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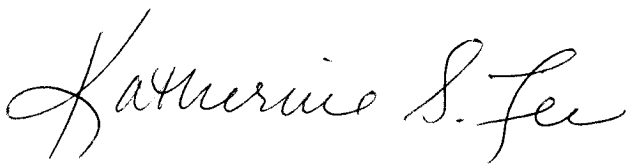

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Abstract

Title of Dissertation:

The host immune response to *Streptococcus pneumoniae*: bridging innate and adaptive immunity

Katherine Shi-Hui Lee

Thesis directed by:

Clifford M. Snapper, M.D.

Professor, Department of Pathology

Streptococcus pneumoniae (Pn) remains the primary cause of community-acquired pneumonia throughout the world, leading to high morbidity and mortality rates in the young and elderly. A better understanding of the host response to the organism would aid in the development of more effective antibiotics and vaccines. Toll-like receptors (TLRs) play an important role in the initial recognition of pathogens by binding conserved moieties known as pathogen associated molecular patterns (PAMPs). This interaction is translated through the induction of signaling cascades that ultimately results in the production of various chemical mediators necessary for the activation of the adaptive arm of the immune response. Presentation of processed antigen in the context of

major histocompatibility complex (MHC) by antigen presenting cells occurs, leading to effective help by primed T cells (T_{eff}) to naïve B cells in a process known as linked recognition. The activation and proliferation of B cells into mature plasma cells results in the development of appropriate antibody responses that are critically important in the clearance of extracellular bacteria such as Pn. These responses, however, need to be modulated so that inappropriate immune activation does not lead to anergy or overresponsiveness. The naturally occurring thymic population of $CD4^+CD25^+$ regulatory T cells (Tregs) survey and monitor the actions of T_{eff} to prevent such inappropriate responses.

In the following dissertation, I have used a murine model to study the host response to Pn. Specifically, I have focused on previous findings in the lab that suggested a possible role for Tregs as well as TLRs in our model system. I found that Tregs do not appear to play a part in modulating acute humoral responses to a bacterial pathogen such as Pn. The length of antigen stimulation may determine the need for regulation by Tregs, but since Pn is rapidly cleared by the immune system, Treg modulation does not appear to be necessary in such an infection model. However, I was able to show that cooperation between TLR2 and TLR4, as well as TLR2 and TLR9, is important for the induction of MyD88-dependent innate immune responses to Pn.

The host immune response to *Streptococcus pneumoniae*: bridging innate and adaptive immunity

by

Katherine Shi-Hui Lee

Dissertation submitted to the Faculty of the

Emerging Infectious Diseases Interdisciplinary Graduate Program of the

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To my parents, who have always been there for me. You will never know how much I appreciate your love, guidance, and support. Thank you from the bottom of my heart. To Elliott, you were my inspiration when times were tough. Tom, I look forward to our exciting future ahead.

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Chapter One

Introduction

Preface

Streptococcus pneumoniae still remains one of the major causes of community-acquired pneumonia, resulting in increased morbidity and mortality of both children under the age of two and the elderly. Although two vaccines are available to prevent invasive disease, they either do not cover all the current serotypes in circulation, or do not offer protection in certain age groups. Futhermore, the rise in antibiotic-resistant strains emphasizes the urgent need to better understand the pathogenesis of pneumococcal infection, with particular attention to the host response. The introduction to this dissertation will be broken down into a number of sections. An overview of the organism will first be presented, with a focus on the epidemiology, pathogenesis, virulence factors, and current protective measures. Next, the host immune response to Pn will be discussed, with detail given to the study of regulatory T cells and Toll-like receptors. Third, a brief description of the model used in the lab will be described. Finally, the hypotheses and specific aims of this dissertation are laid out.

Streptococcus pneumoniae

Epidemiology

A major human pathogen, *Streptococcus pneumoniae* (Pn) is a Gram-positive facultative anaerobe. Originally named *Diplococcus pneumoniae* for its predilection to grow in pairs, the bacteria can also form chains, and belong to the α-streptococci family (Fig. 1). On blood agar, colonies characteristically produce a zone of alpha (green) hemolysis, indicative of partial cell lysis (Fig.1). Despite advances in treatment and prevention, Pn still remains a major cause of morbidity and mortality worldwide, due to infections resulting in sepsis, otitis media, pneumonia and meningitis (Kadioglu and Andrew, 2004; Musher *et al.*, 2000). Globally, Pn is the most common cause of bacterial acute respiratory infections, resulting in over one million childhood deaths each year (Obaro, 2002; Stansfield, 1987) (Briles *et al.*, 2000; Jackson, 2002). In the US, Pn is a major cause of pneumonia in the elderly, estimated to result in over 500,000 cases (Klein, 2000) and 40,000 deaths per year (Obaro, 2002). Pn is also a main culprit of bacteremia and meningitis cases in children 6 months to 5 years of age (Jackson, 2002).

Pathogenesis

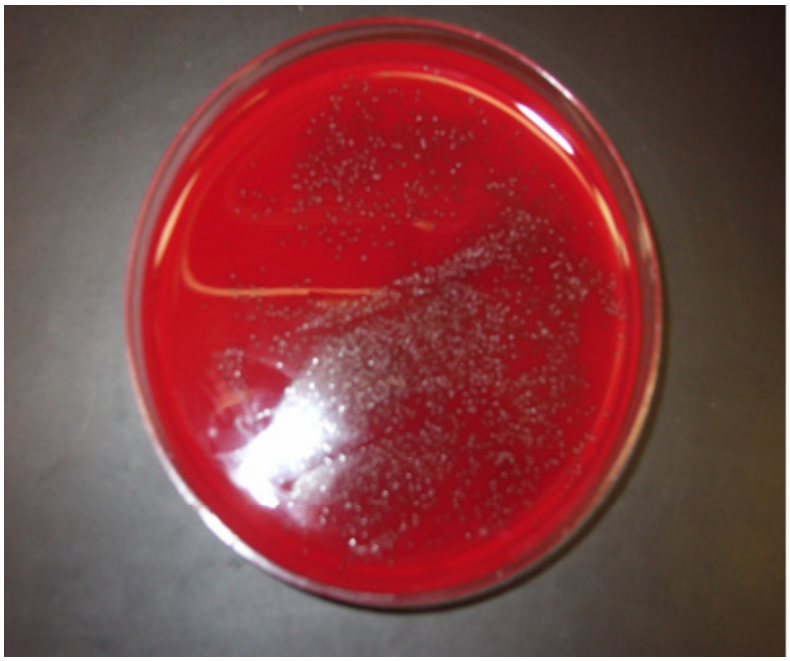
The pneumococcus is a part of the normal flora of the nasopharynx (AlonsoDeVelasco *et al.*, 1995; Kristinsson, 2000; Musher *et al.*, 2000). Carriage is estimated to occur in 40% of the population, resulting in no adverse side effects. Binding of the bacteria occurs via sialylated lactosamines, which are universally expressed by the host (Tuomanen and Masure, 2000). Various risk factors, such as immune status, age and underlying medical conditions such as diabetes, sickle cell anemia and HIV, can affect

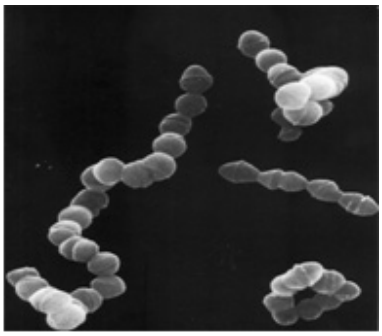
Figure 1. *Streptococcus pneumoniae* exhibit alpha hemolysis on blood agar.

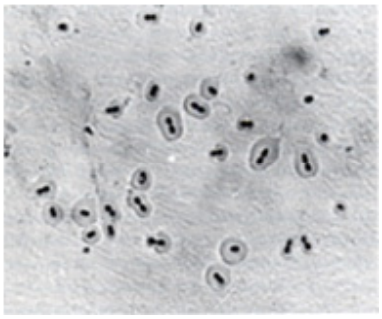
Left: The presence of a green zone is indicative of partial lysis of erythrocytes. This is due to the production of peroxide by the bacteria, which results in a greenish discoloration of the agar.

Right, top: An electron micrograph of chains of pneumococci (http://genome.microbio.uab.edu/strep/info/strep5.gif).

Right, bottom: Quellung reaction to determine the presence of a specific capsular type, or serotype (http://textbookofbacteriology.net/SpQuellung.jpeg). The addition of type-specific antibodies results in capsular swelling.







the outcome of this usually transient colonization (Kristinsson, 2000). Invasive disease, as illustrated in Fig. 2, occurs when the bacteria progress to the interstitium and alveoli of the lungs, the middle ear, or across the blood-brain barrier.

Virulence factors

Streptococcus pneumoniae possesses a number of virulence factors that facilitates the development of invasive disease. The polysaccharide capsule has been shown to be necessary for virulence since non-encapsulated strains are avirulent (Austrian, 1981). The capsule interferes with phagocytosis by preventing C3b opsonization of the bacteria (Musher *et al.*, 2000). Likewise, PspA, or pneumococcal surface protein A, also reduces complement-mediated clearance and phagocytosis (Yuste *et al.*, 2005) (Ren *et al.*, 2004a) (Musher *et al.*, 2000; Ren *et al.*, 2004b). PspA is a member of the choline binding protein family, which allows it to dock on phosphorylcholine (PC) moieties of teichoic (TA) and lipoteichoic acid (LTA) (Snapper *et al.*, 2001). PspA plays an important function by binding lactoferrin, thus preventing the *apo* form from binding the bacteria (Shaper *et al.*, 2004). Apolactoferrin depletes iron, restricting bacterial growth. The thick peptidoglycan layer, as well as TA and LTA, form a matrix that provides elasticity, strength and structure to the bacterium. PC, besides acting as an adhesin, also enables invasion by the bacteria. This occurs through binding to platelet activating factor receptors, which aids in transcellular migration (Tuomanen and Masure, 2000). Pneumolysin is an intracellularly stored toxin that is released upon bacterial lysis,

Figure 2. Steps leading to invasive disease.

Streptococcus pneumoniae is a natural colonizer of the nasopharynx (1). However, certain factors such as age and changes in immune status can cause the bacteria to spread to the brain, resulting in meningitis (2), the middle ear, leading to otitis media (3), the lungs, giving rise to pneumonia (4), and even into the bloodstream, causing septicemia (5). The immune system tries to control infection by eliciting neutrophils and macrophages to phagocytose the bacteria which have made their way to the interstitium and alveoli (Bergeron *et al.*, 1998), the middle ear (Giebink *et al.*, 1980), or the meninges (Takahata *et al.*, 2002). Key chemical mediators are released during this inflammatory process, such as IL-6, TNF, IL-1, and nitric oxide. Further infection into the bloodstream results in overwhelming systemic responses or septic shock, severe disruption of normal tissue architecture and cell counts, and can ultimately lead to death (Bergeron *et al.*, 1998).

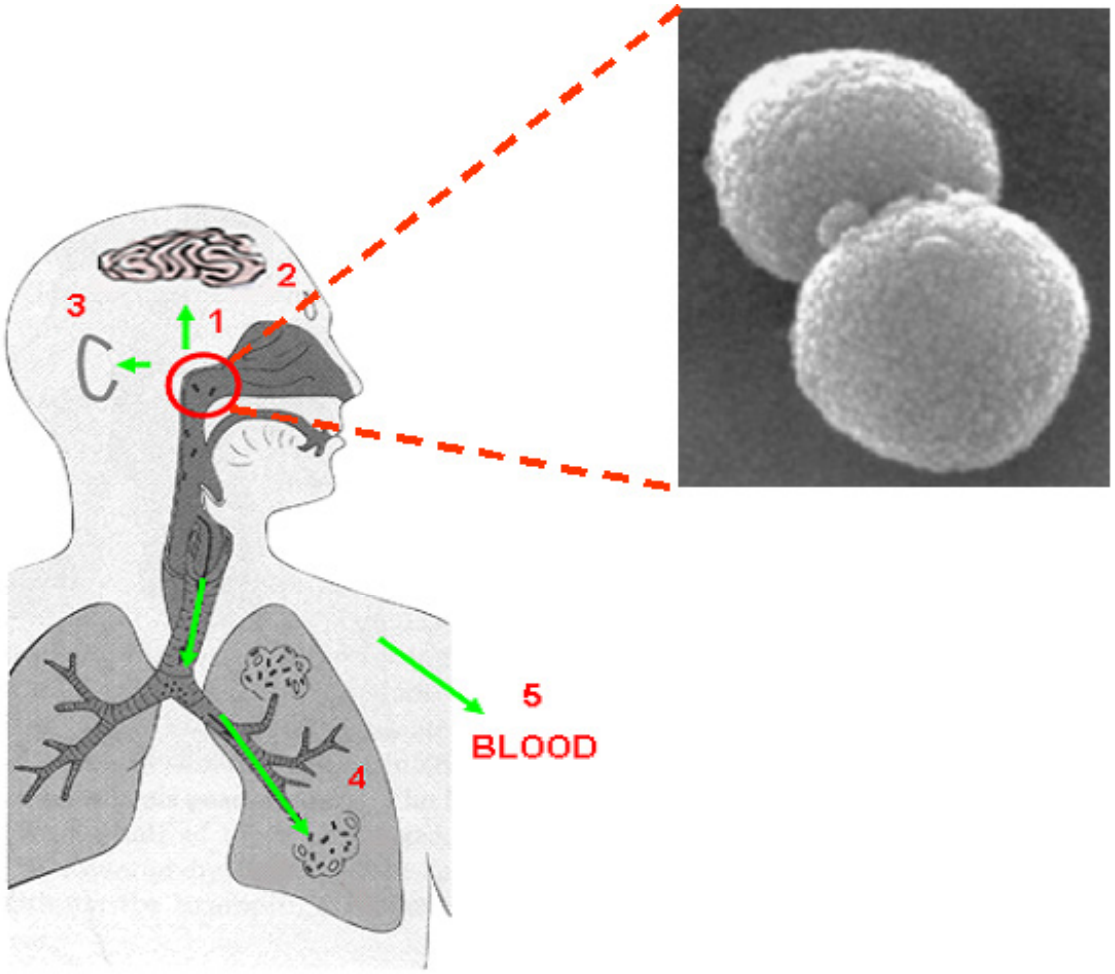
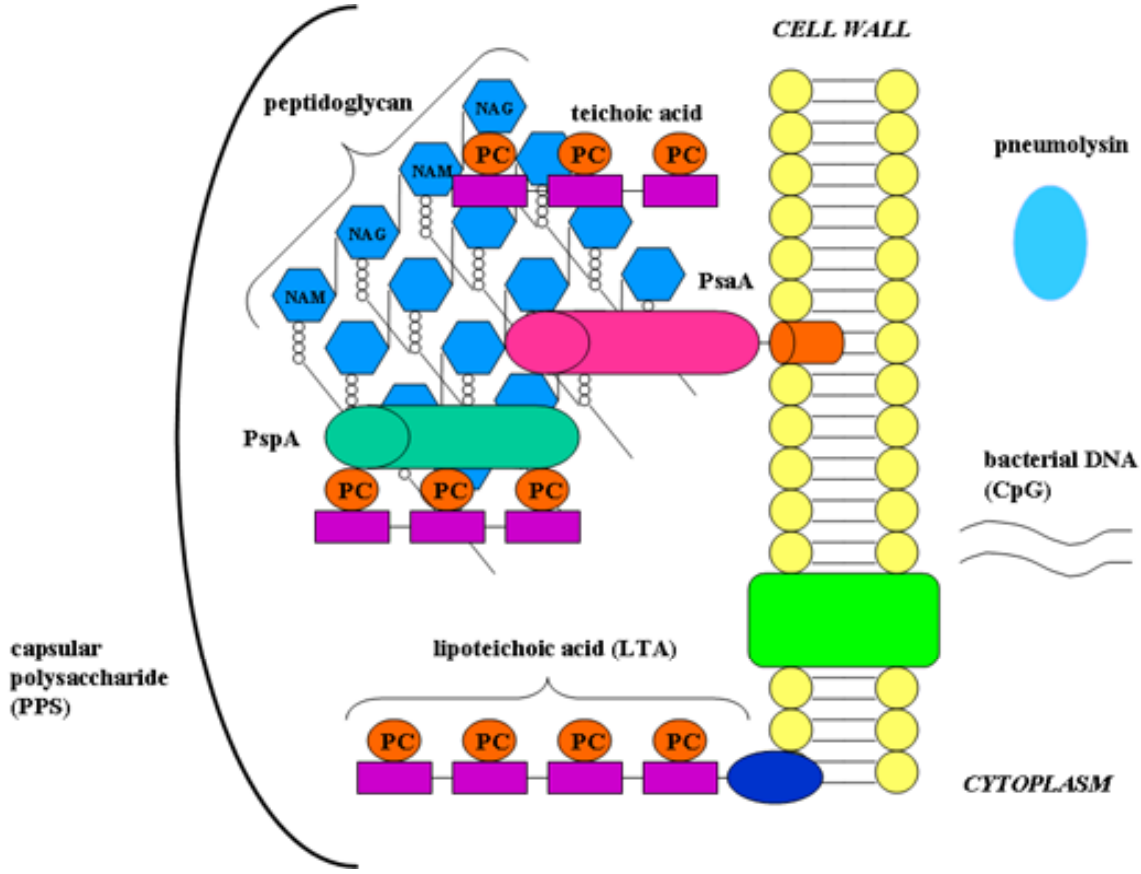

3
2
1
5
BLOOD
4

Figure 3. Important virulence factors of *Streptococcus pneumoniae.* Peptidoglycan, teichoic acid (TA), and lipoteichoic acid (LTA) provide strength and structure to the exterior surface of the bacteria. The capsular polysaccharide also acts as an outer barrier by enveloping the bacteria, and along with PspA, can prevent complement-mediated opsonization and clearance. PsaA as well as PC act as adhesins to allow initial attachment to the host cell. Additionally, PC can bind platelet activating factor receptors and facilitate transepithelial cell migration, furthering the ability of the organism to cause invasive disease. Pneumolysin, a secreted toxin with an affinity for cholesterol in host membranes, can inhibit the beating of cilia, leading to the development of pneumonia. Bacterial DNA contains many CpG motifs.


CELL WALL
peptidoglycan
NAG
teichoic acid
PC
PC
PC
NAM
NAG
NAM
PsaA
PspA
PC
PC
PC
pneumolysin
bacterial DNA
(CpG)
capsular
polysaccharide
(PPS)
lipoteichoic acid (LTA)
PC
PC
PC
PC
CYTOPLASM

binds cholesterol in the host cell membrane, and forms pores, ultimately resulting in cell death (Malley *et al.*, 2003). Pneumolysin can also inhibit the beating of cilia, aiding in transition to a more invasive disease outcome such as pneumonia (Steinfort *et al.*, 1989).

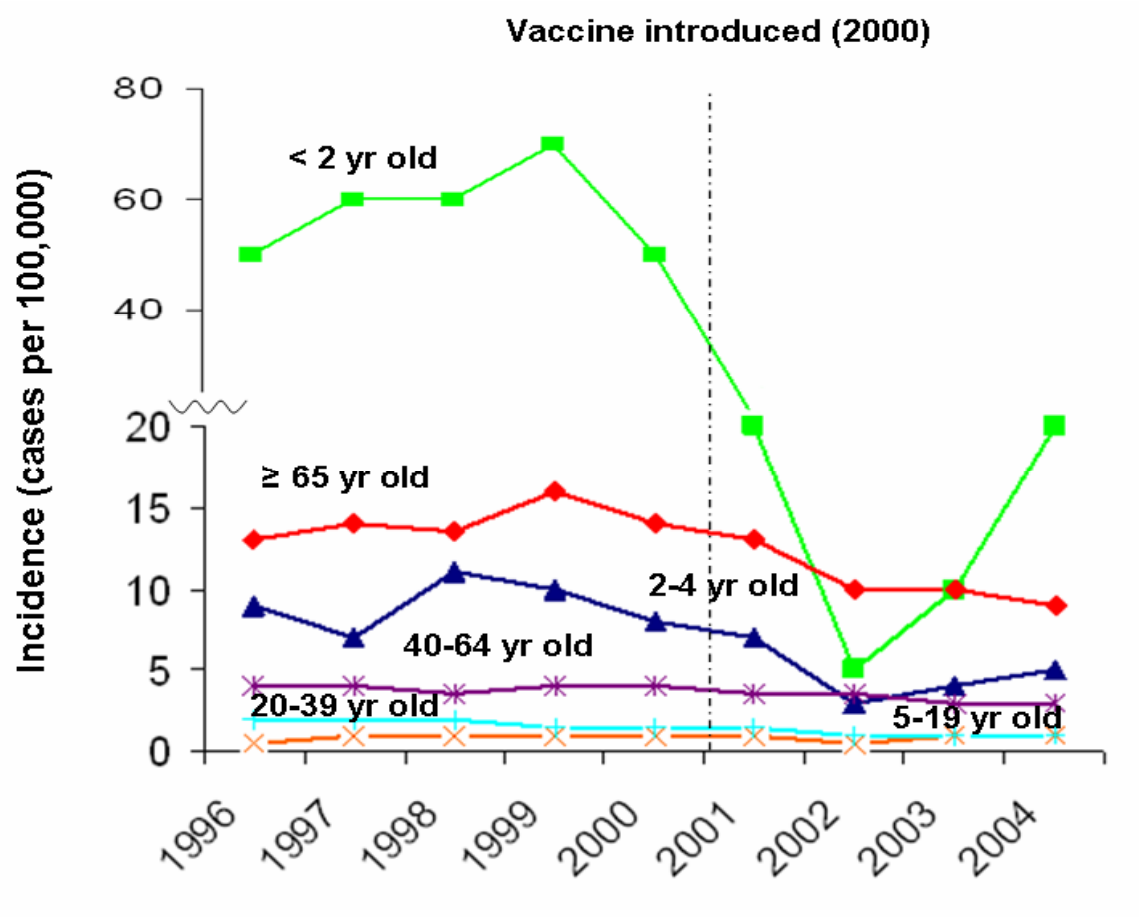
Protection against Streptococcus pneumoniae

In 1912, optochin treatment of pneumococcal-infected mice led to the first documented report *in vivo* of an antibiotic-resistant Pn strain (Klugman, 1990). Over the next few decades, there was an appearance of pneumococcal strains that were resistant to penicillin, erythromycin, trimethoprim-sulfamethoxazole, and tetracycline (Klugman, 1990; Whitney *et al.*, 2000). In 1977, Pneumovax I was developed by Merck Research Laboratories to help reduce the spread of 14 pneumococcal serotypes. Six years later, Merck updated the vaccine to the current 23-valent polysaccharide formulation (Pneumovax II), which represents approximately 90% of serotypes that cause invasive disease in the US (AlonsoDeVelasco *et al.*, 1995; Briles *et al.*, 2000; Kamerling, 2000). However, children under the age of two, a major target group of the bacteria (Jackson, 2002) (Klein, 2000), are unable to mount an effective response to polysaccharide antigens due to their inability to respond to T-independent (TI) antigens (Janeway *et al.*, 2001). This problem can be overcome by conjugating polysaccharides to proteins carriers, thus converting the response from a TI to a T-dependent (TD) antibody response. Even with the advent of the most recent heptavalent conjugate vaccine in 2000 (Butler, 2000; Klein, 2000), in which seven purified polysaccharides have been conjugated to diphtheria toxoid, the use of Prevnar in developing countries is impractical since it is more serotype-restricted than Pneumovax II, and expensive to manufacture (Briles *et al.*, 2000) (Malley

et al., 2005). Additionally, as shown in Fig. 4, although there was an initial decrease in the incidence of disease caused by penicillin-resistant pneumococcal strains in the few years following the introduction of Prevnar, the number of reported cases are once again on the rise (Kyaw *et al.*, 2006). Since over 90 different capsular types, or serotypes, of pneumococci exist (Kamerling, 2000), the task to formulate a comprehensive and protective vaccine has been very difficult. Thus, this fact and the increasing frequency of antibiotic-resistant strains (Kristinsson, 2000; McGee *et al.*, 2000) emphasizes an urgent need for better understanding the mechanisms of pneumococcal pathogenesis, with particular attention on the host response to the organism.

Figure 4. A rise in incidence caused by penicillin-resistant pneumococcal strains in children four years of age or younger despite the introduction of Prevnar.

The graph shows the number of cases of invasive pneumococcal disease identified from January 1, 1996, through December 31, 2004, in eight surveillance areas throughout the US by the Active Bacterial Core of the Centers for Disease Control. The population under surveillance was approximately 15 million, including nearly 500,000 children less than two years of age. The incidence of invasive disease, as identified by the presence of penicillin-resistant pneumococci isolated from normally sterile sites, such as blood, cerebrospinal fluid, or pleural fluid, initially decreased after the introduction of Prevnar in 2000. However, only two years later, an increase can be seen in children under the age of two (green) and 2-4 yr olds (blue), major target groups of the bacteria. The incidence in 5-19 yr olds (orange), 20-39 yr olds (aqua), 40-64 yr olds (purple), and 65 and older (red) appears to have remained steady. Adapted from (Kyaw *et al.*, 2006).


Vaccine introduced (2000)
Incidence (cases per 100,000)
80
60
40
20
15
10
5
0
< 2 yr old
≥ 65 yr old
2-4 yr old
40-64 yr old
20-39 yr old
5-19 yr old
1996
1997
1998
1999
2000
2001
2002
2003
2004

The Immune Response to *Streptococcus pneumoniae*

Overview

Cooperation between the two components of immunity, innate and adaptive, is crucial in the generation of protection against Pn (Fig. 5). Innate immunity is the arm of the immune response that occurs within hours of initial bacterial introduction, and is therefore, non-specific. It is comprised of physical, mechanical, and chemical barriers, as well as a cellular component. Recognition of conserved pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) found on cells such as macrophages and dendritic cells (DCs) results in a series of signaling events, leading to the production of numerous cytokines and chemokines. One of the major PRRs in the mammalian species is the Toll-like receptor (TLR) family of proteins, and will be further discussed below. The chemical mediators recruit and activate neutrophils and macrophages to kill and phagocytose the bacteria. DCs, which have specialized processing abilities, will migrate from to the periphery to secondary lymphoid organs and mature, resulting in their ability to efficiently prime T cells for adaptive responses. The interaction between DCs and T cells initiates the adaptive arm of the immune response, which occurs in an antigen-specific manner, and thus takes more time to develop. Activated T cells can facilitate B cells into becoming antibody-secreting plasma cells. It has long been regarded that adaptive immunity is crucial to the clearance of extracellular pathogens such as Pn (Janeway *et al.*, 2001; Kamerling, 2000). Antibodies can neutralize the bacterium by preventing it from binding its cellular target or opsonize it, thus promoting its uptake by phagocytes such as macrophages (Janeway *et al.*, 2001). Pneumococcal-targeted antibodies to both polysaccharide (PS) and protein antigens have

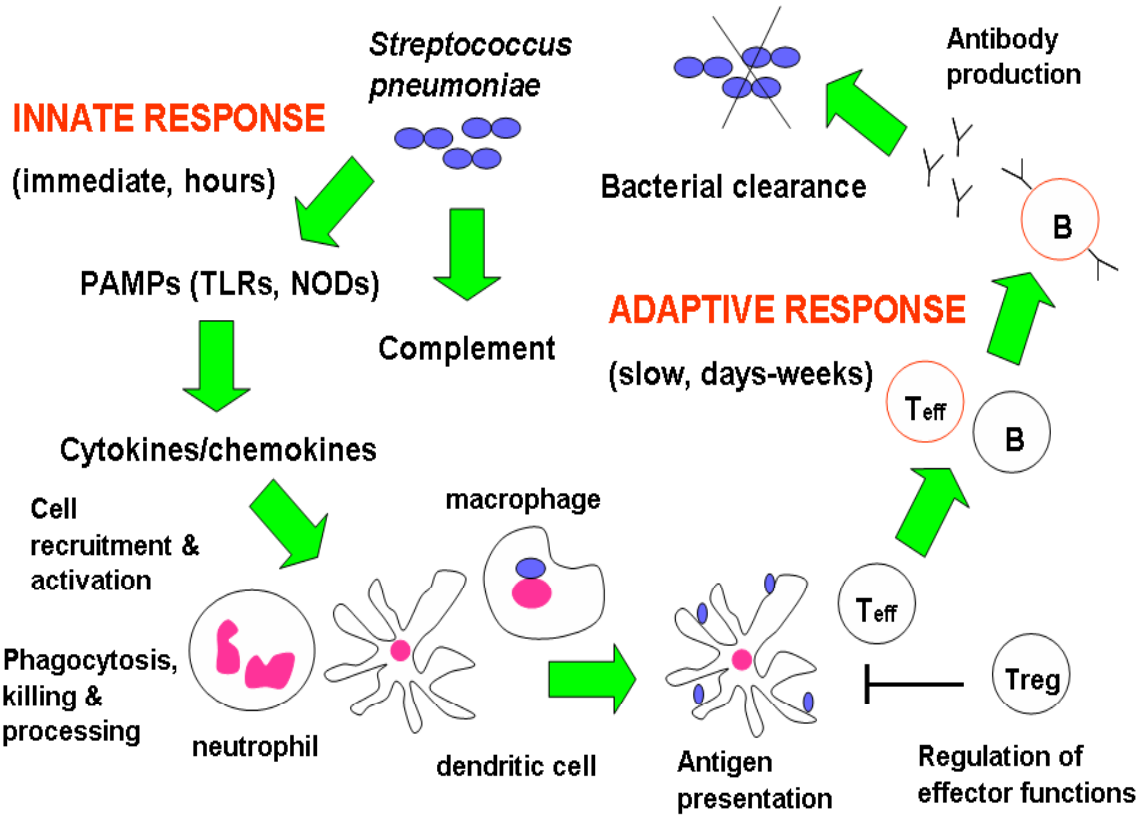
been shown to protect the host from infection with otherwise lethal Pn strains (AlonsoDeVelasco *et al.*, 1995). The binding of antibody to the bacterial surface can activate complement either through the classical or alternative pathways (Janeway *et al.*, 2001), and this activation is necessary to achieve effective clearance of the organism (Kamerling, 2000).

Distinct immunoglobulin (Ig) isotypes possess overlapping and unique effector functions on the basis of the particular Fc region expressed. Thus, the pattern of Ig isotypes elicited during a bacterial infection, in addition to the epitope specificity and affinity of the Ig, may impact on the level of protection afforded by such an antibody. IgG isotypes such as IgG3, IgG2b, and IgG2a, which are associated with Th1 (gamma interferon [IFN-γ]-dominant) immune responses, are particularly effective at mediating complement fixation and both complement- and Fc-mediated bacterial opsonophagocytosis (Germann *et al.*, 1995). In contrast, IgG1 elicited during Th2 (interleukin-4 [IL-4]-dominant) immune responses may serve a role in neutralizing pathogenic proteins without inducing complement activation and inflammation (Rijkers *et al.*, 1993).

Although it has been accepted that adaptive immunity to extracellular bacteria is largely conferred by antibody, a recent report by Malley, *et al*. provides evidence for an antibody-independent mechanism of protection from pneumococcal infection (Malley *et al.*, 2005). Their findings suggest that $CD4^+$ T cells were sufficient to induce immunity, which is supported by the observation that HIV-infected individuals are a higher risk of getting pneumococcal disease (Frankel *et al.*, 1996).

Figure 5. An overview of the immune response to extracellular bacteria such as *Streptococcus pneumoniae*.

The innate response, which is non-specific and occurs within hours of initial bacterial introduction, involves the activation of chemical barriers such as the alternative complement cascade, as well as the activation of various PRRs such as TLRs. As a result, proinflammatory cytokines and chemokines are produced, which leads to the recruitment and activation of neutrophils, macrophages and dendritic cells that aid in the killing, phagocytosis, and processing of the bacteria. At this point, the adaptive arm of the immune responses is initiated, when dendritic cells present antigen in the context of MHC to naïve T cells. These T cells become activated, and can further facilitate B cells that also recognize the same antigen though a process known as linked recognition. This interaction results in the activation of B cells, and allows their proliferation and development into antibody-producing plasma cells. Antibodies are crucial to the clearance of extracellular bacteria such as Pn. More recently, a specialized population of $CD4^+$ T cells, known as regulatory T cells (Tregs), have been reported to play an important role in the regulation of DC-T cell interactions.


Streptococcus pneumoniae
INNATE RESPONSE
(immediate, hours)
PAMPs (TLRs, NODs)
Complement
Cytokines/chemokines
Cell recruitment & activation
Phagocytosis, killing & processing
neutrophil
macrophage
dendritic cell
Antigen presentation
Teff
Treg
Regulation of effector functions
Teff
B
ADAPTIVE RESPONSE
(slow, days-weeks)
B
Antibody production
Bacterial clearance

A special population of $CD4^+$ T cells, known as the naturally occurring population of regulatory T cells (Tregs), plays an important role in the regulation of effector functions. They modulate and oversee the interactions between DCs and T cells to maintain homeostasis and tolerance to self.

Regulatory T cells

The naturally occurring population of $CD4^+CD25^+$ regulatory T cells (Tregs) was first described by Sakaguchi and his colleagues almost a decade ago and found to play a critical role in the maintenance of self-tolerance (Sakaguchi, 1995). They observed that adoptive transfer of thymocytes depleted of Tregs into athymic recipients resulted in the development of various autoimmune diseases such as gastritis, oophoritis, thyroiditis, sialoadenitis, adrenalitis, insulitis, and glomerulonephritis. Due to their broad specificity range, Tregs can control immune responses to both self and foreign antigens (Sakaguchi, 2004; Shevach, 2002).

Functionally, Tregs can downregulate both Th1 and Th2 immune responses (Xu *et al.*, 2003), and can inhibit the function of $CD8^+$ T cells (Suvas *et al.*, 2003), as well as $CD4^+$ T cells. The mechanism of Treg suppression appears to require direct physical contact between the Treg and the effector T cell, and can occur independently of IL-10 or TGF-β release, thus distinguishing Tregs from certain other suppressor T cell subpopulations (Sakaguchi, 2004; Shevach, 2002). Nevertheless, under some experimental conditions, the ability of Tregs to suppress immune responses *in vivo* may require their subsequent induction of these cytokine-secreting T suppressor cells, and some reports have shown that Treg themselves may express IL-10 or TGF-β (Annacker

et al., 2001; Asseman *et al.*, 1999; Nakamura *et al.*, 2001; Nakamura *et al.*, 2004). It is believed that Tregs, through their constitutive expression of CTLA-4, have also been shown to induce tolerogenic dendritic cells (DCs) via binding of B7 on the DC surface; such DCs in turn favor induction of new Tregs (Fallarino *et al.*, 2003; Mahmke, 2002). Recently, it has been reported that CTLA-4 ligation of B7 can lead to the upregulation of the catabolic enzyme indoleamine 2,3 dioxygenase (IDO) in DCs, causing a decrease in tryptophan levels (Mellor *et al.*, 2004). As a result, clonal expansion of effector T cells is inhibited due to their rapid apoptosis.

Tregs are generated in the thymus like effector $CD4^+$ T cells, but are different in that they express the gene Foxp3 (Fontenot *et al.*, 2003). Once in the periphery, Tregs account for 5-10% of the T cell subset. Tregs can be distinguished from naïve precursors of effector $CD4^+$ T cells by a characteristic phenotype that has functional relevance for mediating their suppressor activity under certain conditions (Sakaguchi, 2004; Shevach, 2002). In addition to CTLA-4, CD25 (IL-2Rα) is constitutively expressed on Tregs and serves as a useful marker for these cells, whereas CD25 expression is only induced on effector $CD4^+$ T cells after activation. IL-2 appears to be critical for Treg development and maintenance as IL-$2^{-/-}$ and IL-2Rα$^{-/-}$ mice are markedly deficient in this population (Sadlack *et al.*, 1993; Willerford *et al.*, 1995). Injection of anti-IL-2Rα mAb (PC61) (Lowenthal *et al.*, 1985) has been shown to selectively deplete Tregs *in vivo* and abrogate suppression (Shimizu *et al.*, 1999). Another molecule that is constitutively expressed on Tregs is glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Like CD25, GITR expression can be induced on activated effector $CD4^+$ T cells, as well as $CD8^+$ T cells, and at lower

levels on B cells and macrophages. More recently, the ligand for GITR was identified and shown to be selectively and constitutively expressed on antigen-presenting cells (Tone *et al.*, 2003). An agonistic GITR-specific mAb, DTA-1, can abrogate the suppressor activity of Tregs both *in vitro* and *in vivo* (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). Of interest, GITR can also serve as a costimulatory molecule for $CD4^+$ effector T cells (Tone *et al.*, 2003).

There have been a number of reports citing a role for Tregs in chronic cell-mediated immune responses such as those seen in autoimmunity (Uraushiharam *et al.*, 2003; Vasu *et al.*, 2003) (Mqadmi *et al.*, 2005), tumor immunity (Sakaguchi, 1995); Onizuka, 1999 #47; Shimizu, 1999 #46}, transplantation tolerance (Edinger *et al.*, 2003; Waldmann and Cobbold, 2001), and infections caused by *Plasmodium yoelii* (Hisaeda *et al.*, 2004), *Leishmania major* (Belkaid *et al.*, 2002), *Onchocerca volvulus* (Satoguina *et al.*, 2002), hepatitis B virus (HBV) (Stoop *et al.*, 2005), human immunodeficiency virus (HIV) and cytomegalovirus (CMV) (Aandahl *et al.*, 2004). Furthermore, the Treg population has been shown to influence humoral immune responses, such that in their absence, autoantibodies are generated (Sakaguchi, 2004; Shevach, 2002). This ability is further supported by observations that $CD4^+CD25^+$ T cells could inhibit the elicitation of anti-double-stranded DNA antibodies when administered to non-autoimmune mice (Seo *et al.*, 2002) or to lupus-prone mice (Bagavant and Tung, 2005). In addition, mice that are transgenic for both B and T cells elicit a hyper IgE response to the relevant foreign antigens, which is inhibited by transfer of $CD4^+CD25^+$ T cells (Curotto de Lafaille *et al.*, 2001). In another recent report, removal of Tregs increased the incidence of autoimmune

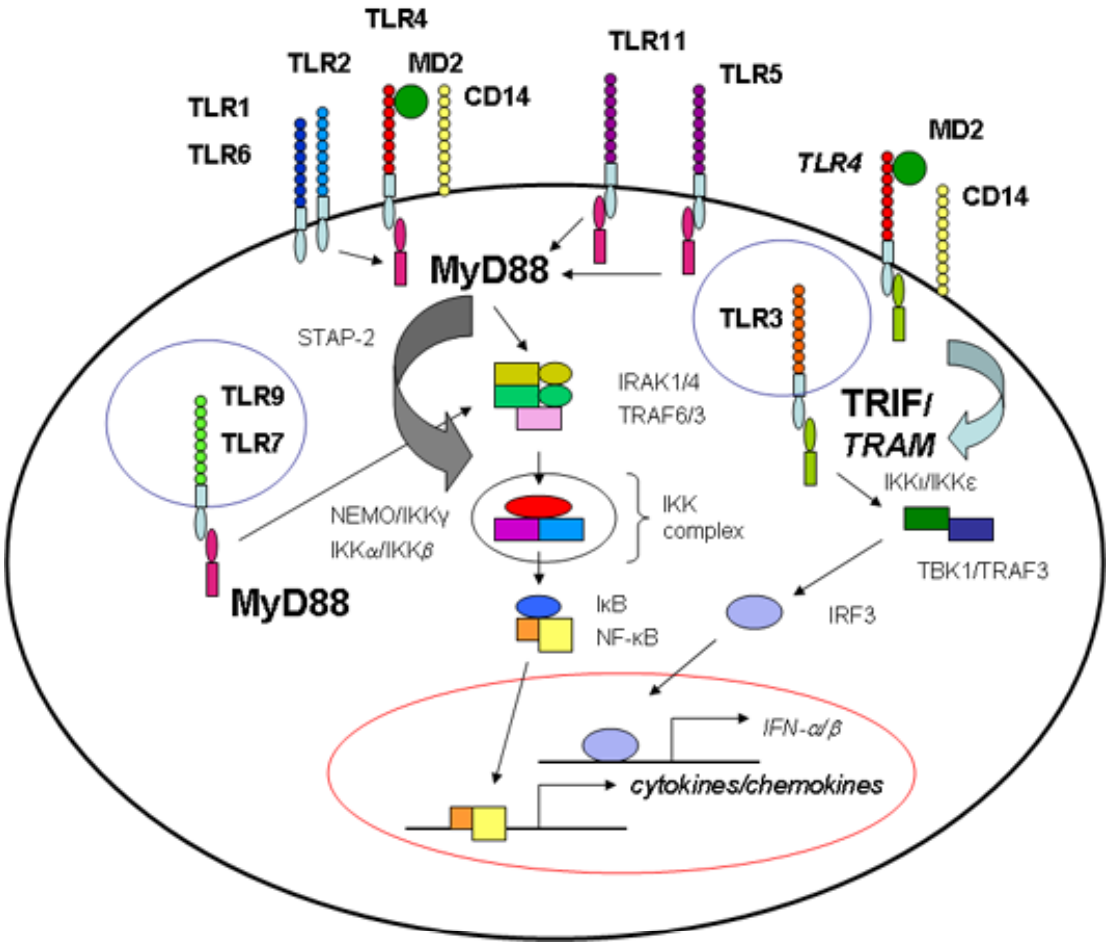
hemolytic anemia, an illness where autoantibodies destroy red blood cells (Mqadmi *et al.*, 2005).

Toll-like receptors

Toll-like receptors (TLRs) are members of the pattern recognition receptor (PRR) family known to recognize conserved moieties on pathogens, also known as pathogen associated molecular patterns or PAMPs (Janssens and Beyaert, 2003). First described in *Drosophila*, the Toll protein was initially shown to be important for dorsoventral patterning (Anderson *et al.*, 1985; Gerttula, 1988; Hashimoto *et al.*, 1988) and later recognized to play a role in the immune response of the adult fly (Lemaitre *et al.*, 1995; Lemaitre *et al.*, 1996). The eleven identified TLRs are single spanning type I transmembrane proteins located at either the cell surface (TLRs 2, 4 [along with 1 or 6], 5, 10, 11) or in endosomes (TLRs 3, 7, 8, 9) (Akira *et al.*, 2001; Nishiya and DeFranco, 2004; Parviz, 2002) (Fig. 6). They contain leucine rich repeats in the N terminus and a TIR (Toll/IL-1R) domain in the C terminus, named for the similarities shared with the mammalian IL-1R (O'Neill, 2000; O'Neill and Greene, 1998). TLRs can homodimerize as well as heterodimerize with other TLRs, and can interact with a number of different adapter molecules. Based on these characteristics, investigators hypothesized that TLRs might therefore play a role in the immune response of humans (Bowie and O'Neill, 2000; Medzhitov *et al.*, 1997; Rock *et al.*, 1998; Wang *et al.*, 1996). More specifically, TLRs were thought to act as a link between innate and adaptive immunity by translating nonclonal pattern recognition signals into clonal antigen-specific immune responses (Hoebe *et al.*, 2004; Pasare and Medzhitov, 2005b).

Figure 6. Toll-like signaling in the mouse.

The eleven identified TLRs are single spanning type I transmembrane proteins located at either the cell surface (TLRs 2, 4 [along with 1 or 6], 5, 10, 11) or in endosomes (TLRs 3, 7, 8, 9). TLR8 is not expressed in the mouse. All but TLR3 and TLR4 are completely dependent upon the adaptor protein MyD88 for signaling. TLR4 can utilize MyD88, Trif, or Tram for cell signaling, whereas TLR3-mediated signaling largely depends on Trif alone. Upon ligand binding, MyD88 is recruited to the C terminal domain of the TLR, resulting in a series of phosphorylation events that leads to the subsequent release of NF-κB into the nucleus and the transcription and production of various cytokines and chemokines. TLR-mediated activation via Trif results in phosphorylation and activation of IRF3 for induction of type I interferons.


TLR4
TLR2
MD2
TLR11
TLR5
TLR1
TLR6
CD14
MD2
TLR4
CD14
MyD88
TLR3
STAP-2
IRAK1/4
TRAF6/3
TRIF/
TRAM
TLR9
TLR7
IKKi/IKKε
NEMO/IKKγ
IKKα/IKKβ
IKK
complex
TBK1/TRAF3
MyD88
IκB
NF-κB
IRF3
IFN-α/β
cytokines/chemokines

TLRs can be divided into a MyD88 (myeloid differentiation factor 88)-dependent or independent signaling pathway (Akira *et al.*, 2000; Akira *et al.*, 2001; Vogel *et al.*, 2003) (Fig. 3). In the MyD88-dependent pathway, MyD88 is recruited to the C terminal TIR domain of the TLR, resulting in the recruitment of IRAK proteins and TRAF6 to its N terminal death domain. Activation of this complex consequently activates the IKK complex, which results in the degradation of IκB. This causes the subsequent release of NFκB into the nucleus and the transcription and production of various chemokines and cytokines (Muzio *et al.*, 1997; Muzio *et al.*, 1998; Wesche *et al.*, 1997). Of all the TLRs identified to date, all but TLR3 are MyD88-dependent. Rather, TLR3 signals through the adaptor TRIF (Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2003a), also known as TICAM-1. This pathway depends on the kinases IKKι/IKKε and TBK1 (McWhirter *et al.*, 2004; Youn *et al.*, 2005), which phosphorylate IRF3 and allows its translocation into the nucleus, leading to the subsequent transcription of genes such as type I interferons. TLR4 can also utilize a MyD88-independent pathway through TRIF, and a unique MyD88-independent pathway through the adaptor TRAM (Fitzgerald *et al.*, 2003; Yamamoto *et al.*, 2003b). A recent finding provides evidence that signal-transducing adaptor protein-2 (STAP-2) plays a role in TLR4 signaling by binding MyD88 and IKKα/IKKβ, forming a functional complex that enhances NFκB activity (Sekine *et al.*, 2006). Additionally, TRAF3 was identified and shown to be recruited along with TRAF6 in MyD88-dependent signaling pathways, and also important for the organization of TBK1, and possibly IKKι, into TIR signaling complexes in TRIF-dependent, MyD88-independent pathways (Hacker *et al.*, 2005).

TLRs have been reported to be found on a number of cell types beside dendritic cells and macrophages. There are a number of publications that suggest a role for these proteins on $CD4^+$ T cells (Crellin *et al.*, 2005) (Caron *et al.*, 2005), including Tregs (Caramalho *et al.*, 2003; Sutmuller *et al.*, 2006). Additionally, B cells also express TLRs (Dasari *et al.*, 2005) (Peng, 2005) (Pasare and Medzhitov, 2005a). Although the role of TLRs on these two cell types is not fully understood, findings suggest that expression of TLRs on T cells may play an important function in regulating Treg suppressive activity (Pasare and Medzhitov, 2003) (Crellin *et al.*, 2005). On B cells, TLRs may be necessary for the development of T-dependent antibody responses (Pasare and Medzhitov, 2005a).

TLRs recognize a variety of conserved moieties on pathogens (PAMPs). These can range from dsRNA such as poly(I:C) expressed by viruses to profilin expressed by uropathogenic bacteria. TLRs 2/6 recognize lipoteichoic acid and zymosan, while TLRs 1/2 recognize lipopeptides and GPI-anchored proteins. TLR3 recognizes dsRNA, while LPS is recognized by TLR4. Flagellin binds TLR5, and TLR7 and TLR8 (functional only in humans) interact with ssRNA and small synthetic molecules such as imiquimod and loxoribine. Bacterial DNA, or unmethylated CpG-ODN sequences, is recognized by TLR9, and most recently, TLR11, expressed only in mice, is important for recognition of uropathogenic bacteria (Zhang *et al.*, 2004) and profilin, an actin-binding protein of *Toxoplasma gondii* ((Lauw *et al.*, 2005). TLR10, which is only expressed in humans (Chuang and Ulevitch, 2001), specifically on lung and immune cells, may play a potential role in asthma (Lazarus *et al.*, 2004). It can homodimerize as well as heterodimerize with TLR1 and TLR2, and is believed to signal through MyD88 (Hasan *et al.*, 2005).

There are a number of TLR ligands that are of particular importance in the context of a pneumococcal infection. The MyD88-dependent TLRs which may play an important role in the recognition of Pn are TLR2, TLR4, and TLR9. TLR2 recognizes lipoteichoic acid and peptidoglycan (in conjunction with CD14) expressed on the bacterial surface (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999), while TLR9 binds CpG DNA which becomes exposed as the bacteria is endocytosed and processed (Hacker *et al.*, 2000; Hemmi *et al.*, 2000), respectively. More recently, Malley, *et al*. described pneumolysin, to be a ligand for TLR4 (Malley *et al.*, 2003).

Our Model in the Lab

Because mice exhibit symptoms and responses similar to that seen in humans, we utilize them to study systemic infection (Frimodt-Moller *et al.*, 1986; Schiffman, 1981; Shinefield *et al.*, 1966). While there are a number of prevalent Pn serotypes in circulation that cause invasive disease, we have chosen to use serotype 14, or Pn14, in our lab. Because the LD_{50} is higher than some other circulating serotypes, it is extremely useful in our murine model. Doses of live bacteria as high as 10^7 do not cause lethality in wild-type mouse, thus allowing us to study the immune aspect of the infection. Pn14 is also one of the more common serotypes that are found in carriers and reported to cause disease (Kristinsson, 2000). Although we use heat-killed Pn (hk Pn) for most of our studies, it still serves as a useful immunogen for the elicitation of an immune response, which is primarily the focus of our interests. Additionally, when we look at *in vivo* effects of the bacteria, we administer the inoculum via an intraperitoneal (i.p) route versus an intranasal, intrathoracic or intrapulmonary route. This route of infection stimulates a systemic, rather than, a mucosal response. Importantly, the i.p. route serves as a model for systemic infection, which is relevant to an organism that can cause bacteremia.

Since what is known about Ig responses focuses mainly on soluble antigens, we wanted a better appreciation and understanding of responses to an intact pathogen. Using hk Pn14, we are able to study responses to the major antigenic factors expressed by the bacteria, such as the capsular polysaccharide, the PC determinant of teichoic acid, also known as the cell wall C-polysaccharide, and PspA (Fig. 3). In summary, we have found

that anti-polysaccharide responses are regulated differently than anti-protein responses. We have shown that the anti-PC response occurs more rapidly than the anti-PspA response. Specifically, anti-PC titers are first seen on day 4 and peaks at day 6, while anti-PspA titers are first seen on day 6 and peak on day 10 (Wu *et al.*, 1999). Additionally, only PspA-specific memory is generated. We also found that while both anti-PC and anti-PspA require $CD4^+$ TCR-$\alpha\beta^+$ T cells, the anti-PC response occurs in a TCR-nonspecific manner and in the absence of germinal center formation, which is consistent with the observed lack of PC-specific memory (Wu *et al.*, 2002). Later, we showed that IgG, but not IgM, anti-capsular responses (anti-PPS), like anti-PspA responses, are $CD4^+$ T cell dependent and TCR specific (Khan *et al.*, 2004). However, in contrast to the anti-PspA response, we did not observe any apparent memory, but did see accelerated kinetics of primary Ig induction and a more rapid delivery of $CD4^+$ T cell with the anti-PPS response.

Hypotheses and Specific Aims

The goal of this project is to gain a better appreciation and understanding of the host response to *Streptococcus pneumoniae* (Pn). This includes both the innate as well as adaptive aspects of the immune response, and how the two arms are linked. The hypotheses and specific aims are as follows:

Hypothesis: **Regulatory T cells (Tregs) play a role in modulating the acute humoral immune response to intact Pn.**

We previously reported that both *in vivo* protein- and polysaccharide (PS)-specific IgG responses to intact *Streptococcus pneumoniae* (Pn) were dependent on TCR-α/β^+, $CD4^+$ T cell help (Khan *et al.*, 2004; Wu *et al.*, 1999), and could be elicited by adoptive transfer of Pn-pulsed dendritic cells (Colino *et al.*, 2002). We further demonstrated that induction of both anti-protein and anti-PS Ig responses to Pn *in vivo* was inhibited by endogenous IL-10, and enhanced by endogenous pro-inflammatory cytokines such as IL-12, IL-6, TNF-α, and IFN-γ (Khan *et al.*, 2002). Collectively, these data led us to examine a potential role for $CD4^+CD25^+$ regulatory T cells in limiting the T cell-dependent (TD) IgG anti-protein and anti-PS responses to intact Pn *in vivo*. Specifically, we would like to: 1) determine the effect of Treg depletion on primary and secondary anti-protein and anti-polysaccharide responses to Pn, 2) determine whether adoptive transfer of Treg-depleted $CD4^+$ T cells would enhance primary antibody responses to Pn, and 3) determine whether removal of Tregs in MyD88-deficient mice would enhance primary antibody responses to Pn.

Hypothesis: **TLR2, TLR4 and TLR9 mediate MyD88-dependent innate immunity to intact Pn**

We have shown previously that MyD88-deficient ($MyD88^{-/-}$) mice are unable to mount an appropriate innate response upon intra-peritoneal (i.p.) challenge with heat-killed *Streptococcus pneumoniae* type 14 (hk Pn14) which we characterized by splenic cytokine protein release and, consequently, the mice succumbed to infection from an i.p. injection of live Pn14 (Khan *et al.*, 2005). However, in TLR2-deficient ($TLR2^{-/-}$) mice, normal induction of cytokines was observed, which correlated well with their ability to survive an i.p. challenge with live Pn14 (Khan *et al.*, 2005). These findings prompted us to determine what MyD88-dependent TLRs, in addition to TLR2, are important for the elicitation of innate cytokines and protective immunity to Pn. More specifically, we wanted to: 1) observe the effects of single and double knockouts of the three TLRs on *in vitro* cytokine production and 2) determine whether mimicking a triple knockout recapitulates the MyD88 phenotype, both *in vitro* and *in vivo*.

Chapter 2

The role of regulatory T cells in the humoral response to *Streptococcus pneumoniae*

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Abstract

IgG anti-protein and anti-polysaccharide responses to intact *Streptococcus pneumoniae* (Pn) are $CD4^+$ T cell-dependent, and therefore might be under the negative control of $CD4^+CD25^+$ regulatory T cells (Tregs). Injection of anti-IL-2Rα mAb to deplete Tregs, agonistic anti-GITR mAb to inhibit Treg function, or adoptive transfer of Treg-depleted $CD4^+$ T cells into athymic nude mice, each had no effect on either the primary or secondary protein- or polysaccharide-specific IgG response to intact Pn. Surprisingly, anti-IL-2Rα mAb also had no effect on the IgG response to intact Pn in $MyD88^{-/-}$ mice or to a soluble protein-polysaccharide conjugate injected into wild-type mice in the absence of adjuvant. Collectively, these data are the first to suggest that, in contrast to their role in limiting chronic, cell-mediated immunity, Tregs may play no significant role in an acute humoral immune response to an intact extracellular bacterial pathogen.

Introduction

Endogenous $CD4^+CD25^+$ T regulatory cells (Tregs) account for 5-10% of peripheral $CD4^+$ T cells, and due to their broad range of antigen specificities, can limit immune responses to many different self, as well as, foreign antigens (Sakaguchi, 2004; Shevach, 2002). Although many publications have described a role for Tregs in down-regulating chronic cell-mediated immune responses such as those seen in autoimmunity (Uraushiharam *et al.*, 2003; Vasu *et al.*, 2003), tumor immunity (Onizuka *et al.*, 1999; Sakaguchi, 1995; Shimizu *et al.*, 1999), transplantation tolerance (Edinger *et al.*, 2003; Waldmann and Cobbold, 2001), and infections caused by *Plasmodium yoelii* (Hisaeda *et al.*, 2004), *Leishmania major* (Belkaid *et al.*, 2002), *Onchocerca volvulus* (Satoguina *et al.*, 2002), human immunodeficiency virus (HIV) and cytomegalovirus (CMV) (Aandahl *et al.*, 2004), very little is known regarding a potential role for Tregs in an acute humoral response to an extracellular bacteria.

The potential for Tregs to influence humoral immune responses is suggested by the emergence of autoantibodies in the absence of a functional Treg population (Sakaguchi, 2004; Shevach, 2002) and the observation that Tregs could inhibit the elicitation of anti-double-stranded DNA antibodies when co-administered with $CD4^+$ T helper cells to non-autoimmune mice (Seo *et al.*, 2002). In addition, immunization of mice expressing transgenes for both specific B and T cell antigen receptors with the relevant, linked foreign antigens, elicited a hyper IgE response that was inhibited by transfer of Tregs (Curotto de Lafaille *et al.*, 2001). Finally, FoxP3 transgenic mice over-expressing scurfin, a protein implicated in inducing the Treg phenotype (Fontenot *et al.*,

2003; Khattri *et al.*, 2003), showed a markedly reduced TNP-specific Ig response to TNP-KLH in CFA/IFA (Kasprowicz *et al.*, 2003).

CD25 (IL-2Rα) is constitutively expressed on Tregs. Injection of anti-IL-2Rα mAb (PC61) (Lowenthal *et al.*, 1985) has been shown to selectively deplete Tregs *in vivo* and abrogate suppression (Shimizu *et al.*, 1999). Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is also constitutively expressed on Tregs (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). An agonistic GITR-specific mAb, DTA-1, can abrogate the suppressor activity of Treg both *in vitro* and *in vivo* (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). GITR expression can be induced on activated effector $CD4^+$ T cells, where it can act as a costimulatory molecule (Tone *et al.*, 2003).

We previously reported that both *in vivo* protein- and polysaccharide (PS)-specific IgG responses to intact *Streptococcus pneumoniae* (Pn) were dependent on TCR-α/β^+, $CD4^+$ T cell help (Khan *et al.*, 2004; Wu *et al.*, 1999). These data led us to examine a potential role for Tregs in limiting the T cell-dependent (TD) IgG anti-protein and anti-PS responses to intact Pn *in vivo*. To our knowledge, this is the first report to explore the potential role of Tregs in an acute humoral response to an intact extracellular bacterium *in vivo*.

Materials and Methods

The preparation of Pn capsular type 14 (Pn14), soluble conjugates of PPS14-PspA and C-PS-PspA, and other reagents used in this study have been described by us previously (Khan *et al.*, 2004). Rat IgG2a anti-mouse GITR mAb (clone DTA-1) (Shimizu *et al.*, 2002), a kind gift from Dr. Shimon Sakaguchi (Kyoto University, Kyoto, Japan), rat IgG1 anti-mouse CD25 (IL-2Rα) mAb (clone PC61) (Lowenthal *et al.*, 1985), purchased from the ATCC, and isotype mAb controls (rat IgG2a anti-E. coli β-galactosidase [clone GL117] and rat IgG1 anti-*E. coli* β-galactosidase [GL113]), kind gifts of Dr. Fred D. Finkelman (U. Cincinnati Medical Center, Cincinnati, OH) were purified from ascites by ammonium sulfate precipitation and passaged over a protein G column. DTA-1-biotin was kindly provided by Dr. Ethan Shevach (N.I.H., Bethesda, MD).

Determination of antigen-specific serum titers of various Ig isotypes by ELISA, magnetic bead cell sorting, flow cytometry, adoptive transfer studies, and statistical analysis, were also performed as described previously (Khan *et al.*, 2004; Wu *et al.*, 2002). Female BALB/c and athymic nude mice were purchased from the National Cancer Institute (Frederick, MD). Mice were used between 6 and 8 wk of age and were maintained in a pathogen-free environment at the Uniformed Services University of the Health Sciences (Bethesda, MD). MyD88$^{-/-}$ mice were obtained from Dr. S. Akira (Osaka U., Osaka, Japan) and bred and genotyped in our facility (Khan *et al.*, 2005). For spleen cell proliferation *in vitro*, spleen cells (1 x 10^6/ml) were treated with varying

concentrations of either GL117 (control mAb) or DTA-1 for various times and incorporation of [^{3}H] thymidine (1 μCi/well) was measured during the last 6 hr of culture.

Results and Discussion

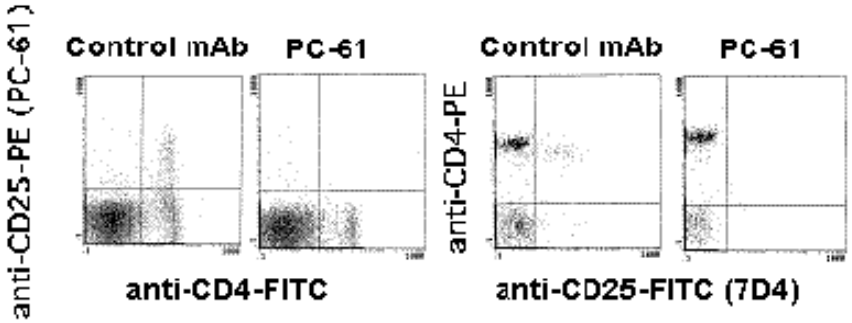
Treatment with anti-IL-2Rα mAb (PC61), to selectively deplete $CD4^+CD25^+$ T cells (Tregs), has no effect on the humoral response to varying doses of live intact Pn14. Administration of PC61, an anti-IL-2Rα mAb, results in the selective depletion of Tregs (Lowenthal *et al.*, 1985). Flow cytometric analysis was performed using spleen cells from mice treated with either PC61 or control mAb, GL113 and stained with anti-CD4, and either PC61 or another anti-CD25 mAb, 7D4, that recognizes a different epitope of CD25. In accordance with other studies (Shimizu *et al.*, 1999), we found that Tregs were markedly reduced when PC61 was given 1 day prior to immunization with live Pn capsular type 14 (Pn14) (Fig. 7A). We injected 3 doses of live Pn14 (5×10^6, 1×10^7, or 5×10^7 CFU per mouse) i.p. that led to induction of varying serum titers of anti-PPS14 (capsular polysaccharide type 14), anti-PC (phosphorylcholine determinant of C-polysaccharide [C-PS]), and anti-PspA (pneumococcal surface protein A). PC61 or isotype-matched control mAb, GL113, was injected 16 h prior to immunization and sera were collected on day 0 (pre-bleed), day 7 (anti-PPS14 and anti-PC) and day 14 (anti-PspA). As illustrated in Fig. 7B, PC61 had no significant effect on the primary serum titers of IgG anti-PPS14, anti-PC or anti-PspA relative to mice treated with GL113, at any of the 3 immunization doses used.

Treatment with an agonistic anti-GITR mAb (DTA-1) does not alter the *in vivo* anti-protein or anti-polysaccharide IgG isotype response to heat-killed intact Pn14. Agonistic anti-GITR mAb (DTA-1) inhibits Treg function both *in vitro* and *in*

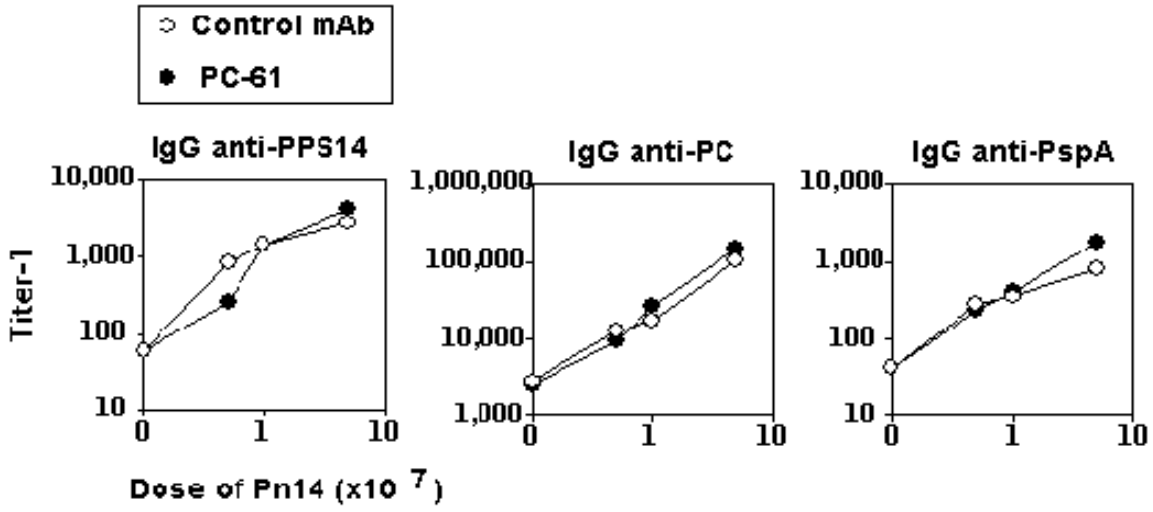
Figure 7. PC61 treatment does not alter the humoral response to various doses of *Streptococcus pneumoniae* capsular type 14.

(A) Flow-cytometric analysis of spleen cells from mice treated with 1 mg PC61 i.p. 1 day prior to sacrifice. Left two panels, spleen cells from control (GL113) and PC61-treated mice stained with anti-CD4-fluorescein isothiocyanate (FITC) and anto-CD25-PE (PC61); right two panels, spleen cells from control and PC61-treated mice stained with anti-CD25-FITC (7D4) and anti-CD4-PE. (B) Seven mice per group were injected i.p. with either 1 mg PC61 or 1 mg GL113 and 16h later with the respective dose of live *S. pneumoniae* capsular type 14. Sera were obtained on days 0, 7, and 14 for determination of antigen-specific IgG titers by ELISA. Data are presented as serum titers of anti-PPS14 and anti-PC (day 7) and titers of anti-PspA (day 14). Values are arithmetic means ± standard errors of the means. Data from one of three representative experiments are shown.

A.


Control mAb
PC-61
Control mAb
PC-61
anti-CD25-PE (PC-61)
anti-CD4-PE
anti-CD4-FITC
anti-CD25-FITC (7D4)

B.

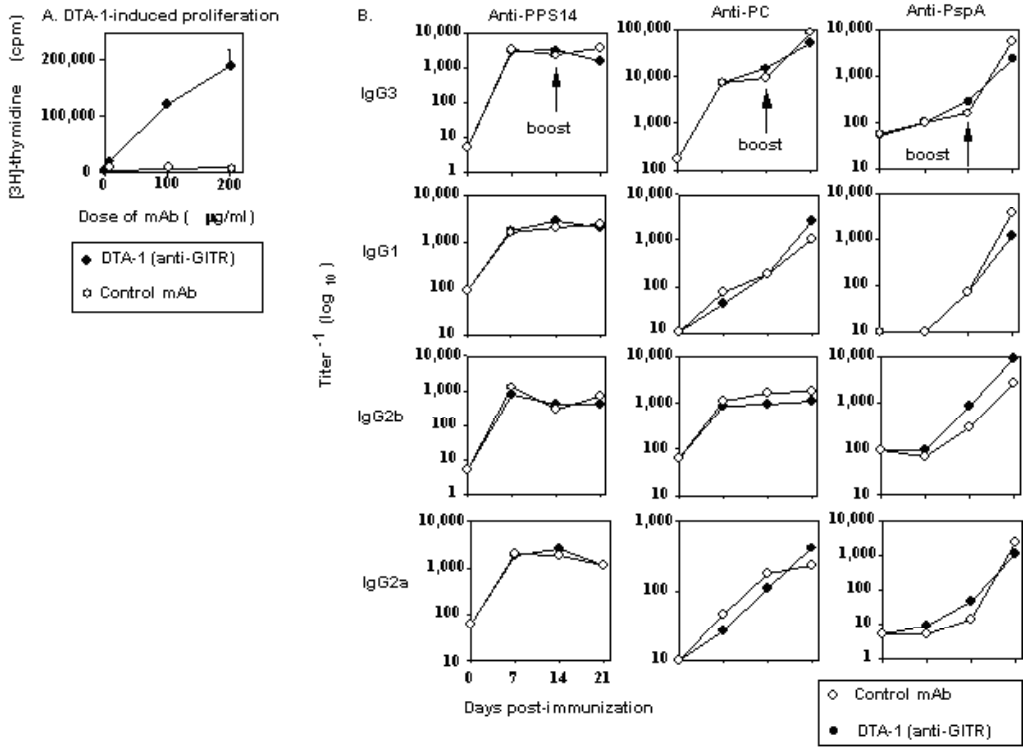

Control mAb
PC-61
IgG anti-PPS14
IgG anti-PC
IgG anti-PspA
Titer-1
10,000
1,000
100
10
1,000,000
100,000
10,000
1,000
0
1
10
Dose of Pn14 (x10 7)

vivo (McHugh *et al.*, 2002; Shimizu *et al.*, 2002). We confirmed that our purified unlabeled DTA-1 preparation was capable of binding to GITR by demonstrating that it specifically blocked staining of activated T cells with biotin-labeled DTA-1 (+ avidin-PE), the latter kindly provided by Dr. Ethan Shevach (NIH, Bethesda, MD) (data not shown). Purified DTA-1 was also confirmed by ELISA to be a rat IgG2a antibody as previously reported (McHugh *et al.*, 2002; Shimizu *et al.*, 2002) (data not shown). Finally, we confirmed *in vitro* that DTA-1 had functional, anti-suppressor activity by demonstrating spontaneous proliferation of spleen cells *in vitro* upon addition of DTA-1 but not with the isotype control, GL117 (Fig. 8A), as previously reported (Shimizu *et al.*, 2002). We next immunized mice i.p. with 2 x 10^8 CFU heat-killed Pn14, 16h after i.p. injection of 1 mg each of either control mAb, GL117 or DTA-1, and boosted with an equal amount of Pn14 alone on day 14. Sera were obtained prior to immunization, and 7, 14, and 21 days later. Administration of DTA-1 had no significant effect on the primary or secondary PspA-, PC-, or PPS14-specific IgM (data not shown) or IgG isotype responses to Pn14 as compared to control GL117 (Fig. 8B) or saline (data not shown). Injecting a combination of PC61 and DTA-1 at the time of primary immunization with heat-killed Pn14 also had no significant effect on the primary or secondary humoral response (data not shown). Further, DTA-1 given only at the time of secondary immunization with heat-killed Pn14, had no affect on the elicitation of the memory IgG response in mice previously primed with Pn14 alone (data not shown).

Adoptive transfer of splenic $CD4^+CD25^-$ T cell into athymic nude mice restores the IgG anti-PspA, anti-PC, and anti-PPS14 responses to heat-killed, intact

Figure 8. DTA-1 treatment does not alter the primary or secondary humoral immune response to heat-killed *Streptococcus pneumoniae* capsular type 14.

(A) Spontaneous proliferation of spleen cells (1 x 10^6/ml) in the presence of various concentrations of control MAb (GL117) or DTA-1. (B) Seven mice per group were injected i.p. with either 1 mg DTA-1 or 1 mg control MAb (GL117), and 16h later with 2 x 10^8 CFU of heat-killed *S. pneumoniae* capsular type 14 i.p. Mice were boosted on day 14 with a similar dose of heat-killed *S. pneumoniae* capsular type 14. Sera were obtained on days 0, 7, 14, and 21 for determination of antigen-specific Ig isotype titers by ELISA. Values are arithmetic means ± standard errors of the means. Data are from one of three experiments.


A. DTA-1-induced proliferation
[3H]-thymidine (cpm)
200,000
100,000
0
0
100
200
Dose of mAb (μg/ml)
DTA-1 (anti-GITR)
Control mAb
B.
Anti-PPS14
Anti-PC
Anti-PspA
IgG3
IgG1
IgG2b
IgG2a
boost
Titer -1 (log 10)
10,000
1,000
100
10
1
0
7
14
21
Days post-immunization
Control mAb
DTA-1 (anti-GITR)

Pn14 to levels no greater than that observed using total $CD4^+$ T cells. We previously reported that adoptive transfer of $CD4^+$ T cells into Pn14-immunized, T cell-deficient, athymic nude mice, could restore the IgG responses specific for PspA, PC, and PPS14 (Khan *et al.*, 2004). To directly test a potential role for $CD4^+CD25^+$ T cells in suppressing a Pn14-induced humoral response, spleen cells from wild-type mice were magnetically sorted into two populations, $CD4^+$ (both $CD25^+$ + $CD25^-$) or $CD4^+CD25^-$ (Fig. 9A), and then adoptively transferred into athymic recipients 16h prior to immunization with Pn14. Seven and 14 days after immunization, sera were collected to measure PC-, PspA-, and PPS14-specific IgG titers. Figure 9B shows that the removal of Tregs had no effect on the $CD4^+$ T cell-mediated enhancement of IgG titers specific for PPS14, PC, or PspA.

Treatment with anti-IL-2Rα mAb (PC61), to deplete Tregs, has no effect on the humoral response to heat-killed, intact Pn14 in $MyD88^{-/-}$ mice or to soluble protein-polysaccharide conjugates injected in the absence of adjuvant. Intact Pn expresses a number of ligands for Toll-like receptors (TLRs) (Schwandner *et al.*, 1999). TLR-mediated immune activation has been shown to abrogate Treg activity, thus allowing for initial induction of immunity (Pasare and Medzhitov, 2003, 2005b). We previously demonstrated that $MyD88^{-/-}$ mice are markedly defective in their innate immune response to Pn and elicit a striking reduction in type 1 IgG isotypes (IgG3, IgG2b, and IgG2a) specific for PPS14, PC, and PspA (Khan *et al.*, 2005). We thus reasoned that Treg activity would still be operative in Pn-immunized $MyD88^{-/-}$, but not wild-type mice, and that Treg depletion would enhance humoral immunity in the former, but not the latter. As shown in Figure 10A, although specific IgM and IgG responses to

Figure 9. Adoptive transfers of $CD4^+CD25^-$ T cells to athymic nude recipients restores *Streptococcus pneumoniae* capsular type 14-induced PspA-, PC- and PPS14-specific IgG titers to levels no greater than that observed for total $CD4^+$ T cells.

(A) Flow-cytometric analysis of spleen cells that underwent magnetic bead selection for $CD4^+$ (both $CD25^+$ and $CD25^-$) (left) and $CD4^+$ $CD25^-$ (right) T cells. (B) Seven athymic nude mice each were injected intravenously with either phosphate-buffered saline, 3 x 10^6 CFU of heat-killed *S. pneumoniae* capsular type 14. Values are arithmetic means of the peak titers for PspA (day 14)-, PC (day 7)-, and PPS14 (day 7)-specific-IgG titers plus standard errors of the means. Data are from one of two representative experiments.

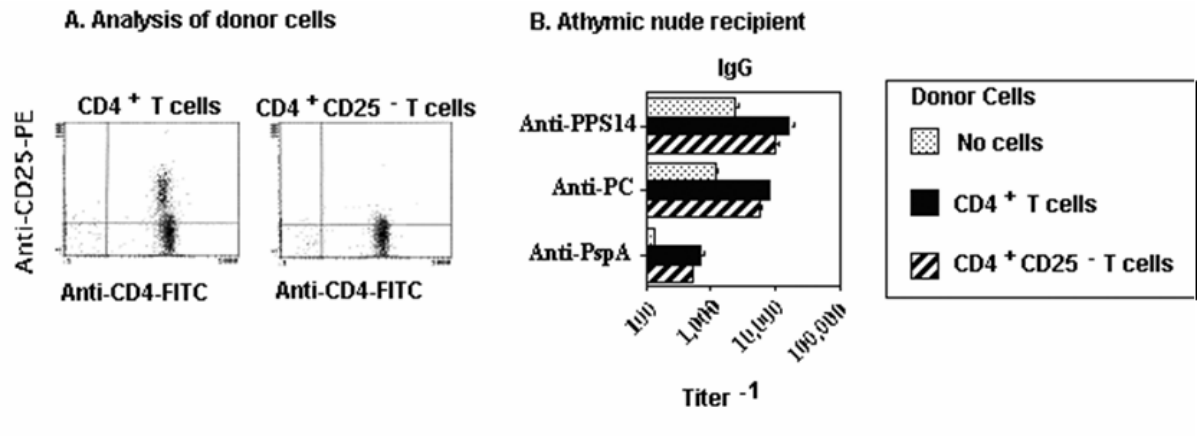
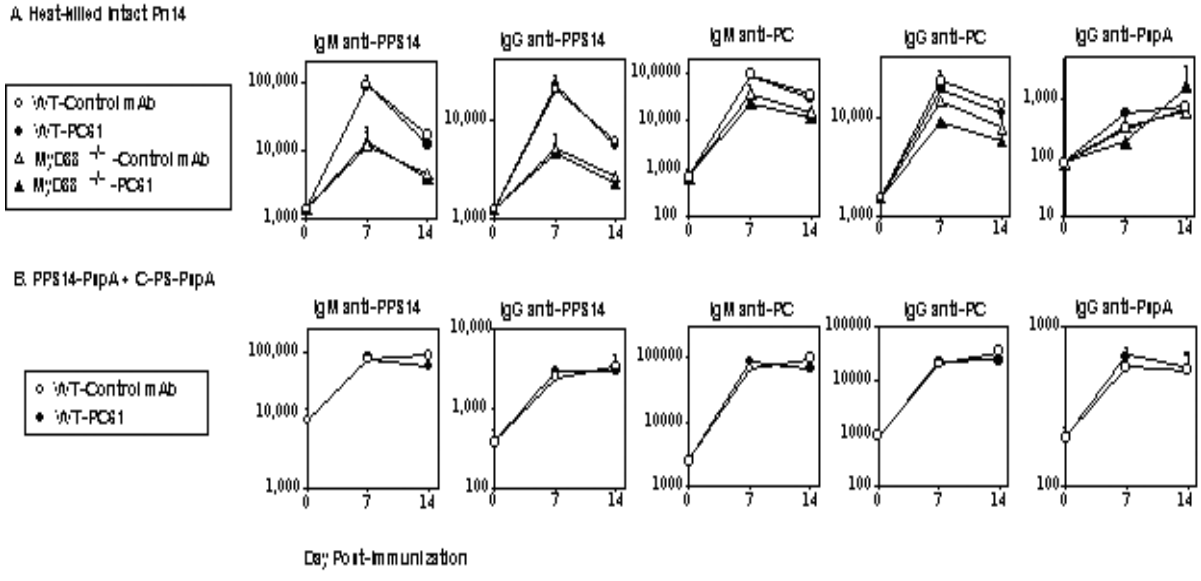

A. Analysis of donor cells
CD4 + T cells
CD4 + CD25 - T cells
Anti-CD25-PE
Anti-CD4-FITC
Anti-CD4-FITC
B. Athymic nude recipient
IgG
Anti-PPS14
Anti-PC
Anti-PspA
1,000
10,000
100,000
Titer -1
Donor Cells
No cells
CD4 + T cells
CD4 + CD25 - T cells

Figure 10. PC61 treatment does not alter the humoral response to intact *Streptococcus pneumoniae* capsular type 14 in MyD88$^{-/-}$ mice or to soluble protein-polysaccharide conjugates injected in saline in to wild-type mice.

(A) MyD88$^{-/-}$ and wild-type mice (seven per group) were injected i.p. with 1 mg of PC61 or 1 mg of GL113 followed 16h later by 2 x 10^8 CFU heat-killed *S. pneumoniae* capsular type 14 i.p. Sera were obtained on days 0, 7, and 14 for determination of antigen-specific Ig isotype titers by ELISA. (B) Wild-type mice (seven per group) were injected i.p. with 1 mg of PC61 or 1 mg of GL113 followed 16h later by 1 μg each of PPS14-PspA and C-PS-PspA in saline i.p. Sera were obtained on days 0, 7, and 14 for determination of antigen-specific Ig isotype titers by ELISA. One experiment each was performed.


A. Heat-killed Intact Pn14
IgM anti-PPS14
IgG anti-PPS14
IgM anti-PC
IgG anti-PC
IgG anti-PspA
B. PPS14-PspA + C-PS-PspA
Day Post-Immunization

heat-killed Pn14 were lower in MyD88$^{-/-}$, relative to wild-type mice, PC61 had no significant effect on the humoral response in either group of mice. Further, Treg depletion using PC61 had no effect on induction of IgM or IgG anti-PPS14, anti-PC, or anti-PspA in wild-type mice immunized with a mixture of soluble PPS14-PspA + C-PS-PspA conjugates in saline (Figure 10B). The ability of PC61 to deplete Treg *in vivo* was independently confirmed prior to these latter experiments (see Figure 6A).

In summary, we used a number of complementary, conventional approaches to assess the potential role of Tregs *in vivo* in order to mitigate against the potential pitfalls inherent in any one approach. Our data suggest that, in contrast to what is observed during many distinct types of chronic cell-mediated immune responses, including infections with intracellular pathogens, Tregs may not play a significant role in acute humoral responses to extracellular bacteria. Extracellular bacteria elicit early TLR-dependent innate immunity followed by a T cell-dependent adaptive humoral response, but are typically eliminated rapidly with no subsequent chronic phase. However, under conditions of chronic immune stimulation often observed with intracellular pathogens, and typically associated with a more limiting level of microbial stimulation, effector T cells may re-establish a more homeostatic balance with Tregs to limit excessive T cell-driven immunity and tissue damage, while allowing for continued T cell immunity (Belkaid *et al.*, 2002).

Chapter 3

TLR cross-talk in the innate immune response to *Streptococcus pneumoniae*

Submitted as:

Lee, K.S., C.A. Scanga, E.M. Bachelder, Q. Chen, and C.M. Snapper. TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent innate immune response to intact *Streptococcus pneumoniae*.

Abstract

We previously demonstrated that splenic cytokine and chemokine release and innate host protection in response to intact *Streptococcus pneumoniae* (Pn) is MyD88-, but not strictly TLR2-dependent, suggesting a role for additional TLRs. In this study, we investigated the role of TLR2, TLR4, and/or TLR9 in mediating splenic cytokine release as well as host protection in response to intact Pn. We utilized the Q-plex™ mouse cytokine array, a chemiluminescent-based ELISA approach, to determine the *in vitro* production of a panel of 16 cytokines and chemokines. We show that a single deficiency in TLR2, TLR4, or TLR9 has only modest, selective effects on cytokine secretion and no effect on lethality in response to Pn. However, $TLR2^{-/-}$ x $TLR9^{-/-}$ and $TLR2^{-/-}$ x $TLR4^{-/-}$ mice each show substantial defects in cytokine release, though not as severe as in $MyD88^{-/-}$ mice, but still with no increase in lethality to live Pn. Chloroquine, which inhibits the function of intracellular TLRs, including TLR9, completely abrogates detectable cytokine release in spleen cells from $TLR2^{-/-}$ x $TLR4^{-/-}$ mice, similar to what is observed for mice deficient in MyD88. Surprisingly, chloroquine-treated $TLR2^{-/-}$ x $TLR4^{-/-}$ mice, in contrast to $MyD88^{-/-}$ mice, continue to resist infection with Pn. These data demonstrate, for the first time that TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Pn, and further suggest that MyD88 signaling may prime the host for enhanced innate immune function prior to pathogen encounter and/or critically interact with non-TLR adaptor proteins involved in innate immunity, following infection.

Introduction

Toll-like receptors (TLRs), which recognize conserved moieties on pathogens, also known as pathogen associated molecular patterns, are critical mediators of innate and adaptive immunity (Janssens and Beyaert, 2003). TLRs are type I transmembrane proteins located at either the cell surface (TLRs 2 [with or without TLR1 or TLR6], 4, 5, 10, and 11) or in endosomes (TLRs 3, 7, 8, and 9) (Akira *et al.*, 2001; Nishiya and DeFranco, 2004; Parviz, 2002). Of the 11 identified TLRs, all but TLR3 and TLR4 are completely dependent upon the adaptor protein MyD88 for signaling (Akira *et al.*, 2000; Akira *et al.*, 2001; Hacker *et al.*, 2000; Takeuchi *et al.*, 2000b; Vogel *et al.*, 2003). TLR4 utilizes both MyD88 and another TLR adaptor protein, Trif/Ticam-1, for cell signaling, whereas TLR3-mediated signaling largely depends on Trif/Ticam-1 alone (Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2003a). Upon ligand binding, MyD88 is recruited to the C terminal domain of the TLR, resulting in a series of phosphorylation events that leads to the subsequent release of NF-kappaB into the nucleus and the transcription and production of various cytokines and chemokines (Muzio *et al.*, 1997; Muzio *et al.*, 1998; Wesche *et al.*, 1997). TLR-mediated activation via Trif/Ticam-1 results in phosphorylation and activation of IRF3 for induction of type I interferons (Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2003a).

The Gram-positive diplococcus, *Streptococcus pneumoniae* (Pn), is a major cause of global morbidity and mortality in the young, elderly, and immunosuppressed due to its ability to cause invasive disease such as otitis media, meningitis, pneumonia and

bacteremia (Cockeran *et al.*, 2005; Kadioglu and Andrew, 2004; Musher *et al.*, 2000). The increasing frequency of antibiotic-resistant strains (Kristinsson, 2000; McGee *et al.*, 2000) has created an urgent need to better understand the mechanisms of pneumococcal pathogenesis, including the host response to the pathogen. There are a number of structures expressed by Pn that are recognized by TLRs. TLR2 recognizes lipoteichoic acid and peptidoglycan (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999), both of which are exposed at the bacterial surface, whereas pneumolysin, a pore-forming cytoplasmic cytotoxin released by Pn upon its lysis, is a ligand for TLR4 (Malley *et al.*, 2003). Both TLR2 and TLR4 mediate signaling at the cell surface of the responding cell (Akira *et al.*, 2001). Pn also expresses unmethylated CpG-containing DNA and single-stranded RNA that could potentially bind TLR9 and TLR7/8, respectively, in an intracellular, endosomal location (Heil *et al.*, 2003; Latz *et al.*, 2004). In this regard, inhibition of endosomal acidification using agents such as chloroquine blocks TLR9- and TLR7/8-, but not TLR2- and TLR4-mediated signaling (Heil *et al.*, 2003; Lee *et al.*, 2003; Macfarlane and Manzel, 1998).

We have previously shown that MyD88$^{-/-}$ mice exhibit a profoundly defective innate splenic cytokine response upon intra-peritoneal (i.p.) challenge with intact Pn associated with 100% mortality to an otherwise sub-lethal infection (Khan *et al.*, 2005). In contrast, TLR2$^{-/-}$ mice exhibited splenic cytokine induction similar to wild-type mice, which correlated with their ability to survive an i.p. challenge with live Pn (Khan *et al.*, 2005). The striking differences observed in the induction of the early splenic cytokine response and host protection in TLR2$^{-/-}$ versus MyD88$^{-/-}$ mice in response to Pn prompted

us to determine which MyD88-dependent TLRs in addition to, or instead of, TLR2 were important for mediating innate immunity. We demonstrate, for the first time, that TLR2, TLR4, and TLR9 act collectively to mediate the MyD88-dependent splenic cytokine response to Pn. Surprisingly, mice exhibiting a profound defect in the Pn-induced innate inflammatory response, due to the loss of acute TLR signaling, continue to resist i.p. infection with Pn, in distinct contrast to mice genetically deficient in MyD88. These data suggest that MyD88 signaling may prime the host for enhanced innate immune function prior to pathogen encounter and/or critically interact with non-TLR adaptor proteins involved in innate immunity, following infection.

Materials and Methods

Mice

C3H/HeJ (TLR4-mutant), C3H/HeOuJ (wild-type), and B6129PF2/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). MyD88$^{-/-}$ (C57BL/6 background), TLR2$^{-/-}$ (B6129 background), and TLR9$^{-/-}$ (B6129 background) mice were obtained from S. Akira (Osaka University, Osaka, Japan), and bred in our facility. TLR2$^{-/-}$ x TLR9$^{-/-}$ mice (B6129 background) were generated in our laboratory by crossing of TLR2$^{-/-}$ and TLR9$^{-/-}$ mice. The above mice were maintained in a pathogen-free environment at the Uniformed Services University of the Health Sciences [U.S.U.H.S.] (Bethesda, MD). TLR2$^{-/-}$ x TLR4$^{-/-}$ mice (C57BL/6 background) were obtained from P. Matzinger (National Institutes of Health [N.I.H.], Bethesda, MD) and housed in a pathogen-free environment at the N.I.H. All mice were used between 6 and 8 wk of age. The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare (National Institutes of Health) 78-23.

Genotyping of mice

All mice used in these experiments were first confirmed by genotyping. DNA was prepared from mouse tail snips. The primers and conditions used for genotyping by PCR are shown in Table 1. All products were separated on a 1% agarose gel.

Reagents

Lipopeptide Pam_3Cys-Ser-Lys_4 (Pam_3Cys) and purified lipopolysaccharide from *E. coli* K12 strain (LPS) were purchased from InvivoGen (San Diego, CA). Phosphorotriated 30-mer (CpG-ODN) (AAA AAA AAA AAA AAC GTT AAA AAA AAA AAA) and suppressive 16-mer (sODN) (CCT CAA GCT TGA GGG G) were synthesized in the Biomedical Instrumentation Center (U.S.U.H.S). Chloroquine was purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of heat-killed Pn14 (hk Pn14)

Streptococcus pneumoniae capsular type 14 (Pn14) was prepared as previously described (Khan *et al.*, 2004), aliquoted at 10^{10} CFU/ml, and frozen at -80°C until their use.

Fluorescence-activated cell sorting (FACS) analysis of splenic cells

Red blood cells were removed from spleen cell suspensions using ACK lysing buffer. Cells were stained with various combinations of the following mAbs which, unless indicated, were obtained from BD Pharmingen (San Diego, CA): PE-labeled anti-CD3ε (clone 145-2C11), anti-F4/80 (clone BM8, eBioscience, San Diego, CA), anti-NK-1.1 (clone PK136), anti-CD45R/B220 (clone RA3-6B2), or anti-CD11c (Caltag, Burlingame, CA); biotin-anti-IgM (clone R6.60.2) + FITC-streptavidin, FITC-anti-Thy1.2 [CD90.2] (clone 53-2.1), FITC-anti-I-A^b (clone AF6-120.1), biotin-anti-CD49b (clone DX5) + FITC-streptavidin, FITC-anti-CD11b (clone M1/70). The cells were

sorted electronically using an EPICS-FACAria (Beckman Coulter, Fullerton, CA), and the negative population of cells was collected for *in vitro* stimulation.

In vitro stimulation of cultured spleen cells

Spleen cells were cultured at a density of 5 x 10^5 cells/well in 48-well tissue culture plates. After a 24 hour stimulation period with various doses of heat-killed Pn14 or TLR ligands, the cells were pelleted by centrifugation for 10 min at 1200 rpm and the supernatants were collected for measurement of cytokine and chemokine concentrations.

Measurement of cytokine concentrations in spleen cell culture supernatant or sera

The Q-plex™ mouse cytokine array (BioLegend, San Diego, CA) was used, according to manufacturer's instructions, to measure the concentration of 16 cytokines and chemokines from culture supernatant. Briefly, 1:2 serial dilutions were made of the various cytokines and chemokines of known concentration in order to generate a standard curve. Standards and samples were added to the plate for 1h at RT, and then washed. The detection mix was added for 1hr at RT and washed. HRP-streptavidin was added for 15 min at RT, washed, and the substrate mix was added and the plate was read for exposure times of 30sec-1min using a Fuji LAS-1000 CCD camera. Relative pixel intensity was determined with image analysis software provided by the manufacturer. The concentration of IL-6 in culture SN or sera was also measured by an optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture monoclonal antibodies, biotinylated monoclonal antibodies used for detection,

and streptavidin-alkaline phosphatase (AP) were purchased from BD Pharmingen (San Diego, CA). Streptavidin-AP was used in combination with *p*-nitrophenyl phosphate, disodium (Sigma, St. Louis, MO) as substrate to detect the specific binding.

Lethality studies

Pn14 was grown to mid-log phase. Bacterial numbers were determined by colony counts on blood agar (Becton Dickinson, Cockeysville, MD). Mice received an i.p. injection of either 2×10^8 or 4×10^7 cfu live Pn14 in PBS. Mice were observed daily for up to 5 days after infection. In some mice, 400ug chloroquine was injected i.p. 30min prior to injection of live Pn14.

Statistics

Data are expressed as the arithmetic mean ± SEM of the individual values. Significance of the differences between groups was determined by Student's t test. $p < 0.05$ was considered statistically significant.

Results

Spleen cells from MyD88$^{-/-}$ mice are markedly defective in secretion of multiple cytokines and chemokines in response to Pn14 in vitro

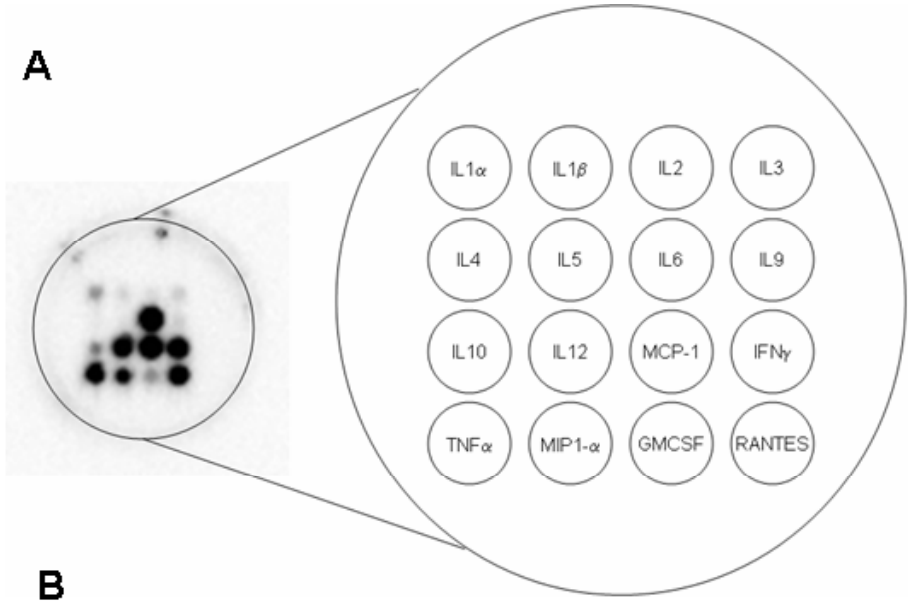
We previously reported that mice deficient in the TLR adaptor protein, MyD88 (MyD88$^{-/-}$) are markedly defective in eliciting a number of cytokines and chemokines (IL-1, IL-6, IL-12, IFN-gamma, TNF-alpha, MIP-1alpha, and MCP-1) in response to heat-killed (hk) Pn14 *in vitro* and/or *in vivo*. This was associated with enhanced lethality following i.p. challenge with live Pn14 (Khan *et al.*, 2005). To confirm and extend these findings to additional cytokines and chemokines, we used the ELISA-based chemiluminescent Q-plex™ mouse cytokine array, which allows simultaneous quantitation of 16 cytokines and chemokines (Fig. 11A). As shown in Fig. 11B, MyD88$^{-/-}$ mice indeed exhibit an essentially complete and global loss in their ability to elicit cytokine and chemokine responses to hkPn14, arguing against a significant MyD88-independent component to the Pn14-mediated splenic response *in vitro*.

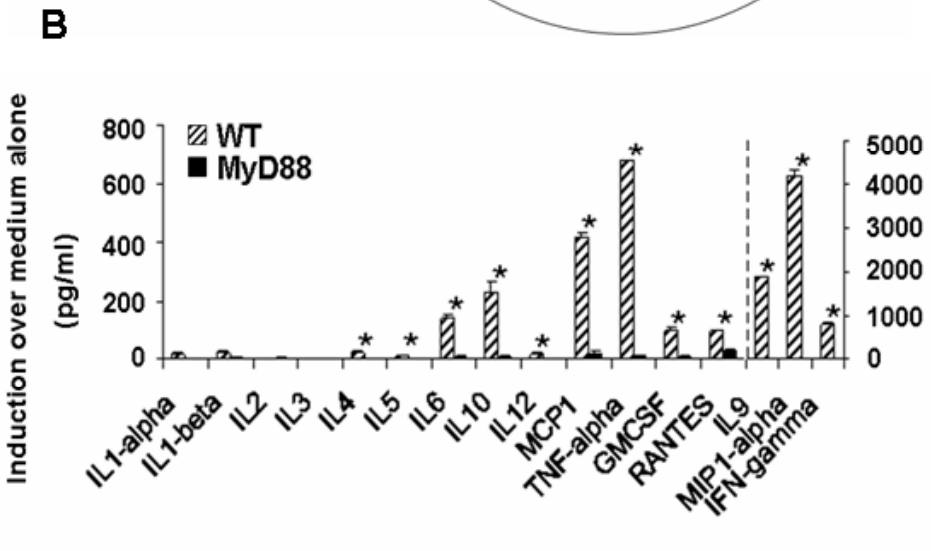
In vitro cytokine and chemokine secretion in response to Pn14 is largely due to splenic macrophages and dendritic cells

We next wished to determine which specific cell populations in the spleen were responsible for the observed in vitro cytokine and chemokine response to hkPn14. For this purpose, we used electronic cell sorting to individually remove T cells, B cells, NK cells, DCs, or macrophages from whole spleen cells obtained from wild-type mice. Each

Figure 11. MyD88$^{-/-}$ spleen cells are defective in their ability to release innate cytokines and chemokines *in vitro* in response to Pn14.

(A) An example of a well from a Q-plex™ mouse cytokine array (Q-plex™ assay) using cell SN from wild-type spleen cells stimulated for 24h *in vitro* with hkPn14. Each well of a 96-well plate is imprinted with 16 cytokine- or chemokine-specific capture mAbs. Cytokine concentrations in cell SN are measured by quantitative chemiluminescence, using recombinant cytokines and chemokines of known concentration, as standards. (B) Cytokine and chemokine concentrations in cell SN were measured by the Q-plex™ assay after 24h of treatment of wild-type and MyD88$^{-/-}$ spleen cells *in vitro* with 10^7 cfu/ml of hkPn14. The arithmetic mean of triplicates and SEM are shown. *p<0.05.


A
IL1α
IL1β
IL2
IL3
IL4
IL5
IL6
IL9
IL10
IL12
MCP-1
IFNγ
TNFα
MIP1-α
GMCSF
RANTES


B
Induction over medium alone
(pg/ml)
WT
MyD88
800
600
400
200
0
5000
4000
3000
2000
1000
0
IL1-alpha
IL1-beta
IL2
IL3
IL4
IL5
IL6
IL10
IL12
MCP1
TNF-alpha
GMCSF
RANTES
IL9
MIP1-alpha
IFN-gamma

cell population was identified on the basis of dual, positive staining with two distinct mAbs as illustrated in Figure 12. The various depleted spleen cell populations were then stimulated for 24h in vitro with hkPn14, and the concentration of secreted cytokines and chemokines was measured by the Q-plex™ assay and compared to sorted, whole spleen cells. We observed that macrophages and dendritic cells collectively contributed to most of the secreted cytokines and chemokines induced by hkPn14 *in vitro* (Figure 12). Of note, neither B cells nor T cells made any measurable contribution to this early splenic response to Pn14, whereas NK cells appeared to play a significant role in induction of IL-1beta.

Dual, but not single, deficiencies in TLR2, TLR4, and TLR9 result in significant, but not absolute, loss of the in vitro splenic cytokine and chemokine response to hk Pn14

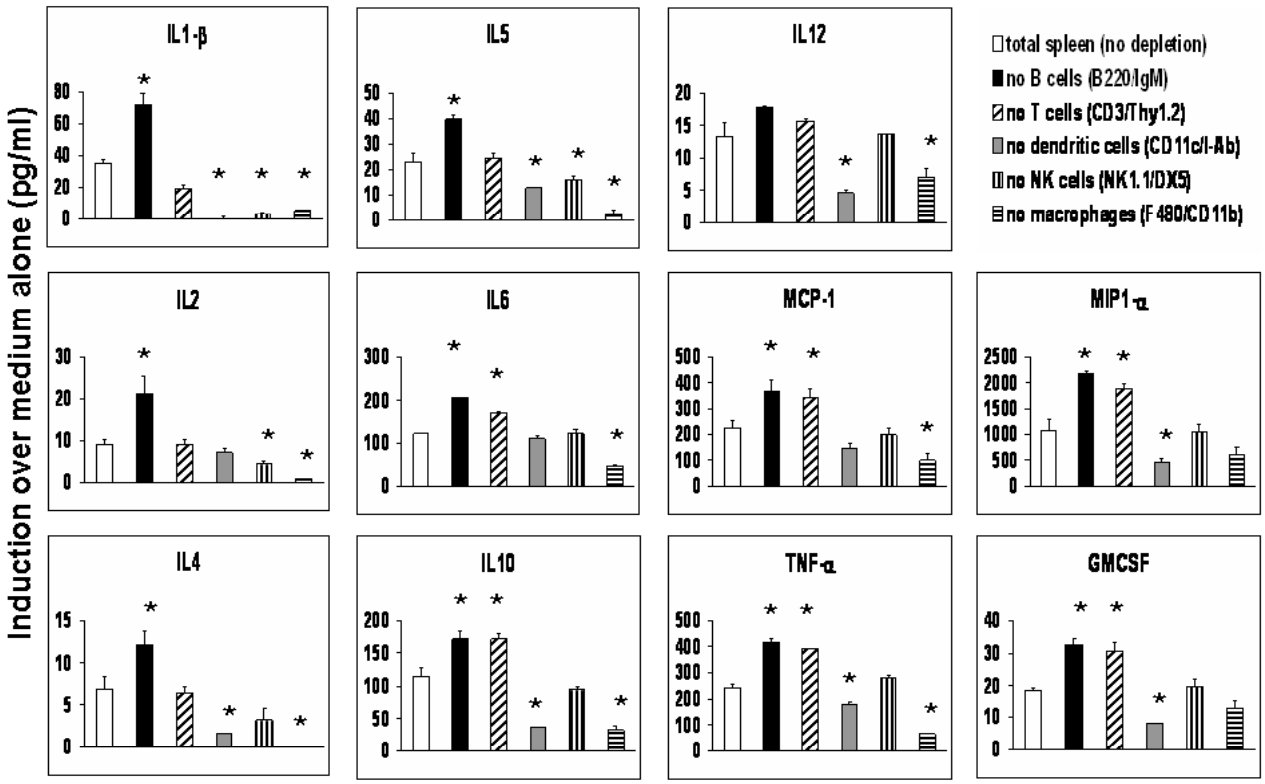
Of the MyD88-dependent TLRs, three are particularly pertinent to the initial recognition of Pn. Specifically, TLR2 recognizes lipoteichoic acid and peptidoglycan, TLR4 recognizes pneumolysin, and TLR9 recognizes bacterial DNA containing unmethylated CpG motifs. In order to determine whether innate cytokine and chemokine induction by Pn is dependent upon signaling via TLR2, TLR4 and/or TLR9, we measured Pn14-induced cytokine and chemokine production by spleen cells from mice deficient in one or two of these receptors (i.e. $TLR2^{-/-}$, C3H/HeJ (TLR4-mutant), $TLR9^{-/-}$, $TLR2^{-/-}$ x $TLR4^{-/-}$, or $TLR2^{-/-}$ x $TLR9^{-/-}$). TLR agonists (LPS [TLR4], Pam_3Cys [TLR2], and CpG-ODN [TLR9]) were used as controls in each experiment to functionally confirm that mice were correctly genotyped (Table 1, Figure 13). Additionally, we confirmed the ability of

Table 1. Primer sequences and PCR conditions used for genotyping TLR-deficient and MyD88$^{-/-}$ mice.

Gene	Primers	PCR cycle	Notes
MyD88	A: 5' TGG CAT GCC TCC ATC ATA GTT AAC C 3'; B: 5' GTC AGA AAC AAC CAC CAC CAT GC 3'; C: 5' ATC GCC TTC TAT CGC CTT CTT GAC G 3'	94°C for 3 min; 35 cycles of: 94°C for 40 s, 65°C for 40 s, and 72°C for 50 s; and 72°C for 10 min	The amplified products are both about 500 bp.
TLR2 (B6129)	A: 5'GTT TAG TGC CTG TAT CCA GTC AGT GCG 3'; B: 5' AAT GGG TCA AGT CAA CAC TTC TCT GGC 3'; C: 5' ATC GCC TTC TAT CGC CTT CTT GAC GAG 3'	94°C for 1 min; 35 cycles of: 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min; and 72°C for 10 min	For detection of the mutated allele, we used primers B and C. For the wild-type allele, we used primers A and B. The amplified products are both about 1,200 bp.
TLR2 (C57BL/6)	A: 5' CAT TGA CAA CAT CAT CGA T 3'; B: 5' GTA GGT CTT GGT GTT CAT T 3'	94°C for 3 min; 12 cycles of: 94°C for 20 s, 64°C for 30 s, and 72°C for 35 s, followed by 25 cycles of: 94°C for 20 s, 58°C for 30 s, and 72°C for 35 s; and 72°C for 2 min	
TLR4 (HeJ)	A: 5' TGT CAG TGG TCA GTG TGA TTG 3'; B: 5' TCA GGT CCA AGT TGC CGT TTC 3'	94°C for 5 min; 30 cycles of: 94°C for 1 min, 59°C for 30 s, and 70°C for 1 min; and 72°C for 2 min	The amplified product of the wild-type allele will appear as a double band at 300-350 bp, and that of the mutant allele will appear as a smaller and larger band at 300 and 400 bp.
TLR4 (C57BL/6)	A: 5' AGG ACT GGG TGA GAA ATG 3'; B: 5' GAT TCG AGG CTT TTC CAT C 3'	94°C for 3 min; 12 cycles of: 94°C for 20 s, 64°C for 30 s, and 72°C for 35 s, followed by 25 cycles of: 94°C for 20 s, 58°C for 30 s, and 72°C for 35 s; and 72°C for 2 min	
TLR9	A: 5' GAA GGT TCT GGG CTC AAT GGT CAT GTG 3'; B: 5' GCA ATG GAA AGG ACT GTC CAC TTT GTG 3'; C: 5' ATC GCC TTC TAT CGC CTT CTT GAC GAG 3'	94°C for 1 min; 35 cycles of: 94°C for 30 s, 67°C for 30 s, and 74°C for 1 min; and 74°C for 10 min	For detection of the mutated allele, we used primers B and C. For the wild-type allele, we used primers A and B. The amplified products are both about 1,200 bp.

Figure 12. Macrophages and dendritic cells are responsible for most of the observed cytokine induction by WT splenic cells *in vitro* in response to hk Pn14.

Spleen cells from WT mice were stained with pairs of mAbs exhibiting the specificities shown in parentheses, and cells staining positively for both mAbs were subsequently removed by electronic cell sorting. The remaining cells were treated with 10^7 cfu/ml of hkPn14 for 24hr and cytokine and chemokine concentrations in culture SN were determined using the Q-plex™ assay. The arithmetic mean of triplicates and SEM are shown. *$p<0.05$


Induction over medium alone (pg/ml)
IL1-β
IL5
IL12
IL2
IL6
MCP-1
MIP1-α
IL4
IL10
TNF-α
GMCSF
total spleen (no depletion)
no B cells (B220/IgM)
no T cells (CD3/Thy1.2)
no dendritic cells (CD11c/I-Ab)
no NK cells (NK1.1/DX5)
no macrophages (F480/CD11b)

chloroquine, an inhibitor of endosomal acidification, to selectively block signaling via intracellular TLRs (e.g. TLR9) while leaving unaffected responses through the membrane TLRs (e.g. TLR4 and TLR2) (Figure 13). A single deficiency in TLR2, TLR4, or TLR9 caused only selective and relatively modest reductions or enhancements in cytokine and chemokine production by Pn14-activated spleen cells (Figure 14, left panels). However, cytokine production was more dramatically and globally reduced in spleen cells from TLR2$^{-/-}$ x TLR4$^{-/-}$ and TLR2$^{-/-}$ x TLR9$^{-/-}$ mice, with the former exhibiting a more marked defect (Figure 14, right panels). These data strongly suggest that TLR cross-talk is necessary for the optimal induction of innate release of multiple splenic cytokines and chemokines in response to Pn.

The combination of TLR2, TLR4, and an intracellular TLR(s) recapitulate the MyD88$^{-/-}$ phenotype for splenic cytokine and chemokine induction in response to Pn14

Since the defect in splenic cytokine and chemokine release, even in TLR2$^{-/-}$ x TLR4$^{-/-}$ mice, was not as absolute as that observed in MyD88$^{-/-}$ mice, we wished to determine whether a combination of TLR2, TLR4, and intracellular TLR(s) [e.g. TLR9] could recapitulate the MyD88$^{-/-}$ phenotype. While bacterial DNA contains immunostimulatory CpG motifs that signal through TLR9, recent reports have shown that certain oligodeoxynucleotides rich in poly G or GC sequences are suppressive (sODN) and interfere with this interaction (Barrat *et al.*, 2005; Gursel *et al.*, 2003; Huang *et al.*, 2005; Lenert, 2005). The activity of sODN is dominant over TLR9 stimulation by CpG-ODN (Yamada *et al.*, 2002). However, we observed that sODN, used at optimal doses, failed to completely block CpG-ODN-induced cytokine and chemokine release in

Figure 13. IL-6 secretion by various TLR-deficient and chloroquine-treated WT spleen cells in response to TLR agonists *in vitro*.

TLR agonists were used as controls in each experiment to ensure that the mice were correctly genotyped. LPS [TLR4] (1 ug/ml), Pam_3Cys [TLR2] (Pam) (300 ng/ml) or CpG-ODN (CpG) [TLR9] (4 ug/ml) were used to stimulate spleen cells from the indicated mice for 24h *in vitro*. IL-6 concentrations were measured by ELISA. A representative result is shown. The arithmetic mean of triplicates and SEM are shown. *$p<0.05$

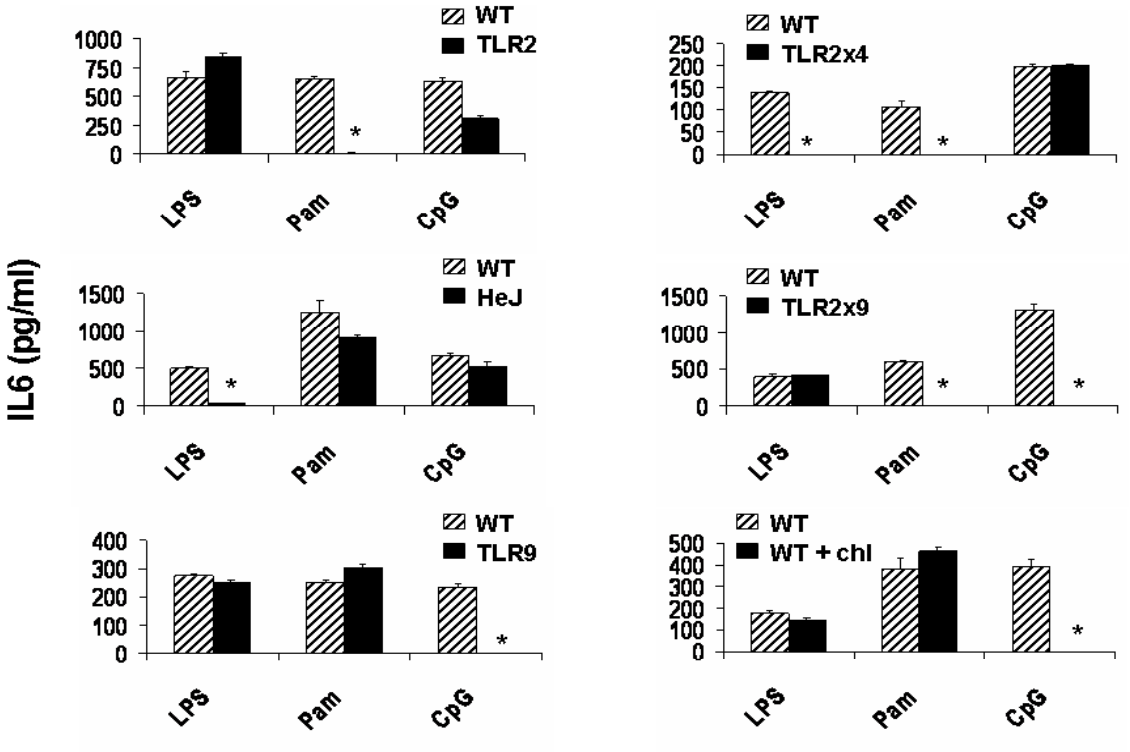
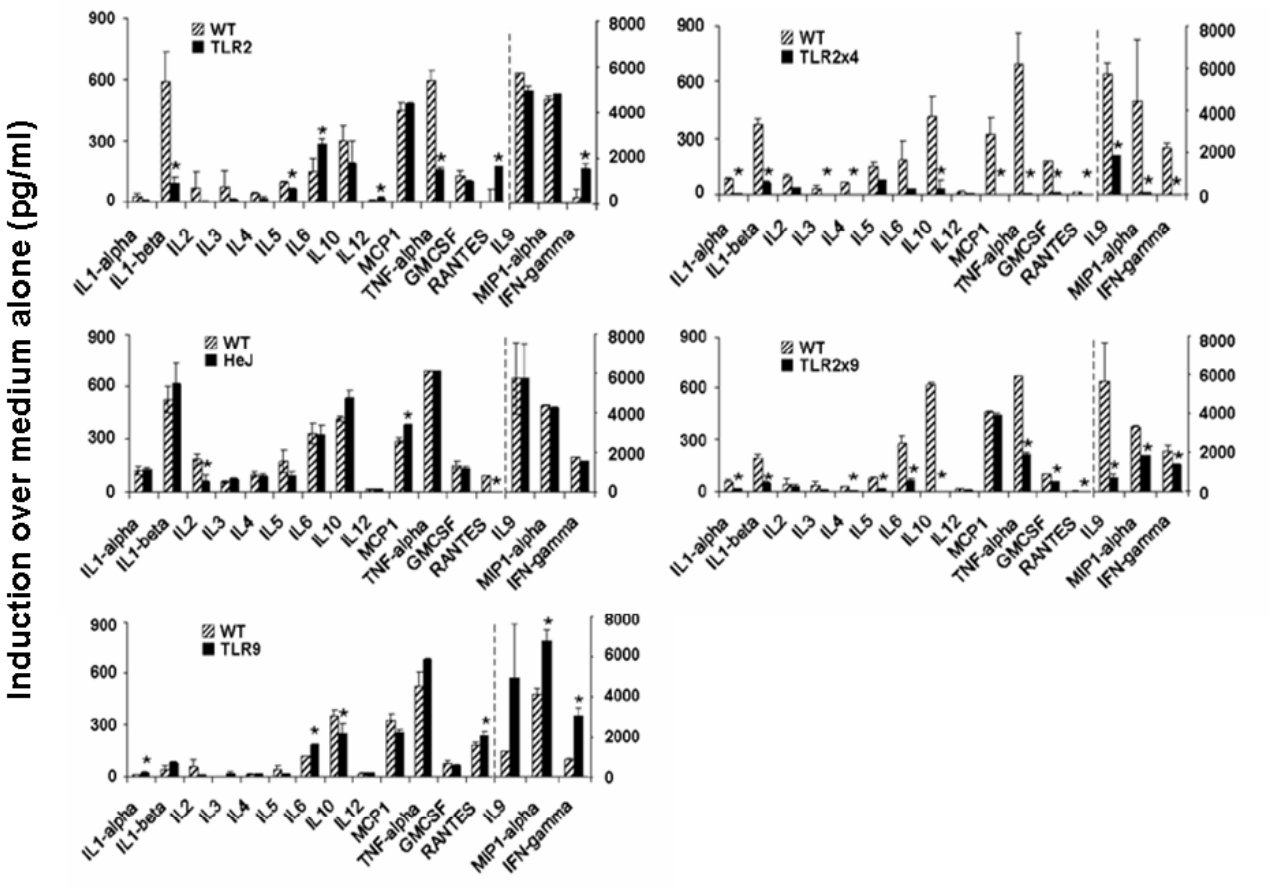

IL6 (pg/ml)
WT
TLR2
1000
750
500
250
0
LPS
Pam
CpG
WT
TLR2x4
250
200
150
100
50
0
WT
HeJ
1500
1000
500
0
WT
TLR2x9
WT
TLR9
400
300
200
100
WT
WT + chl
500
400
300
200
100
*

Figure 14. Spleen cells from mice with a double, but not single, deficiency in TLR2, 4 and/or 9, are defective in innate cytokine secretion in response to hk Pn14 *in vitro*.

Cytokine and chemokine concentrations in culture SN from spleen cells obtained from the indicated mice were measured using the Q-plex™ assay following 24hr of treatment with 10^7 cfu/ml hkPn14. The arithmetic mean of triplicates and SEM are shown. *$p<0.05$


Induction over medium alone (pg/ml)
WT
TLR2
TLR2x4
HeJ
TLR2x9
TLR9
900
600
300
0
8000
6000
4000
2000
0
IL1-alpha
IL1-beta
IL2
IL3
IL4
IL5
IL6
IL10
IL12
MCP1
TNF-alpha
GMCSF
RANTES
IL9
MIP1-alpha
IFN-gamma

wild-type cells, and had no significant effect on Pn14-induced stimulation of spleen cells from either wild-type or TLR2$^{-/-}$ x TLR4$^{-/-}$ mice (data not shown).

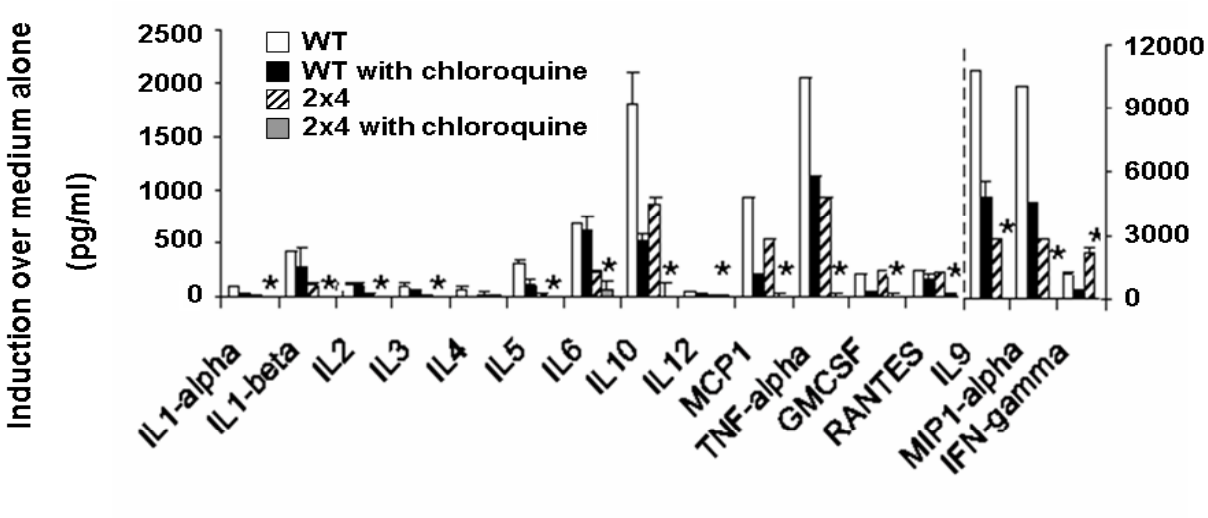
The antimalarial drug chloroquine blocks endosomal acidification (Brown *et al.*, 1984), a process specifically critical for productive interactions between intracellular TLRs (TLR3, TLR7/8, and TLR9) with their respective ligands Macfarlane, 1998 #257; Lee, 2003 #203; Heil, 2003 #258}. Treatment of spleen cells from TLR2$^{-/-}$ x TLR4$^{-/-}$ mice with chloroquine resulted in a complete loss of the detectable splenic cytokine and chemokine response to hkPn14 *in vitro* (Figure 15), similar to what we observed using MyD88$^{-/-}$ mice (Figure 11B). Of interest, whereas TLR9$^{-/-}$ mice exhibited no significant defect in splenic-mediated release (Figure 14), chloroquine treatment induced a modest, but significant reduction in secretion of several cytokines and chemokines from wild-type spleen cells (Figure 15). These data suggest a possible role of other intracellular TLRs (e.g. TLR7 interaction with single-stranded RNA) (Diebold *et al.*, 2004; Heil *et al.*, 2004) in the Pn14-mediated response.

Chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$, in contrast to MyD88$^{-/-}$, mice resist i.p. infection with live Pn14, despite a profound loss in the early cytokine and chemokine response

We have shown previously that MyD88$^{-/-}$ mice succumb to i.p. challenge with otherwise sub-lethal doses of live Pn14 (Khan *et al.*, 2005). Specifically, 2 x 10^8 cfu of live Pn14 but not 4 x 10^7 cfu, will kill wild-type mice by day 2, whereas all MyD88-/-

Figure 15. Chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$, like MyD88$^{-/-}$ spleen cells, are completely defective in cytokine and chemokine release in response to hkPn14 *in vitro*.

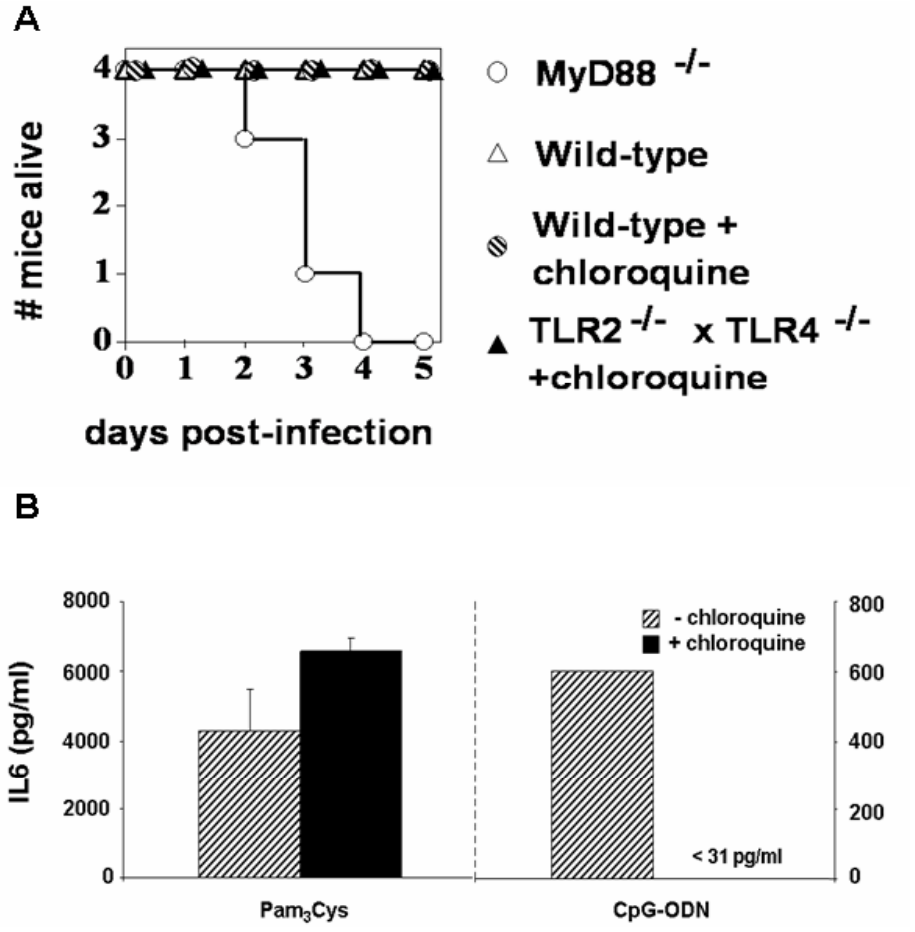
Spleen cells from WT or TLR2$^{-/-}$ x TLR4$^{-/-}$ mice were stimulated *in vitro* for 24h with 10^7 cfu/ml of hkPn14 in the absence or presence of 3 ug/ml chloroquine. The arithmetic mean of triplicates and SEM are shown. *p<0.05 TLR2$^{-/-}$ x TLR4$^{-/-}$ versus TLR2$^{-/-}$ x TLR4$^{-/-}$ + chloroquine.


Induction over medium alone
(pg/ml)
2500
2000
1500
1000
500
0
12000
9000
6000
3000
0
WT
WT with chloroquine
2x4
2x4 with chloroquine
IL1-alpha
IL1-beta
IL2
IL3
IL4
IL5
IL6
IL10
IL12
MCP1
TNF-alpha
GMCSF
RANTES
IL9
MIP1-alpha
IFN-gamma

mice succumb to either 2 x 10^7 or 4 x 10^7 cfu of live Pn14 by day 4 (Khan *et al.*, 2005). We observed that neither TLR2$^{-/-}$, C3H/HeJ (TLR4-mutant), TLR9$^{-/-}$, TLR2$^{-/-}$ x TLR4$^{-/-}$, or TLR2$^{-/-}$ x TLR9$^{-/-}$ mice succumbed to an i.p. challenge with 4 x 10^7 cfu of live Pn14 up to 5 days post-infection, and showed no signs of illness (data not shown). Surprisingly, as shown in Fig. 16A, unlike MyD88$^{-/-}$ mice that all succumb to live Pn14 challenge, no chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice died post-infection up to 5 days, nor showed signs of illness. To confirm the ability of chloroquine to selectively block intracellular TLR signaling *in vivo*, we injected either CpG-ODN or Pam_3Cys into wild-type mice following chloroquine treatment and showed that chloroquine specifically blocked TLR9-, but not TLR2-mediated, induction of serum IL-6 (Fig. 16B). Thus, chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice continued to resist i.p. infection with live Pn14, despite evidence for a profound loss in the early innate cytokine and chemokine response.

Figure 16. Chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$, in contrast to MyD88$^{-/-}$ mice, resist i.p. challenge with live Pn.

(A) WT and TLR2$^{-/-}$ x TLR4$^{-/-}$ mice (4 mice/group) were treated with 4x10^7 cfu live Pn14 i.p. alone, or with 400 ug/mouse chloroquine i.p. 30 min prior to bacterial challenge. MyD88$^{-/-}$ and WT mice also received 4x10^7 and 2x10^8 cfu, respectively, of live Pn14 i.p. Survival was monitored for 5 days. (B) Pam_3Cys (2.0 ug/mouse) or CpG-ODN (25 ug/mouse) were injected i.p. into WT mice in the absence or presence 400ug/mouse chloroquine given 30 min prior to i.p. injection of the TLR agonists. Sera were obtained 1 hr following TLR agonist injection, and the concentration of IL6 was measured by ELISA. The arithmetic mean of triplicates and SEM are shown. *p<0.05.


A
mice alive
days post-infection
MyD88 -/-
Wild-type
Wild-type + chloroquine
TLR2 -/- x TLR4 -/- +chloroquine
B
IL6 (pg/ml)
- chloroquine
+ chloroquine
< 31 pg/ml
Pam3Cys
CpG-ODN

Discussion

We previously demonstrated that MyD88$^{-/-}$ mice exhibit a profound defect in splenic cytokine and chemokine expression, and increased lethality to i.p. infection with Pn14, whereas TLR2$^{-/-}$ mice, in this regard, were largely similar to wild-type mice (Khan *et al.*, 2005). These data suggested that additional TLRs were critically involved in the innate immune response to this bacterium. Pn is known to express ligands for TLR2 (Schwandner *et al.*, 1999), TLR4 (Malley *et al.*, 2003), and TLR9 (Dalpke *et al.*, 2006). Utilizing mice with single or dual deficiencies in these TLRs, we demonstrate significant cross-talk between TLR2 and TLR4, and TLR2 and TLR9, for Pn14-induced splenic cytokine and chemokine release. The ability of chloroquine (Brown *et al.*, 1984), an inhibitor of signaling mediated by intracellular TLRs (TLR3, TLR7/8, and TLR9) (Macfarlane and Manzel, 1998), to completely abrogate mediator release by TLR2$^{-/-}$ x TLR4$^{-/-}$ spleen cells in response to Pn14 strongly suggests that TLR2, TLR4, and intracellular TLRs (TLR9 and possibly TLR7) collectively mediate the MyD88-dependent response, likely in a synergistic manner. Despite the profound defect in acute TLR-dependent splenic-mediated release, chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice continue to resist i.p. infection with Pn14, in distinct contrast to MyD88$^{-/-}$ mice.

We earlier reported that intact Pn induces a mixed type 1 and type 2 cytokine response in wild-type mice *in vivo* (Khan *et al.*, 2002). Our more extended Q-plex™ cytokine analysis showing splenic release of mediators associated with type 1 (IL-12, IFN-gamma, and MIP-1alpha) and type 2 (IL-4, IL-5, IL-9, and MCP-1) responses

(Luther and Cyster, 2001; Murphy and Reiner, 2002) to intact Pn14 is consistent with this observation. In this regard, we further demonstrated that TLR2$^{-/-}$ mice exhibit a significant reduction in polysaccharide- and protein-specific type 1 IgG isotypes (IgG3, IgG2b, and IgG2a), but not the type 2 IgG isotype, IgG1, in response to i.p. immunization with intact Pn14 (Khan *et al.*, 2005). In distinct contrast, co-injection of purified TLR2 agonists, such as Pam_3Cys or *Porphyromonas gingivalis* LPS, with protein antigen promoted type 2 responses (Dillon *et al.*, 2004; Pulendran *et al.*, 2001; Redecke *et al.*, 2004). These latter studies differed from ours in their use of purified TLR2 agonists, whereas we utilized intact Pn14, which as demonstrated here induces significant cross-talk between TLR2 and TLR4, and to a lesser extent, TLR2 and TLR9. In light of the ability of TLR2, TLR4, and TLR9 agonists to induce distinct cytokine profiles (Cowdery *et al.*, 1999; Hermann *et al.*, 2002; Kreutz *et al.*, 1997; Sing *et al.*, 2000), the effect of TLR2 signaling on the overall innate response may differ depending on the simultaneous, relative activity of other TLRs. Indeed, MyD88$^{-/-}$ mice immunized with intact Pn14, in contrast to TLR2$^{-/-}$ mice, exhibited a significantly elevated type 2 (IgG1) response (Khan *et al.*, 2005; Schnare *et al.*, 2001) and increased CD4^{+} T cell priming for IL-5 and IL-13 (unpublished data), similar to previous observations using purified antigens (Schnare *et al.*, 2001). The observation of defective type 1 IgG isotype production in TLR2$^{-/-}$ mice in response to Pn14 despite a relatively normal innate cytokine response suggests that TLR2 signaling may be required at the level of the B cell (Pasare and Medzhitov, 2005a).

The pattern of cytokine and chemokine release from TLR9$^{-/-}$ spleen cells stimulated with intact, hkPn14 was essentially identical to that observed using wild-type

cells, and this was associated with innate resistance of TLR9$^{-/-}$ mice to i.p. challenge with live Pn14. The *in vitro* splenic response to Pn14 was mediated largely by macrophages and dendritic cells, which readily internalize the bacteria. Hence, Pn14 has access to the endosomal compartment necessary for TLR9-mediated activation by CpG-containing DNA (Hacker *et al.*, 1998). In contrast, IL-12p40, IFN-gamma, and IFN-alpha induction in response to heat-killed *Brucella abortus* was shown to be either entirely or partly dependent on TLR9 depending on the cytokine and cell source (Huang *et al.*, 2005). Similarly, TLR9$^{-/-}$ mice exhibited defective induction of IL-12p40 and IFN-gamma in response to aerosol challenge with live *Mycobacterium tuberculosis* (Bafica *et al.*, 2005). In addition, induction of splenic and hepatic IFN-gamma following i.v. injection with heat-killed *Propionibacterium acnes* was defective in TLR9$^{-/-}$ mice (Kalis *et al.*, 2005). DNA from distinct bacterial species express different proportions of unmethylated CpG which correlates directly with the TLR9-dependent immunostimulatory properties of their DNA (Dalpke *et al.*, 2006; Neujahr *et al.*, 1999). Likewise, the proportion of unmethylated CpG within the DNA of plasmid vaccines was associated their immune-activating properties (Klinman *et al.*, 1997; Yew *et al.*, 2000). Relatively high concentrations of DNA, and thus large numbers of bacteria, are necessary to directly induce isolated TLR9-mediated effects (Dalpke *et al.*, 2006; Nonnenmacher *et al.*, 2003; Sparwasser *et al.*, 1997), conditions that may not often be encountered physiologically. Of interest, the proportion of CpG-containing DNA in *M. tuberculosis* (12.7%) (Dalpke *et al.*, 2006), *P. acnes* (9.6%) (Dalpke *et al.*, 2006), and *B. abortus* (9.2%) (Huang *et al.*, 2005) is substantially higher than that found in *S. pneumoniae* (2.7%) (Dalpke *et al.*,

2006). This could explain the critical impact of an isolated deficiency in TLR9 on the innate responses to the former bacteria, in contrast to what we observe for Pn14.

TLR2$^{-/-}$, in contrast to MyD88$^{-/-}$ mice, elicited a largely intact innate splenic cytokine response, although Q-plex™ analysis revealed a moderate reduction in secretion of IL-1beta and TNF-alpha. This correlated with a resistance of TLR2$^{-/-}$ mice to i.p. infection with live Pn14, in contrast to MyD88$^{-/-}$ mice. In this regard, although TLR2$^{-/-}$ mice are more susceptible to experimental Pn meningitis, a substantial part of the inflammatory response is TLR2-independent (Echchannaoui *et al.*, 2002; Koedel *et al.*, 2003). Additionally, TLR2$^{-/-}$ mice inoculated intranasally with live Pn displayed only a modestly reduced inflammatory response in the lungs, and normal host immunity relative to wild-type mice, despite defective cytokine production from freshly-isolated TLR2$^{-/-}$ alveolar macrophages (Knapp *et al.*, 2004). TLR2 was found, however, to be required for efficient clearance of Pn from the upper respiratory tract (van Rossum *et al.*, 2005). The modest susceptibility of TLR2$^{-/-}$ mice to pneumococcal infection could, in part, be secondary to a defect in neutrophil phagocytosis and oxidative bactericidal activity (Letiembre *et al.*, 2005). In contrast to TLR2$^{-/-}$ mice, MyD88$^{-/-}$ mice exhibit a more severe defect in innate cytokine production and host defense in experimental meningitis (Koedel *et al.*, 2004) and pneumonia models (Albiger *et al.*, 2005), as well as reduced clearance of pneumococci from the upper respiratory tract (Albiger *et al.*, 2005). Thus, in these models as well as in our own, TLR(s) in addition to TLR2 appear to be important for anti-pneumococcal innate immunity.

Mice deficient in TLR4 signaling (C3H/HeJ) (Poltorak *et al.*, 1998), like mice with single genetic deficiencies in TLR2 or TLR9, exhibited an essentially normal innate splenic cytokine and chemokine response to hkPn14, and resistance to i.p. challenge with live Pn14. Pneumolysin, a cytoplasmic, cytotoxic protein expressed by Pn (Paton, 1996) and released upon autolysis, is a TLR4 ligand (Malley *et al.*, 2003). Thus, pneumolysin can stimulate IL-6 and TNF-alpha from macrophages (Malley *et al.*, 2003) and induce macrophage and epithelial cell apoptosis in a TLR4-dependent manner (Srivastava *et al.*, 2005). In this regard, $TLR4^{-/-}$ mice are more susceptible to lethal infection after intranasal colonization with pneumolysin-positive pneumococci (Malley *et al.*, 2003; Srivastava *et al.*, 2005). In contrast, a separate study found no role for TLR4 in pneumococcal colonization, although pneumolysin was found to induce a localized inflammatory response and promote clearance of colonized Pn in a TLR4-independent manner (van Rossum *et al.*, 2005). Further, induction of meningitis by Pn did not require pneumolysin, nor did the absence of pneumolysin expression affect the inflammatory response (Friedland *et al.*, 1995; Wellmer *et al.*, 2002). Heat-inactivation destroys the cytotoxic and cytokine-inducing activity of pneumolysin (Houldsworth *et al.*, 1994; Malley *et al.*, 2003), arguing against a key role for pneumolysin in the *in vitro* splenic cytokine and chemokine response in our study. Of interest, as discussed below, spleen cells from $TLR2^{-/-}$ x $TLR4^{-/-}$, but not $TLR2^{-/-}$ or C3H/HeJ mice, exhibited a striking reduction in secretion of cytokines and chemokines in response to hkPn14, suggesting that Pn expresses a novel, heat-resistant TLR4 ligand in addition to the heat-sensitive pneumolysin.

TLR2$^{-/-}$ x TLR4$^{-/-}$, and to a lesser extent, TLR2$^{-/-}$ x TLR9$^{-/-}$ mice exhibited striking reductions in secretion of splenic cytokines and chemokines in response to hkPn14 *in vitro*, in contrast to mice deficient in only one of these TLRs. These data thus demonstrate significant cross-talk or synergism between TLR2 and TLR4, and to a lesser extent, TLR2 and TLR9, in the Pn-induced cytokine and chemokine response in splenic macrophages and dendritic cells. These data are in contrast to findings by Koedel, *et al*. in which IFN-gamma-primed murine macrophages from mice with a dual deficiency in TLR2 and TLR4 signaling elicited a normal TNF-alpha response to Pn (Koedel *et al.*, 2003). Our observation of functional synergism between TLR2 and TLR4 in response to Pn is consistent with the finding that these two receptors, which have differing cytoplasmic tails, mediate distinct, although overlapping, cytokine response patterns in macrophages (Carl *et al.*, 2002; Hirschfeld *et al.*, 2001; Jones *et al.*, 2001a; Jones *et al.*, 2001b) and dendritic cells (Re and Strominger, 2001). Distinct TLR ligands have indeed been shown to act synergistically to promote release of inflammatory mediators by macrophages (Gao *et al.*, 1999; Sato *et al.*, 2000; Yi *et al.*, 2001) and dendritic cells (Gautier *et al.*, 2005; Napolitani *et al.*, 2005). Consistent with these findings, whereas TLR4 alone appears to play a dominant role in macrophage release of IL-6 and TNF-alpha in response to a variety of Gram-negative bacteria that elicit acute infections, concomitant deficiency in both TLR2 and TLR4 resulted, synergistically, in a more defective phenotype (Lembo *et al.*, 2003). Likewise, whereas TLR4$^{-/-}$ but not TLR2$^{-/-}$ mice were more susceptible to acute infection with the Gram-negative bacterium, *Salmonella typhimurium*, TLR2$^{-/-}$ x TLR4$^{-/-}$ mice exhibited the most defective host response (Weiss *et al.*, 2004). Of interest, in one study, TLR2$^{-/-}$ x TLR4$^{-/-}$ macrophages

elicited normal IL-6 and TNF-alpha responses to the Gram-positive bacteria, *Staphylococcus aureus* (Lembo *et al.*, 2003), although another study demonstrated reduced IL-6 and TNF-alpha in TLR2$^{-/-}$ macrophages in response to this bacterium (Takeuchi *et al.*, 2000a). Synergy between TLR2 and TLR9 (Bafica *et al.*, 2005) and between TLR2 and TLR4 (Tsuji *et al.*, 2000; Uehori *et al.*, 2003) has also been observed in the host response to the intracellular bacterium, *Mycobacterium tuberculosis*.

Surprisingly, chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice, in contrast to MyD88$^{-/-}$ mice, continued to resist i.p. infection with live Pn14 despite an essentially undetectable acute splenic cytokine or chemokine response. These data suggest the possibility that a genetic deficiency in MyD88, resulting in chronic TLR dysfunction, as well as defective IL-1 (Adachi *et al.*, 1998; Muzio *et al.*, 1997; Wesche *et al.*, 1997) and IL-18 signaling (Adachi *et al.*, 1998), may lead to defects in innate immunity not observed subsequent to acute TLR blockade. Thus, fusion of phagosomes and lysosomes upon uptake of particles lacking TLR ligands is defective in MyD88$^{-/-}$ macrophages (Yates and Russell, 2005). Further, focal adhesion kinase-dependent macrophage secretion of TNF-alpha in response to protein I/II from *Streptococcus mutans* is dependent upon MyD88, but apparently not on TLR stimulation (Zeisel *et al.*, 2005). In addition, IFN-gammaR1 associates with MyD88 upon binding IFN-gamma resulting in stabilization, although not induction, of IFN-gamma induced mRNA (Sun and Ding, 2006). Finally, commensal bacteria that are recognized by TLRs under normal steady-state conditions play a critical role in the maintenance of intestinal epithelial homeostasis and protection against gut

injury and associated mortality (Rakoff-Nahoum et al., 2004), and could in theory have an impact on innate immunity.

Chapter 4

Discussion

Toll-like Receptors

Our previous findings that MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice exhibit defects in innate cytokine production and host defense to *Streptococcus pneumoniae* (Pn) suggested a role for additional MyD88-dependent TLRs (Khan *et al.*, 2005). Accordingly, we accessed the role of three potential TLRs for which pneumococcal ligands have been reported to recognize and bind. We show here that cross-talk between TLR2, TLR4 and TLR9 in spleen cells collaboratively mediate the MyD88-dependent response to Pn. However, in contrast to MyD88$^{-/-}$ mice, those with a functional deficiency in the three TLRs continue to exhibit resistance to live Pn challenge.

There are a number of explanations for the discrepancy in our *in vitro* and *in vivo* observations. First, as shown in Figure 15, chloroquine treatment of wild-type mice led to some reductions in cytokine and chemokine responses. This finding and the fact that a deletion of TLR9 did not result in any noticeable differences in innate responses (Fig. 14) may imply that other intracellular TLRs such as TLR7 may be playing a role. Even though we used heat-killed bacteria in these experiments, we cannot rule out the possible involvement of bacterial RNA binding to TLR7 (Kariko *et al.*, 2005). However, because chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice were able to resist challenge with live bacteria, where all intracellular TLR signaling should have been inhibited, this data strengthens the thought that a genetic deficiency in MyD88 could lead to defects in innate immunity not observed subsequent to acute TLR blockade. In support of this hypothesis, investigators have found that commensal bacteria recognized by TLRs under steady-state conditions play a critical role in the maintenance on intestinal epithelial homeostasis, and

subsequently, innate immunity (Rakoff-Nahoum *et al.*, 2004). Furthermore, it appears that MyD88-dependent TLR siganling is important for the development of a specialized subset of CD8αα TCRαβ and TCRγδ intestinal intraepithelial lymphocytes, whose interactions with commensal organisms are important for intestinal homeostasis (Yu *et al.*, 2006).

It is also possible that Pn expresses ligands that have yet to be identified that are recognized by TLRs other than the three investigated here that would provide another pathway for the elicitation of innate immune responses. However, our *in vitro* finding in chloroquine-treated TLR2$^{-/-}$ x TLR4$^{-/-}$ mice (Fig. 15) suggests that TLR2, TLR4, and TLR9 collectively reproduce the MyD88 phenotype, pointing to the involvement of other mechanisms *in vivo*. In addition to the reports on MyD88 and maintenance of intestinal homeostasis, a number of other observations describe a role for MyD88 in pathways that do not depend on signaling through TLRs. Zieisel, *et al*. described a MyD88-dependent, but TLR2, TLR4 and TLR6-independent, pathway that involves focal adhesion kinase for cytokine responses to a ligand expressed by *Streptococcus mutans* (Zeisel *et al.*, 2005). Similarly, transcription of TNF and IP10 was dependent on a physical interaction between IFN-gamma receptor 1 and MyD88 (Sun and Ding, 2006). Furthermore, MyD88$^{-/-}$ mice display decreases in phagosome/lysosome fusion in macrophages that functions independently of TLR signaling pathways (Yates and Russell, 2005) as well as decreases in natural serum IgM and IgG levels (Pasare and Medzhitov, 2005a). The generation of a conditional knockout in which MyD88 is selectively knocked out at the time of bacterial challenge would help in distinguishing whether earlier developmental defects make these mice more susceptible to infection. The Cre/LoxP technology could

be used to make two strains of mice. One strain would contain a plasmid with the MyD88 gene flanked by *loxp* sites, and the other strain would contain a plasmid that expresses the Cre recombinase under the control of cell-type specific promoters, such that crossing the two strains would result in the generation of a mouse that lacks MyD88 at a specific time and in specific cell types, i.e. macrophages and dendritic cells.

Another possibility is that MyD88$^{-/-}$ mice succumb to opportunistic infections that would otherwise be managed by intact innate immune responses. It is apparent that these mice cannot control infection; however, they are not dying of septicemia since they do not exhibit any innate cytokine release. There could be a number of explanations for this observation. It has been shown that MyD88$^{-/-}$ mice exhibit a decrease in leukocyte infiltration, most likely as a consequence of little to no production of chemokines (Albiger *et al.*, 2005; Koedel *et al.*, 2004). The absence of this crucial step in controlling initial pathogenic insult allows the bacteria to replicate unmonitored. Additionally, MyD88$^{-/-}$ mice have lower levels of IL-6, which is important for the induction of hepcidin (Nemeth *et al.*, 2003; Schaible and Kaufmann, 2004). Hepcidin reduces the concentration of free iron, which is necessary for bacterial growth. However, MyD88$^{-/-}$ mice show no decrease of free iron and no enhancement of hepcidin (Albiger *et al.*, 2005). Koedel, *at al*. also show that MyD88$^{-/-}$ mice have lower levels of complement factors, which also allows the bacteria to escape opsonization and phagocytosis (Koedel *et al.*, 2004). The toxic effects of pneumolysin, PspA and PC facilitate the development of invasive disease, causing the mice to have difficulties with breathing, and ultimately succumb to massive organ failure (Koedel *et al.*, 2004). By keeping the mice in barrier

facilities, it is possible to minimize opportunistic infections so that an inherent deficiency or external factor could be tested and verified as the cause of death.

Pathogens typically express multiple TLR ligands, as well as additional agonists, that collectively regulate the subsequent innate host response. By acting together, activation through numerous TLRs initiates a cascade of distinct responses that are necessary for the control and clearance of microbes. A number of reports have described similar roles of TLRs in immunity to *Pseudomonas aeruginosa* (Ramphal *et al.*, 2005), *Brucella abortus* (Huang *et al.*, 2005; Weiss *et al.*, 2005), *Mycobacterium tuberculosis* (Bafica *et al.*, 2005; Shi *et al.*, 2003) and *Legionella pneumophila* (Sporri *et al.*, 2006). We show that the cooperative interaction or "cross-talk" between TLR2, TLR4 and TLR9 is necessary for innate cytokine production against the gram-positive organism, *Streptococcus pneumoniae*, but that host defense is not so simplistic and likely depends on innate mechanisms that may not be explained by the collective action of TLRs alone.

Regulatory T cells

The naturally occurring population of regulatory T cells (Tregs) has recently acquired some attention for their role in controlling immune response to both self as well as foreign antigens. A number of reports have described the importance of Tregs in chronic cell-mediated immune responses such as those seen in autoimmunity (Uraushiharam *et al.*, 2003; Vasu *et al.*, 2003) (Mqadmi *et al.*, 2005), tumor immunity (Onizuka *et al.*, 1999; Sakaguchi, 1995; Shimizu *et al.*, 1999), transplantation tolerance (Edinger *et al.*, 2003; Waldmann and Cobbold, 2001), and infections caused by *Plasmodium yoelii* (Hisaeda *et al.*, 2004), *Leishmania major* (Belkaid *et al.*, 2002), *Onchocerca volvulus* (Satoguina *et al.*, 2002), hepatitis B virus (HBV) (Stoop *et al.*, 2005), human immunodeficiency virus (HIV) and cytomegalovirus (CMV) (Aandahl *et al.*, 2004). Given our findings that humoral responses to Pn were dependent on TCR-α/β^+, $CD4^+$ T cell help (Khan *et al.*, 2004; Wu *et al.*, 1999), and that Pn does not cause chronic disease, we were interested in determining whether Tregs would be a subset of $CD4^+$ T cells that plays a role in controlling immune responses to acute infection by Pn.

We wanted to determine whether removal of the Treg population using mAbs to CD25 and GITR would have any effect on the humoral responses to Pn. It was shown that the greater the degree of activation of effector $CD4^+$ T cells, the more resistant they are to Treg-mediated suppression (Baecher-Allan *et al.*, 2002). This prompted us to determine whether the dose of bacteria used could influence the suppressive effects of Tregs. As it turns out, the bacterial dose did not have any effect on the humoral response to Pn (Fig. 7B). In parallel to the mAbs studies, we performed adoptive transfers of total

$CD4^+$ and $CD4^+CD25^-$ populations into athymic nude recipients, which lack T cells. We had previously reported that IgG antibody responses are restored in these recipients upon $CD4^+$ T cell transfer (Khan *et al.*, 2004). Therefore, we asked the question of whether transfer of Treg-depleted $CD4^+$ cells could further enhance these antibody responses. As shown in Figure 9B, although transfer of total $CD4^+$ T cells restored IgG responses, as previously described, transfer of $CD4^+CD25^-$ T cells did not further enhance the responses.

In light of a recent report showing that production of IL-6 after TLR stimulation abrogates Treg suppression (Pasare and Medzhitov, 2003), we wanted to determine what the effect of Treg-depletion would be in $MyD88^{-/-}$ mice. Since $MyD88^{-/-}$ mice cannot respond to TLR stimulation, and subsequently do not produce IL-6 (Khan *et al.*, 2005), Treg activity should still be operative. We therefore asked whether humoral immunity could be enhanced in these mice, which exhibit a striking defect in their type 1 IgG responses (Khan *et al.*, 2005), after Treg depletion. Figure 10A shows that despite generally lower responses in $MyD88^{-/-}$ mice as compared to their wild type counterparts, PC61 treatment did not alter the humoral responses to Pn. Because hk Pn14 contains a number of TLR ligands which can act as adjuvants, we wanted to test the effect of PC61 in a situation where no adjuvants would be present, and therefore, where Tregs would still be able to mediate their suppressive effects. For this purpose, we immunized mice with two protein-polysaccharide conjugates in saline, and tested the ability of PC61 to alter antibody responses to these antigens. Once again, removal of the Treg population, even in the absence of adjuvants, did not alter the humoral responses to the two conjugates (Fig. 10B).

By using a number of methods to deplete $CD25^+/GITR^+$ Tregs, our data suggest that, in contrast to what is observed during many distinct types of chronic cell-mediated immune responses, including infections with intracellular pathogens, $CD4^+CD25^+$ Tregs may not play a significant role in acute humoral responses to extracellular bacteria. Two recent reports cite that the presence of Tregs dampens responses to herpes simplex virus (HSV) (Suvas *et al.*, 2003) and hepatitis B virus (HBV) (Furuichi *et al.*, 2005) in both the acute and memory phases. This discrepancy can be explained by the fact that HSV and HBV are viruses, which require cytolytic $CD8^+$ T cells to control infection. It is possible that Tregs have different effects on acute infections by extracellular, antibody-controlled pathogens like Pn and intracellular cell-mediated pathogens such as viruses. Additionally, persistent antigenic stimulation may explain why Tregs are involved in chronic but not acute infections (Anderson *et al.*, 2006; Zheng *et al.*, 2006). The presence of antigen requires a mechanism in which cellular interactions are modulated and kept in check, such as that provided by Tregs. A reason we did not find a role for Tregs in our model system could have to do with the fact that Pn is rapidly cleared by the immune system, bypassing a need for regulation by Tregs.

Another reason we did not find a role for Tregs may be that other types of T cells with regulatory functions may be operative in our model. In support of this idea, a role for IL10-dependent Tr1 regulatory cells has been described in an acute model of *Bordetella pertussis* infection, which involves a novel subversion strategy to evade protective immune responses (McGuirk *et al.*, 2002). However, the Th3 cells that function through TGF-β production do not appear to play a role in controlling infectious diseases, only tumor immunity and autoimmunity (Inobe *et al.*, 1998; Kitani *et al.*, 2000).

Thus, it might be worthwhile to investigate the potential role of both Tr1 and Th3 regulatory T cell populations in our Pn model.

Regulatory T Cells and Toll-like Receptors: Linking Innate and Adaptive Immunity

Although we did not find a role for Tregs in our Pn model of acute humoral immunity, the literature does suggest that there is an important interaction between TLRs and $CD4^+CD25^+$ Tregs. It was initially described by Britz, *et al*. that mycobacterial activation (presumably through TLRs) of DCs led to the activation of an effector T cell population that is resistant to suppression (Britz *et al.*, 1982). Furthermore, administration of LPS and poly:IC to mice resulted in increased anti-DNA levels (Izui *et al.*, 1977). More recently, it was shown that production of IL-6, as a consequence of TLR signaling, abrogates Treg-mediated suppression by acting on responder $CD4^+CD25^-$ T cells (Pasare and Medzhitov, 2003) and similarly, IL-12 can release effector T cells from Treg-mediated suppression (King and Segal, 2005). Specifically, Liu, *et al*. (Liu *et al.*, 2006) and Sutmuller, *et al.* found that signaling through TLR2, but not TLR4 or TLR9 (Sutmuller *et al.*, 2006), on T cells enhances proliferation of and IL-2 production by $CD4^+CD25^-$ effector cells. This process transiently silences Tregs until the amount of TLR2 stimulation decreases after the pathogen is eliminated (Liu *et al.*, 2006). At this point, it is likely that Tregs regain their suppressive abilities and control of unnecessary immune activation.

From past and current literature, it appears that the mechanism in which Treg activity is regulated depends on the presence of antigen, as suggested by a number of investigators (Anderson *et al.*, 2006; Zheng *et al.*, 2006). TLRs expressed on antigen presenting cells (APCs) play a critical role by sensing pathogens, and through production

of cytokines such as IL-6 and IL-12, can suppress Treg function to allow adaptive immune responses that will help in the clearance of the pathogen. Once the infection is controlled, and antigen is not longer available to stimulate TLRs, cytokine levels decrease, releasing Tregs from the blockade. At this point, Tregs can once again regulate the interaction between effector T cells and APCs.

In conclusion, I hope the research I have presented in this dissertation is convincing of the fact that Tregs and TLRs are intricately linked, and indeed bridge innate and adaptive immunity. Through elegantly regulated mechanisms, immune homeostasis is maintained by keeping cellular responses in check.

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