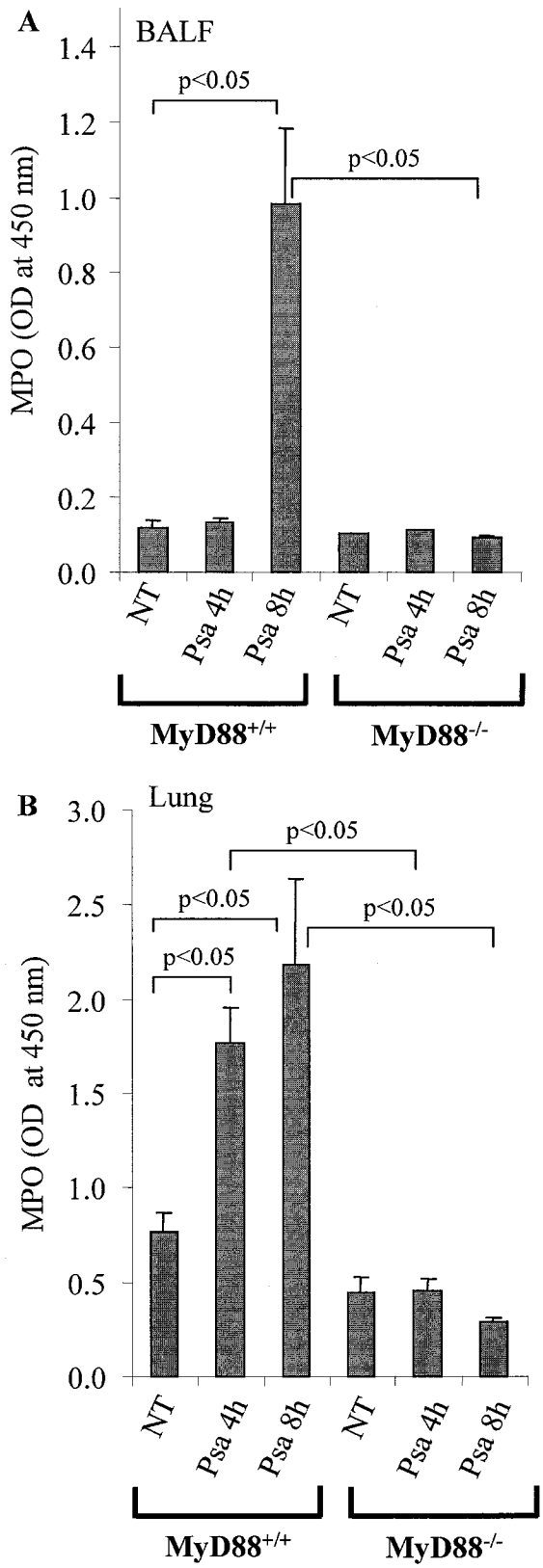
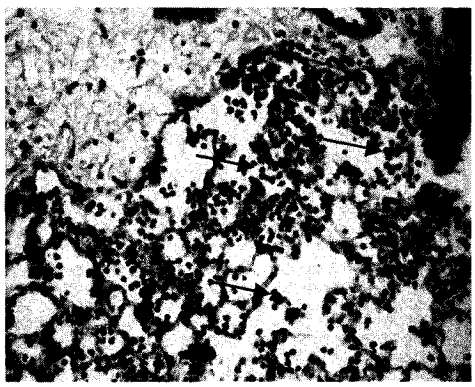


FIGURE 3.1 Impaired clearance of *P. aeruginosa* from the lungs of MyD88$^{-/-}$ mice. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were challenged intranasally with *P. aeruginosa* (mucoid strain 8821). After 24 h, the BALF (A) and the right lungs (B) were collected for bacterial colony counting. Data are the mean ± S.E. of 6 mice per group.

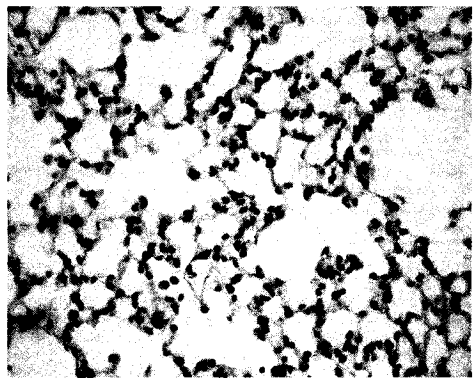
FIGURE 3.2 Defective neutrophil recruitment into the airways of MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1 x 10^7 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). (A) and (B), after 4 or 8 h, BALF and lung tissue were collected for the determination of MPO activities. Data are the mean ± S.E. of 7 – 11 mice/group (*, $p < 0.05$, compared with the NT group). (C-F) 8 h after infection, mice were sacrificed, and the upper lobe of the left lung was collected for hematoxylin-eosin staining. (C) MyD88$^{+/+}$ mouse lung at x40; (D) MyD88$^{+/+}$ mouse lung at x100; (E) MyD88$^{-/-}$ mouse lung at x40; (F) MyD88$^{-/-}$ mouse lung at x100.


A
BALF
MPO (OD at 450 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
B
Lung
MPO (OD at 450 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p<0.05
p<0.05
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-

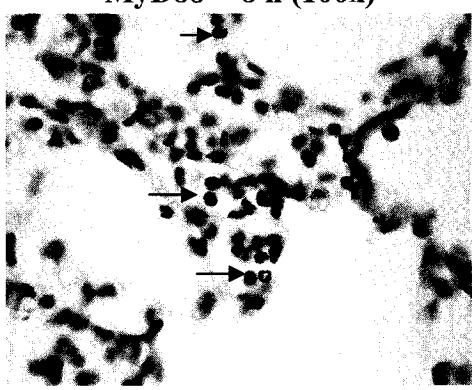
C MyD88$^{+/+}$ 8 h (40x)



E MyD88$^{-/-}$ 8 h (40x)



D MyD88$^{+/+}$ 8 h (100x)



F MyD88$^{-/-}$ 8 h (100x)

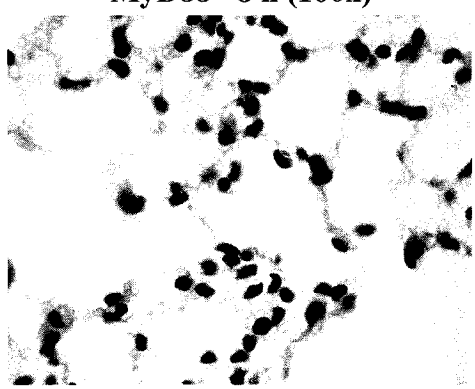


FIGURE 3.3 Diminished CXCL2/MIP-2 and CXCL1/KC in MyD88 deficient mice following *P. aeruginosa* lung infection. (A) lung tissue was collected from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice 4 h after intranasal administration of *P. aeruginosa* (*Psa*) strain 8821 (1 x 10^7 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). Total RNA isolated from the lungs was subjected to real-time RT-PCR analysis for CXCL2/MIP-2 expression. Data are expressed as -fold increase relative to NT-treated MyD88$^{+/+}$ mice. Data are the mean ± S.E. of 3 – 6 mice per group. (B) and (C), 4 or 8 h after *P. aeruginosa* lung infection (strain 8821, 1 x 10^7 CFU/mouse), BALF and lung tissue were collected for the determination of CXCL2/MIP-2 protein by ELISA. (D) 4 or 8 h after *P. aeruginosa* lung infection (strain 8821, 1 x 107 CFU/mouse), lung tissues were collected for the determination of CXCL1/KC protein by ELISA. Data are the mean ± S.E. of 7 – 11 mice per group.

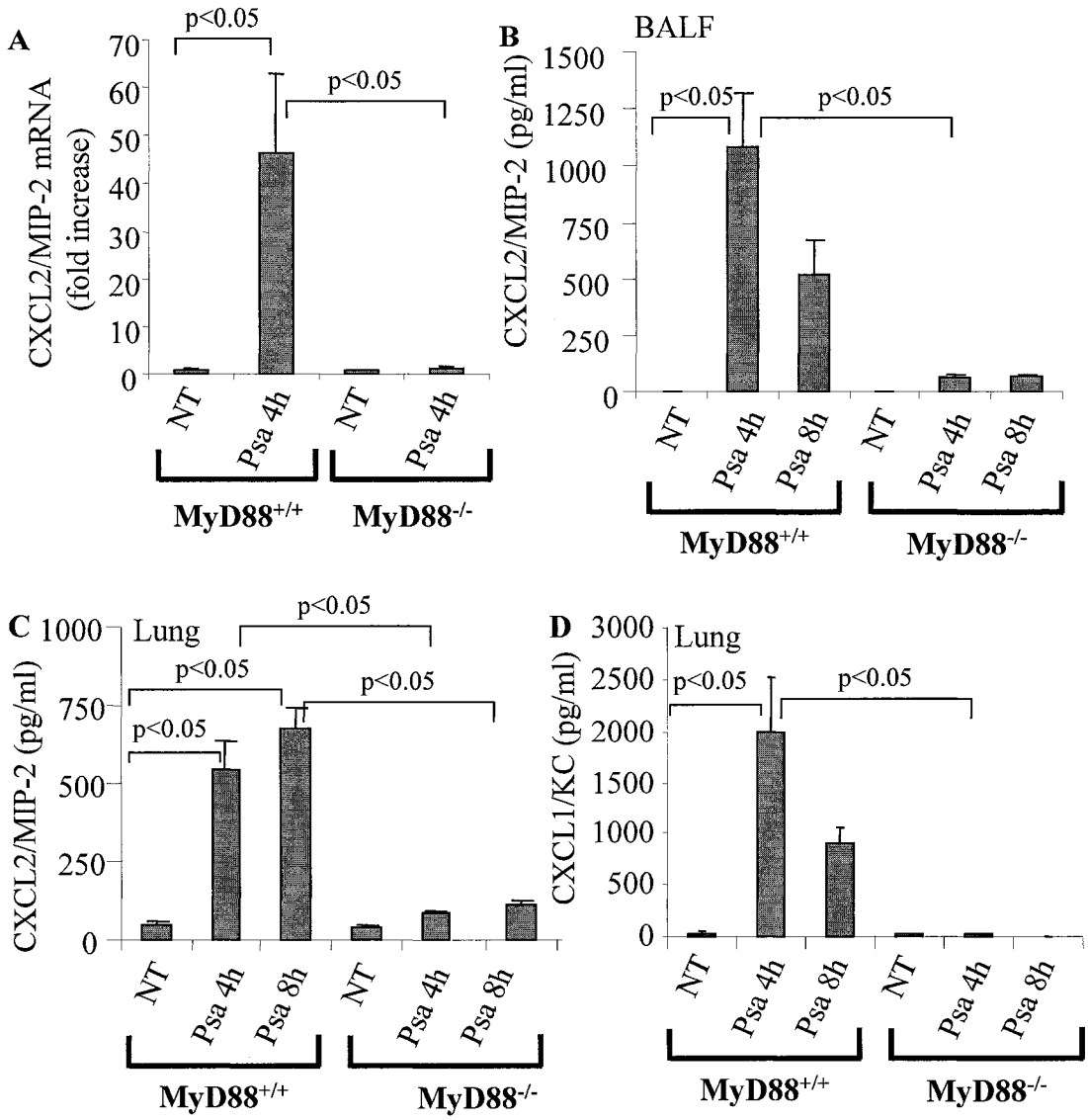
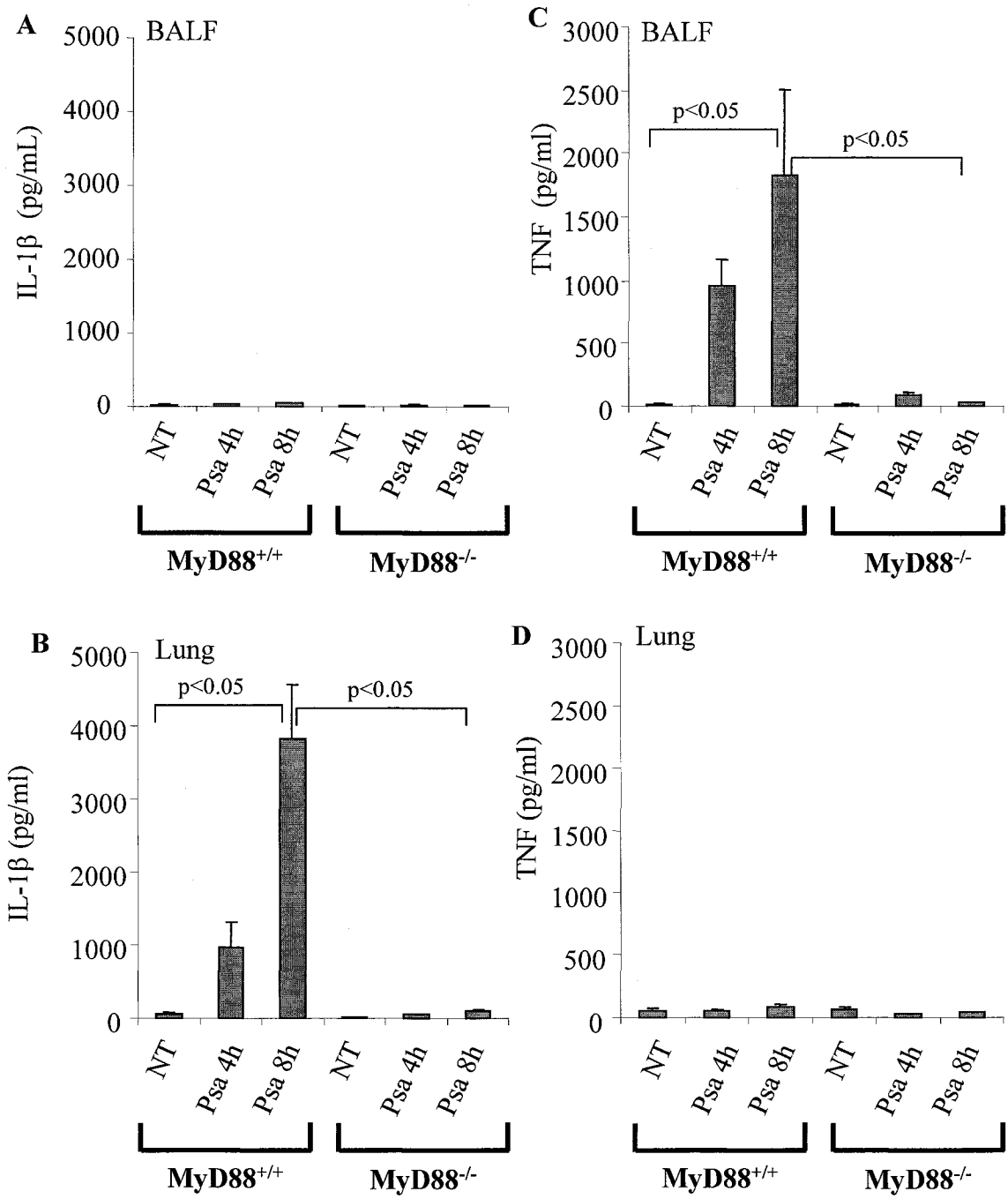

A
p<0.05
p<0.05
CXCL2/MIP-2 mRNA (fold increase)
70
60
50
40
30
20
10
0
NT
Psa 4h
NT
Psa 4h
MyD88+/+
MyD88-/-
B
BALF
CXCL2/MIP-2 (pg/ml)
1500
1250
1000
750
500
250
0
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
C
Lung
CXCL2/MIP-2 (pg/ml)
1000
750
500
250
0
p<0.05
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
D
Lung
CXCL1/KC (pg/ml)
3000
2500
2000
1500
1000
500
0
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-

FIGURE 3.4 Defective IL-1β and TNF production in MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1 x 10^7 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). After 4 or 8 h, BALF and lung tissue were collected for the determination of IL-1β (A and B) and TNF (C and D) by ELISA. Data are the mean ± S.E. of 5 – 10 mice per group.


A
BALF
IL-1β (pg/mL)
5000
4000
3000
2000
1000
0
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
C
BALF
TNF (pg/ml)
3000
2500
2000
1500
1000
500
0
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
B
Lung
IL-1β (pg/ml)
5000
4000
3000
2000
1000
0
p<0.05
p<0.05
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-
D
Lung
TNF (pg/ml)
3000
2500
2000
1500
1000
500
0
NT
Psa 4h
Psa 8h
NT
Psa 4h
Psa 8h
MyD88+/+
MyD88-/-

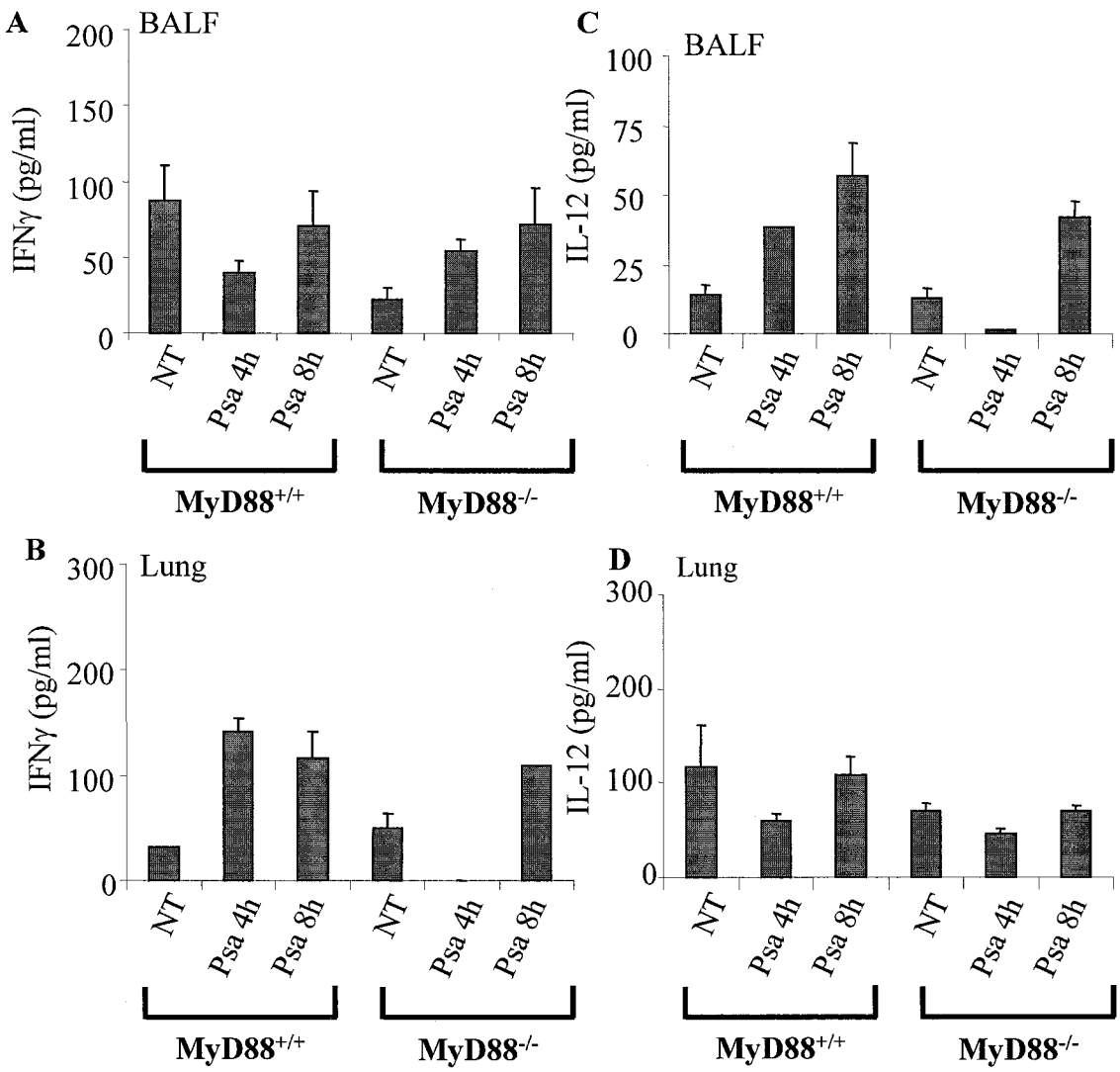


FIGURE 3.5 IFNγ and IL-12 production in MyD88$^{+/+}$ and MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1 x 10^7 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). After 4 or 8 h, BALF and lung tissue were collected for the determination of IFNγ (A and B) and IL-12 (C and D) by ELISA. Data are the mean ± S.E. of 5 – 10 mice per group.

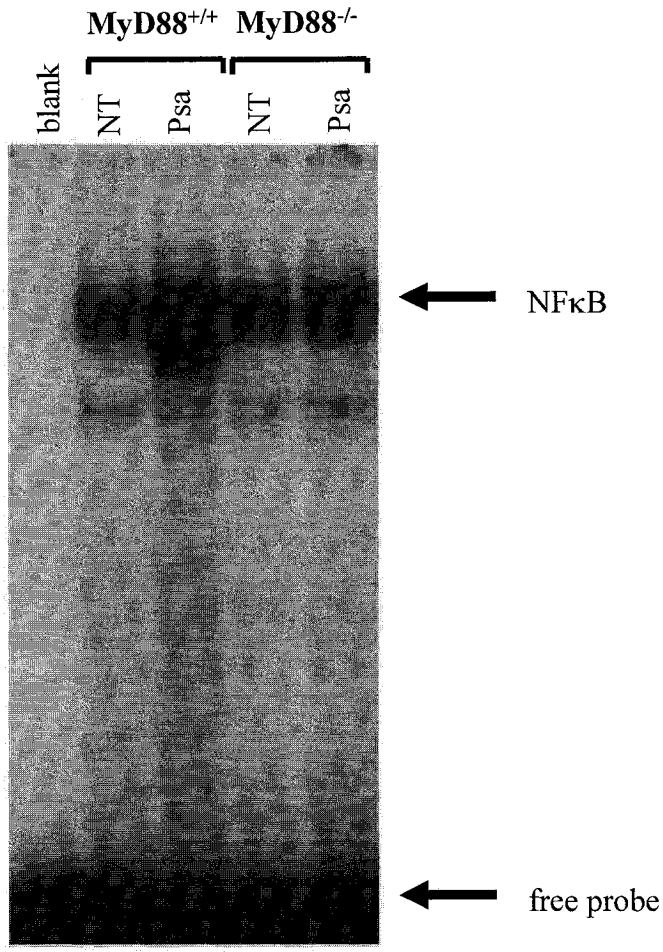
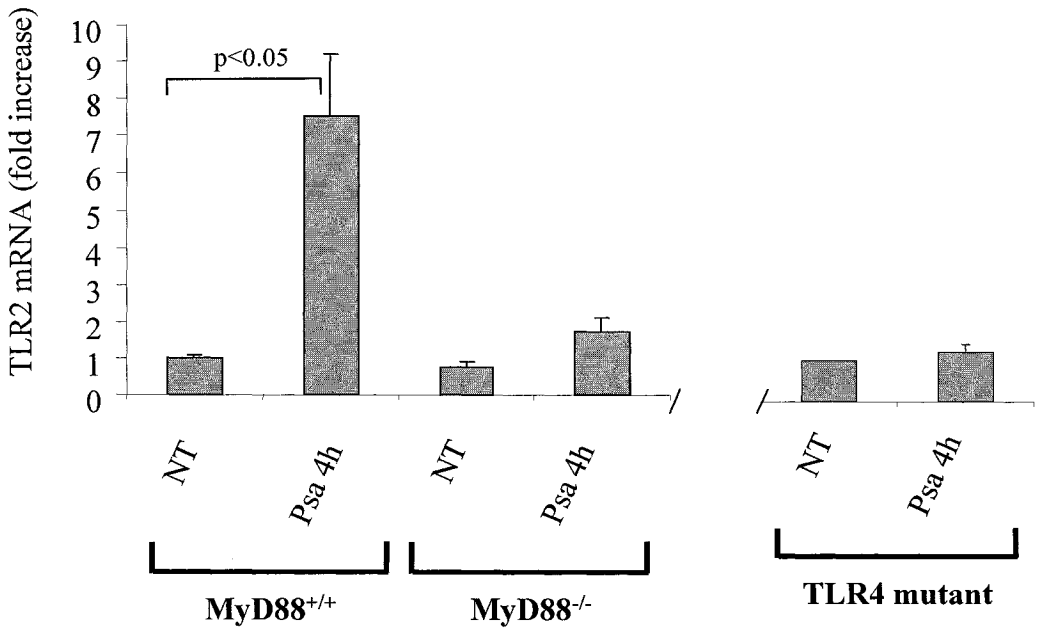


FIGURE 3.6 Impaired NFκB activation in MyD88$^{-/-}$ mice after *P. aeruginosa* lung infection. Lung tissue collected from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice 4 h after intranasal administration with *P. aeruginosa* (*Psa*) strain 8821 (1 x 10^7 CFU/mouse) or saline was homogenized. Nuclear proteins were isolated and used to determine NFκB activation by electrophoretic mobility assay. Figure provided by Dr. Yongde Peng and Fang Lui.

FIGURE 3.7 Expression of TLR2 and TLR4 in MyD88$^{+/+}$, MyD88$^{-/-}$, or TLR4 mutant mice following *P. aeruginosa* lung infection. Lung tissue was collected from MyD88$^{+/+}$ (C57BL/6), MyD88$^{-/-}$, or TLR4 mutant mice 4 h after intranasal administration of *P. aeruginosa* strain 8821 (1 x 10^7 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). Total RNA isolated from the lungs was subjected to real-time RT-PCR analysis for TLR2 or TLR4 expression. For comparison between MyD88$^{+/+}$ and MyD88$^{-/-}$ mice, data are expressed as -fold increase relative to MyD88$^{+/+}$ control mice (normal basal expression). In the study of TLR2 expression in TLR4 mutant mice, data are expressed as -fold increase relative to NT-treated TLR4 mutant mice. Data are the mean ± S.E. of 3 – 6 mice per group. Figure provided by Sandy Edgar.

A


TLR2 mRNA (fold increase)
10
9
8
7
6
5
4
3
2
1
0
p<0.05
NT
Psa 4h
NT
Psa 4h
NT
Psa 4h
MyD88+/+
MyD88-/-
TLR4 mutant

B

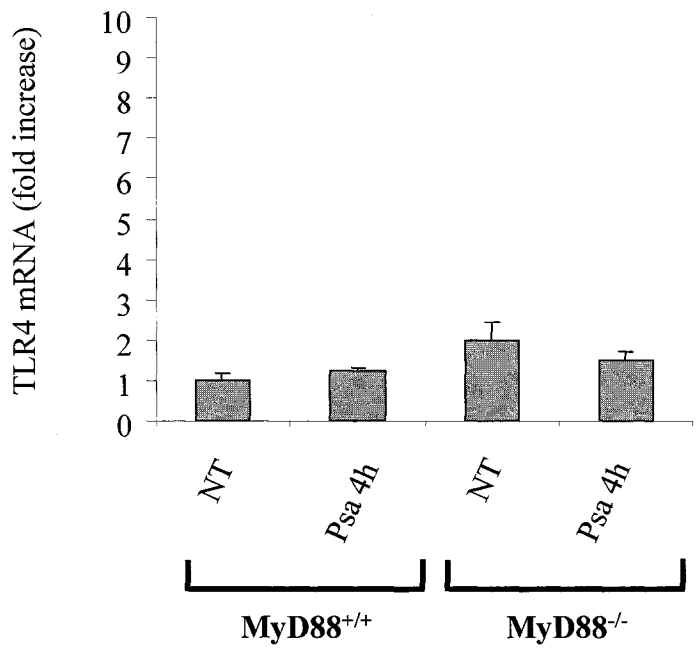
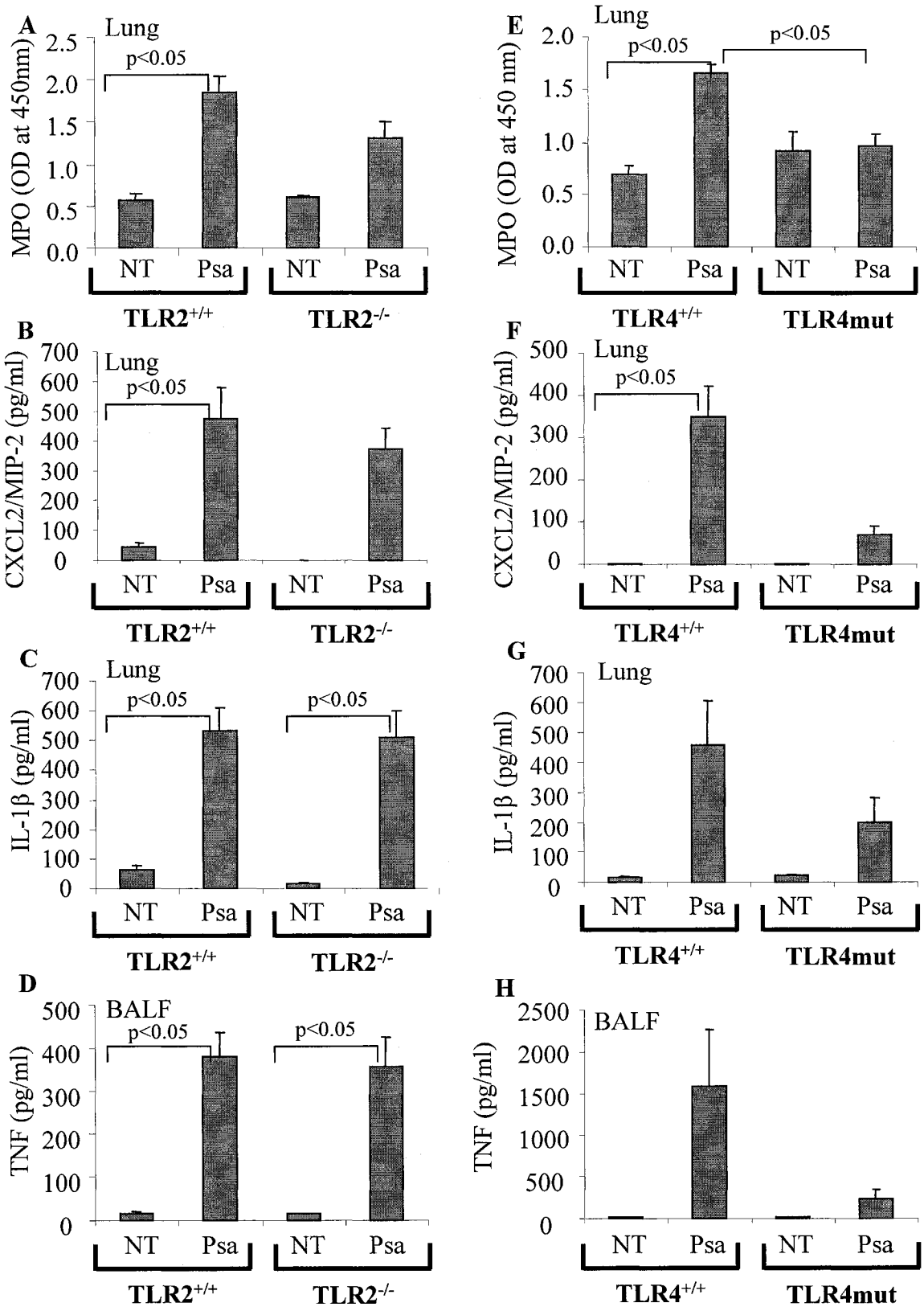

TLR4 mRNA (fold increase)
10
9
8
7
6
5
4
3
2
1
0
NT
Psa 4h
NT
Psa 4h
MyD88+/+
MyD88-/-

FIGURE 3.8 *P. aeruginosa* induced differential immune responses in the lung in TLR2-/- and TLR4 mutant mice. Lung tissue and BALF were collected from TLR2-/- and TLR4 mutant mice and their respective control mice after intranasal infection with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1 x 10^7 CFU/mice) for 4 h. Mice that were not treated with bacteria or those that received saline served as controls (NT). Lung homogenates were used to determine MPO activity (A and E) and CXCL2/MIP-2 (B and F) and IL-1β (C and G) production. TNF (D and H) was measured using BALF samples. Data are the mean ± S.E. of 12 – 16 mice/group.


A
Lung
p<0.05
MPO (OD at 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
NT
Psa
NT
Psa
TLR2+/+
TLR2-/-
B
Lung
p<0.05
CXCL2/MIP-2 (pg/ml)
700
600
500
400
300
200
100
0
NT
Psa
NT
Psa
TLR2+/+
TLR2-/-
C
Lung
p<0.05
p<0.05
IL-1β (pg/ml)
700
600
500
400
300
200
100
0
NT
Psa
NT
Psa
TLR2+/+
TLR2-/-
D
BALF
p<0.05
p<0.05
TNF (pg/ml)
500
400
300
200
100
0
NT
Psa
NT
Psa
TLR2+/+
TLR2-/-
E
Lung
p<0.05
p<0.05
MPO (OD at 450 nm)
2.0
1.5
1.0
0.5
0.0
NT
Psa
NT
Psa
TLR4+/+
TLR4mut
F
Lung
p<0.05
CXCL2/MIP-2 (pg/ml)
500
400
300
200
100
0
NT
Psa
NT
Psa
TLR4+/+
TLR4mut
G
Lung
IL-1β (pg/ml)
700
600
500
400
300
200
100
0
NT
Psa
NT
Psa
TLR4+/+
TLR4mut
H
BALF
TNF (pg/ml)
2500
2000
1500
1000
500
0
NT
Psa
NT
Psa
TLR4+/+
TLR4mut

3.3 Discussion

Depending on the nature of the microbial infection, the mechanism of the host defense varies, including variations in the receptor usage, signaling pathways, cellular participants, and the pattern of gene expression (478, 482). The mechanisms of host defense against *P. aeruginosa* lung infection *in vivo* remain incompletely defined. The lung has a unique relationship with the environment and has developed distinct strategies to defend itself from microbial invasion with innate immune mechanisms that are primarily responsible for the elimination of bacterial organisms (9, 496). *P. aeruginosa*, which generates a mucoid phenotype after colonizing lung tissue, appears to have a specialized relationship with the lung (497). *P. aeruginosa*-induced lung damage is the major cause of death in cystic fibrosis patients (432) and accounts for 40% of the deaths in people with ventilator-associated pneumonia (480). We attempted to determine the role of the TLR-MyD88 pathway in the host defense against *P. aeruginosa* lung infection. Consistent with a recent finding (308), our results suggest that the MyD88 dependent pathway is a central component of the initiation of *P. aeruginosa*-induced early immune responses in the lung, leading to the clearance of this bacterium. Neutrophils play a major role in the clearance of *P. aeruginosa* from the lung, therefore the defective clearance of *P. aeruginosa* seen in MyD88$^{-/-}$ mice is likely caused by a deficient recruitment of neutrophils into the airways. Given that MyD88$^{-/-}$ neutrophils appear to have a normal migratory ability (476), the defective influx of neutrophils into the airways is most likely the result of insufficient production of neutrophil attractants in the lung of MyD88$^{-/-}$ mice as observed in this study. Accordingly, our results support the model in which, during acute *P. aeruginosa* lung infection, MyD88 is absolutely required for the early production of the cytokines and chemokines CXCL2/MIP-2, IL-1β, and TNF, which are responsible for neutrophil recruitment and subsequent bacterial clearance. However, this does not exclude the possibility of the involvement of MyD88 independent pathways such as the TRIF/TRAM pathway in the *P. aeruginosa*-induced host response, especially in the later phase of the infection. Several IFN-regulatory factor (IRF) 3-regulated cytokines and chemokines such as CCL5/RANTES and CXCL10 are induced by the TRIF/TRAM pathway in response to bacterial LPS stimulation (167, 498). CCL5/RANTES and

CXCL10 are up-regulated during *P. aeruginosa* infection (499). Thus, the role for the TRIF/TRAM pathway in *P. aeruginosa*-induced lung infection requires further study.

Given the importance of MyD88 in TLR signaling (469) and the circumstantial evidence of the association between *P. aeruginosa* and TLR2 or TLR4 (488-490), immune responses to *P. aeruginosa* infection were examined in MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR4 mutant mice, with the goal of assessing the relative contribution of these molecules as well as additional TLRs in *P. aeruginosa* infection. Apparently, the pattern of *P. aeruginosa*-induced immune responses in TLR2$^{-/-}$ or TLR4 mutant mice is different from that seen in MyD88$^{-/-}$ mice. These results suggest that neither TLR2 nor TLR4 functions individually as the only component responsible for *P. aeruginosa* induced immune response in the lung *in vivo*. This difference suggests that additional TLRs, such as TLR5 (488, 500), or synergistic effects between different TLRs (501) may be involved. Nevertheless, the increase in TLR2 expression in the lung after *P. aeruginosa* infection and the partial inhibition of immune responses in TLR2$^{-/-}$ mice support a role for TLR2 in *P. aeruginosa*-induced lung inflammation. This is consistent with recent *in vitro* studies (502) demonstrating that TLR2 is involved in the activation of macrophages and monocytes by mannuronic acid polymers, a component of *P. aeruginosa* alginate that is specifically related to the mucoid phenotype (490). Interestingly, TLR2 is mobilized into an apical lipid raft receptor complex in epithelial cells after *P. aeruginosa* stimulation. In addition, TLR2 together with TLR5 and gangliotetraosylceramide have been shown to be involved in *P. aeruginosa* flagella-induced epithelial cell activation *in vitro* (488).

Although the level of TLR4 mRNA was not changed, the inhibition of *P. aeruginosa*-induced neutrophil infiltration and production of CXCL2/MIP-2, IL-1β, and TNF in TLR4 mutant mice supports a role for TLR4 in *P. aeruginosa* lung infection. However, unlike the nearly complete inhibition in MyD88$^{-/-}$ mice, a slight *P. aeruginosa*-induced production of CXCL2/MIP-2, IL-1β, and TNF was observed in TLR4 mutant mice. Other studies (503, 504) have demonstrated *P. aeruginosa*-induced immune responses in C3H/HeJ mice. Thus, other TLRs in addition to TLR4 or synergistic effects between TLRs may be involved in host responses to *P. aeruginosa* lung infection.

The increase in TLR2 mRNA expression and the unchanged TLR4 mRNA level in *P. aeruginosa*-infected lungs suggest that TLR2 and TLR4 may have different roles

during *P. aeruginosa* infection. It is likely that the contribution of TLR2 may increase when the infection progresses, considering the 7.5-fold increase of TLR2 mRNA in the lungs of *P. aeruginosa*-infected wild type mice. The *P. aeruginosa*-induced increase of TLR2 mRNA was not observed in TLR4 mutant mice. This suggests that *P. aeruginosa* up-regulates TLR2 through activation of TLR4. It is widely recognized that TLR4 is associated with LPS-induced tolerance before exposure to bacterial LPS, leading to a state of hyporesponsiveness to subsequent LPS stimulation (505). Thus, it is possible that TLR4 plays a major role during the early phase of *P. aeruginosa* infection, whereas TLR2 may play a greater role as the infection progresses.

It has been well established that MyD88 transduces cell surface signals to transcription factor NFκB, which regulates NFκB dependent gene expression, including TNF and IL-1β (469). *P. aeruginosa*-induced NFκB activation has been reported previously (506) and was confirmed in our study in the infected lung tissue using an electrophoretic mobility shift assay. The defective production of TNF and IL-1β in MyD88$^{-/-}$ mice is likely caused by the blockade of signaling from TLRs to NFκB in these animals, because *P. aeruginosa*-induced NFκB activation in the lung was markedly reduced in MyD88$^{-/-}$ mice. Our model shows that production of TNF and IL-1β seems to be localized differentially within the lung. TNF is found produced in the BALF whereas IL-1β is found in the lung tissue. Specific cells in the lung may be able to produce higher levels of certain cytokines, which may be responsible for the observed differential localization of TNF and IL-1β. However, the removal of the BALF from the airways may provide discrepancies between data obtained from a lung where BALF was taken or not. In this model, BALF was always collected before removal of the lung. Therefore, the differential localization of TNF and IL-1β may in part be due to the nature of the experiment or the locations of cells within the lung that are vital for producing each cytokine.

Interestingly, unlike CCL5/RANTES and CXCL10, which can be regulated in a MyD88-NFκB-independent manner (167), MyD88$^{-/-}$ mice showed an almost complete deficiency in CXCL2/MIP-2 expression at both mRNA and protein levels. CCL5/RANTES and CXCL10 contain IRF binding sites in their promoters and are

regulated through the TRIF-IRF3/7 pathway during TLR activation (167, 173). In contrast, there is no report regarding the existence of an IRF-binding site in the CXCL2/MIP-2 promoter. Although the CXCL2/MIP-2 promoter contains a conserved NFκB consensus motif (507), TLR-mediated CXCL2/MIP-2 production appears to be NFκB independent because the NFκB inhibitor pyrrolidinedithiocarbamate blocks LPS-induced TNF and IL-1β production but has no effect on CXCL2/MIP-2 expression (508). Thus, MyD88 may regulate CXCL2/MIP-2 production through additional transcription factors rather than through NFκB. Alternatively, MyD88 dependent CXCL2/MIP-2 production may be secondary to the production of TNF and IL-1β because IL-1β and TNF are able to regulate CXCL2/MIP-2 expression (509, 510). Thus, the role for MyD88 in the host defense against *P. aeruginosa* lung infection is likely to rely on the interplay of multiple *P. aeruginosa*-induced mediators in the lung, such as CXCL2/MIP-2, TNF, and IL-1β.

In summary, our results suggest that the MyD88 dependent pathway plays an essential role in *P. aeruginosa*-induced early immune responses, including CXCL2/MIP-2, IL-1β, and TNF production and subsequent neutrophil recruitment and bacterial clearance. Multiple MyD88 dependent TLRs, including TLR2 and TLR4, may be involved in the host defense against *P. aeruginosa* lung infection.

CHAPTER 4. The Myeloid Differentiation Factor 88 (MyD88) is Dispensable for the Development of a Delayed Host Response to *Pseudomonas aeruginosa* Lung Infection in Mice

Power M.R., J.S. Marshall, M. Yamamoto, S. Akira, and T.J. Lin. *Clin. Exp. Immunol.* 2006; 146:323-329. (511)

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4.1 Introduction

The lung has a unique relationship with the environment. The lung has developed a complex defence system against microbial infection. An effective defence strategy involves coordinated early and sustained immune responses against a specific pathogen. *Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen which often induces strong and sustained inflammatory responses in the lung (512). However, to our knowledge, no studies to date directly address the contribution of a specific molecule in the initiation of immune responses as opposed to its role in the subsequent delayed inflammatory response to *P. aeruginosa* in the lung.

Toll-like receptors (TLRs) have been implicated in *P. aeruginosa* infection *in vitro* and *in vivo* (488, 489). Based on the usage of the TLR adaptor protein myeloid differentiation factor 88 (MyD88), TLR-mediated signaling events are divided broadly into MyD88 dependent and the MyD88 independent pathways. We and others have demonstrated that the TLR-MyD88-dependent pathway plays an essential role in the development of early host defence against *P. aeruginosa* lung infection, because MyD88 deficient mice have a near complete defect of early cytokine production and neutrophil recruitment and are accompanied with impaired bacterial clearance (307, 308). However, it is not known if an effective immune response to *P. aeruginosa* infection can develop in the absence of MyD88 when the infection progresses. To differentiate the mechanisms involved in the early *versus* delayed phase of *P. aeruginosa* infection may be particularly important, as this bacterium is well known for its chronic features.

In addition to the MyD88 pathway, TLRs also □ransducer signals through a MyD88 independent pathway to induce a late nuclear factor activation and subsequent delayed cytokine production (164). Thus, we hypothesize that a MyD88 independent

mechanism is responsible for the delayed innate immune response in the lung during *P. aeruginosa* infection. Here, we demonstrate that effective delayed innate immune responses in the lung following *P. aeruginosa* infection, including production of neutrophil chemoattractants and neutrophil recruitment, are developed in the absence of MyD88. Importantly, MyD88 deficient mice are able to clear *P. aeruginosa* from the lung after 48 h of infection. These results, together with our previous finding of the role for MyD88 dependent pathway in early immune responses, suggest that a coordinated host defence against *P. aeruginosa* lung infection consists of the MyD88 dependent early initiation of the innate immune response and the MyD88 independent delayed innate immune response.

4.2 Results

4.2.1 MyD88$^{-/-}$ Mice Produce Neutrophil Chemoattractant Mediators in the Airway after 24–48 h of Exposure to *P. aeruginosa*

Previously, we demonstrated that the early production of inflammatory mediators that affect neutrophil recruitment, including CXCL2/MIP-2, TNF and IL-1β in the airway is critically dependent on MyD88 in mice after *P. aeruginosa* lung infection for 4–8 h. To determine the role of the MyD88 independent pathway in the delayed phase of host response to *P. aeruginosa*, MyD88$^{-/-}$ mice and MyD88$^{+/+}$ control mice were challenged with *P. aeruginosa* intranasally. After 24–48 h, BALF and lung tissues were collected for determination of CXCL2/MIP-2 (Figure 4.1), CXCL1/KC (Figure 4.2), IL-1β (Figure 4.3) and TNF (Figure 4.4) production. In contrast to the MyD88 dependent production of these mediators during the early phase of infection (307), CXCL2/MIP-2 levels in the BALF and lung at 24 and 48 h were similar in MyD88$^{-/-}$ mice and MyD88$^{+/+}$ control mice (Figure 4.1). MyD88$^{-/-}$ mice also produce significant amounts of TNF and IL-1β after *P. aeruginosa* challenge ($P < 0{\cdot}01$ when compared to untreated mice) although the levels of these two cytokines were lower than that of MyD88$^{+/+}$ mice (Figures 4.4 and 4.3). CXCL1/KC production was still diminished at 24 and 48 h post infection in the lungs of MyD88$^{-/-}$ mice (Figure 4.2). These data suggest that the MyD88 independent

pathway is involved in delayed inflammatory mediator production following *P. aeruginosa* lung infection.

4.2.2 MyD88$^{-/-}$ Mice Recruit Neutrophils to the Airway after 24–48 h of Exposure to *P. aeruginosa*

Neutrophils are the major effector cells responsible for the clearance of *P. aeruginosa* and are recruited by the neutrophil chemoattractants produced in the airway. Whether MyD88 independent production of neutrophil chemoattractants such as CXCL2/MIP-2, TNF and IL-1β during the delayed phase (24–48 h) of *P. aeruginosa* infection can recruit neutrophils efficiently to the airway was determined. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with 1×10^9 *P. aeruginosa* strain 8821. BALF and lung tissues were collected 24 or 48 h later for the detection of neutrophil infiltration by MPO assay. MyD88$^{-/-}$ mice were able to recruit neutrophils efficiently into the lung after 24 or 48 h of *P. aeruginosa* infection, as did MyD88$^{+/+}$ mice (Figure 4.5). MPO levels in the BALF were lower in MyD88$^{-/-}$ mice than those in MyD88$^{+/+}$ mice, suggesting that less neutrophils reached the airway in MyD88$^{-/-}$ mice.

4.2.3 MyD88$^{-/-}$ Mice Cleared *P. aeruginosa* Effectively after 24 or 48 h of Exposure to this Bacterium

To determine if MyD88 independent immune response in the airway is capable of clearing *P. aeruginosa*, BALF and lung tissues from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were collected for the detection of viable bacteria by CFU counting after intranasal administration for 24 or 48 h of 1×10^9 *P. aeruginosa* strain 8821. When mice were challenged with 1×10^9 CFU/mouse of *P. aeruginosa* for 24 h, MyD88$^{-/-}$ mice showed more bacterial CFU in the BALF and the lung compared to MyD88$^{+/+}$ mice. However, 48 h post-infection, numbers of bacterial counts in the lung were similar in MyD88$^{-/-}$ or MyD88$^{+/+}$ mice (Figure 4.6). These data suggest that during the delayed stage of infection (24–48 h) the MyD88 independent mechanism is effective in clearing *P. aeruginosa* from the lung. However, the CFU number from the BALF of MyD88$^{-/-}$ mice is higher than that of MyD88$^{+/+}$ mice, suggesting a remaining effect of the MyD88 dependent pathway in the delayed response (Figure 4.6).

4.2.4 TLR2 or TLR4 are not Essential for the Delayed (48 h) Host Response to *P. aeruginosa* Lung Infection

To examine a role for TLR2 or TLR4 in *P. aeruginosa*-induced delayed host responses, $TLR2^{-/-}$ mice, $TLR4^{-/-}$ mice and their respective control mice were challenged with *P. aeruginosa* intranasally (8821, 10^9 CFU/mouse) for 48 h. Bacterial CFU, MPO level (neutrophil recruitment) and levels of CXCL2/MIP-2, TNF and IL-1β in the airway (BALF) were determined. No differences in the levels of bacterial CFU, MPO, CXCL2/MIP-2 and IL-1β were observed between TLR2 or TLR4 mice and their respective control animals (Table 4.1). The TNF level in $TLR4^{-/-}$ mice but not in $TLR2^{-/-}$ mice was lower when compared to that in their respective control animals, suggesting that TLR2 and TLR4 may have different roles in *P. aeruginosa* infection. These results suggest that, individually, TLR2 or TLR4 are not essential for the delayed host response to *P. aeruginosa* lung infection.

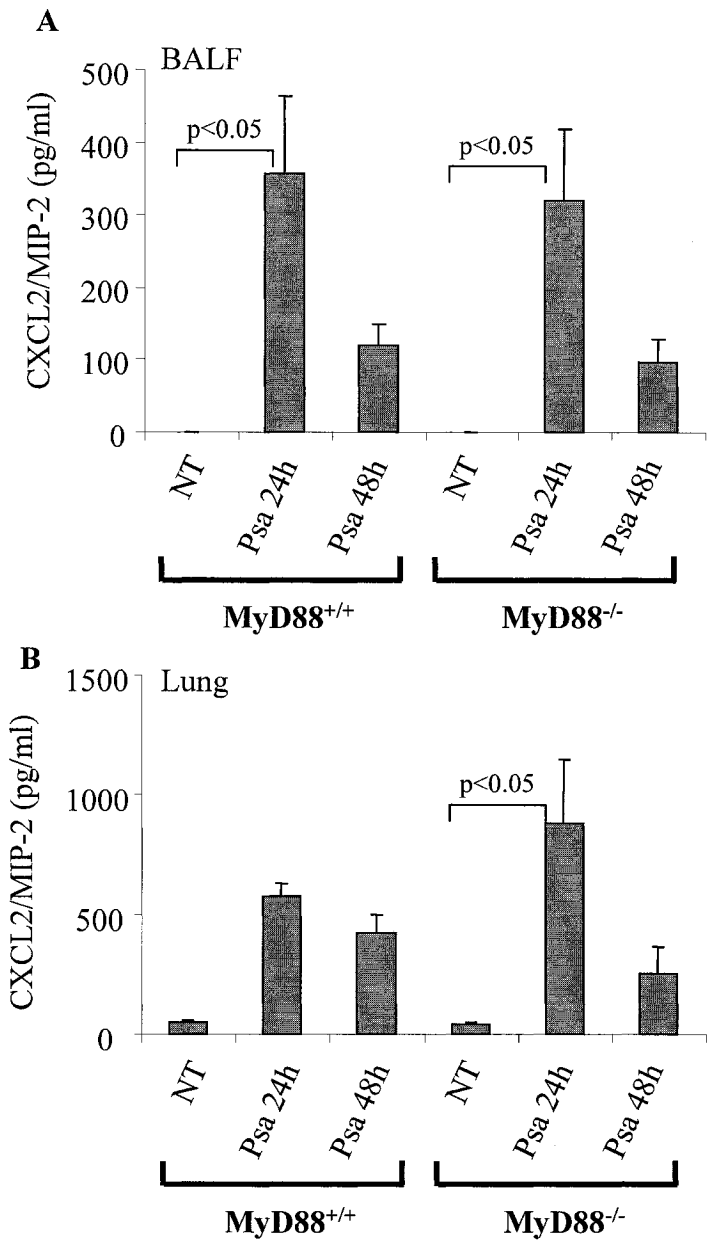

FIGURE 4.1 Late CXCL2/MIP-2 production is MyD88 independent following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1 × 10^9 CFU/mouse, Psa) for 24 or 48 h. Mice that were not treated with bacteria were used as controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of CXCL2/MIP-2 protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 5 - 11 mice/group.

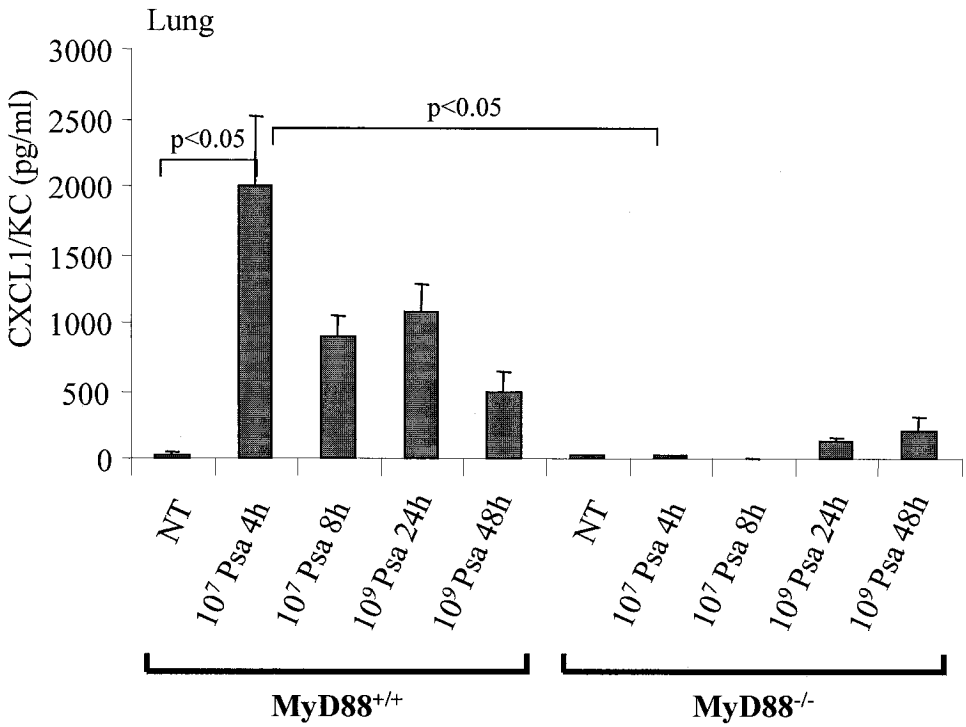

FIGURE 4.2 Abrogated CXCL1/KC production in MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4, 8, 24 or 48 h. Mice that were not treated with bacteria were used as controls. Lung tissues were collected for the determination of CXCL1/KC protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 5 - 11 mice/group.

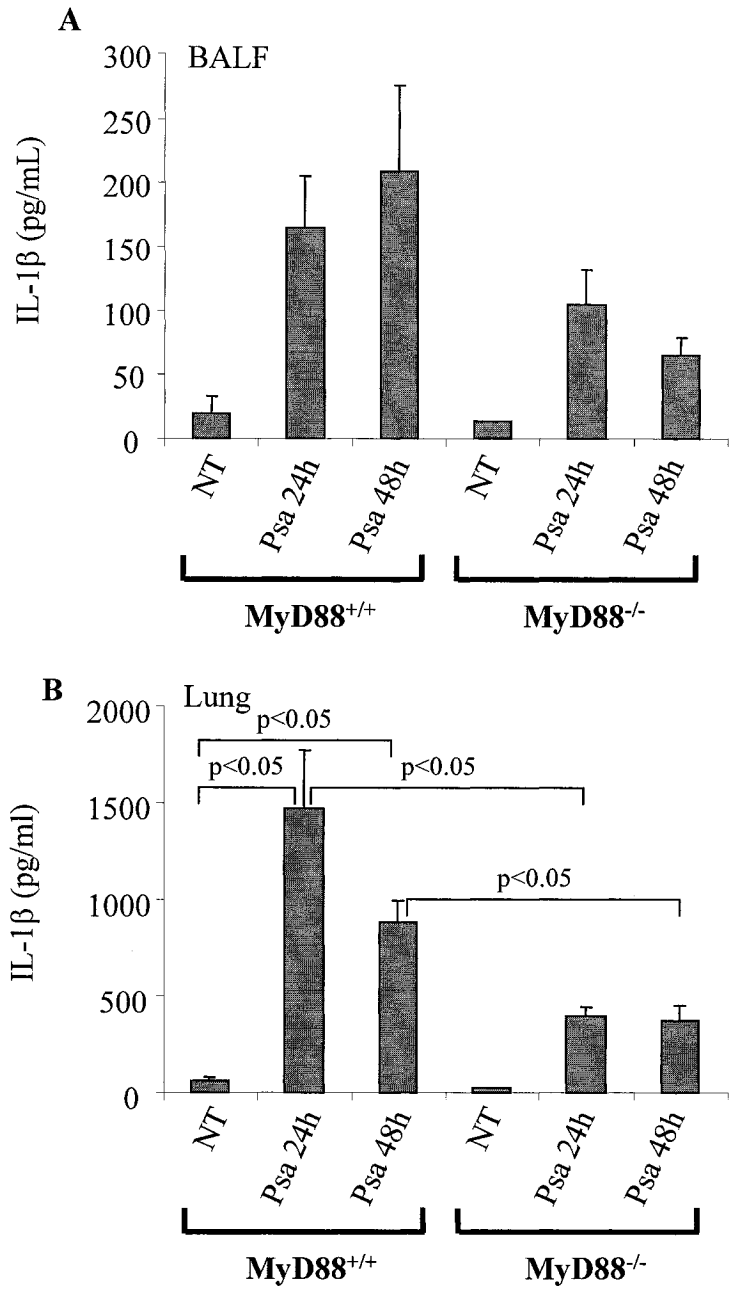

FIGURE 4.3 Delayed IL-1β production in MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1 × 10^9 CFU/mouse, Psa) for 24 or 48 h. Mice that were not treated with bacteria were used as controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of IL-1β protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 5 - 11 mice/group.

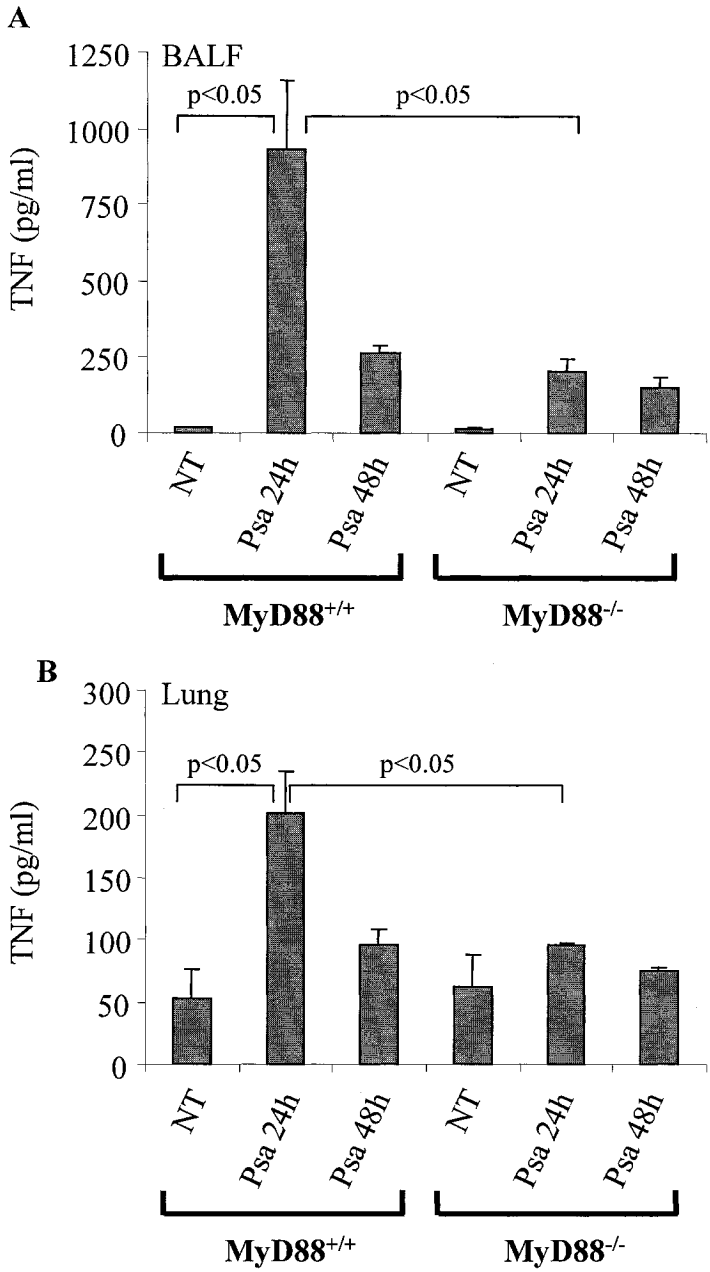

FIGURE 4.4 Delayed TNF production in MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1 × 10^9 CFU/mouse, Psa) for 24 or 48 h. Mice that were not treated with bacteria were used as controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of TNF protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 5 - 11 mice/group.

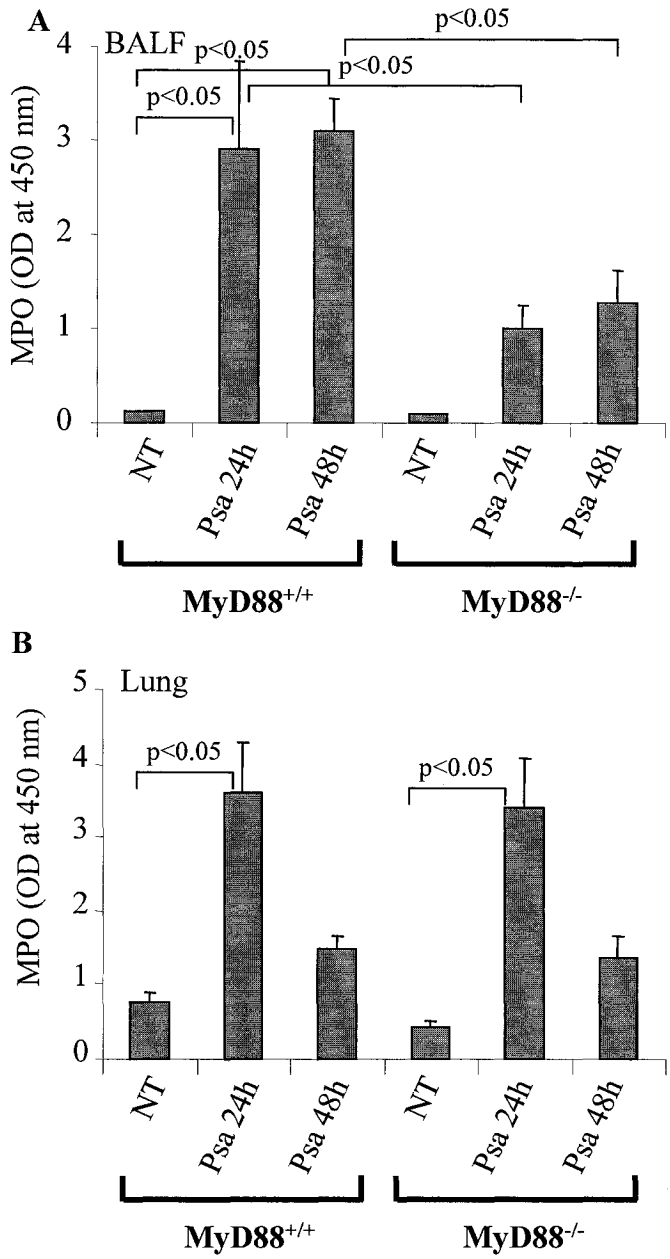

FIGURE 4.5 Significant neutrophil recruitment into the airways of MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1 × 10^9 CFU/mouse, Psa) for 24 or 48 h. Mice that were not treated with bacteria were used as controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of myeloperoxidase (MPO) activities. Data are the mean ± S.E. of 5 - 11 mice/group.

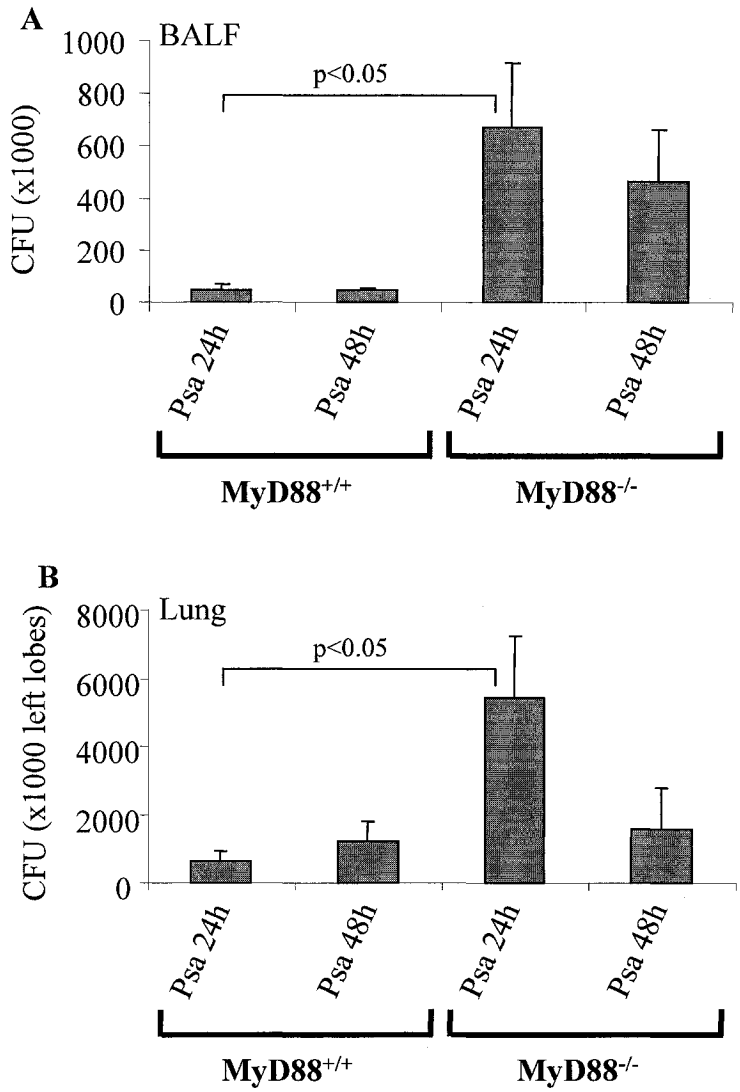

FIGURE 4.6 Clearance of *P. aeruginosa* from the lungs of MyD88$^{-/-}$ mice. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were challenged with *P. aeruginosa* (Psa) (mucoid strain 8821, 1×10^9 CFU/mouse for 24 or 48 h, Psa) intranasally. After 24 or 48 h, the bronchoalveolar lavage fluid (BALF) and the right lung were collected for colony counting. MyD88$^{-/-}$ mice showed impaired clearance of *P. aeruginosa* from the airways at 24 h. However, after 48 h of infection, numbers of bacterial counting in the lung were similar in MyD88$^{-/-}$ and MyD88$^{+/+}$ mice. Data are the mean ± S.E. of 11 mice/group.

TABLE 4.1 TLR2 and TLR4 are not essential for the delayed (48 h) host response to *P. aeruginosa* infection in mice

	$TLR2^{+/+}$		$TLR2^{-/-}$		$TLR4^{+/+}$		$TLR4^{-/-}$	
	NT	***Psa***	**NT**	***Psa***	**NT**	***Psa***	**NT**	***Psa***
CFU (×1000)	N/A	57 ± 37	N/A	100 ± 62	N/A	111 ± 39	N/A	102 ± 58
MPO (OD)	0·2 ± 0·1	3·4 ± 0·8	0·2 ± 0·2	3·9 ± 0·1	0·3 ± 0·2	2·8 ± 0·8	0·2 ± 0·1	2·5 ± 0·9
CXCL2/ MIP-2 (pg/ml)	46 ± 30	248 ± 74	40 ± 3	262 ± 57	n.d.	1469 ± 280	n.d.	868 ± 641
TNF (pg/ml)	15 ± 8	492 ± 113	16 ± 3	450 ± 43	n.d.	859 ± 266	n.d.	240 ± 83*
IL-1β (pg/ml)	n.d.	902 ± 222	n.d.	1395 ± 413	n.d.	1503 ± 151	n.d.	1746 ± 1020

TLR2 deficient mice ($TLR2^{-/-}$), $TLR2^{+/+}$ (C57BL/6), TLR4 deficient mice ($TLR4^{-/-}$, C57BL/10ScN) and $TLR4^{+/+}$ (C57BL/10ScSn) were challenged with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1×10^9 colony-forming units (CFU)/mouse for 48 h) intranasally. Mice not treated with *P. aeruginosa* served as control (NT). There were no CFU detected in NT mice, not applicable (N/A). After 48 h, the bronchoalveolar lavage fluids from the airways were collected for bacterial colony counting (CFU), determination of myeloperoxidase activity (MPO, neutrophil recruitment), and assay for CXCL2/MIP-2, TNF and IL-1β by ELISA. Some samples in the no treatment group (NT) were undetectable for cytokines and chemokine (n.d.). Data are the mean ± s.e. of four mice per group. $^*P < 0{\cdot}05$ compared to $TLR4^{+/+}$ group.

4.3 Discussion

P. aeruginosa, an opportunistic Gram-negative bacillus, is a common cause of nosocomial pneumonia (479, 480) and the major pathogen in cystic fibrosis (CF) patients (432). One of the major features of *P. aeruginosa* lung infection is chronic infection accompanied with acute exacerbation (513). The lung inflammation induced by this bacterium includes initial stimulation and subsequent sustained responses. To the best of our knowledge, no studies have been designed in an effort to directly differentiate the specific contribution of a molecule involved in the initial responses *versus* its role in the subsequent inflammatory events during *P. aeruginosa* lung infection. Recently, we demonstrated an essential role for MyD88 in the development of early host response to *P. aeruginosa* (307). We hypothesized that the MyD88 independent pathway may be responsible for the development of the delayed host response to *P. aeruginosa* lung infection because the MyD88 independent pathway mediates delayed cytokine production (164). This hypothesis was confirmed, as the MyD88 deficient mice developed delayed production of CXCL2/MIP-2, TNF and IL-1β and recruited neutrophils to the lung at a similar level to that seen in $MyD88^{+/+}$ mice after 24–48 h of *P. aeruginosa* lung infection. After 24 h of infection the number of bacterial CFU in the airways of $MyD88^{-/-}$ mice was greater than that in $MyD88^{+/+}$ mice, suggesting an important role for MyD88 in the first 24 h of infection. This finding is consistent with a recent report (514). Importantly, after 48 h of infection the $MyD88^{-/-}$ mice showed a similar capacity for clearing *P. aeruginosa* from the lung to that of $MyD88^{+/+}$ mice. These findings are in contrast to our previous report, that the early immune responses including neutrophil chemoattractant production, neutrophil recruitment and bacterial clearance in the first 4 – 8 h of infection are critically dependent upon the MyD88 pathway (307). Thus, the present findings, together with our previous report (307), suggest that the host defence against *P. aeruginosa* lung infection develops in an ordered, manner that includes a MyD88 dependent early response and a MyD88-independent delayed response.

The molecules involved in the MyD88 independent mechanism remain to be determined. The MyD88-independent TLR–TRAM/TRIF pathway mediates delayed nuclear factor (NF)κB activation and cytokine production (163, 164), a pattern of

immune response similar to that seen in this study during *P. aeruginosa* lung infection. Thus, it is possible that the TLR–TRIF pathway may be responsible for the delayed phase of host defence against *P. aeruginosa* lung infection. Alternatively, other cell surface molecules that are unrelated to MyD88 may also be responsible for the host responses seen in MyD88 deficient mice. For example, several cell surface molecules that do not require MyD88 for signaling have been documented to be involved in the host defence against *P. aeruginosa* infection, including complement receptor 3 (484), gangliotetraosylceramide (asialoGM1) (485), the cystic fibrosis transmembrane conductance regulator (458), CD91 (486) and syndecan-1 (487). In an attempt to examine a role for TLR2 or TLR4 in *P. aeruginosa*-induced delayed host responses, we used TLR2- or TLR4-deficient mice. After 48 h of lung infection, no difference in bacterial CFU, MPO level (neutrophil recruitment) or levels of CXCL2/MIP-2 and IL-1β in the airway (BALF) was observed between $TLR2^{-/-}$ or $TLR4^{-/-}$ mice and their respective control animals. These results suggest that, individually, TLR2 or TLR4 are not essential for the development of delayed host response to *P. aeruginosa* lung infection. These results are consistent with a recent study demonstrating that TLR2 and TLR4 are not essential for the clearance of *P. aeruginosa* from the lung (515).

It is noteworthy that the level of CXCL2/MIP-2 after 24 h of *P. aeruginosa* infection in the airways of $MyD88^{-/-}$ mice is similar to that in $MyD88^{+/+}$ mice. In contrast, the levels of TNF and IL-1β in the airways of $MyD88^{-/-}$ mice are lower than those in $MyD88^{+/+}$ mice. In addition, the level of TNF in the $TLR4^{-/-}$ mice was lower than that in wild-type control mice. Similar findings were also observed by others demonstrating that cytokines and chemokines are regulated differentially during *P. aeruginosa* infection (514). These data suggest that CXCL2/MIP-2, TNF and IL-1β are probably regulated through different pathways, and sufficient neutrophil recruitment and bacterial clearance can be achieved under conditions of low TNF or IL-1β responses in the lung.

It is also noteworthy that the *P. aeruginosa* strain used in this study is a clinical isolate with a mucoid phenotype (466). Multidrug-resistant mucoid strains may have a different capacity in the induction of host immune responses compared to the susceptible *P. aeruginosa* strains (516). Thus, we are cautious in generalizing our findings. The

MyD88 independent pathway in the host defence against other *P. aeruginosa* strains may require additional study.

In summary, we have demonstrated an effective delayed immune response to *P. aeruginosa* lung infection through a MyD88 independent mechanism. This finding, together with our previous report (307), leads us to propose a novel conceptual model that the MyD88 dependent mechanism is responsible for the early host response while the MyD88 independent mechanism is responsible for the delayed host response, which occurs to mount host defence against *P. aeruginosa* lung infection.

This finding may be relevant for understanding clinical conditions in patients who have a compromised immune system. *P. aeruginosa*, a nosocomial pathogen, is often associated with ventilator-associated pneumonia in critically ill patients or systemic infections in neutropenic patients. Both the MyD88 dependent early immune response and the MyD88 independent delayed immune response may not be triggered effectively in these patients. Thus, targeting the MyD88 independent and the MyD88 dependent pathways may serve as an alternative approach for managing *P. aeruginosa* infection in immunocompromised patients.

CHAPTER 5. A Role of Toll-IL-1 Receptor Domain-Containing Adapter-Inducing IFNβ (TRIF) in the Host Response to *Pseudomonas aeruginosa* Lung Infection in Mice

M.R. Power, B. Li, M. Yamamoto, S. Akira, and T.-J. Lin. *J. of Immunol.* 2007; 178(5): 3170-3176. (517)

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5.1 Introduction

Pseudomonas aeruginosa is a major opportunistic pathogen in patients with hospital-acquired pneumonia and in individuals with cystic fibrosis. Airway infection with *P. aeruginosa* triggers both acute and chronic inflammatory responses including elevated production of various cytokines and chemokines (482, 512, 518, 519). It is generally accepted that the host defense against *P. aeruginosa* lung infection is initiated by the recognition of this bacterium by local resident cells, followed by secretion of inflammatory mediators (cytokines/chemokines) that attract and activate immune effector cells for the clearance of this pathogen (482, 512, 518, 519).

Recently, a number of *in vitro* and *in vivo* studies have shown that Toll-like receptors (TLRs) are involved in the recognition of *P. aeruginosa* (307, 488, 490, 515, 520, 521). TLRs are a family of pattern recognition receptors that are critical for cellular responses to microbial products (132). TLRs mediate distinct immune responses depending upon their usage of specific adaptor molecules which transduce signals from TLRs to transcription factors. Increasing evidence suggests that the adaptor proteins, myeloid differentiation factor 88 (MyD88) and Toll/IL-1 receptor (TIR) domain-containing adapter-inducing IFNβ (TRIF, also known as TICAM-1) mediate two distinct TLR signaling pathways (522). MyD88 mediates nuclear factor κB (NFκB) activation and subsequent expression of NFκB-regulated genes, such as TNF and IL-1. TRIF mediates interferon regulatory factor (IRF) 3 and IRF7 activation leading to IFNα/β expression (163). In addition to activation of IRF3/7 pathway, TRIF also mediates a second pathway leading to NFκB activation (209, 210). Global gene expression profiles distinctly regulated by the MyD88 dependent and the MyD88 independent pathways have been characterized (523). In fact, only 21.5% of lipopolysaccharide (LPS) responsive

genes are MyD88 dependent, while the majority of these genes (74.5%) are MyD88 independent (523). Thus, activation of the MyD88-dependant pathway and the MyD88 independent TRIF pathway lead to distinct gene expression profiles.

Depending upon the nature of microbial pathogens, different signaling pathways are used to develop an effective host response to a specific pathogen. We and others have demonstrated that the MyD88 pathway is required for the development of early immune response to *P. aeruginosa* lung infection (307, 308). However, MyD88 is not essential for innate immunity to *Staphylococcus aureus* (308) or *Mycobacterium tuberculosis* (474). In fact, MyD88 deficiency appears to improve resistance against sepsis caused by polymicrobial infection (476). Thus, effective immunity against bacterial infection can be developed through MyD88 independent mechanisms. This notion is supported by a recent study demonstrating that the TRIF pathway but not the MyD88 pathway is needed for P fimbriated *Escherichia coli*-induced signaling in epithelial cells (524).

TRIF is a major TLR adaptor protein that mediates MyD88 independent pathways (471). A role of TRIF in the host defense has been primarily associated with viral infections due to its significant roles in the induction of IFNα/β (525, 526). The contribution of TRIF in the development of host defense against bacterial infection is less clear. *In vitro*, two recent studies showed that TRIF, but not MyD88 is required for *Yersinia*-induced macrophage apoptosis (527) and *Escherichia coli*-induced dendritic cell apoptosis (528). *In vivo*, TRIF appears to be dispensable for the clearance of the Gram-negative bacterium *Haemophilus influenzae* from mouse lung (529). In addition, activation of IRF-IFNα/β pathway impairs host resistance to the Gram-positive intracellular bacterium *Listeria monocytogenes* (530). A role of TRIF in *P. aeruginosa* infection has not been investigated previously.

In vivo, by using TRIF deficient mice, we demonstrated that TRIF deficiency leads to markedly reduced pulmonary eradication of *P. aeruginosa*. This was associated with delayed neutrophil recruitment and a distinct pattern of cytokine and chemokine production in the airways. *P. aeruginosa*-induced production of several NFκB-regulated gene products including CCL5/RANTES, CXCL1/KC and TNF was impaired in TRIF-deficient mice. Thus, TRIF is required for the full development of host defense against

P. aeruginosa lung infection. This is in contrast to the lack of effect or negative role of TRIF pathway in *H. influenzae* or *L. monocytogenes* infection.

5.2 Results

5.2.1 Impaired Cytokine and Chemokine Production in the Airways of TRIF-Deficient Mice Following *P. aeruginosa* Lung Infection

To determine a role for TRIF in the development of immune response to *P. aeruginosa* lung infection *in vivo*, $TRIF^{-/-}$ mice were infected with *P. aeruginosa* strain 8821 at the concentration of 1×10^7 or 1×10^9 CFU/mouse for 4 or 24 h. BALF and lung tissues were collected for the determination of CCL5/RANTES, CXCL1/KC, CXCL2/MIP-2, TNF and IL-1β. *P. aeruginosa*-induced CCL5/RANTES production was completely inhibited in $TRIF^{-/-}$ mice (Figure 5.1*A* and *B*). This appeared to be different from $MyD88^{-/-}$ mice which showed significant CCL5/RANTES production after *P. aeruginosa* (1×10^9 CFU/mouse) infection for 24 or 48 h (Figure 5.2). Similarly, neutrophil chemoattractant CXCL1/KC was also markedly reduced in $TRIF^{-/-}$ mice (Figure 5.1*C* and *D*). However, $TRIF^{-/-}$ mice had little or no defects in the production of CXCL2/MIP-2 or IL-1β in response to *P. aeruginosa* lung infection (Figure 5.3*A*, *B* and *C*). This is in contrast to MyD88 deficiency which leads to significant impairment of CXCL2/MIP-2 and IL-1β production in the lung (307). *P. aeruginosa*-induced TNF production was partially reduced in $TRIF^{-/-}$ mice (Figure 5.3*D*). Taken together, $TRIF^{-/-}$ mice have a specific pattern of cytokine and chemokine production in response to *P. aeruginosa* lung infection, which is distinct from $MyD88^{-/-}$ mice (307, 308, 515). IFNα and IFNβ in the BALF and lung were also tested by ELISA. *P. aeruginosa* infection had little effect on IFNα or IFNβ production in wild type or $TRIF^{-/-}$ mice (Figures 5.4 and 5.5).

To examine *P. aeruginosa*-induced transcription factor activation in the lung, wild type and $TRIF^{-/-}$ mice were challenged with *P. aeruginosa* intranasally for 4 h. Lung tissue was harvested from these mice. Nuclear proteins were extracted and were subjected to EMSA analysis for NFκB (Figure 5.6) or IRF binding (Data not shown). *P. aeruginosa* infection induced NFκB activation in wild-type mice. Interestingly, TRIF-

deficiency caused only a mild reduction of the *P. aeruginosa*-induced NFκB activation in the lung (Figure 5.6).

IRF-7 is an inducible transcription factor important in the TRIF signaling pathway. To examine whether *P. aeruginosa* infection induces changes in IRF-7 expression, lung tissue from *P. aeruginosa*-infected (24 h) wild type (C57BL6) mice was used to determine mRNA levels by real-time RT-PCR. Results were expressed as the -fold increase relative to the level in wild type untreated mice by using the average value from these animals as a calibrator. As shown in Figure 5.7, *P. aeruginosa* infection induced an increase in IRF-7 expression in wild type mice, suggesting that IRF-7 may be involved in *P. aeruginosa* lung infection. However, no IRF binding activity, as assessed by EMSA analysis for IRF-3 binding, could be observed in *P. aeruginosa*-treated or untreated lung tissues (data not shown). Perhaps the induction of IRF-7 after *P. aeruginosa* infection is via a not yet determined pathway. These data indicate that the role of IRF-7 in response to *P. aeruginosa* treatment is not yet clear.

5.2.2 Delayed Neutrophil Recruitment into the Airways in TRIF$^{-/-}$ Mice Following *P. aeruginosa* Lung Infection

Neutrophils are involved in the clearance of *P. aeruginosa* and are recruited by neutrophil chemoattractants produced in the airways (482). The distinct pattern of cytokine and chemokine production in TRIF$^{-/-}$ mice led us to examine if TRIF deficiency affects neutrophil recruitment into the airways. TRIF$^{-/-}$ mice were infected intranasally with 1×10^7 or 1×10^9 *P. aeruginosa* strain 8821. BALF and lung tissues were collected 4, 8 or 24 h later for the detection of neutrophil infiltration by MPO assay. MPO levels in the earlier time points (8 h in the BALF, 4 and 8 h in the lung) in TRIF$^{-/-}$ mice were lower than that in TRIF$^{+/+}$ mice (Figure 5.8*A* and *B*). Similarly, lung histology showed that a limited number of neutrophils reached the alveolar airspace in the earlier stages (8 h) of infection in TRIF deficient mice. However, at the later stages of infection the level of neutrophil infiltration into the alveolar airspace was similar in wild type and TRIF-deficient mice (Figure 5.8*C* - *N*). This result suggests that TRIF deficiency caused a delayed neutrophil recruitment into the airways.

5.2.3 Impaired Clearance of *P. aeruginosa* from the Lungs of TRIF$^{-/-}$ Mice

To determine if TRIF dependent immune responses in the airways have an effect on the clearance of *P. aeruginosa* from the lung, the BALF and lung tissues from TRIF$^{+/+}$ and TRIF$^{-/-}$ mice were collected for the detection of viable bacteria by CFU counting after intranasal administration of *P. aeruginosa* strain 8821 (1×10^9 CFU/mouse) for 24 or 48 h. After infection for 24 h, CFU counts in the lung tissues from TRIF$^{-/-}$ mice were 7 fold higher compared to TRIF$^{+/+}$ mice. This difference between TRIF$^{+/+}$ and TRIF$^{-/-}$ mice in the bacterial clearance appeared to last to 48 h. At 48 h after infection, CFU counts in the lung tissues from TRIF$^{-/-}$ mice were 6.2 fold higher compared to TRIF$^{+/+}$ mice (Figure 5.9). These results suggest that TRIF is required for the clearance of *P. aeruginosa* from the lung.

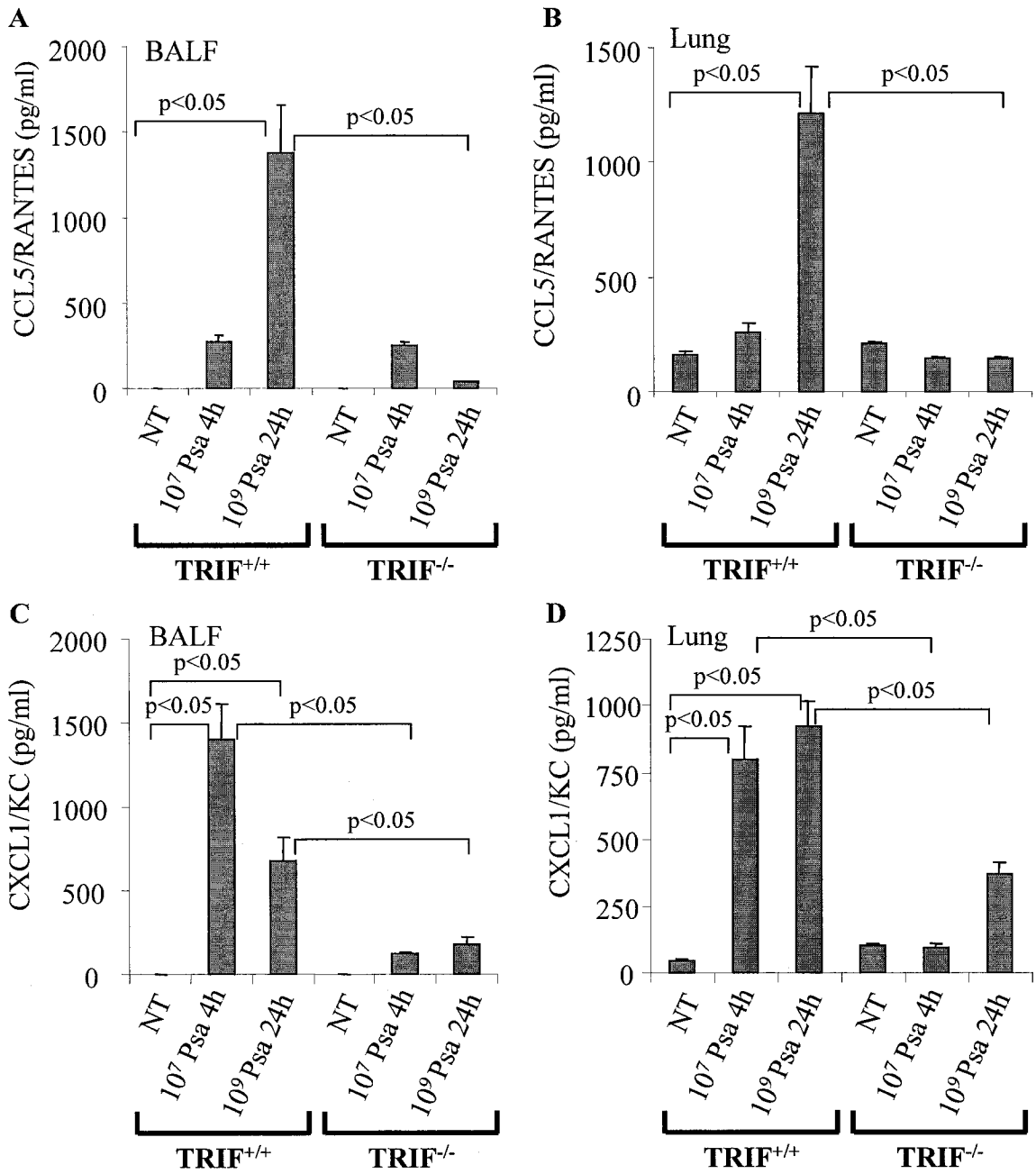


FIGURE 5.1 *P. aeruginosa* induced CCL5/RANTES and CXCL1/KC production in the airways is dependent on TRIF. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4 or 24 h. Mice that were not treated with bacteria were used as controls (NT). BALF and lung tissues were collected for the determination of CCL5/RANTES and CXCL1/KC protein by ELISA. Data are the mean ± SE of 9 - 11 mice/group.

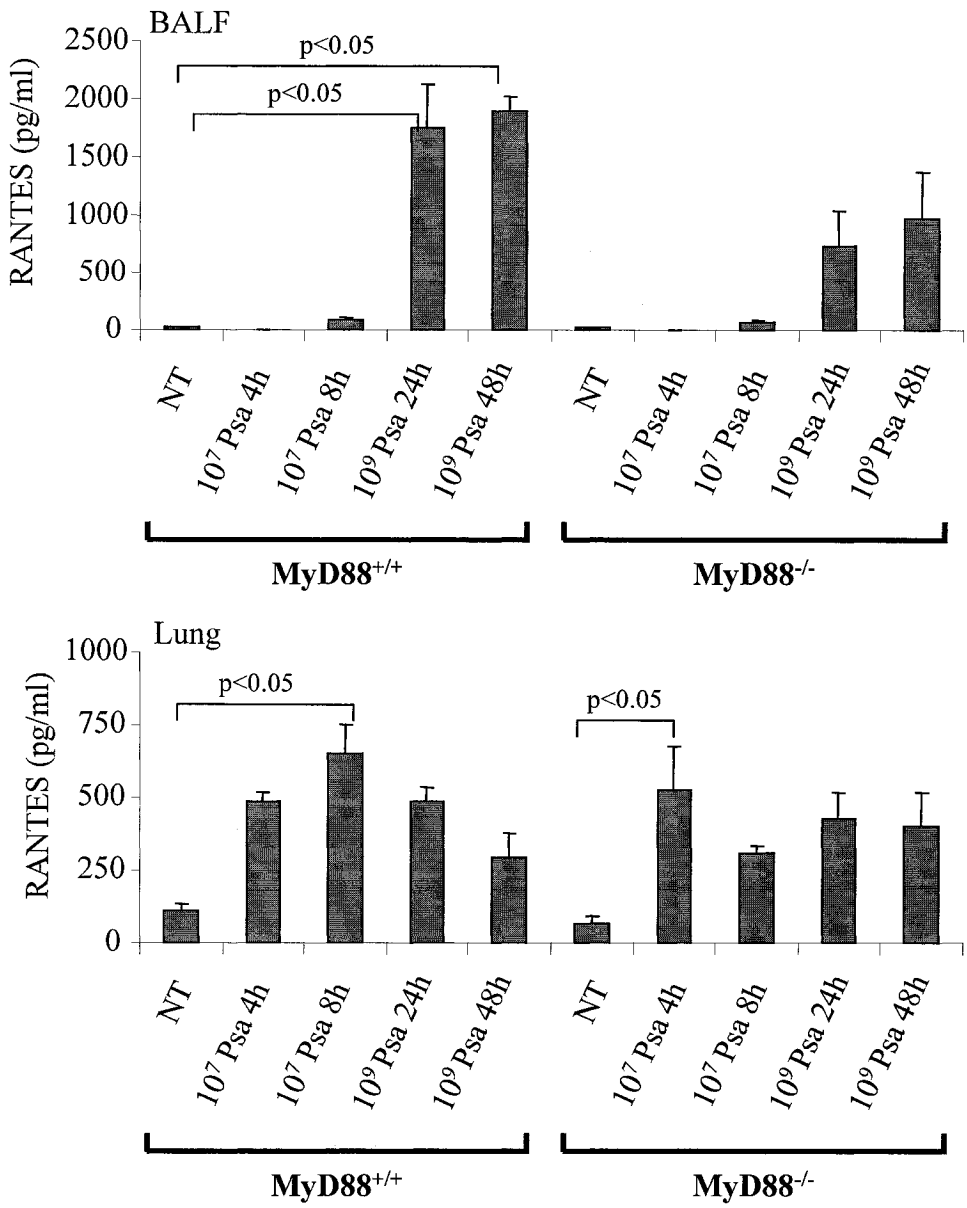


FIGURE 5.2 CCL5/RANTES production in MyD88$^{+/+}$ and MyD88$^{-/-}$ mice following *P. aeruginosa* lung infection. MyD88$^{+/+}$ and MyD88$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4, 8, 24 or 48 h. Mice that were not treated with bacteria were used as controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of CCL5/RANTES protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 5 - 11 mice/group.

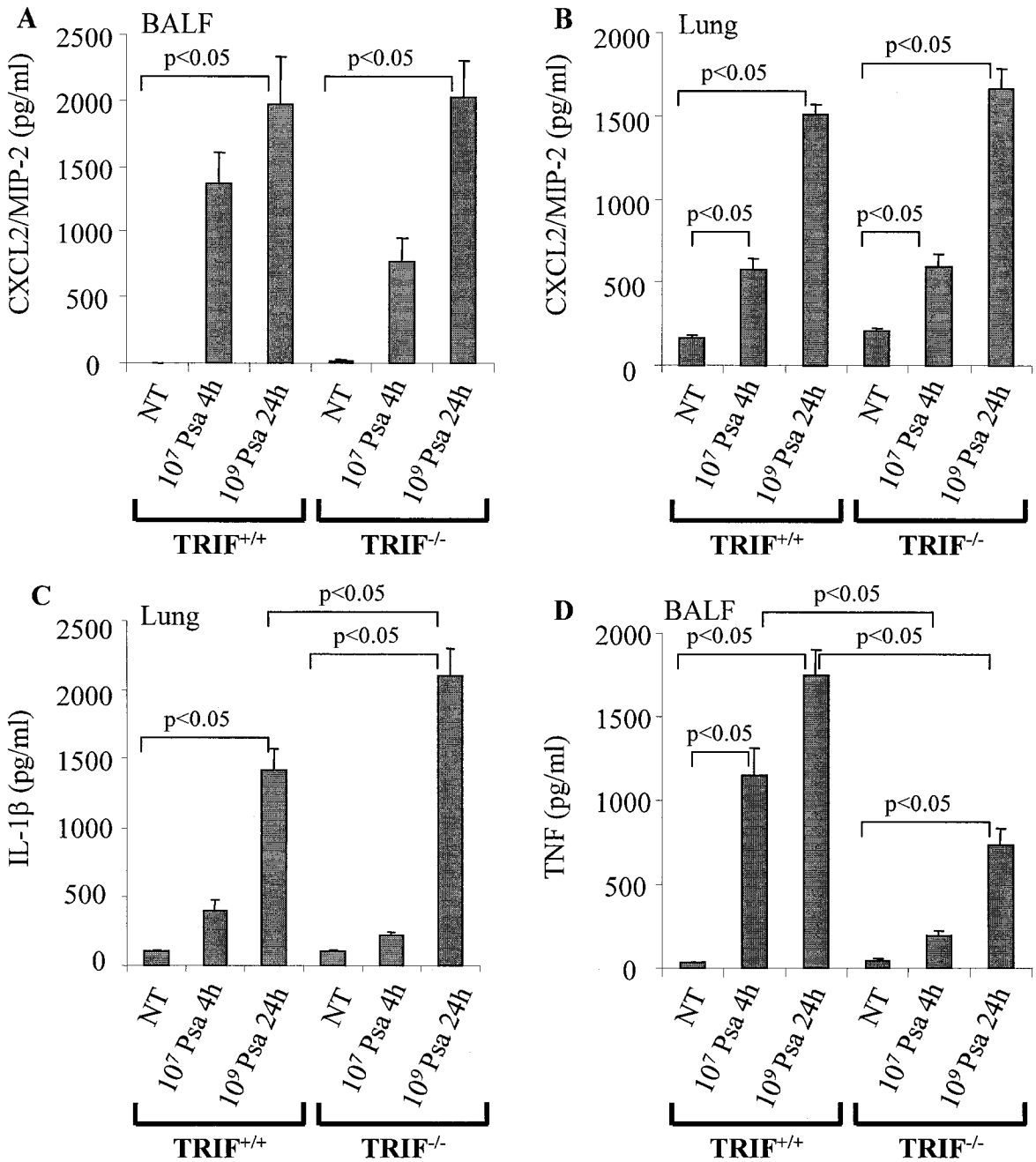


FIGURE 5.3 TRIF deficiency has limited effects on CXCL2/MIP-2, IL-1β and TNF production following *P. aeruginosa* lung infection. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4 or 24 h. Mice that were not treated with bacteria were used as controls (NT). BALF and lung tissues were collected for the determination of CXCL2/MIP-2, IL-1β and TNF protein by ELISA. Data are the mean ± SE of 8 - 11 mice/group.

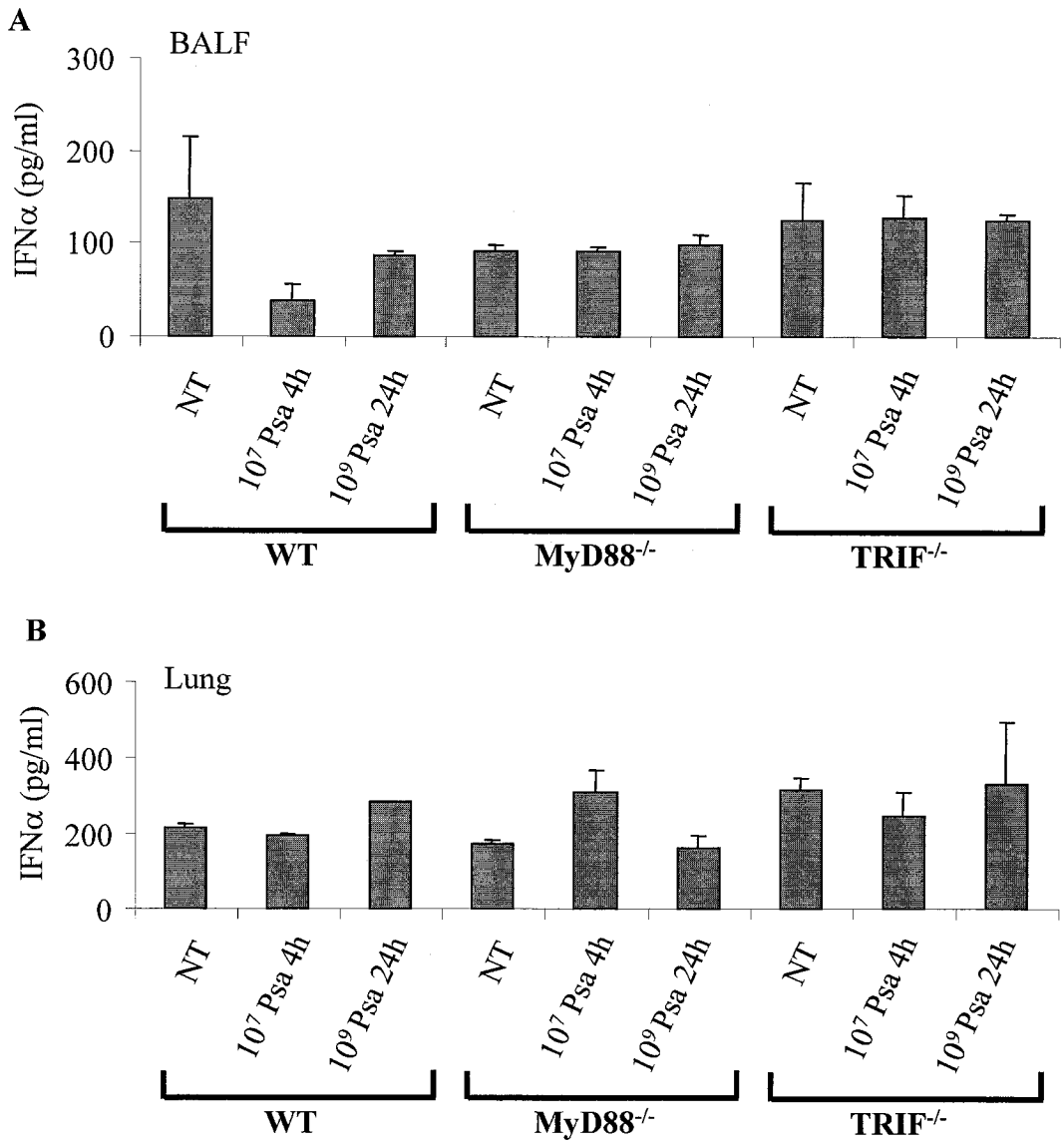


FIGURE 5.4 The levels of IFNα in WT, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice following *P. aeruginosa* lung infection. WT, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4 or 24 h. Mice that were not treated with bacteria were used as NT controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of IFNα protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 3 mice/group.

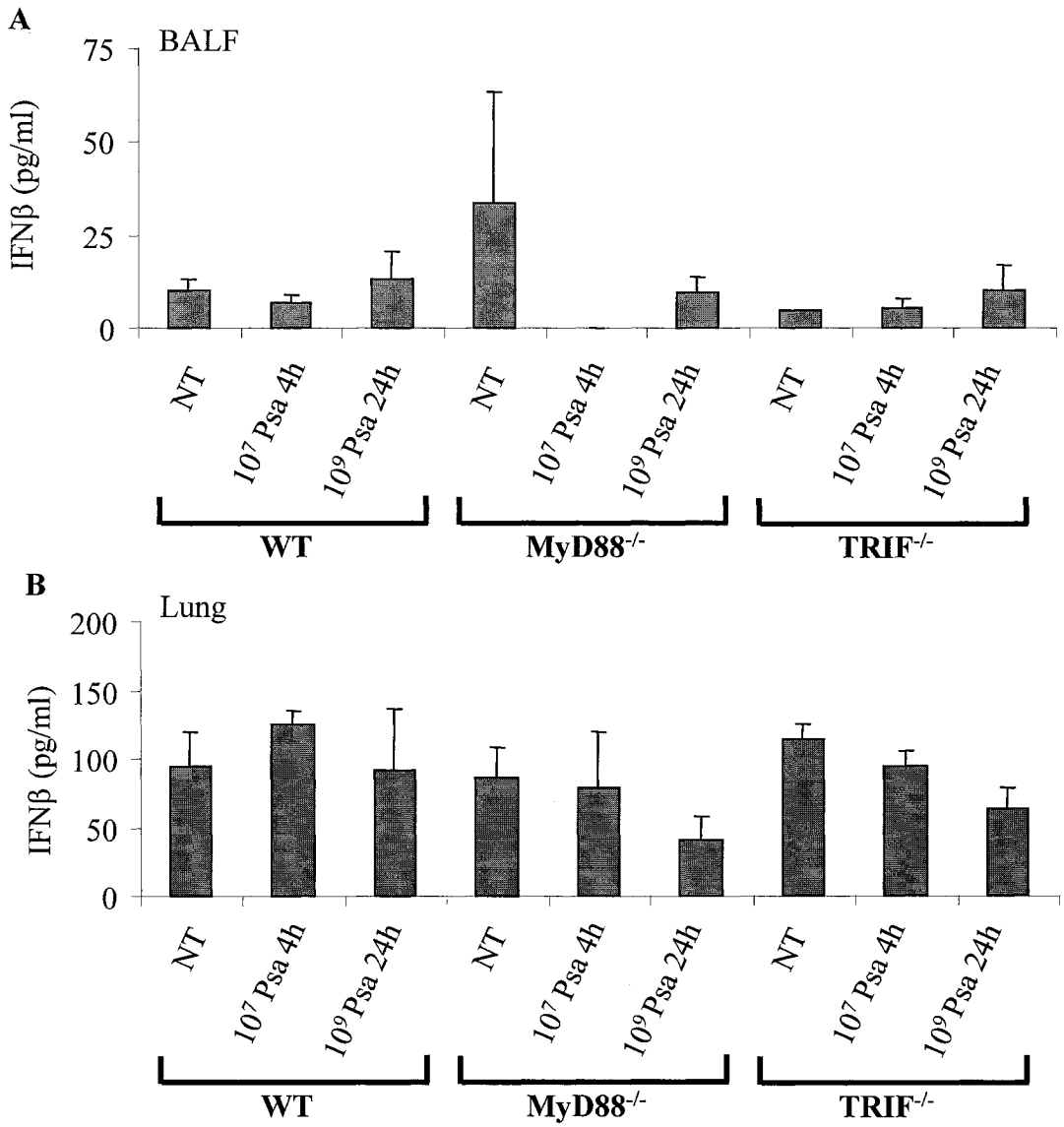


FIGURE 5.5 The levels of IFNβ in WT, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice following *P. aeruginosa* lung infection. WT, MyD88$^{-/-}$ and TRIF$^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (Psa) (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4 or 24 h. Mice that were not treated with bacteria were used as NT controls. (A) Bronchoalveolar lavage fluid (BALF) and (B) lung tissues were collected for the determination of IFNβ protein by enzyme-linked immunosorbent assay (ELISA). Data are the mean ± S.E. of 3 mice/group.

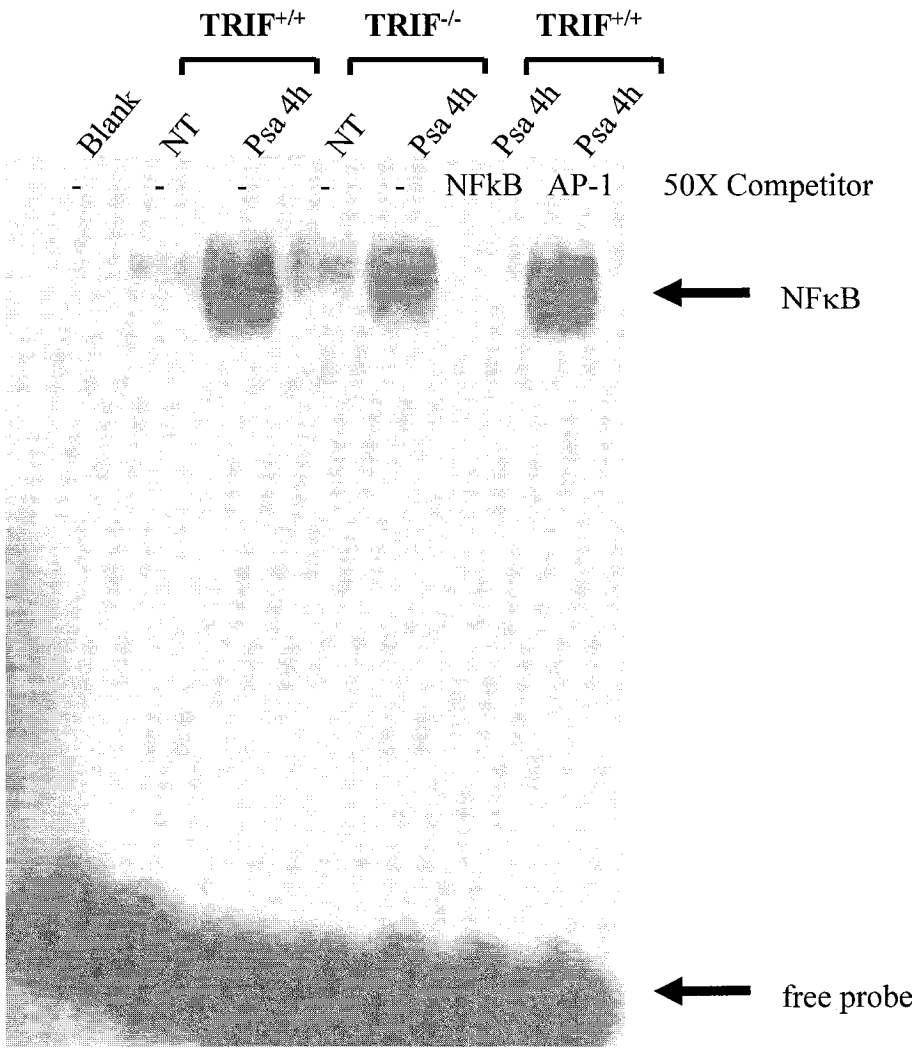


FIGURE 5.6 *P. aeruginosa* induces a reduced NFκB activation in the lung of TRIF deficient mice. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (mucoid strain 8821, 1×10^7 CFU/mouse, Psa) or saline (NT) for 4 h. Nuclear proteins were extracted from lung tissues and were subjected to EMSA by incubation with P32-labeled NFκB DNA probe. Unlabeled NFκB or AP-1 DNA probes (50x) were used for competitive binding assay using nuclear proteins from the lung of *P. aeruginosa* treated wild type mice. Figure provided by Dr. Bo Li.

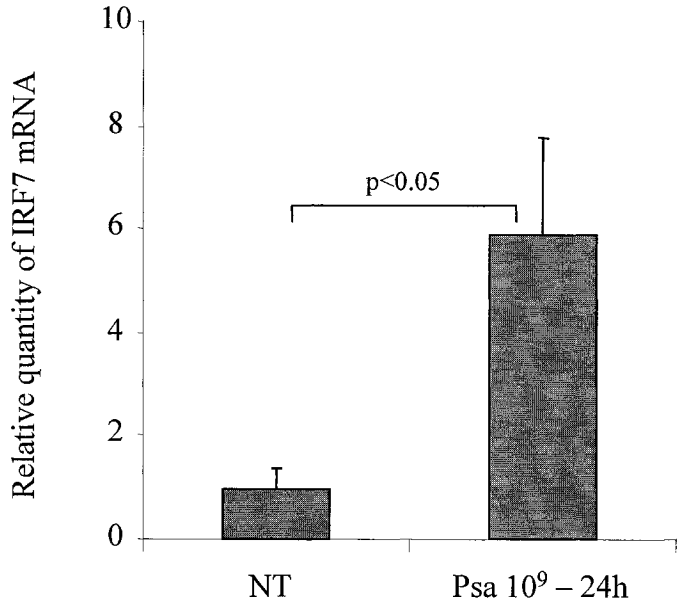
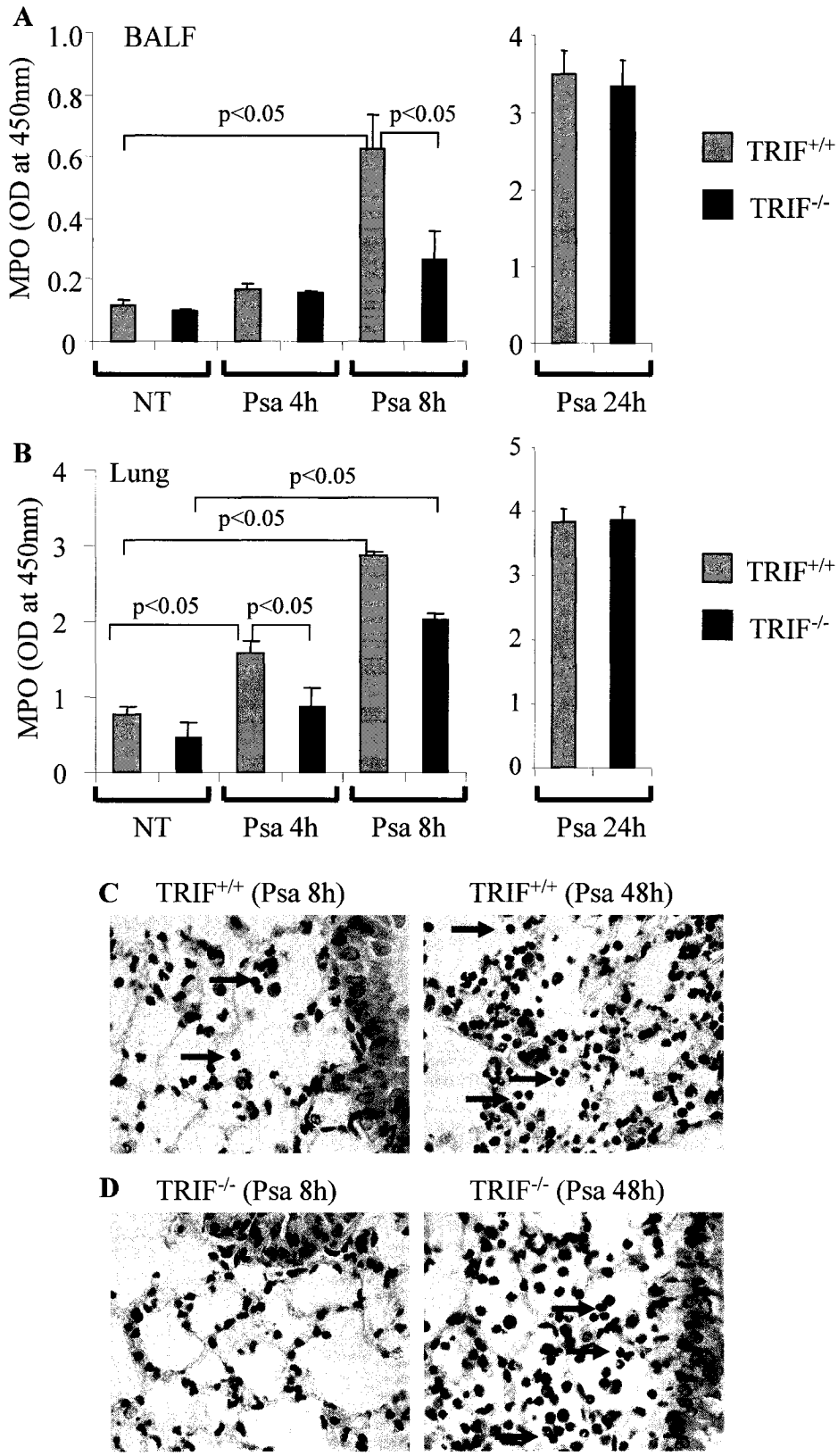


FIGURE 5.7 Increased IRF7 mRNA expression in wild type (C57BL/6) mice following *P. aeruginosa* lung infection. Lung tissue was collected from wild type mice 24 h after intranasal administration of *P. aeruginosa* (*Psa*) strain 8821 (1 x 10^9 CFU/mouse). Mice that were not treated with bacteria were used as controls (NT). Total RNA isolated from the lungs was subjected to real-time RT-PCR analysis for mouse IRF7 expression. Data are normalized to GAPDH and expressed as a fold increase relative to NT wild type mice. Data are the mean ± S.E. of 3 mice/group.

FIGURE 5.8 TRIF deficiency leads to delayed neutrophil recruitment into the airways following *P. aeruginosa* lung infection. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were inoculated intranasally with *P. aeruginosa* (mucoid strain 8821, 1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 4, 8 or 24 h. Mice that were not treated with bacteria were used as controls (NT). **A:** BALF and **B:** lung tissues were collected for the determination of myeloperoxidase (MPO) activities. Data are the mean ± SE of 5 - 11 mice/group. C and D: $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were treated with *P. aeruginosa* (1×10^7 CFU/mouse or 1×10^9 CFU/mouse, Psa) for 8 or 48 h. The upper lobe of the left lung was collected for hematoxylin-eosin staining (original magnification × 100). Arrow indicates neutrophil in the alveolar airspace.


A
BALF
MPO (OD at 450nm)
1.0
0.8
0.6
0.4
0.2
0
p<0.05
p<0.05
4
3
2
1
0
TRIF+/+
TRIF-/-
NT
Psa 4h
Psa 8h
Psa 24h
B
Lung
MPO (OD at 450nm)
4
3
2
1
0
p<0.05
p<0.05
p<0.05
p<0.05
5
4
3
2
1
0
TRIF+/+
TRIF-/-
NT
Psa 4h
Psa 8h
Psa 24h
C
TRIF+/+ (Psa 8h)
TRIF+/+ (Psa 48h)
D
TRIF-/- (Psa 8h)
TRIF-/- (Psa 48h)

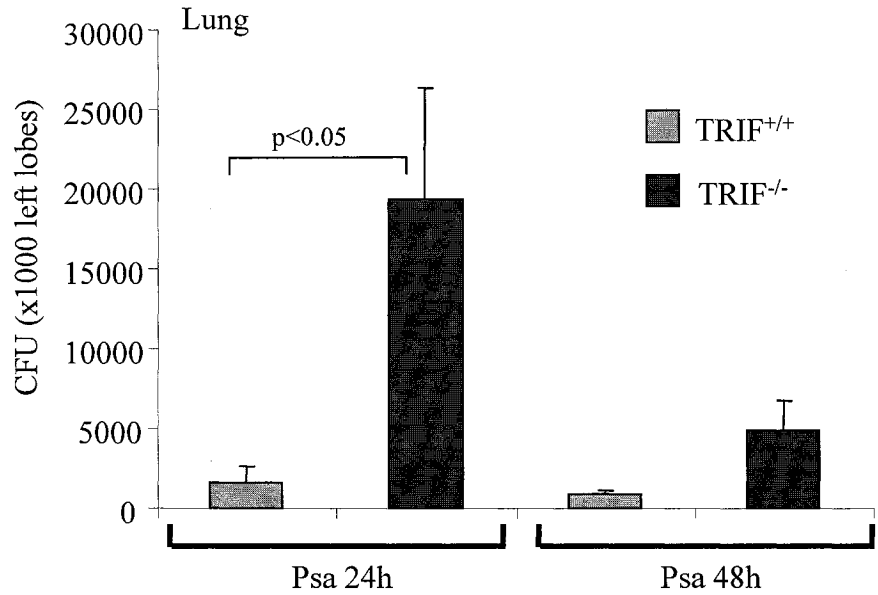


FIGURE 5.9 TRIF deficiency leads to impaired clearance of *P. aeruginosa* from the lung. $TRIF^{+/+}$ and $TRIF^{-/-}$ mice were challenged with *P. aeruginosa* (mucoid strain 8821, 1×10^9 CFU/mouse for 24 or 48 h) intranasally. After 24 or 48 h, the right lobe of the lung was collected and homogenized for colony counting. Data are the mean ± SE of 11 mice/group.

5.3 Discussion

Despite advances in antibiotic therapy, *P. aeruginosa* lung infection remains a major cause of death in cystic fibrosis patients and in immunocompromised individuals. Molecular mechanisms involved in the immune response to this clinically important bacterium are still under investigation. Here we demonstrate for the first time that TRIF is required for the development of host defense against *P. aeruginosa* infection.

P. aeruginosa infection is associated with altered production of a plethora of cytokines and chemokines in the lung (482, 512, 518, 519). Since cytokines and chemokines play a critical regulatory role in the host defense against *P. aeruginosa* infection, it is important to define the molecular pathways responsible for specific patterns of cytokine and chemokine production during *P. aeruginosa* infection. In this study, we demonstrated that TRIF is essential for CCL5/RANTES, CXCL1/KC, and TNF, but not CXCL2/MIP-2 or IL-1β production in response to *P. aeruginosa* infection, a pattern which appears to be distinct from that in MyD88 deficient mice (307).

Previous studies using gene knock-out and dominant-negative mutants suggest that TRIF is involved in TLR2- and TLR4-mediated cellular activation (162, 163, 531). Various TLRs including TLR2, TLR4, TLR5 have been implicated in *P. aeruginosa* infection *in vivo* and *in vitro* (307, 488, 520, 521). Two major TLR signaling pathways including the MyD88 pathway and the TRIF pathway have been described (522). Activation of the MyD88 pathway leads to NFκB activation and subsequent production of various inflammatory cytokines and chemokines such as TNF and IL-1β. An essential role of the MyD88 pathway in the host defense against bacterial infection has been recognized (307, 308, 515). We and others showed that MyD88 deficiency leads to impaired clearance of *P. aeruginosa* from the lung (307, 308, 515). In contrast, the TRIF pathway is responsible for the activation of IRF3/7 leading to type 1 interferon (IFNα/β) production. Accordingly, TRIF has been primarily associated with the host defense against virus infections. In this study, we found that TRIF is required for the development of an effective host response to a Gram-negative bacterium *P. aeruginosa* infection *in vivo*. A similar order of magnitude of bacterial clearance defect was observed in the MyD88 deficient and TRIF deficient mice. After 24 h infection, TRIF deficiency leads to a seven-fold increase of CFU in the lung, while MyD88 deficiency

leads to a nine-fold increase of lung CFU when compared to their control mice. Thus, the full development of host defense against *P. aeruginosa* lung infection likely requires a coordinated effect of both the MyD88 pathway and the TRIF pathway.

TRIF dependent, IRF-regulated, IFNα/β production accounts for a major role in the host defense against various virus infections. A role of TRIF in bacterial infection is less clear. A recent study demonstrated that the IRF3/7-IFNα/β pathway is activated by a Gram-positive intracellular bacterium *Listeria monocytogenes* (530). Contrary to the protective role of IRF3/7-IFNα/β pathway in viral infection, activation of the IRF3/7-IFNα/β pathway by *L. monocytogenes* leads to impaired host defense against this bacterium (530). Considering the significant role of TRIF in the IRF3/7-IFNα/β pathway activation, we examined whether the IRF3/7-IFNα/β pathway is activated during *P. aeruginosa* lung infection. Interestingly, although mRNA levels of IRF7 were increased after *P. aeruginosa* lung infection, no IRF3/7 activation could be observed by electrophoresis mobility shift assay using nuclear extracts from lung tissues after *P. aeruginosa* infection for 4 h (data not shown). However, a better way to examine IRF activation may be to perform Western blotting to examine the dimerization of IRF3 or IRF7. As measured by ELISA, there were no changes in IFNα and IFNβ production in the BALF or lung tissues from *P. aeruginosa*-infected mice (4 and 24 h) (Figures 5.4 and 5.5). These results suggest that the involvement of the IRF3/7-IFNα/β pathway in *P. aeruginosa* infection is not yet clear. The usage of specific signaling pathways seems to be dependent on the nature of a specific pathogen.

In addition to IRF-IFNα/β activation, recent studies suggest that TRIF also activates NFκB through Rip 1 (209, 210). Interaction between MyD88 and TRIF has been reported (532). The importance of a coordinated effect for MyD88 and TRIF in resistance to infection has been suggested in a recent review (533). When both MyD88 and TRIF are absent, mice are more susceptible to infection compared to MyD88 single knock-out mice (533). The MyD88 and TRIF double deficient animals are drastically immunocompromised and showed little or no response to most microbial ligands (533). Thus, it is likely that a coordinated effect of the TRIF pathway and the MyD88 pathway is needed for *P. aeruginosa*-induced NFκB activation.

In contrast to the mild defect in NFκB activation in the lung, *P. aeruginosa*-induced production of several NFκB-regulated genes, including TNF, was severely impaired in the lung in TRIF-deficient mice. This defect was observed at both shorter (4 h) and longer (24 h) time points after *P. aeruginosa* infection, suggesting a non-redundant role for TRIF in the regulation of these genes *in vivo* during *P. aeruginosa* lung infection. It is possible that a full and complete activation of NFκB in the lung is required for *P. aeruginosa*-induced TNF production. Alternatively, since TNF production is also regulated by additional transcription factors such as CREB, C/EBP β, NFATp, ATF-2/Jun, Egr-1, Nrf1, Ets and others (534), TRIF deficiency may directly or indirectly affect activation of additional transcriptional factor other than NFκB. In addition, post-transcriptional regulation including RNA stability and translational regulation also contributes to the expression level of cytokines and chemokines. It is unclear whether TRIF deficiency affects gene expression in the lung through direct post-transcriptional mechanisms.

The promoter of CCL5/RANTES contains both NFκB binding sites and an interferon-stimulated response element that is sensitive to IRF3/7 (298). Expression of CCL5/RANTES requires a co-operative effect between NFκB and IRF3/7 (535). The near complete inhibition of CCL5/RANTES in the lungs of TRIF deficient mice is likely due to an impaired NFκB activation since IRF activation was not observed after *P. aeruginosa* infection in this study. CCL5/RANTES is a potent leukocyte chemoattractant. It promotes leukocyte recruitment and activation (291). CCL5/RANTES activates human neutrophils to release anti-microbial peptide α-defensins and promote neutrophil phagocytic capacity (536). These effects likely contribute to host defense against *P. aeruginosa* infection.

Interestingly, TRIF deficiency had minimal effects on other NFκB regulated genes such as IL-1β and CXCL2/MIP-2. This is in contrast to our previous finding that *P. aeruginosa*-induced IL-1β and CXCL2/MIP-2 production is dependent on MyD88 which functions through NFκB (307). It is likely that additional transcription factors other than NFκB are also involved in TRIF and MyD88 dependent gene activation. Thus, TRIF-deficiency induces a distinct pattern of cytokine and chemokine production that is different from MyD88 deficiency. This is consistent with a recent finding that of the

1,055 genes found to be LPS responsive, only 21.5% were dependent on MyD88 expression, with MyD88 independent genes contributing 74.7% of the genetic response (523).

The impaired production of neutrophil chemoattractants such as CXCL1/KC, TNF and CCL5/RANTES in TRIF deficient mice may contribute to the delayed neutrophil recruitment into the airways. It is apparent neutrophil recruitment was only partially impaired in the early hours (4 or 8 h) of infection. After 24 h infection, TRIF-deficient and wild type mice recruited comparable levels of neutrophils into the airways. However, impaired bacterial clearance in TRIF-deficient mice was observed even after 48 h of infection, suggesting that additional neutrophil independent mechanisms may be involved in TRIF dependent host defense. These data suggest a non-redundant and indispensable role of TRIF in the clearance of *P. aeruginosa* from the lung. Further studies on the roles of TRIF in the bactericidal activity of neutrophils and other immune effector cells such as epithelial cells, NK cells and NKT cells may provide new insights into the function of TRIF in host defense against this clinically important bacterium.

CHAPTER 6. Discussion

6.1 Summary of the Major Findings and Comparison to Current Literature

This study examined an *in vivo* mouse model of *P. aeruginosa* lung infection to determine the roles of TLRs and TLR signaling pathways in the innate immune response to *P. aeruginosa.* TLRs function as pattern recognition receptors that recognize conserved motifs on pathogens (127). The main signaling pathways are through the adaptor molecules MyD88 (159, 160) and/or TRIF (162). These signaling pathways activate the gene expression of many cytokines, chemokines, and adhesion molecules (163, 187, 188). The release of these mediators at the site of infection induces the recruitment of immune cells to clear the pathogen (15). The endothelium is activated by cytokines to increase the expression of adhesion molecules and to keep chemokines like CXCL8/IL-8 (27, 29, 30) on the vascular surface by glycoproteins (320). Chemokines activate firm adhesion of leukocytes such as neutrophils to the endothelium by increasing the affinity of integrin binding to adhesion molecules on the endothelium (99). The neutrophils then extravasate into the site of infection (323) traveling up a chemokine gradient. Neutrophils are important for bacterial clearance (15) because they can phagocytose foreign pathogens (338) and release reactive oxygen intermediates (358, 361-365) and antimicrobial substances (358) that kill pathogens and ultimately clear an infection. Deficiencies in any part of the complex cascade of events may lead to an impaired innate immune response and impaired bacterial clearance. In this study, the roles of TLR2, TLR4 and the TLR signaling molecules MyD88 and TRIF were examined in the innate immune response to *P. aeruginosa* infection.

6.1.1 The Role of TLR in *P. aeruginosa* Infection

6.1.1.1 TLR4

There are multiple bacterial products that may signal through TLR4 after *P. aeruginosa* lung infection, but the major ligand for TLR4 is LPS (126, 142). TLR4 requires CD14 and MD-2 for LPS recognition (150, 151). Another component of *P. aeruginosa* that may signal through TLR4 is the mannuronic acid polymers (poly-M)

from *P. aeruginosa* alginate. Macrophages from TLR4 deficient mice were completely unresponsive to poly-M from *P. aeruginosa* (490). Exoenzyme S is a toxin secreted by *P. aeruginosa* that has been found to be able to activate cells through TLR4/MD2/CD14 (521). There are other bacterial components and host factors that are associated with acute lung injury that may be recognized by TLR4 (537). Despite the multiple components of *P. aeruginosa* that may signal through TLR4, we (Table 4.1) and others (515, 538) have shown that TLR4 is not required for clearance of *P. aeruginosa* from the lung, while we infected mice intranasally with a clinical isolate of *P. aeruginosa* (strain 8821). Skerrett *et al.* (538) infected mice by exposing them to aerosolized *P. aeruginosa* (PAK strain), and Ramphal *et al.* (515) infected mice with *P. aeruginosa* (PAK strain) intratracheally. Ramphal *et al.* and Skerrett *et al.* both demonstrated that the bacterial clearance in TLR4 deficient mice was similar to wild type mice 24 h after infection (515, 538), while we found that TLR4 deficient mice were able to clear *P. aeruginosa* lung infection, 48 h after infection (table 4.1). In contrast, a paper by Faure *et al.* (491) indicates that there is impairment in bacterial clearance and survival of TLR4 deficient mice 3 days after intratracheal infection with the PA103 strain of *P. aeruginosa*. TLR4 may not be vital for bacterial clearance, but it does seem to play a role in the initiation of the innate immune response to *P. aeruginosa* infection.

The early production of cytokines and chemokines and subsequent neutrophil recruitment is important for bacterial clearance (15). We found that the cytokine response and early neutrophil infiltration were impaired in TLR4 mutant mice infected with *P. aeruginosa* (Figure 3.8). These findings are consistent with other reports that examined TLR4 in *P. aeruginosa* lung infection (491, 538). A report by Ramphal *et al.* (515) demonstrated that there were impaired cytokine responses in the airways of TLR4 deficient mice, but no impairment in neutrophil recruitment to the BALF 4 h after *P. aeruginosa* lung infection. We observed no detectable neutrophil recruitment in the BALF of wild type mice 4 h after infection (Figure 3.2). We show that the neutrophil recruitment in the lung homogenate is significantly reduced in TLR4 mutant mice (Figure 3.8). The impaired neutrophil recruitment in TLR4 deficient mice may be due to an impaired cytokine/chemokine production. Ramphal *et al.* (515) reported impairment in the production of TNF, CXCL1/KC, CCL5/RANTES and IL-1β in *P. aeruginosa*

challenged TLR4 deficient mice. We also found significant decreases in TNF and CXCL2/MIP-2 and slight decreases in IL-1β in TLR4 mutant mice (Figure 3.8) but we did not evaluate CCL5/RANTES or CXCL1/KC production in our studies of TLR4 mutant mice. A deficiency in TLR4 leads to a decrease in inflammatory mediator production and the subsequent decrease in neutrophil recruitment after *P. aeruginosa* lung infection. This information is important because it demonstrates that TLR4 is important for recognizing *P. aeruginosa* and initiating the innate immune response. However, other TLRs are also likely to recognize *P. aeruginosa* ligands since a deficiency in TLR4 did not impair overall bacterial clearance.

6.1.1.2 TLR2

TLR2 has been reported to recognize several *P. aeruginosa* ligands including lipoproteins (539), alginate (490), flagellin (488) and Exoenzyme S (521). However, the role of TLR2 in recognition of *P. aeruginosa* components is more subtle than that of TLR4 as we found that the cytokine response to *P. aeruginosa* lung infection was similar in TLR2 deficient and wild type mice (Figure 3.8). These results are similar to findings in other studies (538, 540). For example, Skerrett *et al.* (538) found no significant difference in the effective bacterial clearance in TLR2 deficient mice compared to wild type mice, consistent with our findings (Table 4.1). Therefore, a deficiency in TLR2 alone does not appear to have a significant impact on the production of inflammatory mediators; however, TLR2 may play a minor role in the host response to *P. aeruginosa.*

We found an increase in the production of TLR2 mRNA after *P. aeruginosa* lung infection, which may be dependent upon TLR4 signaling. TLR4 mutant mice did not exhibit an increase in TLR2 expression after *P. aeruginosa* lung infection. Therefore, TLR2 may play a role in the later stages of the innate immune response after infection with *P. aeruginosa* (Figure 3.7). This data suggests that the role of TLR2 is secondary to that of TLR4 and is consistent with another study showing that TLR2 was up-regulated later in infection. TLR4 was expressed on resting macrophages while, TLR2 expression was increased following treatment with *Salmonella* (541). Therefore, signaling through TLR4 may activate the expression of TLR2, and since TLR2 can recognize *P. aeruginosa* ligands, TLR2 may play a role in the later stages of *P. aeruginosa* infection.

6.1.2 The Role of TLR Signaling Pathways in the Innate Immune Response to *P. aeruginosa* Infection

6.1.2.1 MyD88 Signaling Pathway

6.1.2.1.1 Impaired Early Bacterial Clearance in MyD88 Deficient Mice

MyD88 is the adaptor molecule responsible for the downstream signaling and activation of inflammatory mediator production from most TLRs. We reported that MyD88 deficient mice had an impaired clearance of mucoid *P. aeruginosa* 24 h after lung infection (307). This finding was independently reported by Skerrett *et al.* (308), in the same year, and later confirmed by Ramphal *et al.* (515). Skerrett *et al.* (308) exposed mice to aerosolized nonmucoid *P. aeruginosa* strain PAK in a whole animal chamber for 30 min. Ramphal *et al.* (515) infected mice intratracheally with *P. aeruginosa* strain PAK. Consistent with the impaired neutrophil recruitment to the lung we observed 4 and 8 h post *P. aeruginosa* infection (Figure 3.2), Skerrett *et al.* (308) and Ramphal *et al.* (515) found similar deficiencies in the early recruitment of neutrophils to the lungs. Early neutrophil recruitment is important for the phagocytosis and clearance of pathogens. Inflammatory mediators are produced to recruit neutrophils to the site of infection. Our group (Figures 3.3 and 3.4) and Skerrett *et al.* (308) showed that MyD88 deficient mice had significant reductions in TNF, IL-1β, CXCL2/MIP-2 and CXCL1/KC production 4 h after infection. These findings are important because they may be responsible for the observed impairment in bacterial clearance 24 h after infection. Overall, these three different infections models using different strains of *P. aeruginosa* indicate that the results of *P. aeruginosa* infection provide reproducible results. We next wanted to determine whether or not MyD88 deficient mice could eventually clear *P. aeruginosa* infection.

6.1.2.1.2 Delayed Bacterial Clearance in MyD88 Deficient Mice

A delay in bacterial clearance was found 48 h after *P. aeruginosa* lung infection in MyD88 deficient mice. Therefore, MyD88 plays an important role in the early host response to *P. aeruginosa* lung infection, but there are other pathways involved. The

observed bacterial clearance may have been due to an increase in late neutrophil recruitment. In contrast to the severe defect in neutrophil recruitment at early time points, this study (Figure 4.5) and Ramphal *et al.* (515) found that neutrophils were recruited to the lungs of MyD88 deficient mice 24 and 48 h after infection. The observed influx in neutrophils may be related to the production of CXCL2/MIP-2 24 h after infection (Figure 4.1). Low levels of TNF, IL-1β, and no CXCL1/KC were produced (Figures 4.4, 4.3 and 4.2), similar to the results of Skerrett *et al.* (308). Ramphal *et al.* (515) also found that the production of TNF and CXCL1/KC was abolished at 6 and 24 h post *P. aeruginosa* lung infection in MyD88 deficient mice. These findings support the literature reports that were published the same year and later using different strains of *P. aeruginosa* to infect MyD88 deficient mice (308, 515). These studies indicate the vital role for MyD88 in the immediate host response to *P. aeruginosa* lung infection and highlight that a MyD88 independent pathway may also play a role in the innate immune response to *P. aeruginosa* infection.

6.1.2.2 The TRIF Signaling Pathway

There is significant evidence from *in vivo* studies that indicate MyD88 dependent signaling pathways contribute to lung defense against bacterial pathogens; however, the role of the MyD88 independent signaling pathway in initiating the innate immune response to bacterial infection in the lung has not been well studied. It was suspected that a MyD88 independent signaling pathway was important in *P. aeruginosa* lung infection since MyD88 deficient mice had a delay in bacterial clearance but were able to eradicate the infection. TRIF is an adaptor molecule in the MyD88 independent signaling pathway for TLR3 and TLR4 (162, 167). We examined whether there is an important role for TRIF in the MyD88 independent signaling pathway for host defense against lung infection with *P. aeruginosa* (517). Our study revealed that TRIF is vital for the bacterial clearance of *P. aeruginosa* from the lung.

6.1.2.2.1 Novelty of TRIF Being Involved in Gram-Negative Bacterial Infection

We have found a novel role for TRIF in mediating the host response to Gram-negative bacterium *P. aeruginosa.* There is also evidence that the TRIF dependent

signaling pathway is involved in responding to another Gram-negative bacterium, *Escherichia coli* (542). This paper examines the role of TRIF in a mouse *E. coli* lung infection model and was published in the same issue of *J. Immunol.* as our report of TRIF involvement in resistance to *P. aeruginosa* infection. Similar to the paper by Jeyaseelan *et al.*, we show that TRIF is important for host defense against Gram-negative bacteria. The lungs of TRIF deficient mice harbored more *E. coli* (542) or *P. aeruginosa* (517) 24h after infection compared with wild type controls. Together, these findings indicate that TRIF is important for limiting Gram-negative bacterial colonization in the lungs.

6.1.2.2.2 Impaired Neutrophil Recruitment in TRIF Deficient Mice

In *P. aeruginosa* infected mice, neutrophil recruitment to the lungs of TRIF deficient animals were impaired as measured by an MPO assay and histology (Figure 5.8). Interestingly, TRIF deficient mice have impaired neutrophil recruitment similar to that of MyD88 deficient mice after *P. aeruginosa* infection. Similarly, neutrophil recruitment was impaired in TRIF deficient mice 6 h after *E. coli* lung infection (542). There was a decreased neutrophil recruitment, and subsequently impaired bacterial clearance after *P. aeruginosa* lung infection in TRIF deficient mice. Since Jeyaseelan *et al.* (542) demonstrated that TRIF deficient neutrophils and alveolar macrophages have an intact bactericidal capability (542), the defective recruitment of neutrophils and subsequent impaired bacterial clearance in the airways is most likely the result of attenuated inflammatory mediator production in the lungs of TRIF deficient mice.

6.1.2.2.3 Cytokine/Chemokine Profile in TRIF Deficient Mice

The cytokine and chemokine profile produced by wild type and TRIF deficient mice was examined after *P. aeruginosa* infection. The literature suggests that the production of CCL5/RANTES and type I interferons is controlled by TRIF (163). Therefore, we examined these mediators. Of importance, it was found that CCL5/RANTES is produced after *P. aeruginosa* infection in wild type mice (Figure 5.1) and that CCL5/RANTES production was not significantly impaired by deficiency in MyD88 (Figure 5.2). In contrast, the production of CCL5/RANTES was significantly impaired in TRIF deficient mice (Figure 5.1). Therefore, CCL5/RANTES is produced

after *P. aeruginosa* infection and production is regulated by TRIF and not MyD88 mediated signaling.

We (517) and Jeyaseelan *et al.* (542) found that the cytokine and chemokine profile produced after infection of TRIF deficient mice with Gram-negative bacteria was different from that of wild type mice and MyD88 deficient mice. Similar to our findings, Jeyaseelan *et al.* (542) find that the production of TNF was decreased and CXCL2/MIP-2 production was normal in TRIF deficient mice (Figure 5.3); however, they find that the production of CXCL1/KC was normal (542), which is in contrast to our findings. We show that the production of CXCL1/KC was decreased in TRIF deficient mice (Figure 5.1), suggesting that TRIF mediated production of CXCL1/KC significantly contributes to the neutrophil influx in the lungs in response to *P. aeruginosa*. Jeyaseelan *et al.* (542) found that there were fewer neutrophils recruited to the lungs, and that this may be due to a decrease in the production of the neutrophil chemoattractant, LPS-induced CXC chemokine (LIX). The discrepancy between our findings and their observations may be explained by the specific differences in the innate immune response to the different Gram-negative pathogens examined. These findings suggest that TRIF dependent signaling is important for the induction of CCL5/RANTES, CXCL1/KC and TNF in response to *P. aeruginosa* induced lung infection.

6.1.2.2.4 The Role of the IRF Transcription Factors in *P. aeruginosa* Infection

In part, the translocation of NFκB to the nucleus is downregulated in TRIF deficient mice after *P. aeruginosa* infection, therefore, other transcription factors are likely to be involved in signaling. Another important transcription factor downstream of TRIF signaling is IRF3. IRF3 activates the transcription of IFNβ (191), which can then act in an autocrine or paracrine fashion to induce signaling for the transcription of the inducible transcription factor IRF7 (167, 189, 192-195, 198). Significant levels of IFNβ produced after *P. aeruginosa* infection were not detected by ELISA (Figure 5.5); however, this may be due to the low level produced in the lung. The mRNA for IRF7 was increased by real time RT PCR analysis after *P. aeruginosa* infection (Figure 5.7). This indicates that the IRF3 and IRF7 transcription factors may play a role in the host response to *P. aeruginosa* infection.

6.1.2.2.5 Interpreting TRIF Deficient Mouse Data

The observed abrogation in the innate immune response and bacterial clearance in TRIF deficient mice after Gram-negative infection may not be solely due to a defective TLR4 signaling pathway. It is possible and quite likely that TRIF signals downstream of other receptors, including both TLR and non-TLRs. This possibility cannot be excluded at this time and more research into the interaction of TRIF with other proteins will be needed to further discover the role of TRIF in the innate immune response.

We initially hypothesized that MyD88 was involved in the early innate immune response to *P. aeruginosa* and that MyD88 independent signaling was involved at later time points. However, our data suggests that both the MyD88 and TRIF signaling pathways are functional at the same time immediately after *P. aeruginosa* infection. This is novel because previous literature (543) indicated that TRIF signaling after LPS stimulation was delayed. Our data indicates that TRIF is actually critical for the early innate immune response and clearance of *P. aeruginosa*.

6.2 Model of the Role of TLRs, MyD88 and TRIF in *P. aeruginosa* Infection

6.2.1 The Roles of MyD88 and TLR in *P. aeruginosa* Infection

TLR4 and, in part, TLR2 play a role in the immune response to *P. aeruginosa* (Figure 6.1), and the signaling adaptor molecule MyD88 is important in the innate immune response to *P. aeruginosa*. Specifically, the early clearance of *P. aeruginosa* from the lung was impaired, early cytokine and chemokine production of CXCL2/MIP-2, CXCL1/KC, TNF and IL-1β was impaired, and there was a diminished neutrophil recruitment in MyD88 deficient mice after *P. aeruginosa* infection compared to wild type mice.

The work of Skerrett *et al.* (538) showed that multiple TLR deficiencies do not replicate the phenotype of MyD88 deficient mice after *P. aeruginosa* infection. This may be due to the role MyD88 plays in the IL-1R, IL-18R and IL-33R signaling pathways (227), since IL-1β is produced after *P. aeruginosa* infection. A possible role for the autocrine and paracrine effects of cytokines cannot be excluded when comparing the phenotype of the MyD88 deficient mouse to mice deficient in multiple TLR. MyD88

may also act downstream of other unknown receptors that are important in *P. aeruginosa* infection.

When MyD88 deficient mice were infected with *P. aeruginosa* for 48 h, bacterial clearance had begun at this time, indicating that there was a delay in bacterial clearance rather than non-responsiveness. The production of some inflammatory mediators were MyD88 independent at later time points and neutrophils were recruited to the lung 24 – 48 h post infection. Since MyD88 deficiency led to a delayed innate immune response and bacterial clearance, other pathways may be involved and the role of the TRIF signaling pathway in *P. aeruginosa* infection was therefore investigated.

6.2.2 Role for TRIF in *P. aeruginosa* Infection

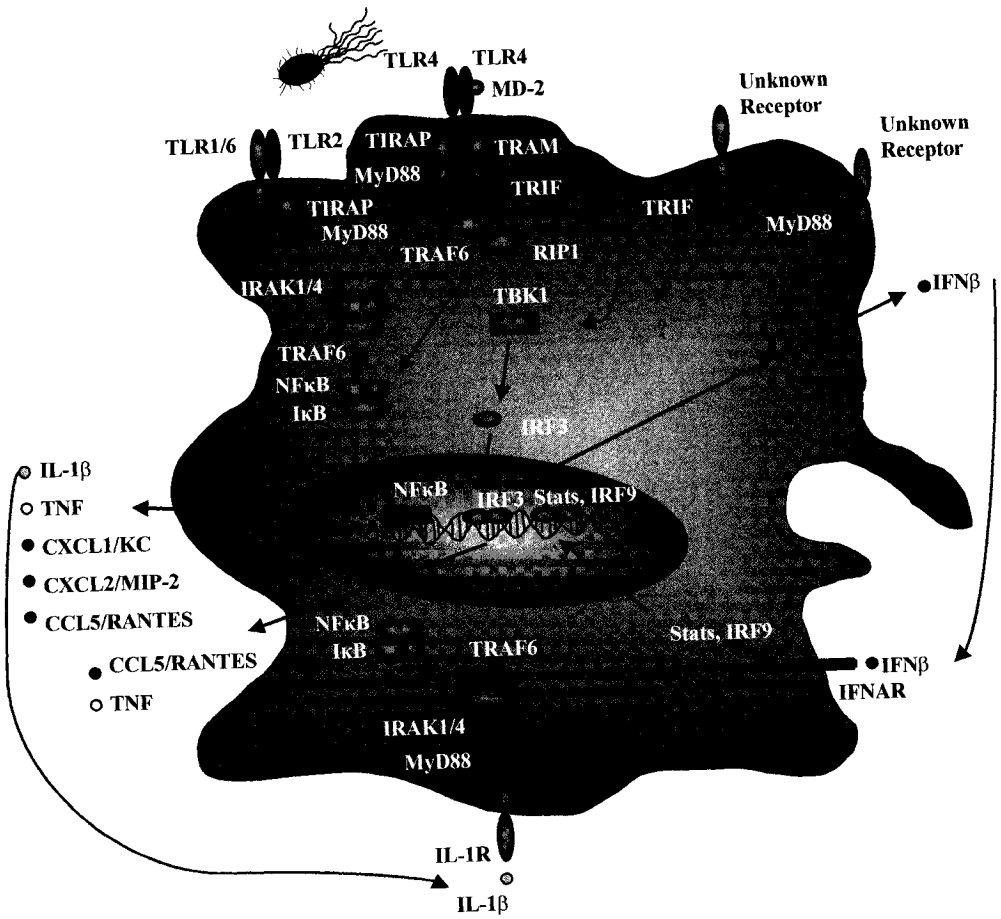
A TRIF deficiency led to an impaired bacterial clearance after *P. aeruginosa* infection. TRIF did play a role in the translocation of NFκB (Figure 6.1) to the nucleus and in the production of CCL5/RANTES, CXCL1/KC and TNF. The abrogation in production of these chemokines and cytokines was accompanied by a decrease in neutrophil recruitment to the lung and impaired bacterial clearance. IRF3 may activate the transcription of IFNβ after *P. aeruginosa* infection, which when produced can then act in an autocrine and paracrine fashion to induce signaling for the transcription of the inducible transcription factor IRF7. However, since we found no evidence of IFNβ production (Figure 5.5) but we did find induction of IRF7 mRNA (Figure 5.7) after *P. aeruginosa* infection there may be an unrecognized mechanism for the activation of IRF7. Further examination of this part of the TRIF signaling pathway in response to *P. aeruginosa* infection is warranted to fully understand whether IRF3 is involved in the innate immune response to *P. aeruginosa* infection.

The defect in the innate immune response in TRIF deficient mice is attributed to defective TLR signaling; however, when examining the TRIF deficient mouse data it has to be acknowledged that TRIF may also interact with other, so far unknown, receptors that may be important in the host response to *P. aeruginosa* infection.

6.2.3 Overview of Model

The MyD88 and TRIF signaling pathways function together for an immune response to a bacterial pathogen (Figure 6.1). A Gram-negative bacterial pathogen, by signaling through TLR4, activates both MyD88 and TRIF signaling pathways. The MyD88 pathway activates NFκB immediately to drive production of inflammatory mediators such as TNF, IL-1β, CXCL1/KC and CXCL2/MIP-2 to recruit neutrophils to the site of infection for bacterial clearance. The TRIF pathway will activate the transcription factors NFκB and possibly IRF3 to induce cytokine and chemokine production including CCL5/RANTES, TNF and CXCL1/KC (idea adapted from (223)). The inflammatory mediator production induces neutrophil recruitment to the site of infection and facilitates bacterial clearance by phagocytosing pathogens and releasing reactive oxygen intermediates. Therefore, TLR signaling after *P. aeruginosa* infection is complex but research in this area is starting to determine the important mechanisms involved.

FIGURE 6.1 Model for the innate immune response and the role of TLR signaling pathways after *P. aeruginosa* infection. TLR4 and, in part, TLR2 plays a role in the immune response to *P. aeruginosa*. The adaptor molecule MyD88 was important in the innate immune response to *P. aeruginosa*. The translocation of NFκB to the nucleus was decreased in MyD88 deficient mice. MyD88 played a role in the production of CXCL2/MIP2, CXCL1/KC, TNF and IL-1β, which are important for neutrophil recruitment. Since MyD88 deficiency led to a delayed innate immune response and bacterial clearance, it was hypothesized that the TRIF signaling pathway may be involved in *P. aeruginosa* infection. TRIF was found to play a role in the translocation of NFκB to the nucleus as well as a role in the production of RANTES, KC and TNF. The role of IRF3 in the response to *P. aeruginosa* infection needs to be examined in the future. IRF3 is a transcription factor that up-regulates IFNβ. IFNβ could not be detected in this model by ELISA after *P. aeruginosa* infection. If IRF3 was activated to up-regulate IFNβ in this system, it could act in an autocrine or paracrine manner to induce signaling for the transcription of the inducible transcription factor IRF7. *P. aeruginosa* did induce IRF7 mRNA expression, as detected by real time RT PCR. Therefore, IRF3 may be involved in response to *P. aeruginosa* infection. In interpreting this model, it needs to be acknowledged that MyD88 and TRIF may be signaling downstream of other unknown receptors, as well as TLRs. For example, MyD88 is involved in the IL-1R signaling pathway. IL-1β is produced after *P. aeruginosa* infection; therefore, a possible role for the autocrine and paracrine effects of this cytokine cannot be excluded when comparing the phenotype of the MyD88 deficient mouse to mice deficient in multiple TLR. Adapted from reviews by (148, 179, 193, 222, 226).


TLR4
TLR4
MD-2
Unknown
Receptor
Unknown
Receptor
TLR1/6
TLR2
TIRAP
TRAM
MyD88
TRIF
TIRAP
TRIF
MyD88
MyD88
TRAF6
RIP1
IRAK1/4
TBK1
IFNβ
TRAF6
NFκB
IκB
IRF3
IL-1β
TNF
CXCL1/KC
CXCL2/MIP-2
CCL5/RANTES
NFκB
IRF3
Stats, IRF9
NFκB
IκB
TRAF6
Stats, IRF9
CCL5/RANTES
TNF
IFNβ
IFNAR
IRAK1/4
MyD88
IL-1R
IL-1β

6.3 Limitations of the Current Study

It is important to recognize that there are limitations associated with all research models since the conditions are never ideal. This research is not an exception and there are limitations to its value.

6.3.1 Taking Animal Infection Models and Making Conclusions about Human Infections

Due to the fact that ethical and methodological difficulties inherent in human research limit the progress of studies in humans, animal models provide an ideal experimental system that mimic many situations observed in humans. It is hoped that findings in animal models can be extrapolated to humans and lead to improved medical treatments (544, 545). Genetic studies have proven useful in examining immunity to infection. Mice with natural mutations and mice with genes of interest knocked out can be used to examine the role of the specific gene with the observed phenotype. (544). Hypotheses can be tested under experimental conditions because multiple mice can be sacrificed at various time points and all organs and body fluids can be examined (544). However, there are differences between human infections and mouse models of infection.

Several differences exist between artificial animal models and human models of infection (544, 545). Some variables include: 1) the microorganism, often an established laboratory strain used in a highly pure inoculum; 2) the host, usually an inbred animal that has a short life span living under fairly sterile conditions in contrast to humans which are mainly outbred with a variable age of infection and a much longer life span; and 3) route of infection, animals are usually an artificial route, while humans come into contact with a microorganism via a natural route and at fairly low dose (544). Therefore, there are inherent differences in using mouse models for infections and these limitations must be taken into account when interpreting results from mouse models and making applications to human disease and treatment.

Although there are similarities in the DNA sequences of murine and human genes, there are several deficiencies in using a mouse model for a human disease. The proteins in mice and humans involved in the immune system are not all identical between species. For example, CXCL8/IL-8 is a neutrophil chemoattractant in humans. Although

there are neutrophil chemoattractants in the mouse, there is no counterpart for CXCL8/IL-8 in mice (545).

6.3.1.1 Limitations in Interpreting Data to the CF Lung

There are limitations in interpreting data from a mouse model to human infections. Not only is the size of a mouse lung extraordinarily smaller than that of a human lung but there are significant differences in the CF lung in comparison to a normal human lung, as well as a normal mouse lung. Therefore, there are inherent difficulties that arise in extrapolating data from a mouse lung infection model to a CF patient. The CF lung has an inequality in the microenvironment due to obstructive changes in the bronchi that naturally happens, leading to differences in the sputum collected from one area of the lung to another (546). Developing a mouse model similar to the CF patient with a defect in CFTR has been difficult because mice with a CFTR deficiency do not spontaneously develop lung infections. Evidence suggests that there is a redundant chloride transporter that is able to function in mice and compensate for a deficiency in CFTR (428). Therefore, a good lung mouse model for CF has been difficult to develop.

6.3.2 Different Routes of Administering Bacteria to the Lung

In examining the literature with respect to mouse lung infection models, the route of infection varies. In the model described in this thesis, mice were infected intranasally, not requiring surgery and providing an "easy" route for administering the *P. aeruginosa.* Another route that provides ease of administration of bacteria is to place animals in a chamber with aerosolized bacteria (538). The aerosolization of *P. aeruginosa* for infection may provide a good model that is more representative of natural routes of infection (426); however, it requires the proper equipment and containment considerations have to be made. Furthermore, it is difficult to standardize dosing as it is dependent on the respiratory rate of the animal. A more direct way of infecting mice is to surgically administer the bacteria to the trachea (515). However, performing surgery to inoculate with *P. aeruginosa* may influence the results. The mice will experience surgical trauma, as well as the airway infection resulting in inflammation in both the trachea and the lung. Differences in the route of administering a pathogen may provide

differences in the outcome of the infection; however, these differences should ideally be minor. Interestingly, in comparing our studies to others (515, 538), there is very little difference between the different models of infection.

6.3.3 Limitation in Interpreting the Real-Time Quantitative PCR Data

In analyzing our real-time RT-PCR data, we used a calculation to indicate a fold increase after treatment. The baseline was made by dividing each sample by the no treatment wild type. The validity of using this method to measure mRNA levels is dependent on the assumption that cells, such as neutrophils, recruited into the lung after infection do not normally express higher levels of the mRNA being tested than the resident lung cells. Therefore, if neutrophils coming into the lung normally express higher levels of TLR2, TLR4 or IRF7, interpretation of these results would need to be revised.

6.3.4 Limitations in Using Mice with Genetic Deficiencies

In an *in vivo* model where a mouse with a genetic deficiency is compared to a genetically normal mouse, one assumes that the only difference in these genetically altered mice is due to the gene defect. However, several minor aspects of the mouse will also be altered. Interactions with intracellular signaling molecules are lost in MyD88 or TRIF deficient mice. Therefore, normal intracellular signaling may be disrupted in ways we cannot understand due to unknown interactions with each of the adaptor molecules. Functions of other proteins inside the cell may be different in the knock out mice due to the missing protein. A similar argument can be made in using the TLR deficient mice. Therefore in mice with a TLR signaling pathway defect there may be unforeseen consequences with respect to the complement cascade, NK cell and neutrophil function.

There is also a limitation due to compensation where one protein is able to take over the function of the missing protein. Unexpected effects on development (547) and hematopoiesis may occur and need to be considered. Some deficiencies are lethal and require conditional expression which may lead to a dysregulated deficiency.

6.3.4.1 Possible Impairments in Neutrophil Function due to TLR Signaling Deficiency

Bacterial clearance may be delayed in TLR signaling deficient mice, in part due to a decrease in neutrophil recruitment; however, the neutrophils that are recruited may also have impaired function with respect to pathogen killing since they cannot recognize pathogens in the same manner as normal neutrophils. Therefore, the mechanisms behind a defect in bacterial clearance may be complex. There is a deficiency in the recruitment of neutrophils to the lungs, possibly due to decreases in cytokine and chemokine production, but there may also be defects in the function of the neutrophils once they do get there (110).

TLR activation plays a role in inhibiting the apoptosis of neutrophils (399). Therefore, in our model where TLR signaling pathways are compromised, the effects on neutrophil function with respect to apoptosis are uncertain. Perhaps neutrophils without TLRs undergo apoptosis and optimal killing of pathogens cannot be maintained.

6.3.4.2 Changes in the Natural Flora due to TLR Deficiency

There are bacteria everywhere, including our skin and gastrointestinal tract. Usually these organisms do not harm the host and are often referred to as the normal flora. TLR signaling needs to be regulated to allow colonization by microflora and avoid excessive inflammation. Therefore, the localization of TLRs is regulated in different parts of the body and within the cell (548). For example, TLR2 and TLR4 are expressed at low levels in the gut and are found to be tolerant to stimulation with agonists (549-551). The mechanisms regulating TLR signaling may be disturbed in a TLR or TLR signaling deficient mouse. Due to the fact that TLRs interact with microbes to recognize and alert the host of a pathogen, it is likely that mice deficient in a specific TLR or TLR signaling pathway may have a significantly different normal flora in comparison to wild type mice.

6.3.4.3 Why can the Phenotype of MyD88 Deficient Mice not be Duplicated in Multiple TLR Deficient Mice After *P. aeruginosa* Induced Lung Infection?

Most studies that examine the role of the TLR signaling pathway after infection with a specific pathogen focus on the signaling pathways downstream of a single TLR.

However, during infection with a pathogen, immune cells most likely detect several PAMPs through multiple TLRs and produce an immune response that is more complex than that mediated by a single TLR (201). The phenotype of MyD88 deficient mice after *P. aeruginosa* lung infection could not be reproduced in mice lacking TLR2, TLR4 and flagella dependent (TLR5) mediated signaling (538). Skerrett *et al.* (538) found that mice deficient in both TLR2 and TLR4 that were infected with flagellin deficient *P. aeruginosa* were able to produce cytokines, although at lower levels, and efficiently clear the *P. aeruginosa* from the lungs. Our work and Skerrett *et al.* (538) show that MyD88 deficient mice have abrogated innate immune responses at 24 h after infection, resulting in an impaired bacterial clearance. Skerrett *et al.* (538) suggest that since these multiple TLR deficient mice are not able to duplicate the observed phenotype of MyD88 deficiency, there are other MyD88 dependent signaling cascades involved in the clearance of *P. aeruginosa* from the mouse lung. They report preliminary data suggesting that TLR9, which recognizes bacterial CpG motifs (133), is not essential for clearance of *P. aeruginosa* because mice lacking TLR2, TLR4 and TLR9 were not more handicapped in their response to flagellin deficient *P. aeruginosa* than mice that were only deficient in TLR2 and TLR4. Therefore, multiple deficiencies in TLRs have not been able to replicate the phenotype of MyD88 deficient mice after *P. aeruginosa* infection.

There may be several reasons for the fact that a combination of receptors are involved in the response to *P. aeruginosa* induced lung infection including TLR2, TLR4, TLR5 but that the combined deficiency of these receptors and TLR9 (538) together do not give the same phenotype as MyD88 deficient mice. *P. aeruginosa* may interact with other non-TLR innate immune receptors that signal through MyD88. For example, MyD88 also signals for IL-1, IL-18 and IL-33 receptors (227).

Since MyD88 also mediates signaling through the IL-1, IL-18 and IL-33 receptors (227, 465), enhancing the activation of NFκB (552), it can be reasoned that MyD88 deficient mice may express a more severe phenotype than mice with single or multiple TLR deficiencies after infection. We (307) and others (308, 553) show that IL-1β production is increased soon after lung infection with *P. aeruginosa*. IL-1β can bind the IL-1R in an autocrine or paracrine manner to up-regulate NFκB to further increase the

production of inflammatory mediators. Without this positive feedback from IL-1R signaling you may anticipate the early abrogation in the inflammatory response we observed. This information highlights that the possible role of the IL-1R cannot be denied in the MyD88 deficient mouse model.

Since, IL-1β is an important inflammatory cytokine that signals through MyD88, one may suspect that since IL-1R signaling does not occur in MyD88 deficient mice that this may be important for the observed phenotype of MyD88 deficient mice after infection. However, the interplay of molecules in the innate immune response is complex; this is indicated by the fact that IL-1R deficient mice were able to clear *P. aeruginosa* more efficiently than wild type mice (553). The role of the IL-1R signaling pathway in response to *P. aeruginosa* infection seems complex. The production of IL-1β, IL-1α, TNF, CXCL1/KC and CXCL2/MIP-2 is similar at 6 h post infection in IL-1R deficient and wild type mice, but cytokine differences are observed at 24 h after infection with *P. aeruginosa* (553). There was a small decrease in the number of neutrophils recruited to the lungs of IL-1R deficient mice 24 h after infection (553). This data begs the question, why would inhibiting IL-1R signaling be beneficial for bacterial clearance? Perhaps limiting inflammation 24 h after infection is beneficial for bacterial clearance. This exemplifies the fact that the role of IL-1R signaling is complex in the development of the innate immune response.

Therefore, in response to a pathogen there may be an important and selective innate immune response responsible for the production of an appropriate balance of cytokines and chemokines. The IL-1R signaling pathway, or other unknown innate immune receptors that signal through MyD88, may contribute to the observed phenotype of MyD88 deficient mice after *P. aeruginosa* infection. Future studies involving an examination of other innate immune receptors and the importance of the IL-1R signaling are required to examine these possibilities.

Even though there are inherent limitations, mouse models have been vital for determining the fundamental mechanisms involved in the development of the immune response, which will impact the course of future immunity and host defense studies to infection(544).

6.4 Future Studies

This research has answered many questions regarding the important components of the TLR signaling pathways that are involved in initiating the innate immune response to *P. aeruginosa*. However, it has also led to a lot of new and very specific questions about the details of the signaling pathways. To further these studies, it would be good to further examine the role of other TLR signaling pathway components.

6.4.1 The Role of IRF3 in *P. aeruginosa* Infection

Of particular interest are the transcription factors IRF3 and IRF7. IRF3 is activated downstream of TRIF and is critical for the induction of IFNβ transcription, which feeds back in an autocrine loop to induce transcription of IRF7 (167, 189, 193, 194). In other studies, LPS has been shown to induce an increase in the protein expression of IRF7 4 to 8 h after stimulation (193). Since an increase in IRF7 mRNA was upregulated after infection with *P. aeruginosa* (Figure 5.7), this suggests that IRF3 may be involved downstream of TRIF after *P. aeruginosa* infection. However, we observed no translocation of IRF3 to the nucleus and little production of IFNβ following *P. aeruginosa* infection, suggesting that a different pathway could be involved. Furthermore, IRF7 induction was not tested in $TRIF^{-/-}$ mice to confirm that it is TRIF dependent. Whether IRF3 dimerizes by western blot in the lungs of wild type mice after infection with *P. aeruginosa* should be examined. Also, there are IRF3 deficient mice that could be used to examine the specific role that this transcription factor plays in cytokine production, neutrophil recruitment and ultimately bacterial clearance. The examination of this part of the TRIF signaling pathway would be important to determine the role of IRF3 and its effects on transcription involved in the cytokine production after lung infection with *P. aeruginosa*.

6.4.2 The Role of IRAK4 in *P. aeruginosa* Infection

MyD88 is the adaptor molecule that interacts with most of the TLRs. Downstream of MyD88 is the signaling molecule IRAK4 (176). Since there are IRAK4 deficiencies in the human population and patients suffer from bacterial infections (554), it would be interesting to determine whether mice that are deficient in IRAK4 also have

defects in the innate immune response to *P. aeruginosa*. Examining a mouse model is a good way to further examine specific aspects of this immunodeficiency that are not possible to test in humans.

6.4.3 The Effect of Mice Deficient in Both MyD88 and TRIF on the Innate Immune Response to *P. aeruginosa*

Since mice deficient in either MyD88 or TRIF have delayed innate immune responses to *P. aeruginosa*, it can be hypothesized that a doubly deficient mouse may have more severe deficiencies in initiating the innate immune response and killing bacteria. Double knockouts of MyD88 and TRIF have been generated, making these experiments possible. However, the double knockouts do not breed well and obtaining large numbers of the appropriate littermates from heterozygotes is difficult.

Examining MyD88 and TRIF double mutant mice would be beneficial to further examine the overall importance of these pathways; however, delayed neutrophil recruitment may still occur in MyD88 and TRIF double deficient mice because recognition of microbes would still occur through collectin binding and activation of complement. Therefore, the host has multiple mechanisms for relaying the message that danger is present. Inhibiting the production of cytokines and chemokines through TLR signaling pathways may not prevent bacterial clearance since bacteria can still be recognized by other ways.

6.4.4 The Role of Mast Cells in *P. aeruginosa* Induced Lung Infection

Mast cells have been shown to be involved in the development of the innate immune response (555). Interestingly, mast cells are present in the lungs (556) and come into contact with the external environment in the airways where they can recognize and interact with pathogens. In response to *P. aeruginosa*, mast cells release NFκB controlled cytokines such as TNF (557), IL-6 (558) and IL-1β (557). These inflammatory mediators are important in the recruitment of neutrophils to the lungs after infection; therefore, the inflammatory mediators released by mast cells may attract neutrophils to the site of infection. One study by Lin *et al.* (557) indicates that mast cells may play a role in *P. aeruginosa* induced neutrophil recruitment through the production

of inflammatory mediators including IL-1β. It would be interesting to test in an *in vivo* model whether cytokines released from mast cells play a role in recruiting neutrophils to the lung during *P. aeruginosa* infection. Preliminary data suggests that mast cells may be involved in neutrophil recruitment in an *in vivo* model (Appendix A). However, more studies are required to further explore this possibility.

6.5 Important Implications of this Study for Disease

This work may be beneficial for patients who acquire *P. aeruginosa* infections while in hospital, as well as immunocompromised patients and patients with CF (559). *P. aeruginosa* induces several types of infections including pneumonias, urinary tract infections, blood stream infections, surgical site infections, skin infections in burn victims and immunocompromised hosts such as patients with diabetes mellitus, ulcerative keratitis, otitis externa, as well as chronic sinopulmonary infection in CF patients. Infections by *P. aeruginosa* are common and antimicrobial resistance has developed in several nosocomial isolates. *P. aeruginosa* infection has been associated with high morbidity and mortality (559). *P. aeruginosa* is the second most common cause of hospital acquired pneumonia and ventilator associated pneumonia. Pediatric patients with CF that have *P. aeruginosa* infection have higher mortality, increased frequency of hospitalization, decreased lung function and lower weight than CF patients without *P. aeruginosa* lung infection (559). *P. aeruginosa* infection can be diverse; the *in vivo* model in this study examines interactions of the innate immune response with *P. aeruginosa* in the context of the lung. This study may give insight into the innate immune response to *P. aeruginosa* lung infection and may enable the development of treatments for patients that suffer from *P. aeruginosa* lung infections.

6.5.1 Relevance to Patients with Immunodeficiencies

So far, evidence of patients that are deficient in MyD88 or TRIF has not been reported. However, it has not been until recently that the importance of these molecules in bacterial infections has been established. There are patients who suffer from specific bacterial infections for unknown reasons (560). Perhaps some of these patients have deficiencies in a specific component of the TLR signaling pathway and have not yet been

tested. Deficiencies in MyD88 and TRIF may lead people to have impaired host defense to specific pathogens and therefore deficiencies may go unnoticed in individuals until they come in contact with a pathogen to which they cannot mount a proper early innate immune response. However, deficiencies in MyD88 and TRIF may be lethal or may be responsible for deaths in neonates that were classified as death due to a bacterial overload. In the past, screening of TLR signaling pathways has not been routine in patients who have recurrent bacterial infections. Therefore, the full extent of deficiencies in TLR signaling may not currently be appreciated and may be relevant in the human population.

This work has been able to establish in an *in vivo* model in order to determine the role of MyD88 and TRIF in immunity to *P. aeruginosa* lung infection. Establishing this model is important for making connections between the genotype of an individual and the observed phenotype (560). The ultimate relevance of the TLR signaling pathways is yet to be determined. There are multiple redundant pathways involved in alerting the host that danger is present. When there is a TLR signaling deficiency *in vivo*, there is still complement activation and production of C3a and C5a that are important in neutrophil recruitment, collectins like MBL still opsonize pathogens for phagocytosis; therefore, the full relevance of these pathways and their interactions is yet to be elucidated.

6.5.1.1 Known Deficiencies in TLR Signaling Pathways

6.5.1.1.1 Deficiencies in NFκB Activation

Mutations found in either IKKγ or IκBα lead to the impaired activation of NFκB in patients. These individuals are susceptible to infections with encapsulated pyogenic bacteria and specific viral and fungal infections (561). Periperal blood mononuclear cells from these patients produce decreased levels of cytokines after stimulation with LPS, IL-1β, IL-18 or TNF (562). Patients with these deficiencies also have NK cell anomalies (563), poor CD40 mediated signaling, and hyper IgM (564).

Studying TLR signaling pathways in controlled settings may be relevant to human diseases that stem from deficiencies in these pathways. TLR signaling may also be relevant in the induction of the adaptive immune response since TLR signaling and

specifically TRIF signaling induced by LPS can induce the up-regulation of co-stimulatory molecules such as CD40 on dendritic cells (128).

6.5.1.1.2 IRAK4 Deficiency

Individuals with a deficiency in a downstream TLR signaling molecule, IRAK4, have been shown to have problems clearing opportunistic pathogens, including *P. aeruginosa* (554). IRAK4 deficiency leads to a decrease in the production of IL-1β, CXCL8/IL-8, IL-12, TNF and IFN-γ following stimulation with TLR agonists, IL-1β or IL-18 (180, 554). Some of these patients have lived into their thirties, which suggests that the adaptive immune response may be able to compensate in the long run for a weak innate immunity (561). Work examining an *in vivo* model of an opportunistic infection in TLR signaling deficient mice may be relevant for determining the clinical relevance of these signaling pathways.

TLRs may be redundant with other mechanisms of pathogen recognition. The identification of patients with mutations in genes involved in the TLR signaling pathways will help determine the function of TLRs in immunity and host defense (561).

6.5.2 Implications of this Work for Neonates

Human newborns are more susceptible to microbial infection than adults (565, 566). Neonates have an impaired production of cytokines such as TNF after stimulation with LPS (567). The diminished production of cytokines in neonates may in part be due to deficiencies in the TLR signaling pathways. Unlike adults, components of the LPS receptor complex such as CD14 and TLR4 are not increased in newborns after stimulation with LPS. Also, expression of the TLR signaling adaptor molecule MyD88 has been found to be significantly decreased in newborns (567). Therefore, defects in cytokine production in neonates may in part be due to defective TLR signaling. It would be of interest to examine the expression of TRIF in newborns to detect whether this pathway is functional in humans at birth. Performing studies in mice with TLR signaling deficiencies may provide a controlled way to examine the deficiencies of the newborn.

6.5.3 Implications of this Work for CF Patients

The fact that *P. aeruginosa* is successful in colonizing the CF lung suggests that the innate immune response that is required for effective clearance of *P. aeruginosa* is defective in CF patients (426). In order to effectively evaluate the deficiency in CF patients with respect to *P. aeruginosa* clearance, the mechanisms that are involved in the normal innate immune response to *P. aeruginosa* must be examined. A short lag in the initial host defense against bacterial infection may seriously alter the outcome of the infection (541). These results indicate that the MyD88 and TRIF pathways are essential for the development of host responses to *P. aeruginosa* infection, leading to the clearance of this bacterium. TLR2 and TLR4 are partially involved in the recognition of *P. aeruginosa* infection. This information will be important for assessing possible deficiencies in CF patients and may help in the development of new treatment strategies for these patients.

Since, bacterial clearance is impaired in CF patients, it can be inferred that there may be a defect in TLR mediated signaling. In order to determine whether there were defects in CF patients with respect to TLR function, researchers have examined the expression of TLRs in epithelial cells of CF patients and healthy individuals. It has been found that the expression of TLRs is similar in CF and non-CF epithelial cells (568, 569). The expression of TLR may be normal in CF patients, but questions remain regarding whether the TLR signaling pathways are fully functional in these patients.

One way to assess the TLR signaling pathways is to examine the cytokine and chemokine profile produced in response to stimulation. In one study, low levels of CCL5/RANTES were found to be secreted from CF epithelial cells, as measured from the sputa of CF patients. Since CCL5/RANTES is a product of the TRIF signaling pathway, it was suggested that the TRIF signaling pathway may be impaired in CF cells (570). The information from the sputa of patients is interesting because it can describe what is happening in the actual patient; however, there are difficulties in trusting negative data because it is possible that CCL5/RANTES was produced in higher concentration lower down in the airways, which would not be detectable in the sputum.

One study showed that CCL5/RANTES expression is altered in CF epithelial cells and that the expression of CCL5/RANTES, but not CXCL8/IL-8, depends on CFTR

(571). Correction of defective CFTR in epithelial cells restored cytokine induction of CCL5/RANTES expression through a NFκB mediated pathway (571). The neutralization of CCL5/RANTES decreased the migration of leukocytes to the site of inflammation but may have a pro-inflammatory effect at the site of established chronic inflammation (572). Since CF patients have chronic inflammation in the lung without effective bacterial clearance (433, 440, 441, 444, 444, 445), it can be hypothesized that an improper cytokine and chemokine profile may in part be responsible.

An improper cytokine and chemokine profile may be problematic for the effective early bacterial clearance. Therefore, further research is needed to determine if the decrease in CCL5/RANTES production is a result of a defective TLR signaling pathway in CF patients.

6.5.4 General Implications for Treating Disease

Investigating polymorphisms and gene mutations in TLR signaling pathways have provided a link between TLR signaling and disease. Investigating TLR signaling pathways and polymorphisms in humans may help in the development of therapeutics to alleviate microbe mediated inflammation. Targeting TLR signaling adaptor molecules may provide a novel avenue to develop antimicrobial drugs to treat chronic inflammation, especially in conjunction with known genetic profiles of patients. TLR signaling studies, including this one, may have implications for treating septic shock and chronic inflammation (112).

6.6 Concluding Remarks

To my knowledge, this study is the first to demonstrate the role of TRIF in the induction of neutrophil recruitment and the bacterial clearance of *P. aeruginosa* induced lung infection. A critical role for both MyD88 and TRIF in *P. aeruginosa* induced lung infection has been established, demonstrating that both MyD88 dependent and MyD88 independent signaling pathways are required for the effective host response against *P. aeruginosa* lung infection. This murine model of infection examined the TLR signaling pathways that form the groundwork understanding the importance of TLRs and signaling molecules in the pathogenesis of *P. aeruginosa* lung infection. These discoveries identify

the important mechanisms involved in the innate immune response to *P. aeruginosa* infections that may aid in the development of treatments for patients, such as patients with CF, who are not able to effectively clear *P. aeruginosa* lung infection. Future work examining the key components of the TLR signaling pathway in *P. aeruginosa* lung infection will further our understanding of the importance of TLR signaling. Investigating the TLR signaling pathways in patients with recurrent unexplained bacterial infections may be important in the treatment of these patients in the future.

Appendix A. Examining a Role for Mast Cells in the Development of Innate Immunity to *P. aeruginosa* Lung Infection

Summary of Findings

WT and mast cell deficient (W/W^v) mice were infected with *P. aeruginosa* to determine the effect of mast cell deficiency on the innate immune response. As detected by myeloperoxidase assay, there was a reduced level of neutrophil recruitment in the lungs of mast cell deficient mice 8 h post infection (Figure A1). To determine whether mast cell deficiency had an effect on the inflammatory mediator production in the lung, we used ELISA to examine the production of TNF, IL-1β, IL-6 and CXCL2/MIP-2 in lung homogenates and BALF (Figures A2 and A3). Mast cell deficiency did not result in any significant change in the observed cytokine/chemokine profile. These results and inquiries from colleagues led us to examine whether W/W^v mice were neutropenic. The literature on W/W^v mice suggests that these mice may in fact be neutropenic (573). If these W/W^v mice are neutropenic it may explain why there is a significant difference in the levels of neutrophils recruited into the lungs after *P. aeruginosa* infection compared to wild type mice but no effect on inflammatory mediator production. There was a slight decrease in the number of peripheral blood neutrophils in W/W^v mice compared to wild type mice, although the difference was not significant (Figure A4). Therefore, further study is needed to indicate what is responsible for the observed decrease in neutrophil recruitment in mast cell deficient mice after *P. aeruginosa* infection.

Questions remain about the involvement of mast cells in response to *P. aeruginosa* infection. Some of the future goals of the lab include further assessment of the roles of mast cells in *P. aeruginosa*-induced cytokine production and neutrophil infiltration into the lung. Perhaps a different strain of mast cell deficient mice, such as the W^{sh}/W^{sh} mouse model, will be more effective in examining the effect of mast cell deficiency in *P. aeruginosa* infection. However, since the production of IL-1β, IL-6, TNF and CXCL2/MIP-2 was not impaired in W/W^v mice, the role mast cells play *in vivo* after *P. aeruginosa* infection may be masked by functional resident macrophages and epithelial cells in the airways that can effectively respond to infection and initiate the innate immune response.

FIGURE A1 Defective neutrophil recruitment into the airways of mast cell deficient (W/W^v) mice following *P. aeruginosa* lung infection. Wild type and W/W^v mice were inoculated intranasally with *P. aeruginosa* (*Psa*) (mucoid strain 8821, 1 x 10^7 or 1 x 10^8 CFU/mouse). Mice that were not treated with bacteria or those that received saline were used as controls (NT). (A) and (B), 8 h after infection with 1 x 10^8 Psa, BALF and lung tissue were collected for the determination of MPO activities. Data are the mean ± S.E. of 6 - 8 mice/group ($p < 0.05$). (C) and (D), 8 h after infection with 1 x 10^7 Psa, BALF and lung tissue were collected for the determination of MPO activities. Data are the mean ± S.E. of 10 - 12 mice/group ($p < 0.05$). (E) and (F) 8 h after infection, mice were sacrificed, and the upper lobe of the left lung was collected for hematoxylin-eosin staining. (E) Wild type mouse lung (original magnification x100), (F) W/W^v mouse lung (original magnification x100). (G) and (H), 12 h after infection with 1 x 10^8 Psa, BALF and lung tissue were collected for the determination of MPO activities. Data are the mean ± S.E. of 6 - 8 mice/group.

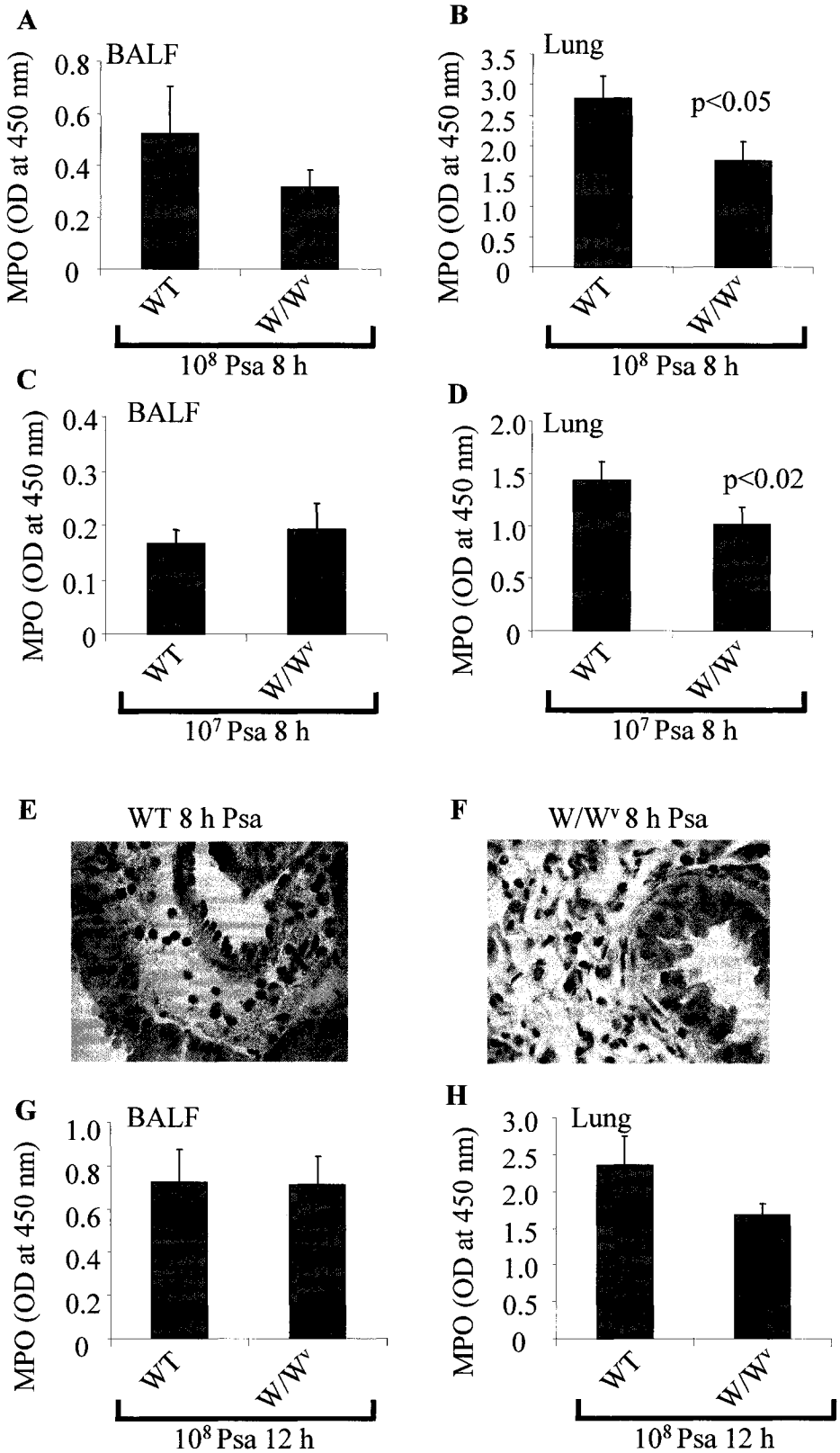

A
BALF
MPO (OD at 450 nm)
0.8
0.6
0.4
0.2
0
WT
W/W^v
10^8 Psa 8 h
B
Lung
p<0.05
3.5
3.0
2.5
2.0
1.5
1.0
0.5
C
0.4
0.3
0.2
0.1
10^7 Psa 8 h
D
2.0
p<0.02
E
WT 8 h Psa
F
W/W^v 8 h Psa
G
1.0
10^8 Psa 12 h
H
3.0

FIGURE A2 Mast cell deficiency has no effect on IL-6, IL-1β, TNF and CXCL2/MIP-2 production following *P. aeruginosa* lung infection. Wild type and mast cell deficient mice (W/W^v) were inoculated intranasally with *P. aeruginosa* (mucoid strain 8821, 1×10^7 CFU/mouse, Psa) for 8 h. BALF and lung tissues were collected for the determination of IL-6, IL-1β, TNF and CXCL2/MIP-2 protein by ELISA. Data are the mean ± SE of 16 mice/group.

8 h after infection with 1 x 10^7 *P. aeruginosa*

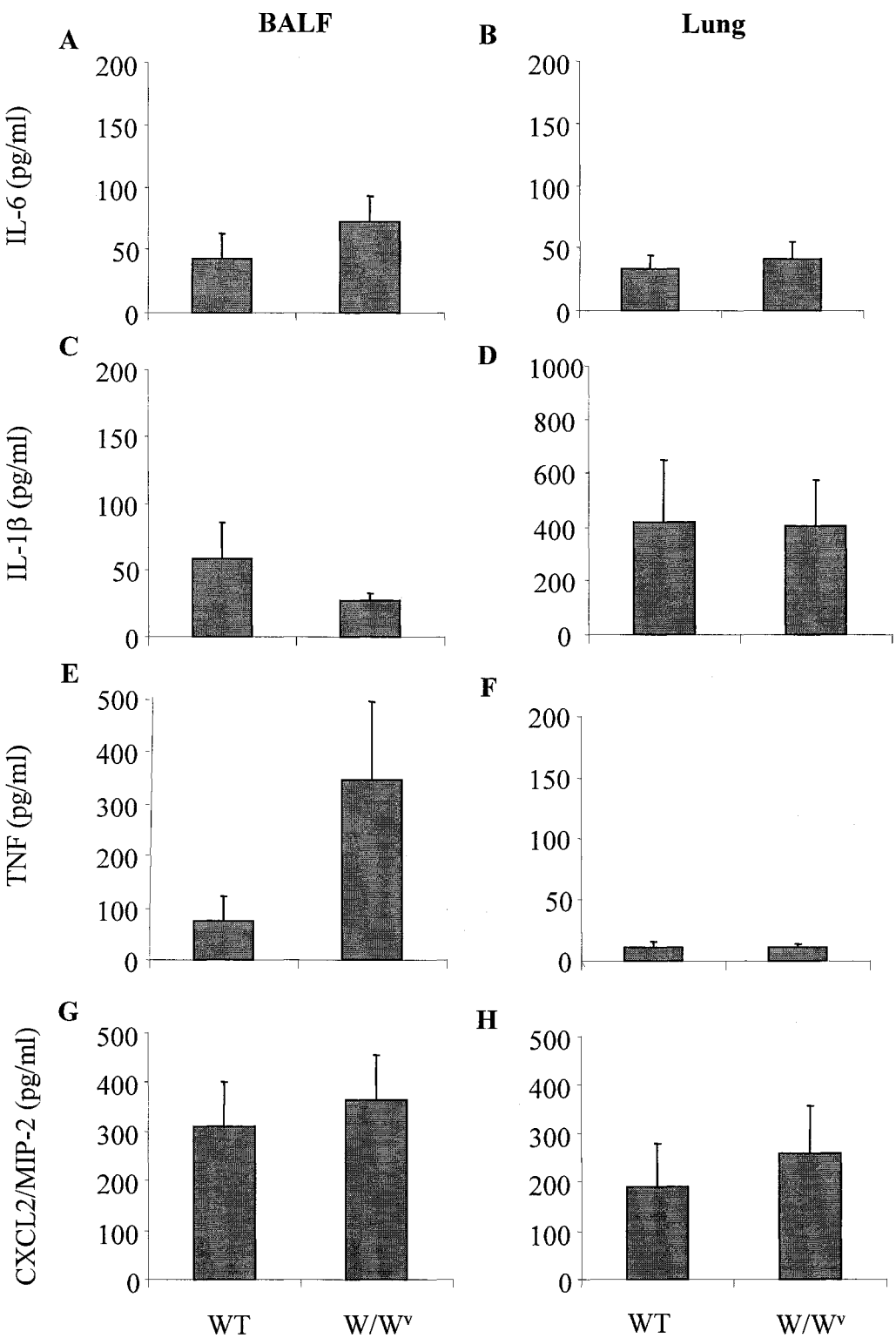
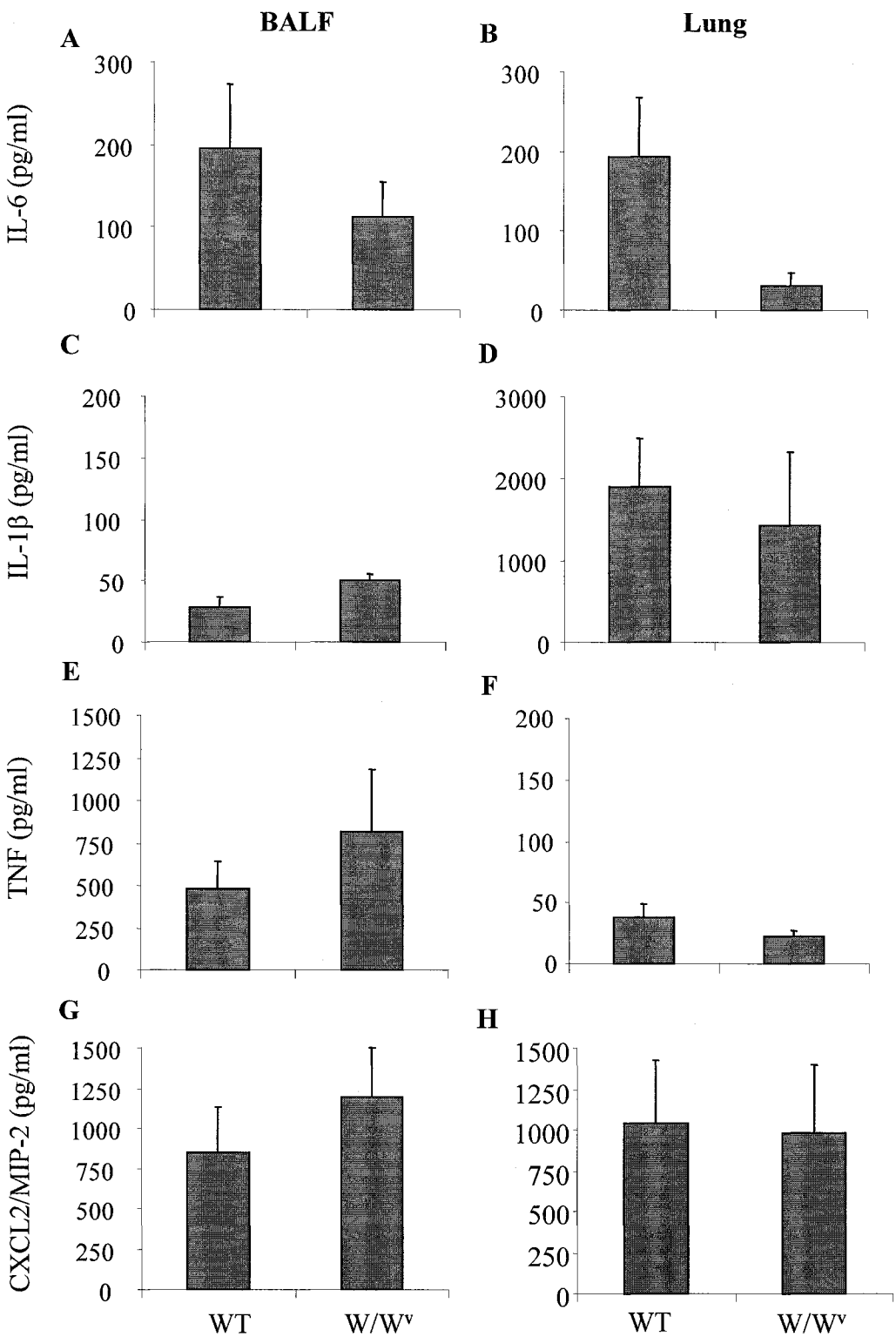


FIGURE A3 Mast cell deficiency has no effect on IL-6, IL-1β, TNF and CXCL2/MIP-2 production following a higher inoculation of *P. aeruginosa* lung infection. Wild type and mast cell deficient mice (W/W^v) were inoculated intranasally with *P. aeruginosa* (mucoid strain 8821, 1×10^8 CFU/mouse, Psa) for 8 h. BALF and lung tissues were collected for the determination of IL-6, IL-1β, TNF and CXCL2/MIP-2 protein by ELISA. Data are the mean ± SE of 4 mice/group.

8 h after infection with 1 x 10^8 *P. aeruginosa*



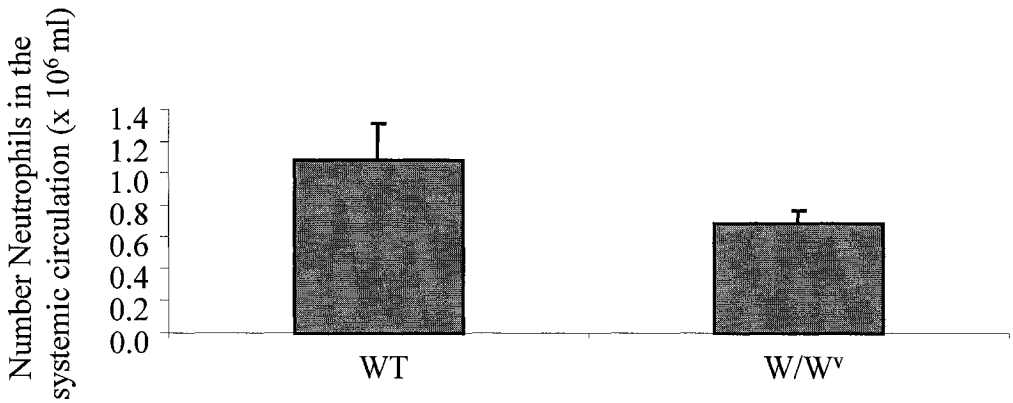


FIGURE A4 The effect of mast cell deficiency on systemic numbers of neutrophils. The number of neutrophils present in the systemic circulation were determined in wild type and W/W^v (mast cell deficient mice). Data are the mean ± SE of 8 mice/group.

Appendix B. Lack of a Role for NFκB in Mast Cell Maturation, Development *In vitro* and *In vivo* and Function *In vivo*

Materials and Methods

Antibodies

FITC-conjugated rat anti–mouse CD117 (c-kit) mAb (IgG2a, CL8936F) and FITC-rat IgG2a (CLCR2A01) were purchased from Cedarlane Laboratories (Hornby, ON). FITC-conjugated rat anti–mouse IgE (IgG1; catalog no. 553415) and FITC-rat IgG1 (catalog no. 553924) were purchased from BD Biosciences (San Jose, CA).

Mast Cell Culture

Murine primary bone marrow–derived mast cells (BMMCs) were cultured as previously described (558). Briefly, bone marrow was harvested in a laminar flow hood from the femurs and tibias of $NF\kappa B^{+/+}$ and $NF\kappa B^{-/-}$ mice. Two or three mice were used per group. The leg bones were kept moist in a dish containing RPMI 1640 medium. The ends of the bones were cut off with sterile surgical scissors, and RPMI medium was run through the shaft using a 30-ml syringe and a 31-gauge needle. Cells were collected each week, centrifuged at 500 × *g* for 5 min at 4 °C, and resuspended at a density of 0.5×10^6 cells/ml (disregarding erythrocytes) in BMMC complete medium (RPMI 1640 medium containing 10% fetal bovine serum, 10% WEHI-3B conditioned medium, 50 units/ml each of penicillin and streptomycin, 50 μM 2-mercaptoethanol, and 200 nM prostaglandin E_2). The nonadherent cells were resuspended in fresh complete medium twice/week and transferred to a fresh flask once/week.

In vivo Method for Passive Cutaneous Anaphylaxis

Passive cutaneous anaphylaxis was detected by measuring the anaphylaxis associated vascular permeability (degranulation). Anesthetized mice were sensitized passively by injecting 20μl of 1μg/ml anti-2,4-dinitrophenol (DNP) IgE in saline intradermally into the dorsal side of the ear, using a 30 gauge needle. An equivalent volume of saline was injected into the contralateral control ear. After 24 hours, mice

were challenged by intravenous injection of 200µl of 0.5mg/ml DNP diluted in 2% Evans blue dye via the tail vein. Thirty minutes after injection mice were sacrificed ears were removed and placed in 5ml polypropylene tubes. Each ear was minced in 2 ml formamide and heated in an 80°C water bath for 2 h. The liquid was collected and spun at 5000 rpm for 5 min at room temperature. The supernatant was collected and the absorbance was read at 590nm.

Summary of Findings

Since NFκB is an important transcription factor involved in the up-regulation of cytokine and chemokine production after stimulation, the role of NFκB in mast cell growth, development and function was examined. Mast cells were cultured from $NF\kappa B^{+/+}$ and $NF\kappa B^{-/-}$ mice. The surface expression of c-kit and the binding of IgE to the IgE receptor on mast cells were examined by FACS. This data indicates that the expression of c-kit and the IgE receptor was not affected by a deficiency in NFκB (Figure A5). This data indicates that NFκB may not be responsible for the development of mast cells. However, NFκB seems to play an important role in the growth of mast cells. Cell counts were lower at every time point in $NF\kappa B^{-/-}$ mast cells compared to $NF\kappa B^{+/+}$ mast cells (data not shown). *In vivo* data indicates that NFκB may not play an important role in the distribution of mast cells and the function of mast cells during passive cutaneous anaphylaxis (Figure A6). Since NFκB is a transcription factor that is up-regulated after stimulation, it is not surprising that a defect in NFκB does not have an effect on mast cell degranulation and therefore no effect on anaphylaxis *in vivo*. Mast cell degranulation occurs immediately after crosslinking of IgE with antigen on its surface and does not require the production of inflammatory mediators to have an effect on vascular permeability, because mast cell granules contain mediators such as histamine that are important for increasing vascular permeability. However, it is interesting to note that NFκB is not vital for mast cell development and function in the context of passive cutaneous anaphylaxis.

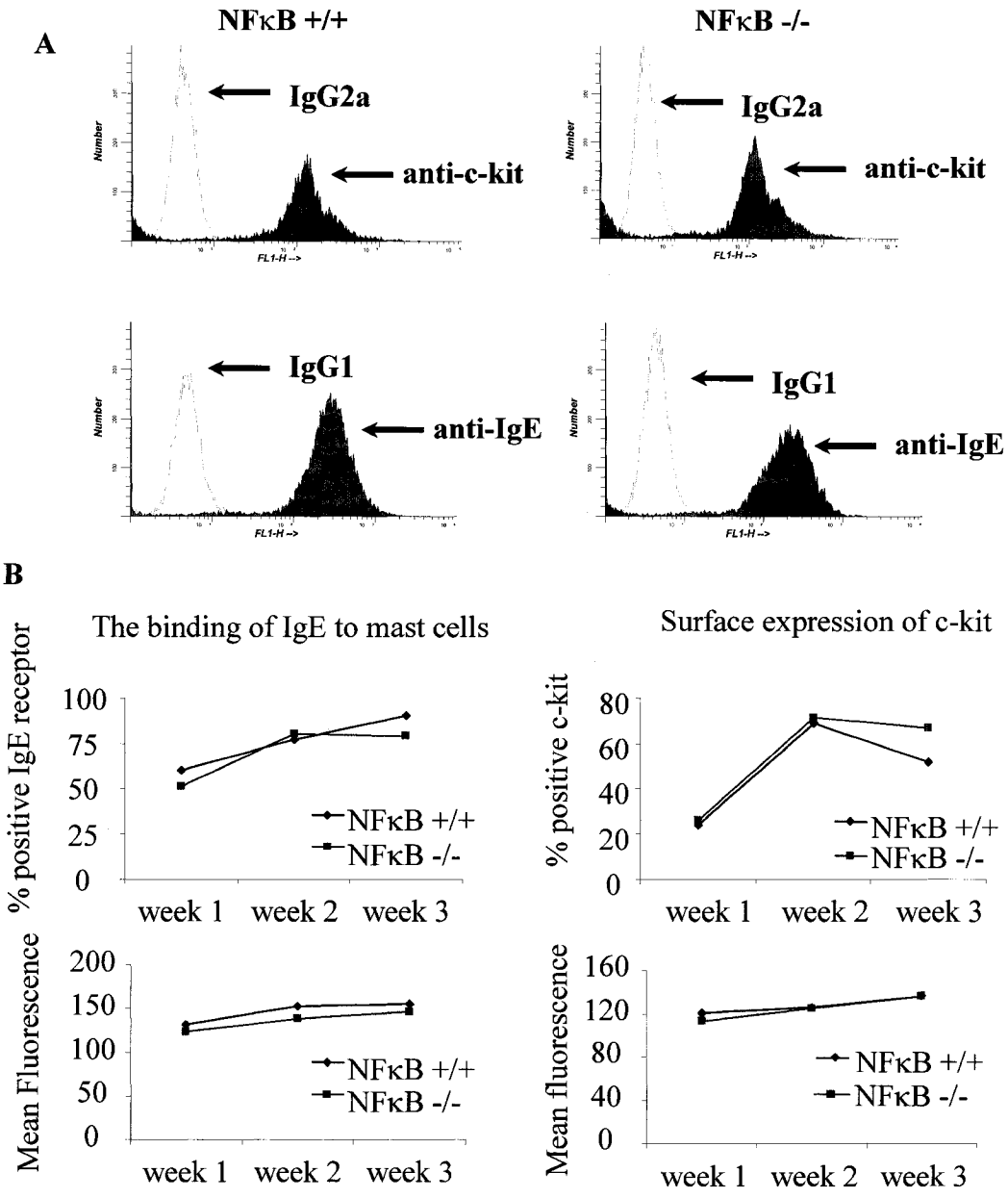


FIGURE A5 There was no observed effect of NFκB deficiency on mast cell maturation. Bone marrow cells from wild type mice or NFκB deficient mice were cultured in conditioned media *in vitro* for 3 weeks and examined by flow cytometry for c-kit and IgE receptor expression. (A) Wild type and NFκB deficient mast cells express similar levels of c-kit and IgE receptor at 3 weeks after culture. (B) Over the 3 weeks of mast cell culture similar levels of c-kit and IgE receptor were detected.

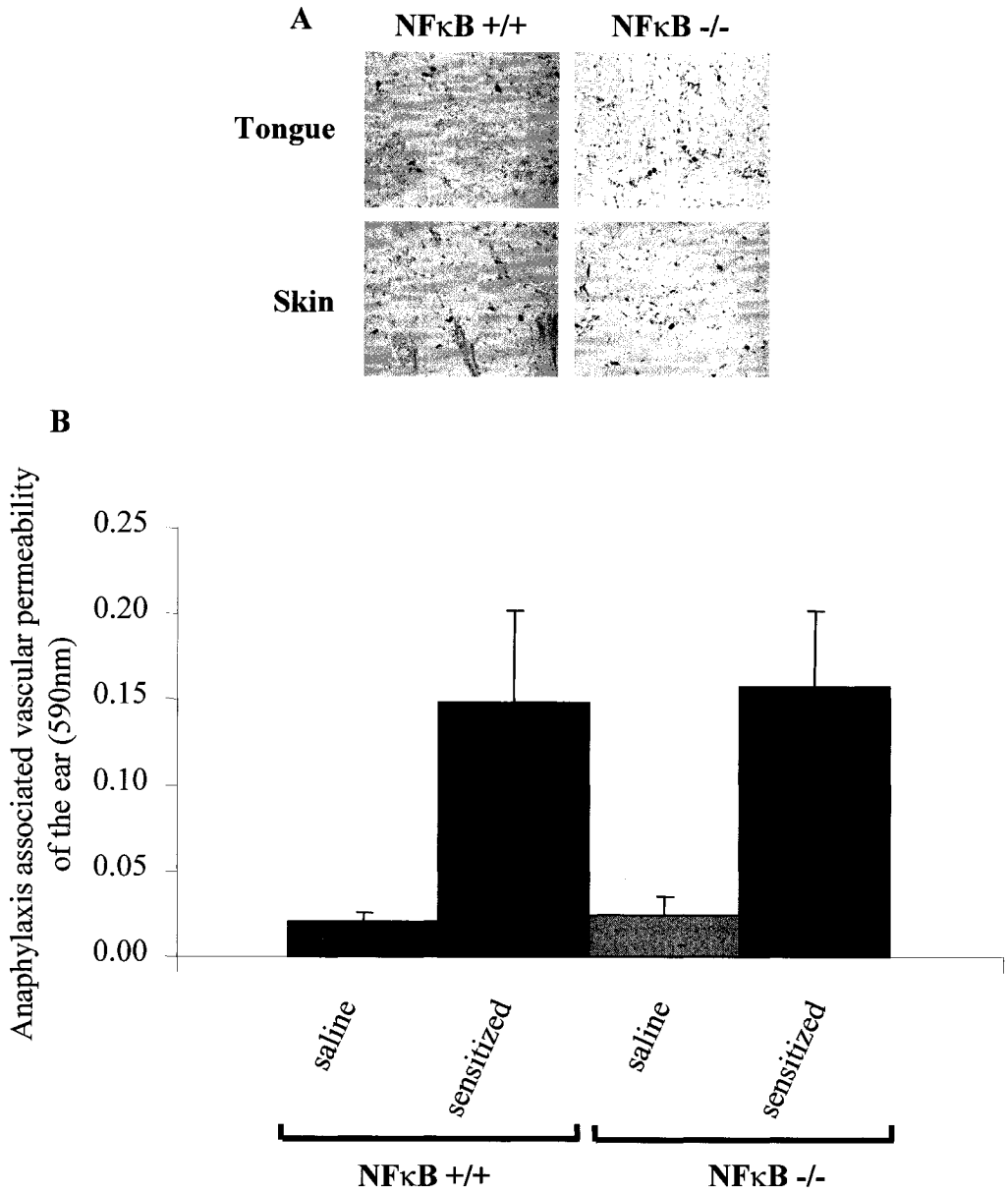


FIGURE A6 There was no effect of NFκB deficiency on mast cell maturation and function *in vivo*. (A) Tongue and skin tissues from wild type mice or NFκB deficient mice were fixed and stained by toluidine blue for mast cells. Original magnification, x 40. (B) Mice sensitized in the ear with anti-DNP and then intravenously challenged with DNP demonstrate that NFκB deficiency does not effect mast cell degranulation resulting in increased vascular permeability *in vivo*. Data are the mean ± SE of 3 mice/group, representative experiment of 5 experiments (results also confirmed in a TNP stimulation mouse model n=13 – 16 mice per group).

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