



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799



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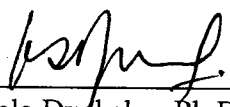
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Name of Candidate: Gouri Chattopadhyay
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Departmental
-Clinical Psychology
-Environmental Health Sciences
-Medical Psychology
-Medical Zoology

Dissertation and Abstract Approved:

Doctor of Public Health (Dr.P.H.)



Gabriela Dveksler, Ph.D.
Department of Pathology
Committee Chairperson

6/19/08

Date

Physician Scientist (MD/Ph.D.)

Master of Science Degrees

-Public Health

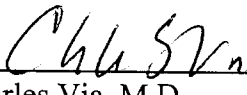


Clifford Snapper, M.D.
Department of Pathology
Committee Member

6/19/08
Date

Masters Degrees

-Military Medical History
-Public Health
-Tropical Medicine & Hygiene

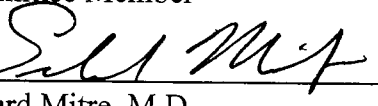


Charles Via, M.D.
Department of Pathology
Committee Member

6/19/08
Date

Graduate Education Office

Dr. Eleanor S. Metcalf, Associate Dean
Janet Anastasi, Program Coordinator
Tanice Acevedo, Education Technician



Edward Mitre, M.D.
Department of Microbiology & Immunology
Committee Member

6/19/08
Date

Web Site

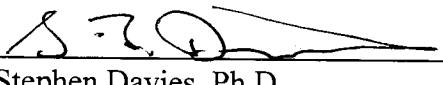
<http://www.usuhs.mil/graded/>

E-mail Address

graduateprogram@usuhs.mil

Phone Numbers

Commercial: 301-295-9474
Toll Free: 800-772-1747
DSN: 295-9474
FAX: 301-295-6772



Stephen Davies, Ph.D.
Department of Microbiology & Immunology
Committee Member

6/19/08
Date

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Gouri Chattopadhyay
Pathology Graduate Program
Uniformed Services University of the Health Sciences

Abstract

Title of Dissertation:

The Regulation of Polysaccharide Specific Humoral Immune Response Against Intact
Streptococcus pneumoniae

Gouri Chattopadhyay, Doctor of Philosophy, 2008

Thesis directed by:

Clifford M. Snapper, M.D.

Professor, Department of Pathology

The adaptive immune response against isolated polysaccharides (PS) is T cell-independent. In contrast, the IgG anti-capsular PS response to both intact *Streptococcus pneumoniae* (Pn), as well as a soluble covalent conjugate of Pn-derived protein and PS, require CD4⁺T cell help, B7-dependent costimulation, and CD40-CD40-ligand interactions. However, the IgG anti-PS response elicited by intact Pn, in contrast to soluble conjugate, peaks more rapidly, entails a shorter period of T cell help, and fails to induce PS-specific memory. Thus, the same antigen present in different physical/biochemical contexts elicits distinctly different humoral responses. The mechanism underlying these differences remains unknown and has important implications for the design of effective anti-PS vaccines against extracellular bacteria.

One potential reason for the limited anti-PS response to Pn is that PS-specific B-cells exhibit greater levels of apoptosis in response to this form of immunogen. We therefore studied mice that constitutively expressed the antiapoptotic protein Bcl-xL or Bcl-2 as a B cell-specific transgene. We show that apoptosis limits a primary PS-specific Ig response to Pn, but does not account for the lack of PS-specific memory.

The humoral immune response to intact Pn versus soluble Pn-derived conjugate is elicited through distinct pathways. We were thus interested in determining whether these pathways were activated independently or were cross-regulatory. We show that when soluble conjugate is co-injected with Pn, the generation of PS-specific memory, as well as the primary and memory protein-specific response, to the conjugate is abrogated. The mechanism of inhibition is an early event and is neither capsular PS- or protein-specific, nor based on the particulate nature of the Pn. These data suggest that during infection with Pn, primary and/or memory responses to released Pn-derived soluble antigens may be inhibited by the concomitant presence of intact Pn within the secondary lymphoid organ.

The Regulation of Polysaccharide Specific Humoral Immunity
Against Intact *Streptococcus pneumoniae*

by

Gouri Chattopadhyay

Thesis submitted to the Faculty of the

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Dedication

To my son, Sambuddha, for your patience and love.

To my parents, back in India, who have always been there for me.

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List of Abbreviations

Ab.....	Antibody
Ag.....	Antigens
APC.....	Antigen presenting cells
DC.....	Dendritic cells
FB.....	Follicular B cells
GC.....	Germinal Center
Ig.....	Immunoglobulin
MZB.....	Marginal Zone B cells
PAMPs.....	Pathogen associated molecular pattern
Pn.....	<i>Streptococcus pneumoniae</i>
PPV.....	Polysaccharide vaccine
PRR.....	Pathogen recognition receptor
PS.....	Polysaccharide
TLRs.....	Toll like receptor

Chapter One

Introduction

The term “Immunity” derived from the Latin word “Immunitas” is defined as “the exemption from various civic duties and legal prosecution”. This exemption was offered to “Roman Senators” during their periods as officials. The term “immunity” quite appropriately gained its access into biomedical science as meaning “defense against the numerous infectious diseases”. The immune system protects the body against pathogenic microorganisms with layered defenses of increasing specificity. If pathogens, such as bacteria and viruses, evade the physical barriers of the host, then the innate immune system is activated to provide a rapid, but non-specific response. Vertebrates possess a third layer of protection, the adaptive immune system, which is more specific and generates immunological memory which results in the ability of the immune system to mount faster and more prolonged attacks each time this particular pathogen is encountered. The word “antigen” originated from the notion that it can stimulate **antibody generation**. Antigens are usually proteins or polysaccharides. Here, I will focus preferentially on humoral immune responses against polysaccharide antigens.

T-dependent versus T-independent Antigens

Antigens (Ag) can be divided into two major groups, T cell-dependent (TD) and T cell-independent (TI) (Mond, Lees et al. 1995). TD Ags are generally proteins that are internalized by Ag presenting cells (APC) and processed into smaller peptides that associate with major histocompatibility complex-II (MHC-II) molecules on the APC surface for presentation to specific CD4⁺T helper cells (MacLennan 1994; Clark, Foy et al. 1996; Snapper 2006). In response to pathogen exposure and initial T cell stimulation by APCs, the APCs also upregulate cell surface co-stimulatory molecules, which are

critical for the complete activation of the CD4⁺T cell for effector function. These functions include T cell stimulation of specific B cell proliferation, immunoglobulin (Ig) secretion and class switching, and generation of Ag specific memory. Unlike TD Ags, TI Ags, which are often polysaccharides (PS), can generate protective antibody (Abs) responses in the absence of MHC-II-restricted T cell help (Sela, Mozes et al. 1972; Goodlad and Macartney 1995). TI responses typically lack affinity maturation and memory, and are associated with either absent or abortive germinal center (GC) (de Vinuesa, Cook et al. 2000) formation. TI (PS) antigens express repeating, identical antigenic epitopes which results in multivalent crosslinking of membrane Ig molecules on the B cell surface that can initiate potent B cell proliferation (Snapper 2006). PS antigens are generally present on the Gram-positive or Gram-negative bacterial cell surface in the form of a capsule, or as outer membrane, cell wall or cytoplasmic glycoproteins or glycolipids including lipopolysaccharides (LPS), teichoic acids, lipoteichoic acids, and peptidoglycan (Jennings, Lugowski et al. 1980; Tomasz 1981; Garcia de Vinuesa, O'Leary et al. 1999). The repeating subunits of capsular polysaccharides (CPS) are composed of one to eight sugars usually linked by glycosidic bonds. Sugars in the PS exhibit large degrees of antigenic variation depending on the differences in their composition, ringforms, linkage positions, isomer forms, and anomeric center configurations (Weintraub 2003; Mazmanian and Kasper 2006). The hallmark of the TI response to purified PS antigens is induction of an IgM response, with only a modest IgG response that is largely IgG3 (mouse), IgG2c (rat), or IgG2 (human). This is due to minimal Ig class switching from the lack of T cell help.

TI-Ags can be subdivided into TI-type 1 (TI-1) and TI-type 2Ags (Mosier, Mond et al. 1977; Mosier, Zaldivar et al. 1977). TI-1 Ags are PS antigens associated with a polyclonal B cell activator, such as a Toll-like receptor (TLR) ligand (e.g. lipid A [TLR4 ligand] of LPS) that can activate both naïve and mature B cells (Mond, Lees et al. 1995; Weintraub 2003). TI-type 2 (TI-2) Ags are also typically PS antigens, but lack an associated polyclonal B cell activating moiety (Mond, Lees et al. 1995). TI-2 Ags activate only mature B cells by crosslinking membrane Ig molecules on the B cell surface, resulting in the production of antigen specific antibodies (Weintraub 2003). TI-2, in contrast to TI-1 or TD, antigens are unable to stimulate B cells of neonatal mice or CBA/N (xid) mice that contain B cells lacking Bruton's tyrosine kinase (Btk) (Amsbaugh, Hansen et al. 1972), a key mediator of antigen receptor signaling (Mond, Lees et al. 1995). Thus, purified PS (TI-2) antigens are unable to elicit protective antibody responses in infants and are relatively weak immunogen in adults (Murray and Lopez 1997). Therefore, it has been important to develop approaches to boost the immune response to TI-2 antigens in these populations.

Polysaccharide Vaccines

Several PS-based vaccines have been in clinical use for a number of extracellular bacteria, including *Streptococcus pneumoniae* (Pn). Pn has 90 different capsular PS serotypes (Henrichsen 1999). The pneumococcal polysaccharide vaccines (PPV) launched by Merck Research Laboratory, are 14-valent Pneumovax I (in 1977) and 23-valent Pneumovax II, consisting of purified PS of different serotypes, and are recommended for use in immunocompromised individuals, the asplenic and elderly

(Lesinski and Westerink 2001). However, children under 2 years of age, who are a major target of infection by Pn, are unable to mount an effective immune response against the purified PS in the Pneumovax preparations. Since PPV lacks the ability to recruit T-cell help and induce the generation of memory, vaccine strategies against this organism have been focused on converting the TI-immune response to a TD-immune response. This was accomplished with the conjugate vaccine, which consists of a PS antigen covalently coupled to an immunogenic carrier protein.

Converting a TI-response into a TD-response

The conjugation of PS to a carrier protein converts the PS antigen from TI to TD by effecting recruitment of protein-specific CD4⁺ T-cell help. This has markedly improved the efficacy of the PS-based vaccine (Anderson, Bowers et al. 1994; MacDonald, Halperin et al. 1998; Briles, Hollingshead et al. 2000; Kelly, Moxon et al. 2004). Presumably, PS-specific B cells after recognizing the PS component of the conjugate vaccine, internalizes the conjugate, processes the protein part by proteolytic degradation and the degraded peptides are then presented to the carrier protein specific CD4⁺ T-cells by MHC-II molecules (McCool, Harding et al. 1999). As a result, PS-specific B cells can mature into antibody producing short-term plasma cells, as well as participate in the GC-reaction to generate Ig class-switched memory cells and long-lived bone marrow (BM) plasma cells (Schneerson, Barrera et al. 1980).

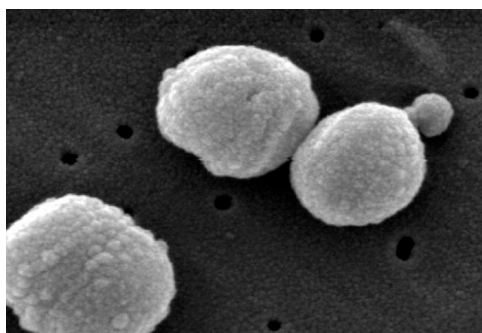
The generation of glycoconjugate vaccines has been one of the greatest success stories in the biomedical sciences. In the year 2000 Wyeth introduced a conjugate vaccine named Prevnar, which consists of 7 pneumococcal capsular polysaccharide serotypes

[2µg each of capsular polysaccharide 4, 9V, 14, 19F, 23F; oligosaccharide from 18C, 4µg of serotype 6B (Bernatoniene and Finn 2005)] covalently conjugated with a modified 20µg of (non-toxic) diphtheria toxin protein (CRM₁₉₇) (cross-reactive malarial) and was found, in contrast to PPV, to confer protection against Pn infections in young children. Indeed, introduction of the conjugate vaccine has dramatically reduced the Pn infection rate in children in the US and Europe. However, in those not protected by pneumococcal vaccine, antibiotic-resistant pneumococcal infections continue to pose a clinical problem due to treatment failure. The most antibiotic resistant infections are caused by five of seven serotypes in the 7-valent conjugate vaccine (6B, 9V, 14, 19F and 23F) (Kyaw, Lynfield et al. 2006). Another potential clinical problem is that although conjugate vaccines largely decrease the invasive disease caused by the serotypes contained within the vaccine, it may cause, emergence of serotypes that are not covered by the vaccine (“serotype replacement”). Both economic and technical concerns have precluded inclusion of more Pn serotypes in the conjugate formulation. Another problem with the current pneumococcal conjugate vaccine is that although they are very effective in children, including the ability to generate boosted secondary responses, they are much less effective in adults (Powers, Anderson et al. 1996) for reasons that have not been clearly elucidated. In light of these various issues, therefore, there is an urgent need to better understand the basic immunology of PS-specific humoral response against Pn, and other extracellular bacteria, which may in turn help in the design of better vaccines.

Studying bacteria: a more physiological approach

In contrast to a large body of experimental work done with purified, soluble, so-called “laboratory PS antigen” to determine the relative T-cell dependency and other parameters mediating the B cell response against PS Ags, our lab has taken the position that a more physiological approach is to study PS-specific immune responses in the context of the intact bacteria that naturally expresses the PS. Our major hypothesis is that immunologically, purified, soluble PS Ags do not adequately mimic PS antigens normally exposed on the bacterial cell surface. Thus, the particulate nature of intact Pn, the association of PS with Pn-derived proteins within the intact bacterial structure, and the presence within bacterial cell wall and cytoplasm of innate stimulatory ligands such as TLR ligands, and scavenger receptor ligands that facilitate Pn uptake by phagocytic cells, may confer on PS in this context certain unique immunologic features not exhibited by purified PS antigens. In this regard, my studies have focused on the anti-PS response to intact Pn bacteria, and how this response may differ from the anti-PS response to a soluble pneumococcal conjugate vaccine.

Streptococcus pneumoniae (Pn)



A.



B.

Figure 1. Electron-microscopic view of *Streptococcus pneumoniae* (A.); Organism's growth on sheep blood agar (B.).

Epidemiology & pathogenesis- Pn is a Gram-positive extracellular coccus grown on blood agar medium and is alpha-hemolytic (Fig.1). Individual cells are between 0.5 to 1.25 micrometers in diameter. It is commonly known in medical microbiology as “pneumococcus” because of its morphology and frequent relation with pneumococcal pneumonia. Pn generally colonizes the upper respiratory tract and can subsequently produce pneumonia (lobar type), paranasal sinusitis, otitis media or meningitis (Shann 1995; Douglas 1999; Peltola 2001). Interestingly, all children eventually become colonized for varying periods of time, and 25% to 75% of infants carry pneumococci at any given time. High colonization rates are promoted by children who are in close proximity with each other in various institutions or childcare settings (Durbin 2004). Pneumococci can spread from person-to-person by large droplets, although clinical disease occurs in only a small percentage of people who are colonized. Transition from colonization to invasive disease depends on both host genetic susceptibility and environmental factors that are only partially understood. Worldwide, pneumococcal septicemia is a major cause of infant morbidity and mortality, especially in developing countries, where it causes approximately 25% of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year (Berkley, Lowe et al. 2005). Each year pneumococcus also causes 70,000 deaths from meningitis and sepsis in young children (Obaro 2001).

Pneumococci colonize the upper respiratory tract, where they are typically asymptomatic. When Pn becomes invasive it initiates a rapid inflammatory response and clinical disease (Kadioglu, Weiser et al. 2008). Pneumonia results from the aspiration of pneumococci from the upper respiratory tract, although a blood-borne route of

dissemination from the upper respiratory tract is also possible (Busse 1991).

Pneumococcal disease begins with the establishment of colonization, termed the “carrier state”. Colonization appears to initiate some degree of immunity. The colonization rate varies among the 90 known serotypes. Pneumococci differ in their colonial morphology, such as opaque, semi-transparent, transparent, depending upon their capsule expression (i.e. transparent lack capsule, opaque possess capsule) (Weiser, Austrian et al. 1994).

Typically bacteria colonizing the nasopharynx have low capsule expression whereas invasion and septicemia is associated with increased capsule expression. For binding, Pn targets various cell surface glycoconjugates and platelet-activating factor (PAF) receptor in the nasopharynx. Pn expresses phosphorylcholine (PC) in its cell wall which specifically binds to the PAF receptor and facilitates adherence and invasion into endothelial cells by virulent Pn (Cundell, Gerard et al. 1995).

Virulence factors-Pn expresses numerous virulence factors (Fig. 2) (Kadioglu, Weiser et al. 2008) including both proteins and PS. Pneumococcal proteins that are common to all serotypes represent potential new protein-based, as opposed to current PS-based, vaccine candidates. Three major groups of cell surface proteins have been classified in Pn: 1) choline-binding proteins, 2) lipoproteins and 3) proteins that are covalently linked to the bacterial cell wall by a carboxy (c)-terminal sortase (LPXTG) motif (Kadioglu, Weiser et al. 2008). Choline-binding proteins (e.g. PspA [pneumococcal surface protein A], PspC [pneumococcal surface protein C] and LytA) share a 20 amino acid stretch homology which is responsible for the attachment of these proteins to the cell surface via binding to

phosphorylcholine (PC) expressed by the cell wall teichoic acid and cell membrane lipoteichoic acid.

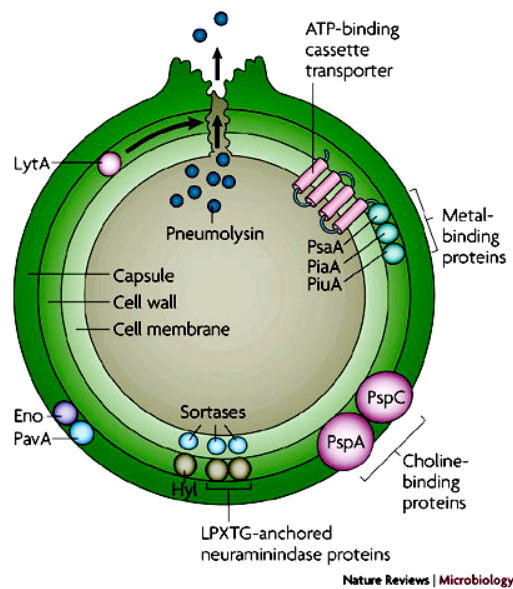


Figure 2: Schematic diagram of Pneumococcal cell showing several virulence factors.

The size of PspA varies from 67-99 KDa, is expressed in all serotypes (Crain, Waltman et al. 1990) and within the host it inhibits complement binding (Jedrzejewski 2001). Fixation of complement to pathogens is important for opsonophagocytosis and is thus a key host defense mechanism. PspC binds to the polymeric Ig receptor that normally transports secretory IgA (Zhang, Mostov et al. 2000). LytA negative mutants show reduced virulence in murine models of pneumonia (Berry, Lock et al. 1989). Divalent metal-ion-binding lipoproteins and LPXTG-anchored proteins are also virulence factors.

Pneumolysin, a 53-KDa protein, is another virulence factor. It is a cytoplasmic enzyme and through its cytotoxic effect can directly inhibit phagocytosis and immune cell function, which leads to a suppression of the host inflammatory and immune response.

Pneumolysin is also a TLR4 ligand that may also serve to enhance host immunity to Pn.

Capsular PS, which is 200-400 nm thick, forms the outermost layer of the bacterial cell surface and protects pneumococci by inhibiting phagocytosis (Tuomanen, Austrian et al. 1995). Cell wall is composed of peptidoglycans and anchors capsular PS (Fig.3) (Snapper, Shen et al. 2001). 90 different structurally, serologically different capsular PS have been identified in Pn (Henrichsen 1995) and spontaneous non-capsulated strains are avirulent. Anti-PS Abs against capsular PS are serotype specific and protective (Austrian, Douglas et al. 1976). SIGN-R1 (a C-type lectin), present on marginal zone macrophages in the spleen, promotes the uptake of both purified capsular-PS and intact Pn (Kang, Kim et al. 2004). In contrast to un-capsulated pneumococci, purified capsular PS shows no inflammatory response when it is directly injected into the lung. Unlike capsular PS, cell-wall PS (CWPS) in its purified form, containing peptidoglycan, is strongly inflammatory and induces inflammation similar to intact Pn (AlonsoDeVelasco, Verheul et al. 1995). Cell membrane LTA and cytoplasmic pneumolysin, as well as bacterial DNA and RNA are also pro-inflammatory.

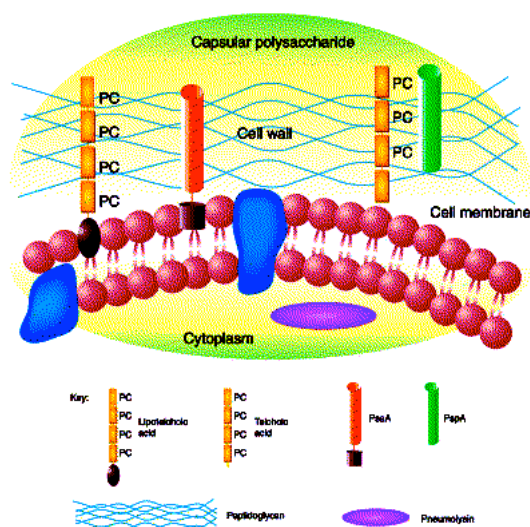


Figure. 3 Outer cell wall & capsule of *Streptococcus pneumoniae* of *Streptococcus pneumoniae*.

Humoral immune response against Pn

Innate and adaptive immunity combine for the optimal clearance of Pn. The innate arm of the immune response includes physical, mechanical as well as cellular components. The innate immune response is both rapid and has limited specificity relative to the adaptive immune response. A major feature of the innate immune response is the influx of neutrophils, and later macrophages, which then phagocytose and kill the organism (Snapper 2006). Initial recognition of Pn is primarily mediated by pattern recognition receptors (PRRs) on the host cell surface including toll like receptors (TLRs) which recognizes conserved molecular patterns (Pathogen Associated Molecular Patterns, PAMPs) on microbes leading to the production of numerous cytokines and chemokines (Akira, Takeda et al. 2001). Host-mediated phagocytosis and killing of Pn is markedly facilitated by opsonization via phagocyte Fc receptors binding to serotype specific antibody, and complement receptors binding to either antibody- or pathogen-bound complement fragments. Of interest, colonization is not directly correlated with the amount of serum or mucosal antibody (Weiser, Austrian et al. 1994; McCool and Weiser 2004). Contrary to popular notions, McCool et al also demonstrated using xid (humoral immune deficient) and μ MT (B cell-deficient) mice that Ab is not required for the T cell-dependent clearance of pneumococcal colonization in mice (McCool and Weiser 2004). On the other hand mice that lack TLR2, the receptor for lipoteichoic acid and/or lipoprotein expressed by Pn, exhibit delayed pneumococcal clearance (van Rossum, Lysenko et al. 2005).

Adaptive immunity starts when APCs, like dendritic cells (DCs) and macrophages, which bind and internalize Ags in the periphery and transport them to the

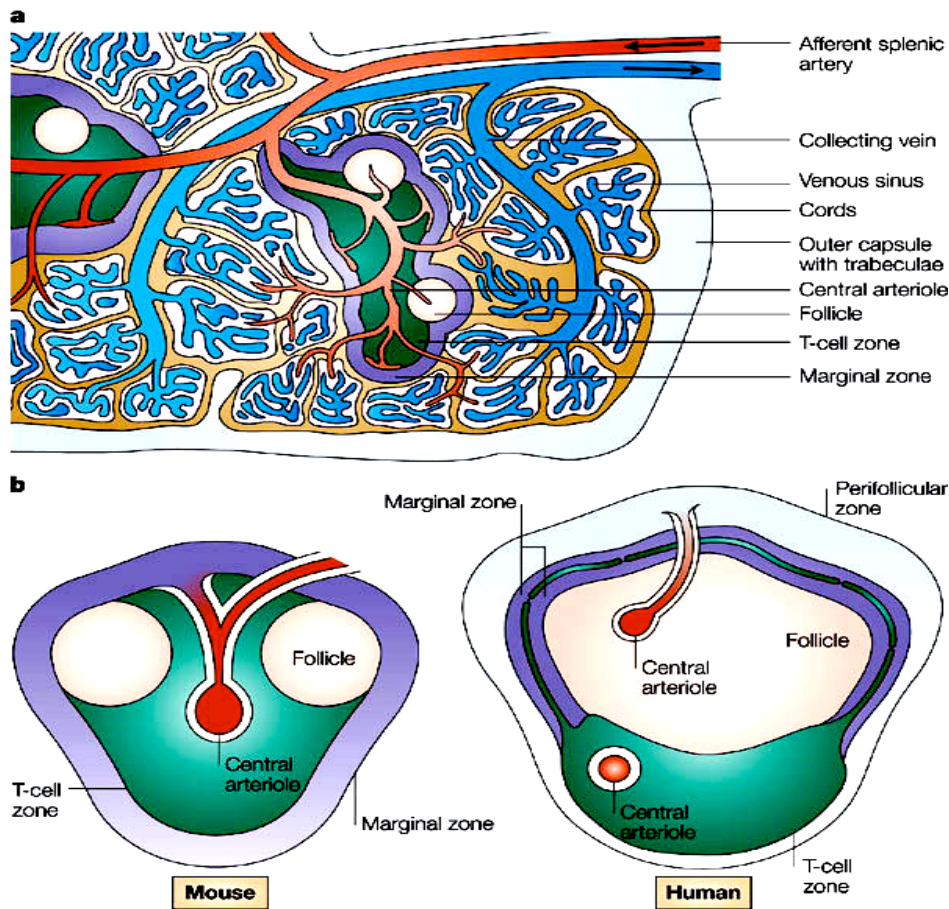
secondary lymphoid organ, where they present them to the helper CD4⁺ T cells.

Activation of B or T lymphocytes by antigen is the critical step to initiate adaptive immunity. B cells acquire particulate antigen in a macrophage rich area within subcapsular sinus in the lymphoid follicle (Carrasco and Batista 2007). B cells, after receiving particulate Ag from marginal zone macrophages, retain Ag for a long period of time before polarizing to the B cell-T cell borders (Carrasco and Batista 2007). Anti-protein responses against intact Pn is comparable with that of purified TD protein Ags but anti-PS response against intact Pn is significantly different than that of purified PS antigen. In fact, both anti-protein and anti-PS responses to intact Pn are CD4⁺T cell dependent (Khan, Lees et al. 2004), need B7-dependent costimulation (Wu, Khan et al. 2000) and CD40-CD40L interactions (Wu, Vos et al. 1999), whereas these requirements are largely lacking in responses to purified PS. The primary, TD, IgG anti-protein response against intact Pn exhibits a relatively prolonged primary kinetics, peaking around day 10 (Wu, Khan et al. 2000) whereas the TI anti-PS IgM and TD anti-PS IgG responses against intact Pn peak on day 6 (Khan, Lees et al. 2004). Both the anti-protein and anti-PS response against intact Pn consists of all four IgG isotypes, whereas that to purified PS is largely IgG3 in the mouse. PS in an intact bacterial context may behave like a TD Ags due to its association within the intact bacterium with immunogenic proteins (AlonsoDeVelasco, Verheul et al. 1995; Snapper 2006). In this regard our lab has been investigating whether intact Pn behaves similarly to a conjugate vaccine for its anti-PS Ig response. The data to date has, in fact, underscored major immunologic differences between these two types of immunogen. Thus, both the anti-protein and anti-PS response against soluble conjugate vaccine is TD and shows significant secondary

memory responses after boosting. In contrast to conjugate vaccine, PS-specific IgG responses against intact Pn fail to show any memory response after secondary challenge (Khan, Lees et al. 2004). Of interest, there is no apparent role of suppressive CD4⁺ T regulatory cells (Tregs) for either the anti-protein and/or anti-PS adaptive immune response against intact Pn probably due to the acute nature of humoral immune response against the extracellular pathogen in contrast to chronic immune states where Tregs have been shown to play a role (Lee, Sen et al. 2005). The differential generation of the memory response for anti-protein vs anti-PS Ig to intact Pn or relative to the conjugate vaccine, cannot be explained by the preferential apoptosis of PS-specific B cells in response to Pn (Chattopadhyay, Khan et al. 2007). Thus, transgenic expression of Bcl-2 or Bcl-xL in murine B cells enhances the primary in vivo anti-PS response, in contrast to anti-protein response, but has no effect on the generation of memory.

Role of the spleen in the generation of the antibody response

Among overwhelming post-splenectomy infections (OPSI) 80% are caused by *Streptococcus pneumoniae* (Pn) (Altamura, Caradonna et al. 2001) which suggests that the spleen is one of the most important protective organs against systemic invasion by Pn. Located in the abdomen, the spleen is the largest filter of blood. It consists of a white pulp (largely containing B and T cells, but also dendritic cells [DC] and macrophages) and red pulp (containing large amounts of blood cells as well as numerous red pulp macrophages, and some DC) (Saito, Yokoi et al. 1988).



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Figure 4. Schematic representation of spleen (A); Comparison of the white pulp of rodents versus human (B)

As shown in Fig.4a, the afferent splenic artery divides into central arterioles which are sheathed by white pulp (specifically T cell) areas. Fig.4b is a schematic comparison of the white pulp structure of human versus rodents. They are more or less similar in structure but have some structural differences (Mebius and Kraal 2005) also. White pulp is subdivided into PALS, follicles and marginal zone (Cesta 2006). The white pulp is circular and mainly consists of B and T lymphocytes (Mebius and Kraal 2005). It functions in a manner similar to the nodules of lymph nodes (LN). White pulp contains

lymphoid follicles where GC reaction occurs. Cell migration within the lymphoid organ undergoes rapid changes upon Ag encounter. Numerous APCs migrate into T-zones and follicles, events induced by TLR signaling and inflammatory cytokines. Ag-reactive lymphocytes quickly shut down their exit program, ensuring that they remain within the Ag-bearing tissues. Ag-specific T cells that have made initial recognition with Ag bearing DCs go on to make extended contacts with DC, and some CD4⁺T cells migrate to the follicle to interact with the activated B cells. Ag-experienced B cells have two major fates; some migrate to the red pulp of the spleen or medulla of the lymph node to become short-term Abs-secreting plasma cells and others participate in the GC reaction within follicle to produce memory cells and long-lived bone marrow plasma cells (Cyster 2005). The extrafollicular response develops relatively rapidly to produce protective antibody early during the immune response. From Ag encounter to Abs generation, extrafollicular responses can be divided into four phases which takes place within separate microenvironments (MacLennan, Toellner et al. 2003). These four phases include Ag encounter with B cells in the blood, B and T cell interaction in T cell zones, plasmablast growth in extrafollicular foci and finally plasma cells gaining access to stroma for a relatively longer survival time (Fig. 5) (MacLennan, Toellner et al. 2003).

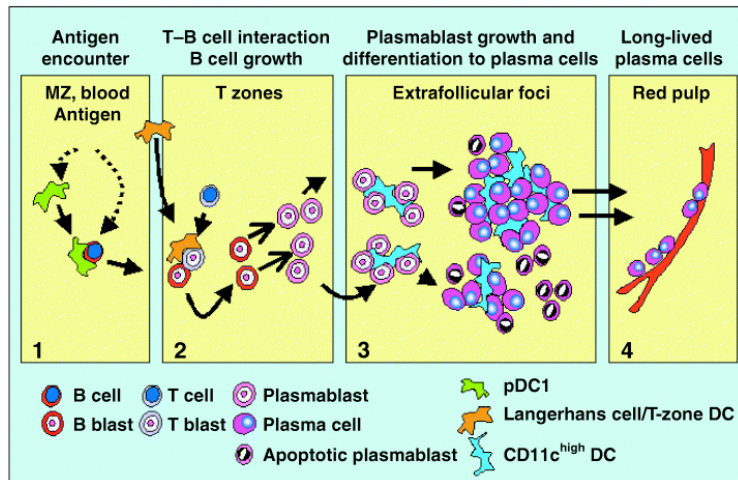


Figure 5. Four microenvironments of extrafollicular Abs production.

Chemokines and chemotactic cytokines play a significant role in the initiation of the adaptive immune response. The correct organization and maintenance of the white pulp is controlled by the interplay between different chemokines that attract T and B cells to their respective domains (Ansel, Ngo et al. 2000). DCs and Ag loaded Ag-specific B-cells interact with T cells in the T-cell zone, and clonal expansion of activated B-cells takes place in the B cell follicles under the influence of chemotactic factors (Mebius and Kraal 2005) as shown in Fig. 6 (MacLennan, Toellner et al. 2003).

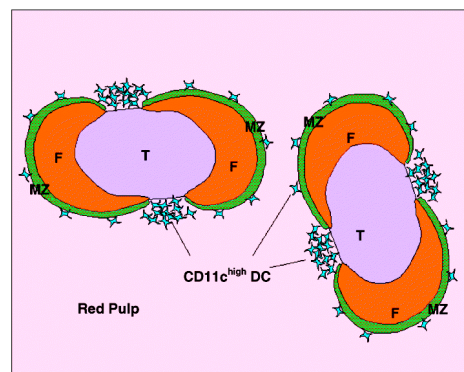


Figure 6. Schematic diagram of a follicle showing T zone, B cell follicle and DC (CD11c)

CXCL13 is required for B cell homing within the follicle whereas CCL19 and CCL21 are involved in attracting T cells and DCs to the T cell zones of the white pulp (Gunn, Kyuwa et al. 1999). B cells in the spleen migrate towards the processes of follicular dendritic cells (FDCs) in search of Ag through chemotactic attractions engaging the receptor CXCR5 on the B cell surface (Cyster 2005). CXCR5 is upregulated when B cell mature in the bone marrow and homing of B cells to lymphoid follicles depends on expression of CXCR5 (Cyster 2005). Follicular stromal cells, including FDCs (which is a specialized subset of fibroblastic reticular cells discovered by ultrastructural studies) produce a protein, CXCL13 which is a strong signal that attracts B cells through CXCR5 on their surface. Follicular B (FB) cells to ensure an efficient survey of FDC are highly motile and move in a “random walk” through the follicle at $\sim 6\mu\text{m}/\text{min}$ (Miller, Wei et al. 2002). Located in the boundary between the follicle and red pulp, and separated from the follicles by a marginal sinus, is the splenic marginal zone which contains marginal zone B (MZB) cells (Kraal 1992; Martin and Kearney 2002). Blood is released in the marginal sinus and filters through this compartment on its way to the red pulp. Entry into the spleen of lymphocytes and other blood cells occurs upon release into the organ from terminal arterioles that open into the marginal sinus or red pulp (van Ewijk and Nieuwenhuis 1985). Most of the naïve T cells express abundant amounts of CCR7 and thus strong chemotactic responses to CCL19 and CCL21. T cells migrate within the T cell zone at an average speed of $12\mu\text{m}/\text{m}$ in a “random walk” like B cells (Miller, Wei et al. 2003; Mempel, Henrickson et al. 2004). Ag can enter into lymphoid tissue either as a free Ag or can be associated with APCs. The relative importance of these two routes of entry is likely to vary depending on the type and amount of Ag. After subcutaneous or

systemic injection of Ag, macrophages in the subcapsular sinus of the draining lymph node or macrophages in the splenic marginal zone, respectively, can bind and transport Ag into lymphoid follicles for Ag delivery to FB cells (Itano and Jenkins 2003). In addition, Ag arriving through blood can be taken up by the MZB cells (specially recognize TI Ag, also capture TD Ag (Cyster 2005) as well as marginal zone macrophages. FB cells capture the particulate Ag from marginal zone macrophages (Carrasco and Batista 2007). LN and spleen also contain a network of collagen fibers which can form a conduit system, allowing for transport of small soluble Ag to the DC-rich area within lymphoid tissue. Injection of pepsin-treated *Streptococcus pneumoniae* is associated with the rapid accumulation of blood DCs in the marginal zone to initiate TI-immune response (Balazs, Martin et al. 2002). Relocalization of Ag-activated B cells near the B cell-T cell interface is governed by the balance between upregulation of CCR7 ligands as well as expression of CXCR5 and CXCL13 (Cyster 2005).

In addition, adaptive immunity within the lymphoid follicle is regulated by the balance between immunity and self tolerance which are mediated by the involvement of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Gavin and Rudensky 2003). Metallophilic macrophages, marginal zone macrophage and MZB form the inner border of the marginal zone of the spleen encircling the white pulp. The marginal zone is a dynamic area with continuous influx and passing of immunocompetent cells (Kraal 1992) and also has some sessile cells. This area has extremely potent phagocytic cells (marginal zone macrophages) which express membrane receptors for bacterial polysaccharides leading to efficient phagocytosis without prior opsonization.

Ag-activated B cells downregulate the expression of CXCR5 and CCR7 but upregulate the expression of CXCR4 which is critical for homing of plasma cells into the red pulp (Hargreaves, Hyman et al. 2001).

GCs are organized into two compartments; the main compartment is the dark zone proximal to the T-zone which contains mainly B cell blasts or centroblasts and the other is the light zone distal to the T-zone that contains centroblast-derived centrocytes, a dense network of germinal center FDCs and helper T cells (MacLennan 1994). Centroblasts undergo somatic mutation within the dark zone and move to the light zone where they compete Ag binding and interact more with helper T cells. Early on, Gowans et al demonstrated that lymphocytes recirculate after entering from blood to lymph node and then from lymph node back to blood (Gowans and Knight 1964). The exit pathway likely involves cells migrating from the white pulp into the red pulp and returning into the blood circulation through venous sinuses involving a G α i-coupled receptor-mediated mechanism (Chaffin and Perlmutter 1991). More recently an immunosuppressant drug named FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) has been identified which inhibits the mixed lymphocyte reaction (MLR) (Fujita, Inoue et al. 1994) and causes rapid loss of lymphocytes from the blood, without affecting NK cells and other myeloid cell members (Chiba, Yanagawa et al. 1998; Yagi, Kamba et al. 2000). Results from several studies suggest that FTY720 acts by inhibiting lymphoid organ egress. MZB cells maintain their positioning within the marginal zone by expressing sphingosine 1-phosphate (S1P) receptor S1P₁ (Cinamon, Matloubian et al. 2004). If the MZB cells lack S1P₁ or are treated with the S1P₁-modulating drug FTY720 they disappear from the marginal zone and relocate within follicles (Cinamon, Matloubian et

al. 2004). Cinamon et al recently showed that via follicular shuttling, MZB cells (which were previously thought to be a sessile population) capture and transport blood borne Ags and deliver them to the FDCs within the white pulp.

Ag encounter promotes transient retention of Ag-reactive B cells within the lymphoid organ (Cyster 2005). Retention occurs via the downregulation of S1P₁ within the Ag-activated cells. For the retention of the Ag-reactive cells, the lymphoid organ undergoes a block or “shutdown” in lymphoid egress either by downregulating S1P₁ or local over production of S1P that disrupt the S1P gradient (Pappu, Schwab et al. 2007).

In light of the central role of the spleen in initiating humoral immune responses following systemic immunizations, we performed select studies on this secondary lymphoid organ to better understand the mechanisms underlying the changes observed in the serum titers of antibodies in our experimental system.

Subsets of B-cells involve in TD vs TI immune response

B cells can be subdivided into two major categories depending on the expression of CD5 molecule. CD5⁻ cells are generally called B-2 cells and CD5⁺ cells are called B-1 cells (Hardy and Hayakawa 2001). However, there is a subset of B cells, most notably in the peritoneal cavity, that share similar phenotypes to CD5⁺ B cells but do not express CD5. So, B-1 cells can be subdivided into B-1a (B220⁺CD11b⁺CD5⁺) and B-1b (B220⁺CD11b⁺CD5⁻). B-1a cells spontaneously produce natural Abs which is necessary for the early protection against invading pathogen. In contrast, B-1b cells play a major role in eliciting a PS-specific adaptive immune response (Haas, Poe et al. 2005). Peritoneum B cells also contain B-2 cells, in addition to B-1a and B-1b. The spleen has

mostly B-2 cells, although 1-2% B-1 cells as well, which can be broadly divided into follicular B cells (FB) ($B220^{+}CD21^{\text{intermediate}}CD23^{\text{hi}}$) and marginal zone B cells (MZB) ($B220^{+}CD21^{\text{high}}CD23^{\text{low}}$). FB-cells typically give rise to TD-immune response whereas MZB-cells are responsible for the TI-immune response. B-1 cells are present mostly in serosal cavities (pleura and peritoneum) but as mentioned are also present as a small percentage in the spleen, and secrete natural Abs (Shapiro-Shelef and Calame 2005; Snapper 2006). Splenic FB and MZB cells differ in function and phenotype (Attanavanich and Kearney 2004). MZB cells express higher levels of the T cell costimulatory molecules, B7.1 (CD80) and B7.2 (CD86) and are able to activate T cells more efficiently (Attanavanich and Kearney 2004). However, FB cells are less efficient than MZB cells for priming T cells. After Ag encounter both MZB cells and FB cells differentiate into plasma cells having limited life span and secrete Ag-specific Abs as part of an extrafollicular response. Another fate of FB cells after Ag encounter is to participate in the GC reaction to generate memory cells and long-lived BM plasma cells (Fig. 7) (Shapiro-Shelef and Calame 2005).

As discussed above different subsets of B cells play distinct roles in humoral immune responses against intact Pn and its soluble conjugate vaccine. Therefore, in our current studies we have emphasized the influence of different B cell subsets in humoral immune responses against intact bacteria. As shown below, when we used Bcl-2/Bcl-x_L Tg mice in comparison to wild type mice, we found a significant increase in B-1b and B-2 peritoneal B cell subsets in Tg mice, which are known to play a major role in TI-immune responses.

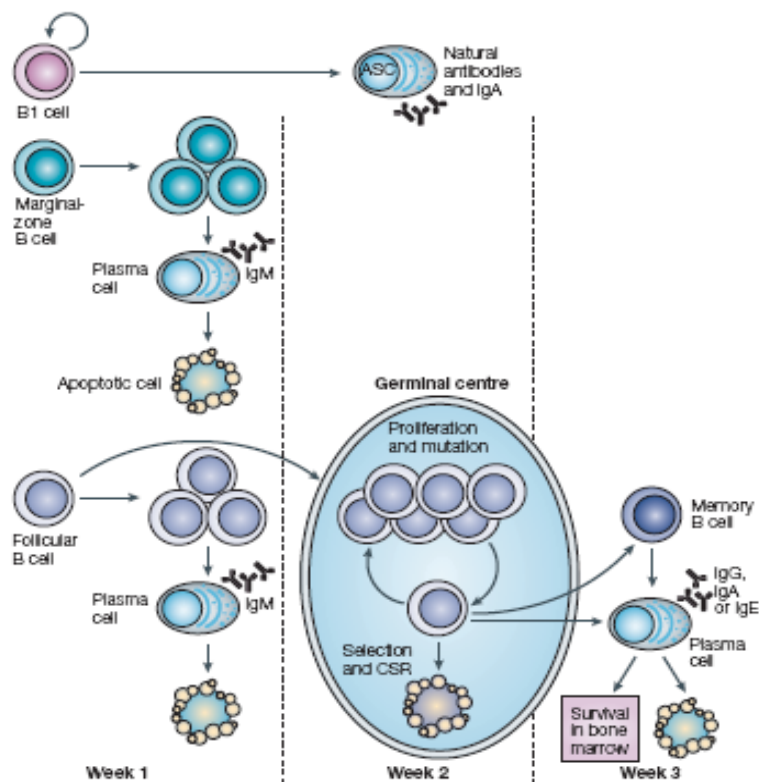


Figure 7. Generation of plasma cell and memory cell after antigen encounter.

Role of Apoptosis in the lifespan of lymphocyte development

Apoptosis is a very important conserved genetic program which is involved in development and homeostasis of the immune system. Apoptosis within lymphoid cells is highly regulated and leads to deletion of cells with non-functional receptors and autoreactive cells. B cell fate during maturation is determined by a balance between survival and death signals that are initiated through the B cell receptor (BCR). In immature B cells or transitional type 1 (T1) B cells, a low basal level of signaling through the BCR is critical for B cell survival, whereas stronger BCR signals mediated by strong binding to self Ag results in apoptosis. In contrast, in mature B cells signals through NF-

kB, AKT or ERK override death signals and prevent apoptosis (Fig.8) (Niir and Clark 2002).

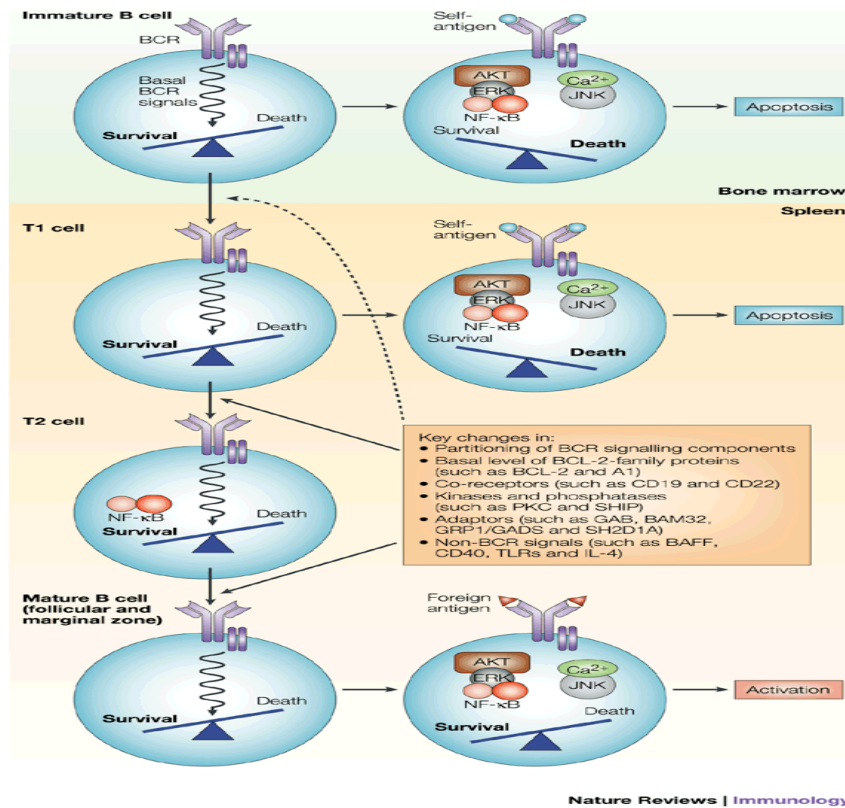


Figure 8. Role of apoptosis in several stages of B cell development

Many of these apoptosis-related checkpoints during lymphocyte development are regulated by the Bcl-2 family of proteins, for example Bcl-2 and Bcl-x_L. These proteins play critical roles in lymphocyte development. Bcl-2 was first identified as an oncogene activated by the characteristic t (14:18) translocation in non-Hodgkin's lymphoma (Cleary, Smith et al. 1986). Bcl-2 is a 25Kda protein primarily localized in the mitochondrial outer membrane, endoplasmic reticulum or in nucleus. Depending on the function Bcl-2 family protein can be divided into anti- and pro-apoptotic protein. Bcl-2

family has four conserved domains which known as Bcl-2 homology (BH) domains, e.g. BH1, Bh2, Bh3 and BH4. BH1 and BH2 are highly conserved located at C-terminal end of these proteins whereas BH3 and BH4 show little similarity (Borner, Martinou et al. 1994). Bcl-2 and Bcl-x_L possess all four BH domains.

Newly-formed B cells that recognize self-Ag are eliminated by apoptosis.

IgM⁺IgD⁺ mature B cells in the periphery undergo T cell dependent affinity maturation of their surface Ag receptors. B cells with high affinity receptors are preferentially selected whereas those displaying low affinity for Ag die by apoptosis (Liu, Joshua et al. 1989). If a B-cell fails to express functional H and L chain on their surface, this leads to cell death (Grillot, Merino et al. 1996). Signaling through IgM is critical for the elimination of B-cells after encounter with a self-Ag. B-cell death in the periphery is mediated by Fas-receptor (Green and Scott 1994). Beside these natural causes of B cell apoptosis during developmental phases, B cell death may result from a wide variety of stimuli including the exposure to cytotoxic drugs, withdrawal of growth factors, γ -irradiation and several others (Nunez, London et al. 1990). Overexpression of Bcl-2 in lymphocytes prevents or delays apoptosis (Vaux, Cory et al. 1988; Nunez, London et al. 1990; Katsumata, Siegel et al. 1992). Bcl-2 expression is markedly diminished in GC B cells where extensive B cell apoptosis is occurring. Among the GC cells, 50% show low mutated Ig genes which undergo apoptosis. GC allows only relatively high affinity germline-encoded Ag receptors to undergo affinity selection. Dysregulated Bcl-2 expression over-rides the need of persistent Ag in the maintenance of B-cell memory (Nunez, Hockenbery et al. 1991). Bcl-2 expression changes during different stages of B cell development. During the maturation of sIgD⁺CD38⁻ naïve B-cells to sIgD⁻CD38⁺ GC B-cells, Bcl-2 protein

content becomes lower whereas Fas/CD95 expression is upregulated (Liu, Arpin et al. 1996). Bcl-2 is required for the maturation of B cells and is critical for mature B-cell survival.

Bcl-x_L-deficient mice died in embryonic stage and their hematopoietic cells in the liver also become apoptotic (Motoyama, Wang et al. 1995). Many B cell precursors fail to complete successful rearrangements of Ig genes and appear to undergo cell death in bone marrow. So, Bcl-x_L affects the life span of immature lymphocytes. Constitutive expression of Bcl-x_L causes accumulation of B cell precursors and mature B cells in animals by enhancing their survival (Grillot, Merino et al. 1996). In vitro stimulation of GC B-cells with CD40-ligand, IL-2 and IL-10 it promotes B-cell survival (Liu, Arpin et al. 1996) and upregulates Bcl-2 (Liu, Joshua et al. 1989). Bcl-x_L was induced after the CD40-CD40L interaction, which in turn promoted mature B cell survival (Hasbold, Johnson-Leger et al. 1994). However, Nunez et al (Nunez, Hockenbery et al. 1991) showed that the effects of Bcl-2 on immune responses is to support long lived B cells involved in Abs production and memory. Bcl-x_L signaling through IgM surface receptor along with CD40 signaling helps mature B cells to overcome apoptosis after Ag induced activation (Grillot, Merino et al. 1996). Collectively, apoptosis in B-cells mediates the removal of early, low affinity Ab-forming cells, unselected GC cells and potentially self-reactive B-cells during plasma cell development, establishing memory B cells and maintaining self-tolerance during an immune response. In our study using Bcl-2 and Bcl-x_L Tg mice we have demonstrated that apoptosis plays a major role by limiting mIg-dependent clonal expansion of PS-specific B cells during a primary immune response to an intact bacterium and also by decreasing the pool of PS-responding B cell subsets.

Models used for experiments

Animal models have proven useful for defining the host and bacterial factors that contribute to pneumococcal colonization that eventually results in the invasiveness that leads to clinical disease. Mice, in particular, represent a convenient and inexpensive model useful for predicting the potential efficacy of vaccine candidates in humans, despite notable caveats (Steinhoff 2007). Thus, Pn Isolates that were previously examined in human experimental colonization models, also colonized inbred adult mice when similar inoculums were used for a similar duration. The immune response against capsular polysaccharide and PspA from these isolates in mice is comparable with that observed in humans (McCool and Weiser 2004). Therefore, mice are useful models to study the complications that arise from bacterial colonization which may lead to pneumonia and bacteremia. Young mice do not respond well to isolated PS antigens due to lack of surface receptor (Lyb5.1) on the neonatal B cell surface and defective BCR signaling in this B cell population (Subbarao, Mosier et al. 1979). Our lab thus employs adult mice for most studies on anti-PS responses. We are primarily interested in the immune response following systemic infection with Pn so our experimental design employs immunization of mice by the intraperitoneally (i.p.) route. In contrast to consistent and reproducible results after i.p. challenge, i.n. challenge tends to produce a greater variability in results in rodent models (Steinhoff 2007).

Among the Pn strain in our laboratory we generally use Pn, capsular PS type 14 (Pn14) as this is the most common strain causing disease and also found in carriers (Kristinsson 1997). Also, purified capsular type 14 PS (PPS14) contains few cell wall contaminants that might otherwise interfere with our specific ELISA assay for detecting

PPS-specific Ig. In select experiments, other serotypes are used including D39 (Pn type 2), WU2 (Pn type 3) and their isogenic mutants R36A (Pn type 2 isogenic mutant), JD11 (Pn type 3 isogenic mutant). We typically use 2×10^8 CFU/200 μ l of Pn14 in PBS per mouse as this dose results in optimal Ig responses; lowering the dose of Pn by 5 or 25 fold results in a significant decrease in the primary IgG titer against the PS antigen (Chattopadhyay & Snapper, unpublished data). Of note, doses of 10^7 live bacteria are not enough to cause lethality in mice but do generate a detectable Ig response. For nearly all in vivo experiments we used heat-inactivated Pn (heat-inactivation was done at 60°C for an hour) since, as discussed above in some detail, we are primarily interested in intact Pn as an immunogenic particle, as opposed to an active infection. As also discussed above we have been interested in comparing the TD responses of intact Pn versus soluble pneumococcal vaccine. The conjugate vaccine used most often in our studies was PPS14-PspA (capsular PS from type 14 and pneumococcal surface protein A) and less often C-PS-PspA (C-PS or teichoic acid expresses phosphorylcholine [PC] determinants; anti-PC antibodies are measured in an ELISA assay). In some studies, PPS14 is also covalently linked to either gp350 (an unrelated EBV protein) or to TT (tetanus toxoid) along and is adsorbed to alum and CpG-ODN (unmethylated CpG DNA of 30 mer) as an adjuvant. The typical protocol is to immunize mice i.p. with either Pn14 in saline or conjugate in alum + CpG-ODN, and boost mice on day-14 (and in select experiments at later time points) with sera typically collected at weekly intervals. Dextran-conjugated anti-IgD monoclonal antibodies (mAbs) ($\alpha\delta$ -dex) were used as an efficient in vitro, polyclonal model of B cell mitogenesis, since the multivalent membrane Ig crosslinking models that

observed for PS-specific B cells contacting their cognate PS antigens (Pecanha, Snapper et al. 1991).

Hypothesis and Specific Aims

The same PS antigen present in different physical/biochemical contexts (intact Pn versus soluble conjugate vaccine) elicits distinctly different, although TD, humoral responses.

The specific aim of these studies includes a better understanding of the mechanisms that distinguish PS-specific humoral response against intact *Streptococcus pneumoniae* versus soluble pneumococcal conjugate vaccine, with a special emphasis on the mechanisms that lead or fail to lead to the polysaccharide-specific IgG memory response.

Hypothesis 1: Enhanced sensitivity to apoptosis of PS-specific versus protein-specific B cells may play a critical role in the failure to generate PS-specific immunologic memory to intact Pn.

Primary IgG anti-capsular polysaccharide (PS) responses to both intact *Streptococcus pneumoniae* (Pn) and a soluble conjugate of Pn-derived protein (pneumococcal surface protein A [PspA]) and PS require specific CD4⁺T cell help, B7-dependent costimulation, and CD40-CD40-ligand interactions (Wu, Vos et al. 1999; Khan, Lees et al. 2004).

However, the IgG anti-PS response elicited by intact Pn, in contrast to the soluble conjugate, peaks more rapidly and fails to induce PS-specific memory. Thus, the same antigen presented to the immune system in different context elicits distinctly different humoral responses. The mechanism underlying these differences remains unknown and has important implications for the design of effective anti-PS vaccines against extracellular bacteria. Thus we hypothesized that the critical difference in the PS-specific memory response between intact Pn and conjugate is the preferential apoptosis of PS-specific B cells against intact Pn resulting in the absence of PS specific memory response

in contrast conjugate vaccine. We thus wished to determine whether B cell specific transgenic over-expression of the anti-apoptotic protein Bcl-2 or Bcl-xL may differentially induce the primary and/or secondary PS-specific humoral response against intact Pn versus soluble conjugate.

Hypothesis 2: Co-immunization of soluble conjugate and intact Pn may lead to cross-regulation of the respective humoral immune responses.

Studies directly comparing the parameters that mediate a PS- and protein-specific Ig response to intact *Streptococcus pneumoniae* (Pn) relative to those that regulate a humoral response to a soluble pneumococcal PS-protein conjugate, have revealed two distinct pathways of immune activation. Specifically, although the IgG anti-PS responses to Pn and conjugate are both dependent upon CD4⁺ T cells, B7/CD28 costimulation, and CD40/CD40L interactions, the IgG anti-PS response to Pn is essentially extrafollicular, with more rapid kinetics of primary induction and failure to generate PS-specific memory, whereas the same response to conjugate is follicular in nature with more prolonged kinetics and the generation of PS-specific memory. Recent data strongly suggest that secretion of PS-specific IgG in response to Pn and conjugate is largely effected by marginal zone (MZB) and follicular (FB) B cells, respectively. In addition, the IgM anti-PS responses to Pn and conjugate are TI and TD, respectively. In contrast, the protein-specific IgG responses to both Pn and conjugate appear to be mediated by FB cells that give rise to a GC reaction followed by the generation of protein-specific memory. Recent, preliminary data from our laboratory appear to indicate that conjugate is more rapidly and extensively transported from the marginal zone into the splenic B cell

follicle and T cell region than intact Pn, following i.p immunization. Whereas large amounts of conjugate localize to splenic DC, intact Pn preferentially accumulates within macrophages. In light of these collective observations we wished to determine the consequences of concomitant immunization with Pn and conjugate via the same i.p. route on the outcome of the PS- and protein-specific Ig responses to the individual immunogens. We believe that this approach may serve as a model for understanding potential cross-regulatory immune pathways mediated by intact pathogens and the soluble products that they secrete.

Chapter two

Transgenic expression of Bcl-x_L or Bcl-2 by murine B cells enhances the in vivo anti-polysaccharide, but not anti-protein, response to intact *Streptococcus pneumoniae*

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Abstract

IgG anti-polysaccharide (PS) and anti-protein responses to *Streptococcus pneumoniae* (Pn) are both CD4⁺ T cell-dependent. However, the primary IgG anti-PS response terminates more quickly, utilizes a shorter period of T cell help, fails to generate memory, and is more dependent on membrane (m)Ig signaling. We thus determined whether this limited anti-PS response to Pn reflected a greater propensity of PS-specific B cells to undergo apoptosis. We utilized mice that constitutively expressed the anti-apoptotic protein Bcl-x_L or Bcl-2 as a B cell-specific transgene. Both Tg mice exhibited increased absolute numbers of splenic B-1, and peritoneal B-1b and B-2 cells, subsets implicated in anti-PS responses, but not in marginal zone B (MZB) cells. Both Tg mouse strains elicited, in an apparently Fas-independent manner, a more prolonged and higher peak primary IgM and IgG anti-PS, but not anti-protein, response to Pn, but without PS-specific memory. A similar effect was not observed using purified PS or pneumococcal conjugate vaccine. In vitro, both splenic MZB and follicular Tg B cells synthesized DNA at markedly higher levels than their wild-type counterparts, following mIg crosslinking. This was associated with increased clonal expansion and decreased apoptosis. Utilizing Lsc^{-/-} mice, the Pn-induced IgG response specific for the capsular PS was found to be almost entirely dependent on MZB cells. Collectively, these data suggest that apoptosis may limit mIg-dependent clonal expansion of PS-specific, B cells during a primary immune response to an intact bacterium, as well as decrease the pool of PS-responding B cell subsets.

Introduction

Purified, soluble polysaccharide (PS), in contrast to protein, antigens can generate comparable Ig responses in wild-type (WT) and T cell-deficient hosts, and are thus referred to as T cell-independent (TI) antigens (Mond, Lees et al. 1995). This, in part, reflects the inability of PS antigens to associate with MHC class II molecules, precluding the recruitment of cognate CD4⁺ T cell help (Harding, Roof et al. 1991; Ishioka, Lamont et al. 1992). Zwitterionic PSs represent a notable exception, but constitute only a minor sub-group of all PSs found in nature (Cobb, Wang et al. 2004; Stephen, Niemeyer et al. 2005). When a PS antigen is covalently linked to an immunogenic protein (i.e. conjugate vaccine), it is transformed into a classical T cell-dependent (TD) antigen. Thus, uptake of the conjugate, and processing of the protein component, by the PS-specific B cell leads to presentation of peptide-MHC class II to specific CD4⁺ T cells (Guttormsen, Wetzler et al. 1998; Guttormsen, Sharpe et al. 1999). As a result, anti-PS responses to conjugate vaccines, in contrast to isolated PS antigens, but similar to isolated proteins, exhibit more prolonged primary kinetics of induction, class switching to IgG isotypes in addition to IgG3, and the generation of PS-specific memory.

Typically, however, the host encounters PS antigens in the context of their expression by an intact microorganism. Thus, in nature, PS antigens are initially presented to the immune system within a particulate structure, associated with numerous distinct proteins, as well as ligands for various innate immune cell receptors that can regulate cellular recognition, uptake, and activation. PSs in this context are therefore

potentially distinct immunogens and hence may elicit Ig responses that are governed by mechanisms that differ from those observed using simplified model antigens. Indeed, accumulating evidence from our laboratory indicates that PS antigens expressed by intact bacteria may be neither classically TI nor TD (Snapper 2006). Thus, immunization of mice with intact *Streptococcus pneumoniae* (Pn), in contrast to an isolated pneumococcal PS antigen, elicits an anti-PS response comprising all 4 IgG isotypes, that is dependent on CD4⁺ T cell help and B7-dependent costimulation, similar to that observed for the concomitant IgG anti-protein response (Wu, Vos et al. 1999; Khan, Lees et al. 2004). However, unlike the anti-protein response, the IgG anti-PS response to intact Pn exhibits a significantly shortened period of primary induction and no apparent memory upon secondary immunization. This is associated with a substantially shorter period of dependence on CD4⁺ T cell help (Khan, Lees et al. 2004) and B7-dependent costimulation (Wu, Khan et al. 2000) for the primary IgG anti-PS, versus anti-protein, response and a greater dependence on B cell membrane (m)Ig signaling, mediated by Bruton's tyrosine kinase (Btk) (Khan, Sen et al. 2006).

Thus, the basis for the relative limitation of the IgG anti-PS, relative to the anti-protein response to intact Pn is not critically related to the absolute ability to recruit CD4⁺ T cell help (i.e. TI vs TD nature of the antigens). In this regard, the strength, duration and/or relative dependence of the mIg signal upon antigen binding, the relative efficiency in recruiting CD4⁺ T cell help, and the involvement of distinct B cell subsets may be more important considerations. One potential consequence is that collectively, these parameters could impact differentially upon the survival/apoptosis of PS- versus

protein-specific B cells, accounting in part for the different primary kinetics and level of induction of the PS-specific Ig response, and perhaps in the failure to generate PS-specific memory.

Two proteins that play a major role in promoting B cell survival are Bcl-x_L and Bcl-2. Although these proteins appear to be equipotent in promoting B cell survival and may act through similar mechanisms (Huang, Cory et al. 1997), they are expressed at different stages of B cell development and activation. Bcl-2 appears to be critical for mature B cell survival (Nakayama, Negishi et al. 1993), whereas Bcl-x_L may be more important in promoting viability of proliferating [immature and activated] B cells (Motoyama, Wang et al. 1995). Thus, resting peripheral B cells constitutively express Bcl-2, whereas Bcl-x_L, although not Bcl-2, is upregulated in these cells upon mlg crosslinking, CD40 signaling, or LPS stimulation resulting in their co-expression (Grillot, Merino et al. 1996). Bcl-2 is then downregulated in B cells within the germinal center (GC), the key site for the generation of memory B cells (Cozine, Wolniak et al. 2005), where extensive apoptosis occurs (Hockenbery, Zutter et al. 1991; Martinez-Valdez, Guret et al. 1996). However, in vitro stimulation via membrane Ig or CD40 promotes GC B cell viability, associated with Bcl-2 upregulation (Liu, Joshua et al. 1989). Additionally, the survival of high-affinity memory B cells can be maintained by Bcl-2 expression (Nunez, Hockenbery et al. 1991). Bcl-x_L, in contrast to Bcl-2, is typically expressed in freshly isolated GC (centrocyte) B cells (Kelsoe 1995; Tuscano, Druey et al. 1996). In light of these observations, we utilized mice that constitutively expressed transgenic Bcl-x_L or Bcl-2, selectively within B cells, to ask whether the sustained

activity of either of these proteins could differentially impact on primary and/or secondary anti-PS and/or anti-protein Ig responses to intact Pn, as well as a soluble pneumococcal conjugate vaccine, or isolated pneumococcal PS.

Materials and Methods

Mice. B cell-specific Bcl- x_L transgenic (Tg) mice kindly provided by Timothy Behrens (University of Minnesota, Minneapolis, MN) were originally made using the human *Bcl-x_L-Tg* cDNA cloned into an expression vector under the regulatory control of the SV40 promoter and IgH enhancer (Grillot, Merino et al. 1996), and were backcrossed 11x onto BALB/c mice. B cell-specific Bcl-2-Tg mice were similarly made using a human *Bcl-2* cDNA inserted into an expression vector containing the IgH enhancer and SV40 promoter (Silva, Kovalchuk et al. 2003) and were backcrossed 20x onto BALB/c mice. Female and male Bcl- x_L and Bcl-2-Tg mice were used between 10-16 weeks of age. Gender and age-matched WT BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used as controls in all experiments. The experiments were conducted under the National Cancer Institute Animal Study Protocols LG023, LG024 and LG025. Mice were genotyped by PCR using the following primers: 1) Bcl- x_L -Tg: Sense: 5' GGCGGGCATTGACCTG 3'; Antisense: 5' TGAGCCCAGCAGAACCACGCCG 3'; Bands: 396 bp transgenic *bcl-x_L*, 321bp endogenous *bcl-x_L* 2) Bcl-2-Tg: Sense: GCAGACACTCTATGCCTGTGTGG; Antisense: GGAAGTGAATGGGAGCAGT; Bands: 361 bp transgenic *Bcl-2*, no band for endogenous *Bcl-2*. *Lsc*^{-/-} mice (Rubtsov, Strauch et al. 2005), used between 8-

12 weeks of age, were generated in, and bred on, a C57BL/6 genetic background, and bred and maintained at the National Jewish Biological Resource Center (Denver, CO). Female *lpr* (Fas-defective), *gld* (Fas-ligand-defective) and WT (C3H/HeJ) mice were purchased from The Jackson Laboratory and were used at 8 weeks of age.

Reagents. Recombinant pneumococcal surface protein A (PspA) was expressed in *Sacharomyces cerevisiae* BJ3505 and purified as previously described (Chen, Sen et al. 2006). Purified PspA was >95% pure by Coomassie blue staining. PC-keyhole limpet hemocyanin (KLH) was synthesized as described previously (Wu, Vos et al. 1999). The resulting conjugate had a substitution degree of 19 PC/KLH. Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from the American Tissue Culture Collection (Manassas, VA). Purified *S. pneumoniae* cell wall C-polysaccharide (C-PS) was purchased from Statens Serum Institut (Copenhagen, Denmark). The soluble C-PS-PspA and PPS14-PspA conjugates were synthesized as previously described (Khan, Lees et al. 2004). Dextran-conjugated anti-IgD ($\alpha\delta$ -dex) was prepared by conjugation of an “a” allotype-specific anti-IgD mAb (clone H δ^a /1) to dextran (2x10⁶ MW) (Brunswick, Finkelman et al. 1988). Agonistic Armenian hamster IgM, κ anti-mouse CD40 mAb (clone HM40-3, no azide, low endotoxin) was obtained from BD Biosciences (San Diego, CA). LPS from *E. coli* serotype 0111:B4 was purchased from Sigma (St. Louis, MO). Goat F(ab')₂ anti-mouse IgM was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Preparation and immunization of *S. pneumoniae*, capsular type 14. A frozen stock of *S. pneumoniae*, capsular type 14 was thawed and sub-cultured on BBL pre-made blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies in blood agar were grown in Todd Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase, collected, and heat killed by incubation at 60°C for 1h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10^9 CFU/ml. Bacteria were then aliquoted at 10^{10} CFU/ml and frozen at -80°C until their use as antigen for mouse immunizations.

Immunizations. Mice were immunized i.p. with 2×10^8 CFU heat-killed Pn14 in saline or PPS14-PspA + C-PS-PspA (conjugates) adsorbed on 13 µg of Alum (Allhydrogel 2% [Brenntag Biosector, Denmark]) mixed with 25 µg of a stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN) (Sen, Flora et al. 2004), and similarly boosted. One microgram each of purified C-PS and PPS14 was injected i.p. in saline. Serum samples for measurement of anti-PPS14, anti-PC, and anti-PspA Ig isotype titers were prepared from blood obtained through the tail vein.

Measurement of serum Ig isotype titers of anti-PC, anti-PPS14, and anti-PspA. ELISA plates were coated with 5 µg/ml (50µL/well) of PC-KLH (Immulon 2 plates, Dynex Technologies, Inc., Chantilly, VA), PPS14 (Immulon 2 or 4), or PspA (Immulon 4) in PBS for 1h at 37°C or overnight at 4°C. Plates were washed 3X with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 30 min at 37°C or

overnight at 4°C. Threefold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS + 0.05% Tween 20 were then added for 1 h at 37°C or overnight at 4°C and plates were washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, and IgG isotype Abs (200 ng/ml final concentration in PBS + 0.05% Tween 20) were then added, and plates were incubated for 37°C for 1 h. Plates were washed five times with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma, St. Louis, MO) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) was then added for 30 min at RT for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Purification of splenic B cells. Single cell suspensions from spleen were prepared, and RBCs were lysed using ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD). B cells were positively selected by magnetic bead sorting using anti-mouse CD45R (B220) micro magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cell purities were checked by flow cytometry following each purification and found to be 90-92% B220+ cells. Purified B cells were used immediately for cell culture studies.

Measurement of DNA synthesis by [³H]-TdR incorporation. Purified B220+ splenic B cells were cultured (2.5 x 10⁵ cells/ml in 0.2 ml) in medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-ME, 50 µg/ml penicillin, and 50 µg/ml streptomycin), in the presence of various stimuli, in flat-

bottom 96 well Costar plates (Corning Incorporated, Corning, NY). After various times in culture at 37°C in a 5% CO₂-containing incubator, [³H]-TdR (2 µCi; specific activity of 25Ci/mmol or 925GBq/mmol, Cat#TRK120) [Amersham Biosciences, Piscataway, NJ] was added to the cultures for an additional 18 h. Cultured cells were then harvested onto glass filter paper (Wallac, Turku, Finland, Cat # 1450-421) using a Harvester 96 (Tomtec, Hamden, CT). Specific incorporation of [³H]-TdR was determined using a 1450 Microbeta, “Wallac” Trilux scintillation counter.

Measurement of cell division by CFDA-SE dilution. 2.5×10^7 cells were pelleted and washed with PBS containing 0.1% BSA (buffer), resuspended in 1 ml of buffer, and incubated at 37°C with 5 µM final concentration of carboxy-fluorescein diacetate, succinimidyl ester (Vybrant CFDA-SE, Molecular Probes, Eugene, OR) for 10 min. After labeling, cells were washed 2x with RPMI 1640 + 10% FBS and resuspended in medium. CFDA-SE-loaded B cells were cultured for varying times at 5×10^5 cells/ml in 24-well plates (2 ml/well) in the presence of αδ-dex (10 ng/ml final concentration). Cells were analyzed using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL) and “proliferation index” (P.I.) based on CFDA-SE dilution was obtained by using Modfit software (Verity Software House, Topsham, ME). Specifically, the P.I. is the sum of the cells in all generations divided by the calculated number of original parent cells.

Measurement of apoptotic cells using propidium iodide. B cells undergoing apoptosis were identified by their reduced relative nuclear DNA content using propidium

iodide incorporation in a hypotonic solution followed by flow cytometric analysis as previously described (Nicoletti, Migliorati et al. 1991). Briefly, B cells were cultured for 4 days at 5×10^5 cells/ml in 24-well plates (2 ml/well) in the presence of $\alpha\delta$ -dex (10 ng/ml final concentration). B cells were then resuspended in a hypotonic solution containing 50 μ g/ml PI (Molecular Probes, Carlsbad, CA), 0.1% Triton X-100 and 0.1% sodium citrate. Samples were stored at 4°C for 16-18 h in the dark and vortexed before analysis using a Coulter Epics XL-MCL flow cytometer. Data were analyzed using “Winlist” software (Verity Software House, Topsham, ME).

Flow cytometric analysis and electronic cell sorting. Splenic and peritoneal cells from WT and Tg mice (three mice per group, each stained separately) were harvested, and B cell subsets were enumerated by flow cytometry as follows: splenic marginal zone B [MZB] ($CD21^{high}CD23^{low/neg}$), follicular B [FB] ($CD21^{intermediate}CD23^{high}$), and B-1 ($B220^{intermed}CD5^{+}$), peritoneal B-1a ($B220^{+}CD11b^{+}CD5^{+}$), B-1b ($B220^{+}CD11b^{+}CD5^{-}$), and B-2 ($B220^{+}CD11b^{-}CD5^{-}$). The following mAbs, purchased from Pharmingen (San Diego, CA) were used: FITC-rat IgG_{2b}, κ anti-mouse CD21/35 (clone 7G6) PE-rat IgG_{2a}, κ anti-mouse CD23 (clone B3B4), FITC-rat IgG_{2a}, κ anti-mouse CD45R/B220 (clone RA3-6B2), biotin-rat IgG_{2a}, κ anti-mouse CD5 [Ly-1] (clone 53-7.3) + streptavidin-PE-Texas Red, and PE-rat IgG_{2b}, κ anti-mouse CD11b (clone M1/70). Cells were analyzed on a “BD-LSR-II” flow cytometer (BD Biosciences, San Jose, CA) using 488 and 635 lasers and results generated using Winlist software. For isolation of MZB and FB cells, RBC-lysed spleen cells were

stained for these B cell subsets as above, and purified using a BD Biosciences FACS Aria flow cytometer cell sorter. Purities of >98% for each B cell subset were obtained.

Statistical Analysis. Data is expressed as geometric mean +/- standard error of the mean (S. E. M.). Significance [*] ($p < 0.05$) between groups was determined using the Student's t-test.

Results

Bcl-x_L-Tg and Bcl-2-Tg mice exhibit significant increases in splenic B-1 and peritoneal B-1b cells. Since B cell subsets may contribute differentially to anti-PS and anti-protein Ig responses, we first wished to determine the percentages and absolute numbers of these cells in the spleen and peritoneum of Bcl-x_L-Tg and Bcl-2-Tg mice, relative to WT mice. Both Tg mouse strains exhibited significant ($p < 0.05$) increases in the total number of spleen cells (Bcl-x_L-Tg: 2.5-fold, Bcl-2-Tg: 2.5-fold) and peritoneal cells (Bcl-x_L-Tg: 5.5-fold, Bcl-2-Tg: 3.8-fold), reflecting in large part, an increase in total B cells (Table 1). Although the percentages of B-1 cells were similar in WT and Tg mice, the absolute number of B-1 cells was significantly increased (Bcl-x_L-Tg: 2.8-fold, Bcl-2-Tg: 3.0-fold). In contrast, the percentages of splenic marginal zone B (MZB) cells in Tg mice were significantly reduced (Bcl-x_L-Tg: 3.2-fold, Bcl-2-Tg: 4.4-fold) but the absolute numbers were not statistically different relative to WT mice. Neither the percentages nor absolute numbers of splenic follicular B (FB) cells were significantly different in WT and

Tg mice. Although the percentages of peritoneal B-1b cells in Tg mice were only modestly higher than in WT mice, the absolute numbers were substantially increased (Bcl-x_L-Tg: 8.4-fold, Bcl-2-Tg: 6.1-fold). Similarly, the absolute numbers of peritoneal B-2 cells were strikingly higher in Tg mice (Bcl-x_L-Tg: 12-fold, Bcl-2-Tg: 16-fold) whereas the percentages were more modestly increased (Bcl-x_L-Tg: 2.2-fold, Bcl-2-Tg: 3.2-fold). In contrast, no significant differences were observed between WT and Tg mice in the absolute numbers of peritoneal B-1a cells, whereas the percentages were significantly reduced in Tg mice (Table 1). Thus, B cell subsets known to play a selective role in T cell-independent anti-PS responses [splenic B-1 cells (Hayakawa and Hardy 2000; Martin and Kearney 2001), peritoneal B-1b cells (Alugupalli, Leong et al. 2004; Haas, Poe et al. 2005), and possibly peritoneal B-2 cells (Hastings, Tumang et al. 2006)] are elevated in Tg mice, whereas those implicated in anti-protein responses (FB cells) are similar between WT and Tg mice.

Bcl-x_L-Tg and Bcl-2-Tg mice elicit higher primary anti-PS, but not anti-protein, responses to intact Pn, relative to WT control mice. We next wished to determine whether Tg mice exhibit differences in their elicitation of primary and/or secondary anti-PS and/or anti-protein Ig isotype responses to intact *Streptococcus pneumoniae*, capsular type 14 [Pn14]). Bcl-x_L-Tg and Bcl-2-Tg mice were immunized i.p. with heat-killed Pn14 suspended in saline, and boosted in a similar fashion 14 days later. Sera were obtained at various time points to determine, by ELISA, the relative serum titers of IgM and/or IgG specific for the type 14 capsular PS (PPS14), the phosphorylcholine (PC) determinant of the C-polysaccharide (teichoic acid), and the cell

wall, PC-binding protein, pneumococcal surface protein A (PspA). As illustrated in Fig. 9A, Bcl-x_L-Tg mice exhibited a significant ($p < 0.05$) 4.1-fold and 9.0-fold increase in peak primary serum titers of IgM and IgG anti-PPS14, respectively, relative to WT mice. A similar significant 6.9-fold and 2.5-fold increase in the peak primary IgM and IgG anti-PPS14 responses was also observed in Bcl-2-Tg mice (Fig. 9B). Importantly, these increases largely reflected a more sustained primary Ig response in Tg mice. Upon secondary immunization, comparable boosts in serum IgM anti-PPS14 titers were observed in Tg and WT mice with Bcl-x_L-Tg (Fig. 9A) and Bcl-2-Tg (Fig. 9B) mice still showing significant 2.5-fold and 4.7-fold higher titers, respectively, relative to WT mice. Secondary IgG anti-PPS14 responses showed little, if any, boosting in serum titers in either Tg or WT mice, with Bcl-x_L-Tg (Fig. 9A) and Bcl-2-Tg (Fig. 9B) mice maintaining 6.8-fold and 5.0-fold higher secondary titers, respectively, relative to WT mice. Further analysis of secondary IgG isotypes in Bcl-x_L and WT mice demonstrated significantly ($p < 0.05$) higher titers of all IgG isotypes (IgG3 [6.3-fold], IgG1 [7.0-fold], IgG2b [14-fold], and IgG2a [3.6-fold]) in Bcl-x_L relative to WT mice (Figure 9C).

Primary and secondary IgM anti-PC responses were also significantly higher in Tg relative to WT mice (Bcl-x_L-Tg: primary 2.2-fold, secondary 2.1-fold; Bcl-2-Tg: primary 3.3-fold, secondary 9.2-fold), similar to that observed for IgM anti-PPS14 titers, although IgG anti-PC responses were largely equivalent (Figure 9A/B). Of note, no significant differences were observed in primary or secondary serum titers of IgG anti-PspA in Bcl-x_L-Tg or Bcl-2-Tg mice relative to WT mice. Similar results were observed for IgG responses specific for two additional pneumococcal proteins (pneumococcal

surface protein C [PspC] and pneumococcal surface adhesin A [PsaA]) (data not shown). These data demonstrate that forced expression of anti-apoptotic proteins within B cells results in a selective enhancement in anti-PS versus anti-protein responses to intact Pn14 largely due to a prolongation of the primary response, without a significant effect on the secondary response following boosting.

Anti-PS responses to PPS14-PspA + C-PS-PspA conjugate vaccine or to purified PPS14 and C-PS are similar in Bcl-x_L and WT mice. As discussed in the *Introduction*, a number of key parameters associated with the induction of the anti-PS response to intact Pn are distinctly different from those associated with the anti-PS response to conjugate vaccine or to purified PSs. In this regard, the intact pathogen behaves as a relatively unique immunogen. To investigate this further, we asked whether constitutive B cell-expression of transgenic Bcl-x_L had a similar enhancing affect on the anti-PS response to either a mixture of 2 soluble pneumococcal conjugates (PPS14-PspA + C-polysaccharide [C-PS]-PspA) or to purified PPS14 + C-PS. Thus, Bcl-x_L Tg and WT mice were immunized i.p. with the conjugate mixture in the presence of adjuvant (alum + CpG-ODN) or i.p. with the 2 isolated PSs in saline. For the conjugates, mice were boosted on days 28 and 56, whereas for the isolated PSs mice were boosted on day 14. As illustrated in Fig. 10A both the primary, secondary, and tertiary IgM and IgG anti-PS (anti-PPS14 and anti-PC) and anti-protein (anti-PspA) responses were similar between WT and Bcl-x_L mice. Likewise, no significant differences between WT and Bcl-x_L-Tg mice were observed for IgM and IgG anti-PC and anti-PPS14 responses to the isolated PSs (Fig. 10B). These data further support the notion that the anti-PS response to intact

Pn14 exhibits unique features relative to that observed following immunization with either conjugate vaccine or isolated PSs.

Lpr (Fas-defective) and gld (Fas-ligand-defective) mice do not elicit elevated anti-PS responses to intact Pn14. The selective enhancement of anti-PS responses to intact Pn14 in mice constitutively expressing either Bcl-x_L or Bcl-2 under the control of the Igh enhancer, suggested that B cell apoptosis in WT mice might limit these responses in vivo. Fas-mediated signaling in B cells is one potential, though non-exclusive, pathway that mediates B cell apoptosis (Mizuno, Zhong et al. 2003). We thus wished to determine whether mice genetically defective in the Fas/Fas-ligand pathway might recapitulate the observations made in the Bcl-x_L-Tg or Bcl-2-Tg mice, in response to intact Pn14. We thus immunized lpr (Fas-defective) and gld (Fas-ligand-defective) mice and their WT controls (C3H/HeJ) i.p. with intact Pn14 in saline, followed by boosting on day 21. Neither lpr nor gld mice exhibited elevations in primary or secondary IgM and/or IgG responses specific for PPS14, PC, or PspA (Fig. 11A/B). A modest decrease in primary IgM and IgG anti-PPS14 responses was observed in gld relative to WT mice. These data suggest that a Fas/Fas-ligand-independent pathway of apoptosis likely plays a significant role in limiting anti-PS response to intact Pn14. Although C3H/HeJ mice, used as a control for lpr and gld mice, have a non-functional TLR4 gene (Poltorak, He et al. 1998) they elicit Ig responses to Pn14 similar to their WT C3H/HeN controls (data not shown).

Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit more sustained DNA synthesis in vitro in response to multivalent mIg crosslinking, but not in response to CD40- or LPS-mediated signaling. We recently reported that anti-PS responses to intact Pn14 were more dependent than anti-protein responses, on btk-dependent membrane (m)Ig signaling (Khan, Sen et al. 2006). We thus wished to determine whether the enhancements in anti-PS responses in Bcl-x_L-Tg and Bcl-2-Tg mice in response to Pn14 might be explained, at least in part, by an increased responsiveness of Tg B to mIg crosslinking. We utilized dextran-conjugated anti-IgD antibodies ($\alpha\delta$ -dex), which we previously demonstrated to be an in vitro model of multivalent mIg crosslinking in response to PS antigens (Snapper and Mond 1996). Purified splenic B cells (B220+) from WT or Tg mice were stimulated in vitro with $\alpha\delta$ -dex and/or LPS or agonistic anti-CD40 mAb, each at a dose that induces optimal B cell proliferation. ³H-thymidine incorporation, as a measure of DNA synthesis, was determined on each of days 1-5 (Fig. 12A). Bcl-x_L-Tg and Bcl-2-Tg B cells exhibited substantially higher peak responses (day 3) than WT (day 2) B cells to $\alpha\delta$ -dex stimulation (Bcl-x_L-Tg: 4.2-fold, Bcl-2-Tg: 4.6-fold). Additionally, Tg B cells maintained high levels of DNA synthesis, relative to the peak response, on days 4 and 5 (Bcl-x_L-Tg: 74% and 58% of peak, Bcl-2-Tg: 43% and 50% of peak, WT: 11% and 11% of peak, respectively). In contrast, WT and Tg B cells activated with either anti-CD40 mAb or LPS made comparable responses, except for 1.6- and 2.7-fold higher responses of LPS-activated Bcl-x_L-Tg and Bcl-2-Tg B cells, respectively, on day 5 (Fig. 12A).

As illustrated in Fig. 12A combined stimulation with a synergistic combination of $\alpha\delta$ -dex + LPS or $\alpha\delta$ -dex + anti-CD40 mAb resulted in roughly comparable peak responses (day 3) between WT and Tg B cells that was higher than that observed for any of the 3 stimulants alone. Of note, whereas responses of WT B cells declined significantly on days 4 and 5, Tg B cell responses continued to rise modestly. Additionally, early during the culture (days 1 and 2) the response of Tg B cells lagged behind that of WT B cells, with WT B cells showing higher levels of DNA synthesis. Intact Pn14 contains a number of distinct TLR ligands. In light of the synergy between $\alpha\delta$ -dex and LPS, for DNA synthesis, we wished to determine whether $\alpha\delta$ -dex and Pn14 also synergized for DNA synthesis and whether the response of Tg B cells to $\alpha\delta$ -dex + Pn14 differed from that of WT B cells. As illustrated in Fig. 12B, both WT and Tg B cells synthesized DNA only modestly to Pn14 alone. Similar to that observed in Fig. 12A, Tg B cells maintained sustained levels of DNA synthesis in response to $\alpha\delta$ -dex alone (days 3-5), whereas the WT B cell response declined dramatically during this period. Of note, combined activation with $\alpha\delta$ -dex + Pn14 was synergistic for DNA synthesis by both WT and Tg B cells, with Tg B cells exhibiting significantly higher responses from days 2-5 relative to WT B cells (Fig. 12B).

Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit greater clonal expansion in response to $\alpha\delta$ -dex. The selective enhancement in DNA synthesis of Bcl-x_L and Bcl-2-Tg B cells in response to $\alpha\delta$ -dex activation, further suggested that Tg B cells also underwent a greater degree of clonal expansion than WT B cells. To determine this, we labeled purified splenic B cells (B220+) from WT and Tg mice with CFDA-SE

and measured the degree of CFDA-SE dilution, by flow cytometry, on various days following $\alpha\delta$ -dex activation. A progressive 50% reduction in CFDA-SE fluorescence follows each round of proliferation. The overall clonal expansion of the B cell population can then be expressed as a “proliferation index” [P.I.] (see Methods). As illustrated in Fig. 13 Bcl- x_L -Tg and Bcl-2-Tg B cells stimulated with $\alpha\delta$ -dex demonstrated a higher P.I. on each of days 1-5 relative to WT B cells. Of note, whereas the PI of WT B cells progressively increased up to day 4 and then decreased slightly on day 5, the PI of Tg B cells continued to increase up to day 5. Thus, the more sustained levels of DNA synthesis of Tg, relative to WT, B cells in response to $\alpha\delta$ -dex correlated with a greater degree of clonal expansion.

Bcl- x_L -Tg and Bcl-2-Tg B cells undergo less apoptosis than WT B cells in response to $\alpha\delta$ -dex. In a final set of analyses we determined whether the enhanced DNA synthesis and clonal expansion of Tg B cells in response to $\alpha\delta$ -dex reflected a decrease in apoptosis relative to WT B cells. To determine this we measured percentages of hypodiploid nuclei of WT and Tg B cells stimulated for 3-5 days with $\alpha\delta$ -dex. As illustrated in Fig. 14A, $\alpha\delta$ -dex-activated WT B cells exhibited a reproducibly higher percentage of hypodiploid nuclei on days 3, 4, and 5 relative to Tg B cells. By day 5, 81% of $\alpha\delta$ -dex-activated B cells were apoptotic on the basis of hypodiploid nuclei, whereas only 37% and 26% of Bcl- x_L -Tg and Bcl-2-Tg B cells, respectively, were apoptotic. Thus, transgenic expression of Bcl- x_L and Bcl-2 inhibited B cell apoptosis in response to $\alpha\delta$ -dex throughout the latter period of culture, which likely contributed to the greater clonal expansion of individual $\alpha\delta$ -dex-activated Tg B cells illustrated in Fig. 13.

The IgG anti-PPS14 response to Pn14 is markedly reduced in Lsc^{-/-} mice.

The increase in absolute numbers of splenic B-1 and peritoneal B-1b and B-2 cells in Tg mice, as well as the enhanced mitogenic response of Tg, relative to WT, B cells following mIg crosslinking suggest two possible, mutually-inclusive, mechanisms for the enhanced anti-PS response to Pn14 in Tg mice. To better resolve the underlying mechanism(s), we first wished to determine the B cell subset(s) that give rise to the anti-PS response to Pn14. Mice genetically deficient in Lsc (Lsc^{-/-}) on the C57BL/6 background exhibit a marked defect in MZB migration from the marginal zone following immunization, precluding MZB interaction with CD4⁺ T cells (Rubtsov, Strauch et al. 2005). Lsc acts selectively on MZB cells (Girkontaite, Missy et al. 2001; Rubtsov, Strauch et al. 2005). Thus, the TD IgM anti-NP response to NP-CG, which is dependent on MZB, is markedly reduced in Lsc^{-/-} mice, whereas the TI anti-NP response to NP-Ficoll is not (Rubtsov, Strauch et al. 2005). The IgG anti-PPS14, anti-PC, as well as the anti-PspA, responses are dependent on CD4⁺ T cells, whereas the IgM anti-PPS14 and anti-PC responses are TI (Wu, Vos et al. 1999; Khan, Lees et al. 2004). We thus utilized the Lsc^{-/-} mouse to determine whether any of these TD IgG responses were derived from MZB cells.

Lsc^{-/-} mice were immunized i.p. with Pn14 and then boosted i.p. with Pn14, 14 days later. Sera were obtained on days 0, 7, 14, and 21 for determination of antigen-specific IgM and IgG titers. As illustrated in Fig. 15, upper panel, Lsc^{-/-} mice exhibited a nearly complete abrogation in the primary and secondary IgG anti-PPS14 response. In contrast, the IgG anti-PC and anti-PspA responses were comparable between Lsc^{-/-} and

WT mice, as well as the IgM anti-PPS14 and anti-PC responses. Thus, these data strongly suggest that the IgG anti-PPS14 response to Pn14 is derived almost entirely from MZB cells. In distinct contrast to Pn14, Lsc^{-/-} mice immunized with a mixture of 2 soluble pneumococcal conjugates (PPS14-PspA + C-polysaccharide (C-PS)-PspA) in alum + CpG-ODN, elicited primary and secondary IgG anti-PPS14 responses comparable to WT mice, in addition to similar IgM and IgG anti-PC, and IgG anti-PspA responses (Fig. 15, lower panel). These data further suggest that the IgG anti-PPS14 response to soluble conjugate vaccine, in contrast to intact Pn14, is derived from FB cells.

Both MZB and FB cells from Bcl-2-Tg, in contrast to WT, mice exhibit markedly higher levels of DNA synthesis late in culture, following mIg crosslinking.

The observation that the IgG anti-PPS14 response to Pn14 appears to derive almost exclusively from MZB cells, and the roughly equivalent absolute numbers of MZB cells in Tg and WT mice, suggests that the prolongation and elevation of this response in Bcl^{x_L}-Tg and Bcl-2-Tg mice, might reflect a more sustained mitogenic response of Tg MZB cells following mIg crosslinking, as we demonstrated for unfractionated splenic B cells (Figs. 12 and 13). To determine this, we purified splenic MZB and FB cells from Bcl-2-Tg and WT mice by electronic cell sorting and cultured them in the presence of $\alpha\delta$ -dex, F(ab')₂ anti-IgM, LPS, or agonistic anti-CD40 mAb, for 4 or 5 days, time points during which the mitogenic response of WT B cells are in substantial decline (see Fig. 12). As illustrated in Fig. 16A, WT MZB cells elicited a significantly higher LPS response than FB cells. In contrast, FB cells responded much more vigorously to mIg crosslinking than MZB cells (Fig. 16A and B), whereas responses to anti-CD40 mAb were comparable.

These responses are consistent with previous reports (Snapper, Yamada et al. 1993; Oliver, Martin et al. 1997). Of note, DNA synthesis in Bcl-2-Tg MZB cells in response to mIg crosslinking ($\alpha\delta$ -dex or F(ab')₂ anti-IgM) was 20-95-fold higher than WT MZB cells on day 4 or 5 (Fig. 16A and B). A smaller, but significant enhancement in DNA synthesis (4.4-fold) was observed in Tg, relative to WT, B cells activated with anti-CD40 mAb (Fig. 16B), whereas LPS responses were comparable (Fig. 16A). DNA synthesis in Tg FB cells activated via mIg crosslinking was also dramatically enhanced (13-68-fold) on day 4 or 5, relative to WT FB cells, roughly comparable to the –fold increases observed in Tg versus WT MZB cells (Fig. 16A and B). A smaller, but significant enhancement in the LPS response of Tg relative to WT FB cells (3.6 and 5.1) on day 4 and 5, respectively was also observed (Fig. 16A), whereas no significant differences were observed in response to anti-CD40 mAb (Fig. 16B). Collectively, these data support the notion that, at least for the IgG anti-PPS response to Pn14, the more prolonged and elevated responses in Bcl-x_L-Tg and Bcl-2-Tg mice reflect a more sustained mitogenic response of PPS14-specific B cells following immunization, likely due to decreased apoptosis.

Discussion

This study makes the following major, novel observations: 1) B cell-specific transgenic expression of either Bcl-x_L or Bcl-2 results in a selective enhancement of the primary IgM and IgG anti-PS, in contrast to the anti-protein, response to intact Pn14; this enhancement in the anti-PS response is not observed in response to isolated PS antigens or to PS-protein conjugate, emphasizing the unique nature of the intact bacterium as an

immunogen. 2) Despite a more prolonged and higher anti-PS response to Pn14 in Tg mice, PS-specific memory is still not generated. 3) Genetic disruption of Fas/Fas-ligand signaling has no enhancing effect on either the anti-PS or anti-protein response to Pn14, strongly suggesting that transgenic Bcl-x_L and Bcl-2 enhance anti-PS responses in a Fas-independent manner. 4) Bcl-x_L-Tg and Bcl-2-Tg mice exhibit higher absolute numbers of splenic B-1 and peritoneal B-1b and B-2 cells relative to WT mice, cells known or suspected to play a selective role in anti-PS responses. Notably, however, Tg and WT mice have comparable absolute numbers of MZB cells, a subset also strongly implicated in PS-specific immunity. 5) Unfractionated Tg B cells undergo more sustained DNA synthesis in response to multivalent mIg crosslinking, but not in response to agonistic anti-CD40 mAb or LPS, associated with greater clonal expansion of Tg B cells and lower levels of apoptosis. 6) Studies in the Lsc^{-/-} mouse strongly suggest a critical role for the MZB, at least for the IgG anti-PPS14 response to intact Pn14, but not soluble pneumococcal conjugate, the latter response likely arising from FB cells. 7) The marked enhancement in DNA synthesis in Tg MZB cells following mIg crosslinking suggests one potential mechanism for the enhanced Pn14-induced IgG anti-PPS14 response.

PS antigens, unlike proteins, contain repeating identical antigenic epitopes capable of multivalent mIg crosslinking (Mond, Lees et al. 1995). Dextran-conjugated anti-IgD mAbs ($\alpha\delta$ -dex), an in vitro polyclonal model for PS-mediated multivalent mIg crosslinking (Pecanha, Snapper et al. 1991), has been shown to deliver potent mitogenic signals to B cells at doses of anti-Ig that are 1000-fold lower than that observed using unconjugated, bivalent anti-Ig antibody (Brunswick, Finkelman et al. 1988).

Additionally, in response to the same set of costimulating cytokines, $\alpha\delta$ -dex often induces a different functional outcome, relative to LPS or CD40-mediated activation (Snapper, Yamaguchi et al. 1994). These data suggest that mIg signaling in response to PS antigens may be quantitatively and qualitatively distinct from that which occurs upon contact with a protein antigen. In this regard, we recently demonstrated that mice with reduced, though not absent, btk-mediated mIg signaling in B cells, while having largely restored B cell development (Satterthwaite, Cheroutre et al. 1997; Satterthwaite, Li et al. 1998) elicit significantly lower IgM and IgG anti-PS, but not IgG anti-protein, responses to intact Pn14 (Khan, Sen et al. 2006). Of note, MZB cells, which are known to play a role in anti-PS responses, including Pn (Martin, Oliver et al. 2001; Pillai, Cariappa et al. 2005), and directly implicated, in this study, in the IgG anti-PPS14 response to Pn14, proliferate relatively poorly in vitro upon multivalent mIg crosslinking alone (Snapper and Mond 1993), relative to FB cells, despite exhibiting higher levels of proliferation upon activation with LPS (Snapper and Mond 1993; Oliver, Martin et al. 1997). Our demonstration that Bcl-x_L-Tg and Bcl-2-Tg B cells activated with $\alpha\delta$ -dex exhibit a more prolonged, high-level of DNA synthesis, associated with enhanced clonal expansion, and decreased apoptosis, relative to WT B cells, suggests one potential mechanism for the selective prolongation and higher peak levels of the primary in vivo anti-PS response in Tg mice. Our further observation of markedly elevated levels of DNA synthesis in Tg MZB, as well as FB, cells following mIg crosslinking, combined with the comparable numbers of MZB cells in un-immunized Tg and WT mice, lend strong support to this notion. This mechanism, and/or the increased numbers of splenic B-1, and/or peritoneal

B-1b and B-2 cells in Tg mice could additionally account for the higher IgM anti-PPS14, and IgM and IgG anti-PC responses also observed in Tg mice.

In contrast to our data, an earlier study failed to demonstrate significant differences in DNA synthesis between Bcl-2-Tg and WT splenic B cells 4 days after in vitro activation with $\alpha\delta$ -dex (Woodland, Schmidt et al. 1996). However, this latter study utilized a different Bcl-2-Tg mouse model, established by Korsmeyer and colleagues (McDonnell, Nunez et al. 1990), which lacked insertion of the E μ enhancer 5' of the *bcl-2* transgene, and additionally was backcrossed onto the CBA/CaJ mouse background. We utilized Bcl-2-Tg mice established by Strasser and colleagues (Strasser, Whittingham et al. 1991) which contained an E μ enhancer 5' to the *bcl-2* transgene and was extensively backcrossed onto the BALB/c mouse background. One potential difference between the two models could be the relative expression of Bcl-2 protein in the B cells, affecting the functional response to $\alpha\delta$ -dex-mediated mIg crosslinking.

A previous report demonstrating that Bcl-2-Tg B cells have an enhanced Ca²⁺ influx relative to WT B cells in response to mIg crosslinking (Brunner, Marinkovic et al. 2003), is consistent with the altered functional responsiveness of Tg B cells to this general mode of activation. Although forced expression of Bcl-x_L and Bcl-2 has been shown to delay cell cycle entry (Mazel, Burtrum et al. 1996; O'Reilly, Huang et al. 1996), the initial kinetics of DNA synthesis and proliferation of WT and Tg B cells activated with $\alpha\delta$ -dex alone are similar. However, some delay in initial DNA synthesis is apparent in Bcl-x_L-Tg and Bcl-2-Tg B cells activated with $\alpha\delta$ -dex + LPS or $\alpha\delta$ -dex + anti-CD40,

and Bcl-2-Tg B cells activated with $\alpha\delta$ -dex + Pn14. Although MZB cells express substantially lower levels of mIgD than FB cells, which could potentially have a differential impact on their response to $\alpha\delta$ -dex, similar observations were also made using F(ab')₂ anti-IgM antibody.

The likelihood that PS-specific B cells are more prone, than protein-specific B cells, to undergo apoptosis in response to Pn14 in vivo, may in part reflect the relatively shorter period during which they receive CD4⁺ T cell help, presumably including CD40-mediated activation, for the IgG anti-PS response, and the T cell-independence of the IgM anti-PS response (Wu, Vos et al. 1999; Wu, Khan et al. 2000; Khan, Lees et al. 2004). Additionally, MZB cells exhibit increased apoptosis in response to mIg crosslinking alone, relative to FB cells (Oliver, Martin et al. 1997). Of note, CD40-mediated activation can prevent both MZB and FB cells (Oliver, Martin et al. 1997), as well as immature and germinal center B cells (Tsubata, Wu et al. 1993; Van Kooten and Banchereau 1996; Choe, Li et al. 2000) from undergoing mIg-induced apoptosis. Thus, the more prolonged period of CD4⁺ T cell help for the anti-protein response to Pn14 may confer increased protection against apoptotic cell death and allow for a more prolonged humoral immune response to occur. The typical provision of a relatively higher level and/or duration of pro-mitogenic stimulation for WT FB cells may account for the similar IgG anti-PspA responses to Pn14, as well as the similar IgG anti-PPS14 and IgG anti-PspA responses to pneumococcal conjugate in WT and Tg mice, despite a higher in vitro proliferative response of Tg FB cells following mIg crosslinking.

Our studies in the *Lsc*^{-/-} mouse directly implicate MZB cells in the IgG anti-PPS14 response to Pn14, but not to soluble pneumococcal conjugate. In the absence of the intracellular protein, *Lsc*, MZB cells do not detach efficiently from integrin ligands within the marginal zone, which likely limits their entry into the T cell zone for receipt of T cell help (Attanavanich and Kearney 2004; Rubtsov, Strauch et al. 2005). Indeed, the TD IgM anti-NP response to NP-CG, which is dependent on MZB, is markedly reduced in *Lsc*^{-/-} mice, whereas the TI anti-NP response to NP-Ficoll is not (Rubtsov, Strauch et al. 2005). *Lsc* deficiency affects MZB cells to a much greater extent than FB cells, likely due to relatively increased levels of *Lsc* protein expressed by MZB cells (Girkontaite, Missy et al. 2001). The *Lsc*^{-/-} mouse strain utilized in this study exhibited similar proportions of MZB and FB cells in the spleen relative to WT mice, although absolute numbers were decreased 50% (Rubtsov, Strauch et al. 2005). In contrast, no significant differences in the numbers of splenic T cells were observed. Although *Lsc*^{-/-} mice were reported to have an 80% decrease in peritoneal B-1 cells (Rubtsov, Strauch et al. 2005), our observations of normal IgM and IgG anti-PC, and IgM anti-PPS14 responses in *Lsc*^{-/-} mice, strongly argue that this reduction did not significantly impact on the overall anti-Pn14 Ig response. B-1 B cells have previously been implicated in anti-PC responses, in particular the TI response to Pn (Martin and Kearney 2001). It is also highly unlikely that the abrogation of the TD IgG anti-PPS14 response in *Lsc*^{-/-} mice was instead, due to defective CD4⁺ T cell help. Firstly, Rubtsov et al (Rubtsov, Strauch et al. 2005) demonstrated a normal TD IgG response to their two TD antigens in *Lsc*^{-/-} mice. Secondly, our data demonstrate a normal IgG anti-PC and IgG anti-PspA response to intact Pn14 and normal IgG anti-PPS14, PC, and PspA responses to soluble conjugate vaccine in *Lsc*^{-/-} mice, responses that are all CD4⁺ T cell-dependent (Wu, Shen et al.

2002; Khan, Lees et al. 2004).

We believe that our use of Lsc^{-/-} mice to establish a role for MZB cells in the IgG anti-PPS14 response was more compelling than the traditional approach of adoptive transfer of B and T cells into lymphopenic mice. As reviewed by Singh and Schwartz (Singh and Schwartz 2006) this latter approach has a number of potential pitfalls. These include homeostatic proliferation, changes in phenotype, changes in function, and alterations in cellular trafficking of the donor cells. Indeed, we observed that the majority of sorted-purified splenic B220⁺CD23⁺CD21^{intermed} cells (FB) transferred into RAG^{-/-} mice had lost expression of CD23 and CD21, but not B220, 2 days following transfer (data not shown). A similar loss of CD21, but not B220, from a majority of B220⁺CD23^{-/-}CD21^{high} cells (MZB) was also observed. Thus, the potential advantage of the Lsc^{-/-} mouse over adoptive transfer into a lymphopenic mouse is that the lymphoid architecture in the Lsc^{-/-} mouse is intact prior to immunization, and there is no *ex vivo* manipulation of lymphocyte cell subsets or effects of transferring cells into a lymphopenic environment. A report by Haas et al (Haas, Poe et al. 2005) using the adoptive transfer approach, demonstrated that the majority of the anti-PPS3 IgM and IgG3 response to purified soluble PPS3 is not mediated by splenic MZ B cells but by peritoneal B1b cells. These data do not necessarily conflict with our own, since the immunogens utilized in these respective studies were significantly different. Thus, the IgG anti-PPS response to soluble PPS is TI, whereas the IgG anti-PPS response to intact Pn is CD4⁺ T cell-dependent. The former antigen is soluble, whereas our immunogen is particulate. Indeed, a major role for splenic MZB, as well as B-1, cells in the IgM anti-PC response to intact Pn has been demonstrated (Martin, Oliver et al. 2001).

The equivalent anti-PS responses to pneumococcal conjugate vaccine in WT and *Lsc*^{-/-} mice, in addition to WT and *Bcl-x_L*-Tg mice, further suggest that this response is mediated by FB cells, as opposed to the involvement of MZB cells in the response to intact Pn14. In this regard, pneumococcal conjugate, but not intact Pn14, elicits memory for the IgG anti-PPS14 response (Khan, Sen et al. 2006). Although MZB cells are potent activators of naïve CD4⁺ T cells, MZB cells appear to be less prone to developing into memory cells than FB cells (Attanavanich and Kearney 2004; Lopes-Carvalho, Foote et al. 2005). Our further demonstration that WT and *Bcl-x_L*-Tg mice elicit equivalent Ig responses to isolated PS antigens, in contrast to Pn14, is consistent with a previous study using NP-Ficoll in *Bcl-2*-Tg mice (Tardivel, Tinel et al. 2004). In this regard, it is possible that Tg expression of *Bcl-x_L* or *Bcl-2* may not compensate for the absence of classical T cell help for Ig responses to purified, soluble PS antigens.

Our observation of normal primary and secondary anti-protein Ig responses in *Bcl-x_L*-Tg mice in response to either intact Pn14 or a soluble pneumococcal conjugate is largely consistent with a previous study by Takahashi et al (Takahashi, Cerasoli et al. 1999). Thus, they reported that in response to i.p. injection of NP-CGG in alum, mice transgenic for *Bcl-x_L* in the B cell compartment briefly expressed higher numbers of splenic NP-specific antibody-forming cells (AFCs) after immunization but did not increase the number or size of GCs, alter the levels of serum antibody, or change the frequency of long-lived AFCs. However, an increase in low affinity NP-specific B cell clones in the spleen, decreased average affinity of both long-lived NP-specific AFCs in the BM, and NP-specific serum Ig was observed in the Tg mice. Similarly, *Bcl-2*-Tg

mice immunized i.p. with TNP-OVA in Alu-Gel-S elicited an anti-TNP Ig response, affinity maturation, and GC formation similar to WT mice (Brunner, Marinkovic et al. 2003). In contrast, it has been demonstrated that Bcl-2-Tg mice immunized i.p. with NP-KLH in alum exhibited an increase in the frequency of AFCs in the spleen and bone marrow, and no decrease in affinity of bone marrow AFCs or serum Ig (Smith, Nossal et al. 1995). Similarly, Bcl-2-Tg mice immunized with NP-CGG in alum elicited higher serum titers of IgM and IgG anti-NP, relative to WT mice (Rahman and Manser 2004). Bcl-2-Tg mice immunized with SRBC also produced an amplified and protracted antibody response (Strasser, Whittingham et al. 1991). The basis for the conflicting results using isolated protein antigens are unclear, especially given that Bcl-x_L and Bcl-2 proteins appear to be equipotent in promoting B cell survival and may act through similar mechanisms (Huang, Cory et al. 1997).

We make the novel observation that the absolute numbers of both splenic B-1 and peritoneal B-1b cells are elevated in both Bcl-x_L-Tg and Bcl-2-Tg mice. Both of these B cell subsets have been implicated in PS-specific and/or T cell-independent Ig responses (Hayakawa and Hardy 2000; Martin and Kearney 2001; Alugupalli, Leong et al. 2004; Haas, Poe et al. 2005). Bcl-x_L-Tg and Bcl-2-Tg mice also exhibit an increase in peritoneal B-2 cells, a B cell subset considered intermediate in function between peritoneal B-1 and splenic B-2 cells, with some capacity to secrete natural Ig (Hastings, Tumang et al. 2006). Bcl-2-Tg mice were earlier shown to exhibit an overall increase in splenic B220⁺ cells and a selective decrease in the percentage, but not the absolute number, of CD21^{high}CD23^{low} B cells (MZB phenotype), relative to WT mice, consistent

with our data (McDonnell, Deane et al. 1989; McDonnell, Nunez et al. 1990; Rahman and Manser 2004; Tardivel, Tinel et al. 2004). In addition, similar to our data, peritoneal B-2 cells were previously shown to be elevated in Bcl-2-Tg mice, whereas total peritoneal B-1a cells were present in normal numbers (Tardivel, Tinel et al. 2004). The numbers of peritoneal B-1b and splenic B-1 cells, found in our study to be elevated in both Bcl-x_L-Tg and Bcl-2-Tg mice, were not reported in this latter study. Bcl-x_L Tg mice were also previously shown to exhibit an increase in B220+IgM+ spleen cells, relative to WT mice (Takahashi, Cerasoli et al. 1999). Collectively, these data suggest that the higher absolute numbers of particular B cell subsets that have been previously implicated in anti-PS responses could contribute at least in part, to the enhanced peak primary anti-PS responses to Pn14 in Tg mice.

Engagement of cell surface Fas/APO-1 (CD95) on B cells can induce B cell apoptosis (Mizuno, Zhong et al. 2003). Whereas, naïve B cells express only low levels of Fas, activation via CD40 or LPS, although not mIg crosslinking, substantially upregulate Fas and subsequent sensitivity to apoptotic cell death. Of interest, concomitant signaling of CD40-activated B cells via mIg significantly decreases Fas-sensitivity relative to B cells activated via CD40 alone, without decreasing Fas expression itself (Rothstein, Wang et al. 1995; Lagresle, Mondiere et al. 1996; Rathmell, Townsend et al. 1996). Resistance to Fas-mediated B cell apoptosis can also be mediated by IL-4 (Foote, Howard et al. 1996), engagement of MHC-II (Catlett, Xie et al. 2001), and TLR9-mediated signaling (Wang, Karras et al. 1997). Thus, B cells exhibit numerous mechanisms for resisting Fas-mediated apoptosis, although germinal center B cells

express relatively high levels of Fas (Smith, Nossal et al. 1995; Martinez-Valdez, Guret et al. 1996) and are susceptible to Fas-mediated apoptosis in vitro (Liu, Barthelemy et al. 1995; Choe, Kim et al. 1996). In this regard, the anti-PS response to intact Pn14 is dependent on cognate CD4⁺ T cell help, CD40- and mIg-mediated activation, TLR-dependent signaling, and is regulated by endogenous IL-4 (Wu, Vos et al. 1999; Khan, Shen et al. 2002; Wu, Shen et al. 2002; Khan, Lees et al. 2004; Khan, Sen et al. 2006), factors that could combine to confer Fas resistance. The ability of Bcl-x_L or Bcl-2 to inhibit Fas-mediated B cell apoptosis is still a matter of controversy based on conflicting reports (Mizuno, Zhong et al. 2003). The *lpr* and *gld* genes encode defective forms of Fas (Watanabe-Fukunaga, Brannan et al. 1992) and Fas-ligand (Takahashi, Tanaka et al. 1994), respectively. In this regard, we show that neither *lpr* nor *gld* mice exhibit significant increases in either the primary or secondary anti-PS or anti-protein response to Pn14, strongly suggesting that the ability of transgenic Bcl-x_L and Bcl-2 to enhance anti-PS responses was not via antagonism of Fas signaling. These data are consistent with a previous report demonstrating a normal anti-NP Ig response to i.p immunization with NP-KLH in alum in *lpr* mice (Smith, Nossal et al. 1995).

In summary, these data are the first to demonstrate that PS-specific B cells responding to an intact bacterium are likely to be more apoptosis-prone, as evidenced by the observation that Bcl-x_L and Bcl-2 can act in a similar fashion to selectively enhance anti-PS, relative to anti-protein, responses to Pn14, when expressed constitutively in a B cell-specific manner. The mechanism for this enhancement in the PS-specific Ig response appears to be Fas-independent, and may reflect a combination of increased clonal expansion of PS-specific Tg B cells in response to PS-mediated multivalent mIg

crosslinking and perhaps, higher absolute numbers of B cell subsets implicated in the anti-PS response.

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3. Abbreviations used in this paper: Pn, intact *Streptococcus pneumoniae*; Pn14, Pn capsular type 14; PspA, pneumococcal surface protein A; PPS14, purified pneumococcal capsular polysaccharide, type 14; PC, phosphorylcholine; $\alpha\delta$ -dex, dextran-conjugated anti-IgD antibodies.

Table 1. B cell subsets in wild-type, Bcl-x_L, and Bcl₂ mice

		% ^a (Total 10 ⁶)			
		Cell Type			
		Total	FOB cells	MZB cells	B-1 cells
Spleen	Wild Type	82±12.4	28±2.3(23±5)	4±0.5*(3±0.8)	3.7±0.15(3.1±0.5)
	Bcl ^{-L}	210±23.1*	19±0.9(40±6)	1.2±0.1*(2.4±0.5)	4.3±0.2(8.8±0.6)*
	Bcl	200±20.8*	19±3.5(39±4)	0.9±0.2*(1.7±0.6)	4.6±0.2(9.2±1.5)*
Peritoneum		Total	B-1a cells	B-1b cells	B-2 cells
	Wild Type	1±0.2	44±2 (0.44±0.08)	31±0.3 (0.3±0.06)	13±1.7 (0.1±0.03)
	Bcl ^{-L}	5±0.6*	17±1.9*(0.95±0.2)	47±1.8*(2.6±0.2)*	29±1.7*(1.6±0.2)*
	Bcl	4±1.1*	11±0.2*(0.55±0.1)	40±0.6*(2±0.3)*	42±1.2*(2±0.4)*

*Significant p<0.05; Tg relative to wild-type

Mean ± SEM of percentage of each B cell subset relative to total number of cells (3 mice per group).

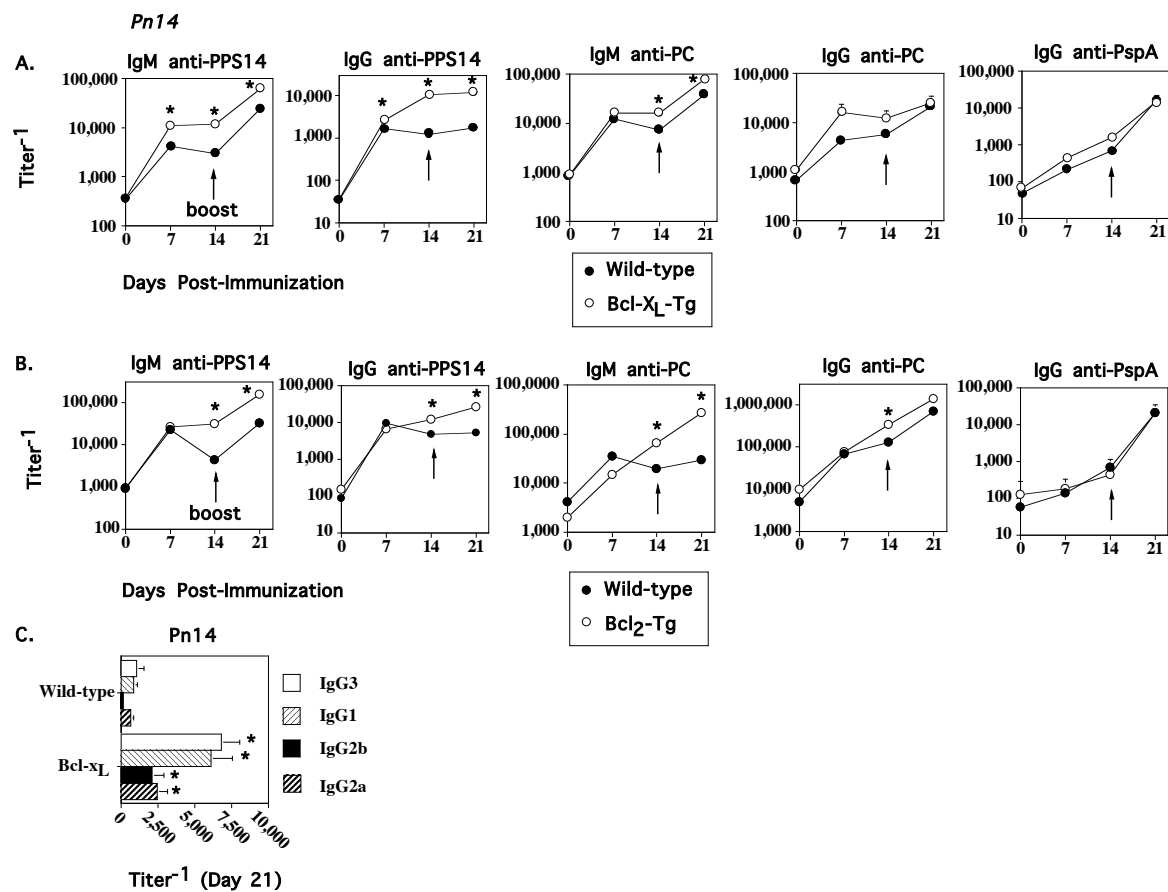


Figure 9

Figure 9. Bcl-x_L-Tg and Bcl-2-Tg mice elicit higher primary anti-PS, but not anti-protein responses to intact Pn, relative to WT control mice. (A, C) Bcl-x_L-Tg and WT (BALB/c) (7 mice per group) and (B) Bcl-2-Tg and WT (BALB/c) (7 mice per group) mice were immunized i.p. with 2×10^8 CFU equivalents of heat-killed Pn14 in saline. Mice were similarly boosted on day 14. Sera were collected on the indicated days for measurement of antigen-specific IgM and IgG isotype titers. Data are presented as geometric mean $\pm$ S. E. M.; *significance $p < 0.05$. One of 3 similar experiments using Bcl-x_L-Tg mice is shown; data on Bcl-2-Tg mice represent a single experiment.

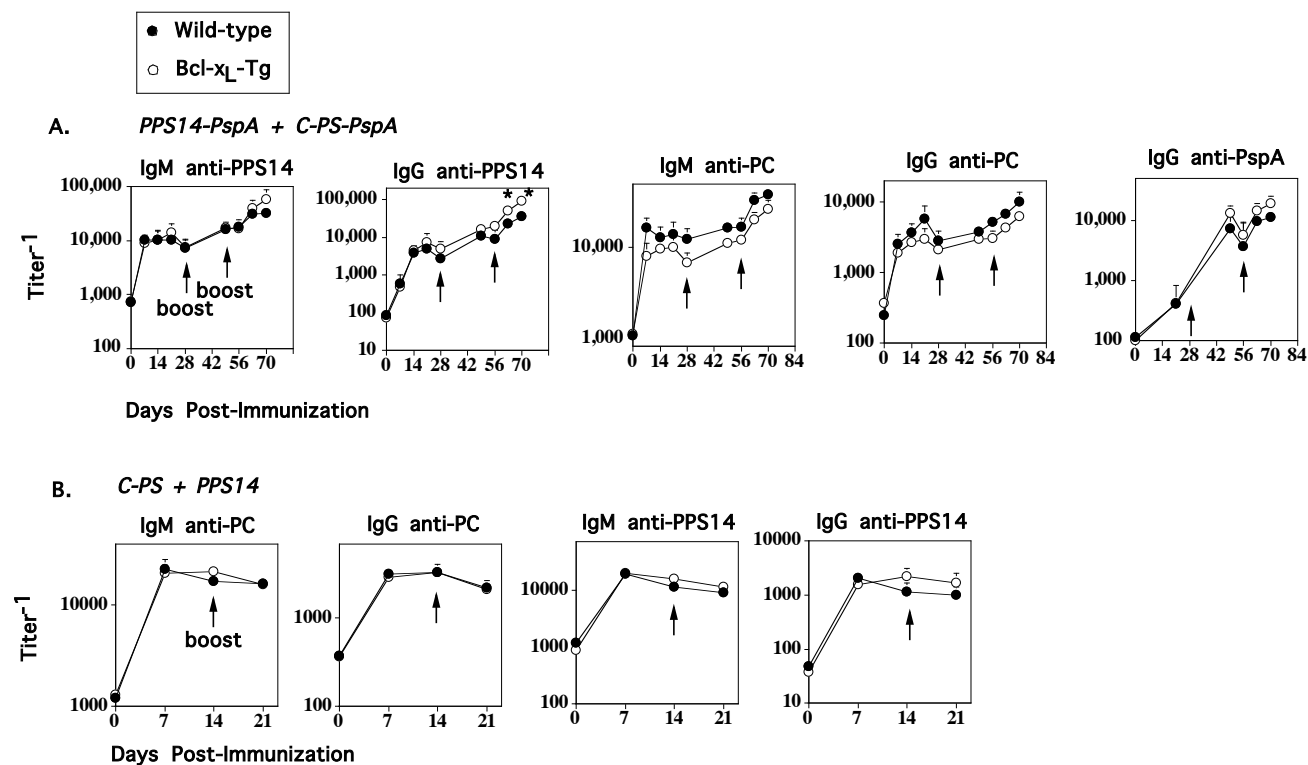


Figure 10. Anti-PS responses to PPS14-PspA + C-PS-PspA conjugate vaccine or to purified PPS14 and C-PS are similar in Bcl-x_L and WT mice. (A) Bcl-x_L-Tg mice (7 mice per group) were immunized i.p. with 1 µg each of PPS14-PspA and C-PS-PspA suspended in alum + CpG-ODN and similarly boosted on day 28 and day 49. (B) Bcl-x_L-Tg mice (7 mice per group) were immunized i.p. with 1 µg each of C-PS and PPS14 in saline and similarly boosted on day 14. Sera were collected on the indicated days for measurement of antigen-specific IgM and IgG isotype titers. Data are presented as geometric mean +/- S. E. M.; *significance $p < 0.05$. One of two similar experiments are shown for both (A) and (B).

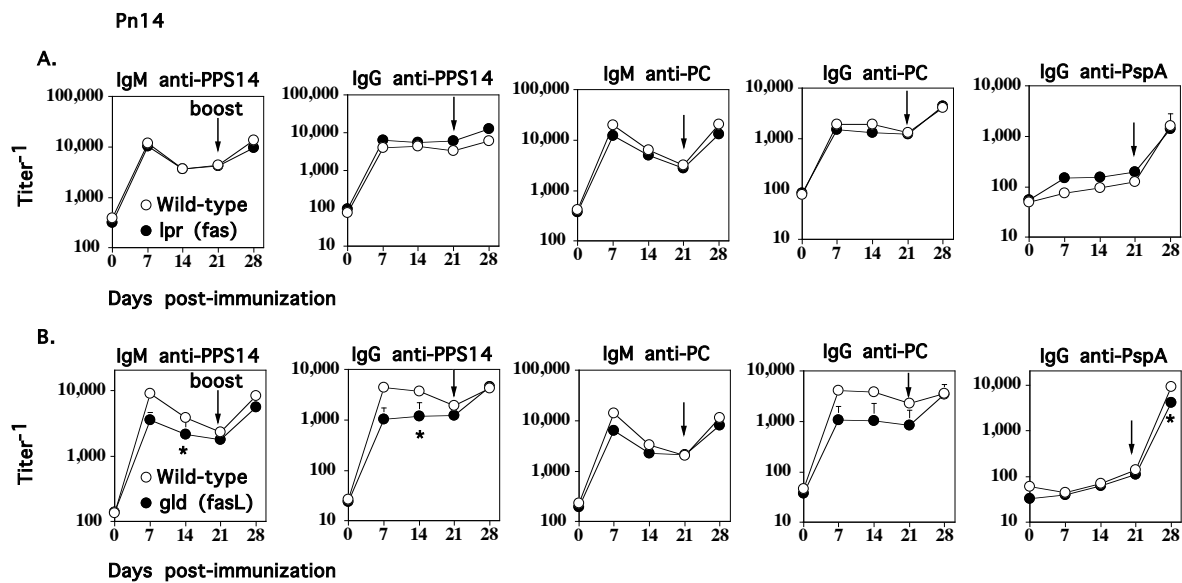


Figure 11. MRL/lpr (Fas-defective) and gld (Fas-ligand-defective) mice do not elicit elevated anti-PS responses to intact Pn14. (A) lpr and WT control [C3H/HeJ] (7 mice per group) and (B) gld and WT control [C3H/HeJ] (7 mice per group) mice were immunized i.p. with 2×10^8 CFU equivalents of heat-killed Pn14 in saline. Mice were similarly boosted on day 21. Sera were collected on the indicated days for measurement of antigen-specific IgM and IgG isotype titers. Data are presented as geometric mean $\pm$ S. E. M.; *significance $p < 0.05$. The experiments illustrated in (A) and (B) were each performed once.

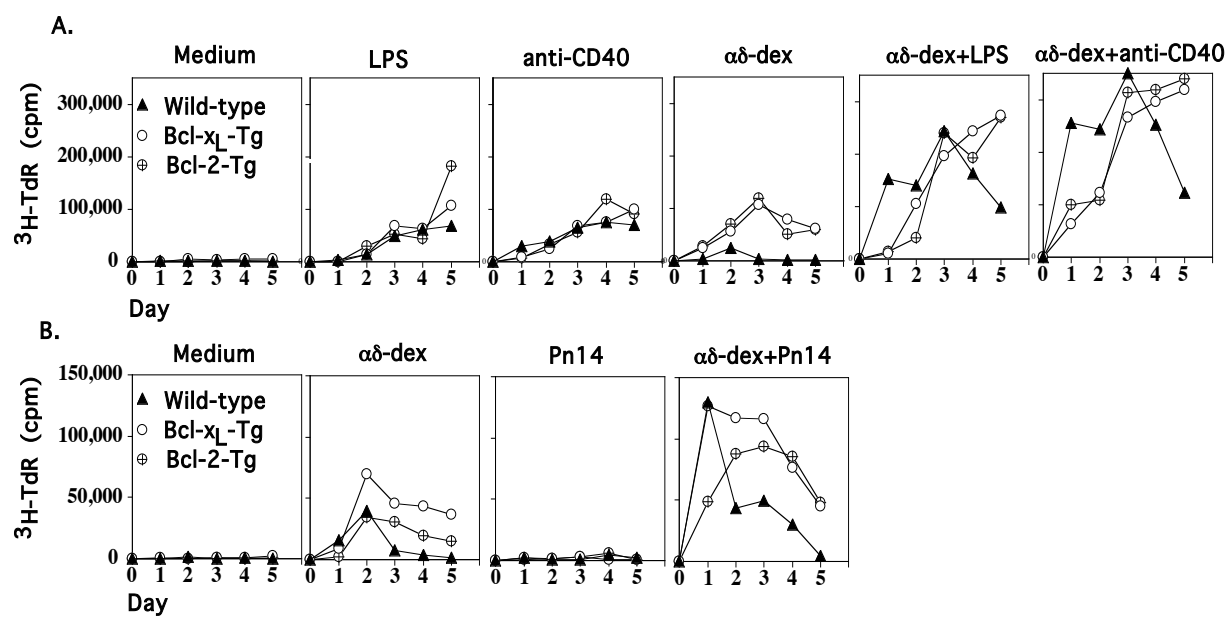


Figure 12.

Figure 12. Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit more sustained DNA synthesis in vitro in response to multivalent mIg crosslinking, but not in response to CD40- or LPS-mediated signaling. (A, B) Purified B220⁺ splenic B cells were cultured at 2.5×10^5 cells/ml (3 wells/group) in the presence of the indicated stimuli (LPS=10 µg/ml, anti-CD40 mAb=10 µg/ml, αδ-dex=10 ng/ml, and/or heat-killed Pn14= 1×10^8 CFU/ml). ³H-TdR was added to independent wells on days 1-5 and cells were harvested 18 hours later for determination of DNA synthesis via incorporated c.p.m. Data are presented as geometric mean +/- S. E. M.; *significance $p < 0.05$. One of two similar experiments are shown for both (A) and (B).

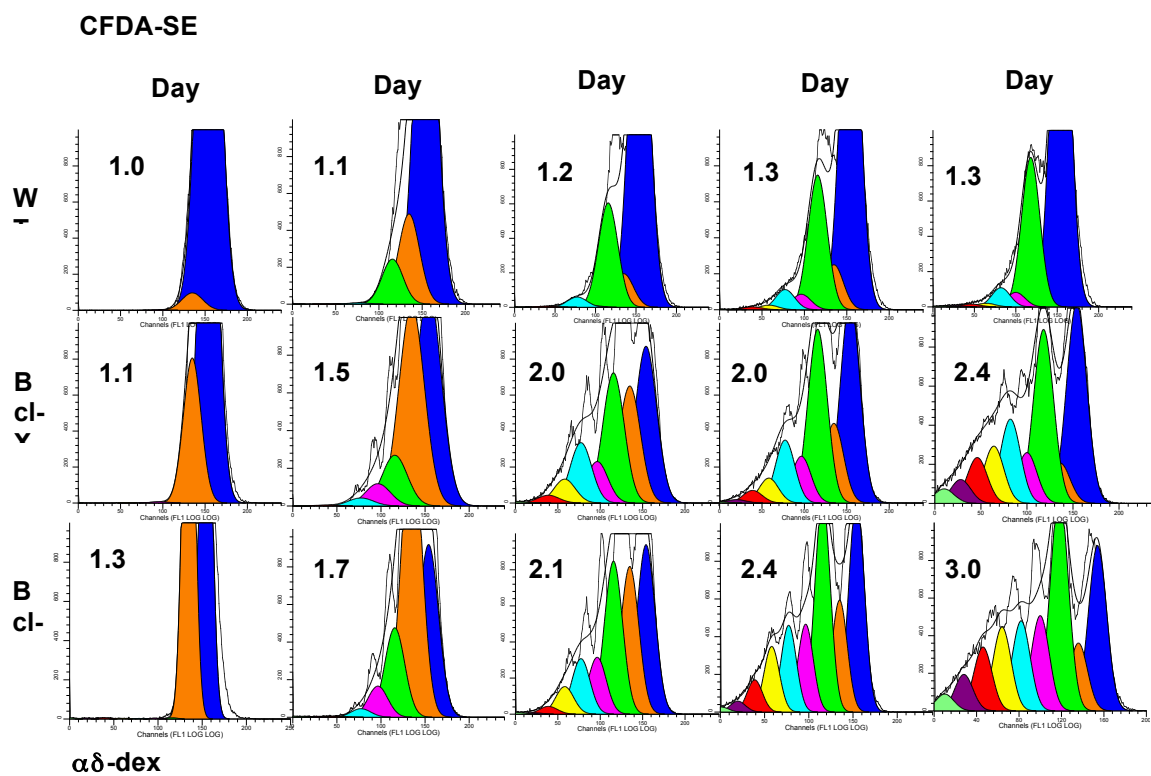


Figure 13.

Figure 13. Bcl-x_L-Tg and Bcl-2-Tg, relative to WT, B cells exhibit greater clonal expansion in response to $\alpha\delta$ -dex. CFDA-SE-loaded, purified B220+ B cells from Bcl-x_L-Tg, Bcl-2-Tg, and WT (BALB/c) mice were cultured for varying times at 5×10^5 cells/ml in the presence of $\alpha\delta$ -dex (10 ng/ml). Cells were analyzed by flow cytometry and “proliferation index” was calculated (upper left). One of two similar experiments are shown.

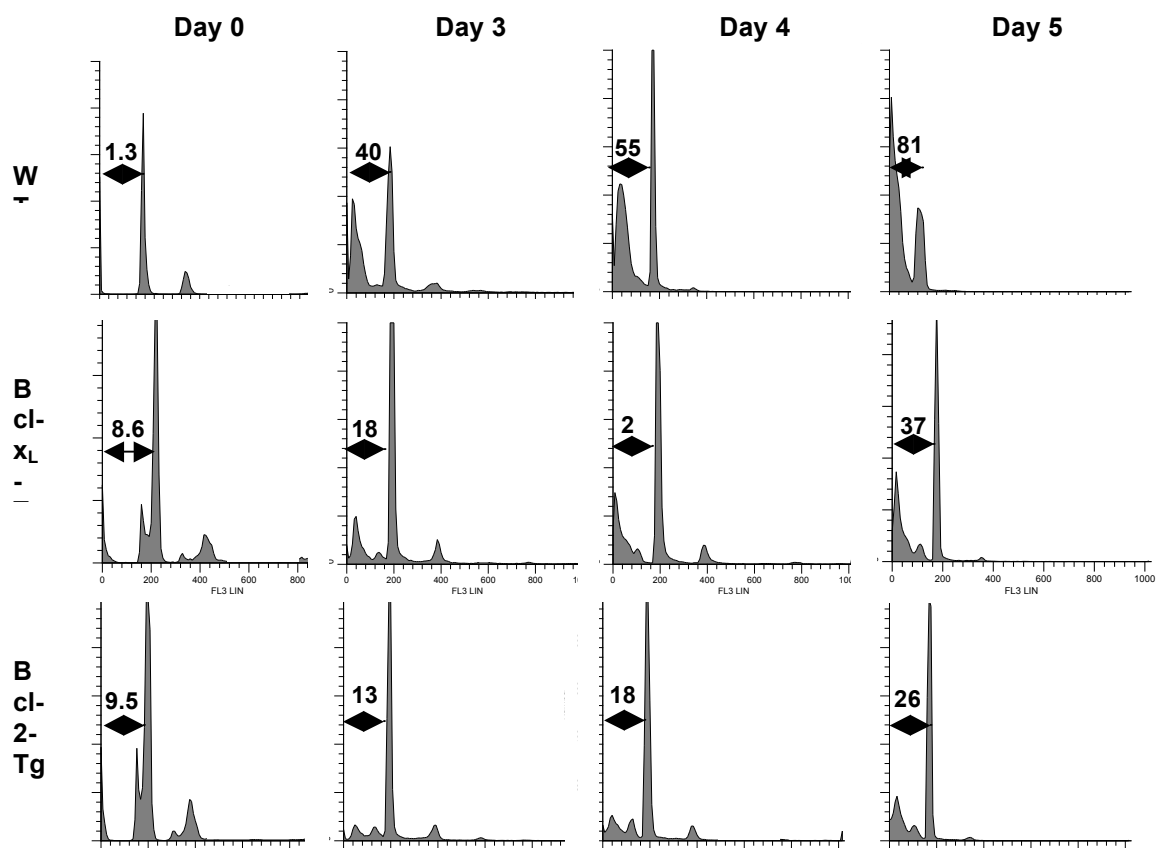


Figure 14.

Figure 14. Bcl-x_L-Tg and Bcl-2-Tg B cells undergo less apoptosis than WT B cells in response to $\alpha\delta$ -dex. Purified B220⁺ B cells from Bcl-x_L-Tg, Bcl-2-Tg, and WT (BALB/c) mice were cultured for 3-5 days at 5×10^5 cells/ml in the presence of $\alpha\delta$ -dex (10 ng/ml). Cells were harvested and nuclei were stained with propidium iodide and analyzed by flow cytometry. The number above the horizontal arrow is the percentage of hypodiploid (apoptotic) nuclei. One of two similar experiments are shown.

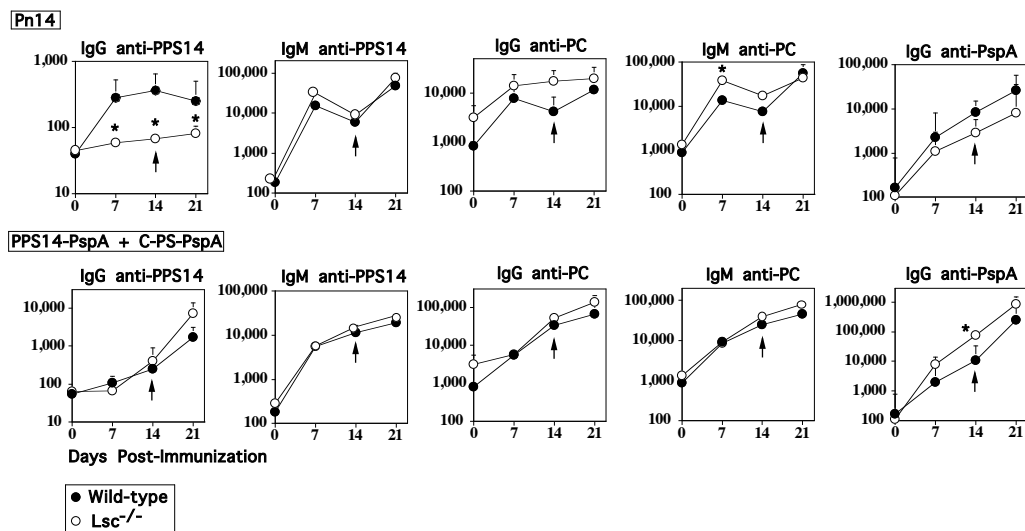


Figure 15.

Figure 15. The IgG anti-PPS14 response to Pn14 is markedly reduced in Lsc-/- mice. Lsc-/- and WT mice (7 per group) were immunized i.p. with either 2×10^8 CFU equivalents of heat-killed Pn14 in saline (upper panel) or i.p. with 1 μ g each of PPS14-PspA and C-PS-PspA suspended in alum + CpG-ODN (lower panel). Mice were similarly boosted with their respective immunogens on day 14. Sera were collected on the indicated days for measurement of antigen-specific IgM and IgG isotype titers. Data are presented as geometric mean $\pm$ S. E. M.; *significance $p < 0.05$. One of 2 similar experiments is shown.

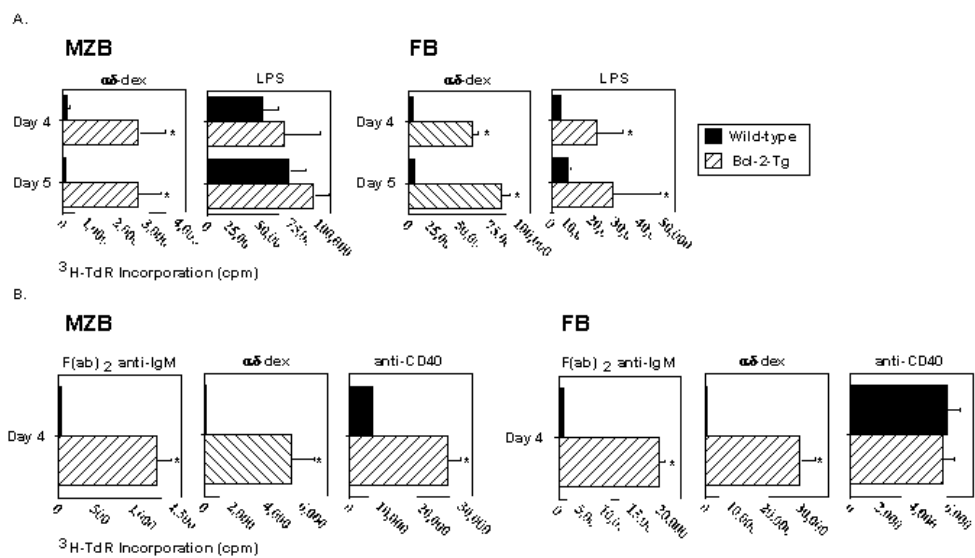


Figure 16.

Figure 16. Both MZB and FB cells from Bcl-2-Tg, in contrast to WT, mice exhibit markedly higher levels of DNA synthesis late in culture, following mIg crosslinking. Purified MZB or FB cells were cultured at 2.5×10^5 cells/ml (3 wells/group) in the presence of the indicated stimuli (LPS=10 μ g/ml, anti-CD40 mAb=10 μ g/ml, $\alpha\delta$ -dex=10 ng/ml, or F(ab')₂ goat anti-mouse IgM (10 μ g/ml) ³H-TdR was added to independent wells on days 4 and/or 5 and cells were harvested 18 hours later for determination of DNA synthesis via incorporated c.p.m. Data are presented as geometric mean +/- S. E. M.; *significance $p < 0.05$.

Chapter three

Concomitant systemic delivery of soluble and intact bacteria-associated antigens results in marked inhibition of the soluble antigen-induced Ig response. A model for humoral immunity during blood-borne bacterial infections

Title:

Concomitant systemic delivery of soluble and intact bacteria-associated antigens results in marked inhibition of the soluble antigen-induced Ig response. A model for humoral immunity during blood-borne bacterial infections

Running Title: Bacterial inhibition of soluble antigen-induced immunity

Authors: Gouri Chattopadhyay^{*}, Quanyi Chen^{*}, Jesus Colino^{*}, Andy Lees[†], and Clifford M. Snapper^{*1}

^{*}Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, and [†]Fina BioSolutions, Rockville, MD 20850

¹Corresponding Author

**Department of Pathology, Uniformed Services University of the Health Sciences,
4301 Jones Bridge Road, Bethesda, Maryland 20814**

Tel: 301-295-3490, FAX: 301-295-1640; E-mail: csnapper@usuhs.mil

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Abstract

T cell-dependent, polysaccharide (PS)-specific murine IgG responses to intact *Streptococcus pneumoniae* (Pn) versus soluble pneumococcal conjugate vaccine exhibit distinct mechanistic features. Co-immunization of Pn with conjugate or soluble protein alone, results in marked inhibition of conjugate-induced PS-specific memory, and/or protein-specific, primary and memory Ig responses. The potency of the carrier protein for conjugate-induced PS-specific IgG inversely correlates with the ability of Pn to inhibit. The mechanism of inhibition is not PS- or protein-specific, occurring with unencapsulated Pn, encapsulated Pn expressing different serotypes of PS than present in the conjugate, or with conjugate containing protein not expressed by Pn. Co-injection of 1 μ m latex beads with adjuvant does not inhibit conjugate-induced memory, indicating that the particulate or innate stimulatory nature of the immunogen per se is not sufficient for inhibition. Memory inhibition by co-injected Pn is long-lasting, and occurs only during the early phase of the immune response, in that delay of Pn injection by 2 days following conjugate immunization results in loss of inhibition. In situ imaging studies of the spleen following Pn and conjugate co-immunization demonstrates that intact Pn inhibits the trafficking of conjugate from the marginal zone to the splenic white pulp, suggesting a potential mechanism for inhibition of memory. These data suggest that during infection with Pn, primary and/or memory responses to released Pn-derived soluble antigens may be inhibited, in a manner inversely proportion to their immunogenic potency, by the concomitant presence of intact Pn within the secondary lymphoid organ.

Introduction

The manner in which antigen is transported within the secondary lymphoid organ, including its binding to, and processing by, distinct immune cell types, may significantly impact on the quality and quantity of the subsequent immune response. The size of the immunogen (Gretz, Norbury et al. 2000; Nolte, Belien et al. 2003; Sixt, Kanazawa et al. 2005), its soluble or particulate nature (Vidard, Kovacsovics-Bankowski et al. 1996); (Nayak, Hokey et al. 2006), the valency (Snapper, Kehry et al. 1995; Thyagarajan, Arunkumar et al. 2003) and biochemical nature of the antigenic epitope (Mond, Lees et al. 1995), and the presence of associated innate immune cell activators, such as TLRs (Barton and Medzhitov 2002; Blander and Medzhitov 2006), and mediators of cellular uptake, such as scavenger receptor ligands (Arredouani, Yang et al. 2004; Kang, Kim et al. 2004; Lanoue, Clatworthy et al. 2004), in turn can influence the outcome of these processes. In particular, the binding of antigen by B cells via their BCR is a critical event for the initiation of a specific humoral immune response. Depending upon the nature of the immunogen and the site of immunization, delivery of intact antigen to B cells within the secondary lymphoid organ can occur in a number of distinct ways, including diffusion through the conduit system (Gretz, Norbury et al. 2000; Nolte, Belien et al. 2003; Sixt, Kanazawa et al. 2005), or transport by immune cells including marginal zone B cells (Ferguson, Youd et al. 2004; Cinamon, Zachariah et al. 2008), macrophages (Groeneveld, Erich et al. 1986; Martinez-Pomares, Kosco-Vilbois et al. 1996; Mueller, Cremer et al. 2001; Karlsson, Guinamard et al. 2003), or dendritic cells (Bjorck, Flores-Romo et al. 1997; Kushnir, Liu et al. 1998; Wykes, Pombo et al. 1998; Bergtold, Desai et al. 2005;

Huang, Han et al. 2005). Additionally, the specific B cell (Attanavanich and Kearney 2004) (Song and Cerny 2003), macrophage (Kang, Yamazaki et al. 2003; Gordon and Taylor 2005) and/or DC subset (Liu, Kanzler et al. 2001; Itano, McSorley et al. 2003; Grdic, Ekman et al. 2005) that initially encounters a particular immunogen may further influence the nature of the subsequent B cell, as well as T cell, signaling and functional outcome. B cell contact with antigen might occur via direct transfer by antigen-transporting cells, or by initial transfer of antigen from these latter cells to FDCs in the B cell follicle, followed by B cell binding to the FDC-bound antigen (Tew, Phipps et al. 1980; Szakal, Kosco et al. 1989).

Many studies have documented cross-regulation between distinct immune cells types and various cellular receptors activated in response to immunization or infection. In this regard, during infections, both soluble products secreted by the pathogen as well as the intact pathogen itself concurrently enter the secondary lymphoid organ where they likely get transported and processed in distinct ways, and activate different, though perhaps overlapping immune cellular pathways. However, little is known whether these pathways evolve independently or are cross-regulatory, and thus how this might impact on the individual immune responses elicited to the pathogen's component antigens. Several studies have demonstrated cross-regulation between various isolated antigens delivered concomitantly. The conjugate vaccine exemplifies a notable example, whereupon covalent linkage of an immunogenic protein to a polysaccharide (PS) antigen, converts the PS from a weak T cell-independent (TI) to a strong T cell-dependent (TD) antigen, including the capacity of the latter to generate PS-specific immunologic memory

(Robbins and Schneerson 1990). Of interest, the presence of free PS in the conjugate has been shown to inhibit the anti-PS response to the conjugate itself (Peeters, Tenbergen-Meekes et al. 1992; Rodriguez, van den Dobbelsteen et al. 1998). Additionally, distinct PS antigens comprising conjugate vaccines may also influence the ability of APCs to present the associated protein antigens to CD4⁺ T cells (Leonard, Canaday et al. 2003).

Studies directly comparing the parameters that mediate a PS- and protein-specific Ig response to intact *Streptococcus pneumoniae* (Pn) relative to those that regulate a humoral response to a soluble pneumococcal PS-protein conjugate, have revealed two distinct pathways of immune activation. Specifically, although the IgG anti-PS responses to Pn and conjugate are both dependent upon CD4⁺ T cells, B7/CD28 costimulation, and CD40/CD40L interactions, the IgG anti-PS response to Pn is essentially extrafollicular, with more rapid kinetics of primary induction and failure to generate PS-specific memory, whereas the same response to conjugate is follicular in nature with more prolonged kinetics and the generation of PS-specific memory (Guttormsen, Wetzler et al. 1998; Guttormsen, Sharpe et al. 1999; Wu, Vos et al. 1999; Wu, Khan et al. 2000; Khan, Lees et al. 2004; Khan, Sen et al. 2006). Recent data strongly suggest that secretion of PS-specific IgG in response to Pn and conjugate is largely effected by marginal zone (MZB) and follicular (FB) B cells, respectively (Chattopadhyay, Khan et al. 2007). In addition, the IgM anti-PS responses to Pn and conjugate are TI and TD, respectively (Guttormsen, Wetzler et al. 1998; Guttormsen, Sharpe et al. 1999; Wu, Vos et al. 1999). In contrast, the protein-specific IgG responses to both Pn and conjugate appear to be

mediated by FB cells that give rise to a GC reaction followed by the generation of protein-specific memory (Chattopadhyay, Khan et al. 2007).

Recent, preliminary data from our laboratory appear to indicate that conjugate is more rapidly and extensively transported from the marginal zone into the splenic B cell follicle and T cell region than intact Pn, following i.p immunization. Whereas large amounts of conjugate localize to splenic DC, intact Pn preferentially accumulates within macrophages. In light of these collective observations we wished to determine the consequences of concomitant immunization with Pn and conjugate via the same i.p. route on the outcome of the PS- and protein-specific Ig responses to the individual immunogens. We believe that this approach may serve as a model for understanding potential cross-regulatory immune pathways mediated by intact pathogens and the soluble products that they secrete. We demonstrate that intact Pn, while exerting little effect on the early primary PS- and protein-specific Ig responses to conjugate, is capable of strongly inhibiting the generation of PS- and protein-specific memory, as well as the longer-term maintenance of the primary response. This inhibition is not mediated in an antigen-specific manner, but instead is associated with the ability of Pn to inhibit the transfer of conjugate from the marginal zone into the B cell follicles and T cell areas.

Materials and Methods

Mice. Female BALB/c mice were purchased from The National Cancer Institute (Frederick, MD) and were used between 7-10 wks of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, revised 1996, and were approved by the Uniformed Services University of the Health Sciences institutional animal use and care committee.

Reagents. Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from American Type Culture Collection (Manassas, VA). Recombinant pneumococcal surface protein A (PspA) was expressed in *Sacharomyces cerevisiae* BJ3505 and purified as previously described (Chen, Sen et al. 2006). A His-tagged, truncated EBV gp350 protein consisting of the first 470 amino acids was expressed from SF9 insect cells transfected with a baculovirus vector containing the truncated gp350 cDNA. The gp350 protein was purified by Ni-NTA affinity chromatography. Tetanus toxoid (TT) was a kind gift from the Serum Institute of India (Pune, India). Soluble conjugates comprising PPS14 (2×10^6 MW) covalently linked to PspA, gp350, or TT were synthesized using CDAP chemistry as previously described (Lees, Nelson et al. 1996). The molar ratios of protein to PPS14 were ~15-20 for each conjugate.

Preparation of *Streptococcus pneumoniae* strains. Frozen stocks of Pn14, D39, R36A, WU-2, and JD11 were thawed and sub-cultured on BBL pre-made blood agar

plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt broth (Becton Dickinson, Sparks, MD) to mid-log phase, collected, and heat killed by incubation at 60°C for 1h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10^9 CFU/ml.

Bacteria were then aliquoted at 10^{10} colony-forming units (CFU)/ml and frozen at -80°C until their use as antigen for mouse immunizations.

Preparation of R36A depleted of choline-binding proteins. Cultures of R36A at the late exponential phase of growth were collected by centrifugation, washed with PBS two times, and the bacterial pellet was treated for 20 min at RT with 2% choline chloride (Sigma, St. Louis, MO) to release the choline-binding protein from the bacterial cell wall (Briles, King et al. 1996). The resulting choline-binding protein-depleted bacteria were washed by centrifugation, heat-killed and stored as indicated above. PspA content of the choline-binding protein- depleted R36A preparations was $\leq 60\text{ng}/10^9\text{CFU}$.

Immunizations. Mice (7 per group) were immunized i.p. with 2×10^8 CFU heat-killed bacteria in saline or 1 μg (weight of polysaccharide) of conjugate (PPS14-PspA, PPS14-gp350, or PPS14-TT) adsorbed on 13 μg of Alum (Allhydrogel 2% [Brenntag Biosector, Denmark]) mixed with 25 μg of a stimulatory phosphorothioated 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN) [5' AAAAAAAAAAAAAACGTTAAAAAAAAAAAAA 3'] (Hemmi, Takeuchi et al. 2000); (Sen, Chen et al. 2006), and similarly boosted. Co-immunization studies were performed

by injecting Pn14 and conjugate + alum/CpG-ODN separately at two different i.p. sites. Biodegradable polystyrene latex beads (1.1 μ m mean particle size) were purchased from Sigma (Catalogue # LB11) and injected i.p. at 2×10^8 particles per mouse. Serum samples for measurement of antigen-specific IgM and IgG titers were prepared from blood obtained through the tail vein.

Measurement of serum antigen-specific Ig isotype titers. Immulon 4 ELISA plates (Dyner Technologies, Inc., Chantilly, VA) were coated (50 μ L/well) with PPS14, PspA or gp350 (5 μ g/ml) in PBS for 1h at 37°C or overnight at 4°C. Plates were washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 30 min at 37°C or overnight at 4°C. Threefold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS + 1% BSA were then added for 1h at 37°C or overnight at 4°C and plates were washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG Abs (200ng/ml final concentration) in PBS + 0.05% Tween 20 were then added, and plates were incubated at 37°C for 1 h. Plates were washed 5x with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) was then added for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

Fluorescence microscopy. Conjugate was labeled with Alexa Fluor-405 (Solulink, San Diego, CA). R36A used as an unlabeled antigen. Following immunization, spleens were removed and incubated at least 6 hours in 15 ml of PLP buffer (0.05 M PBS

containing 0.1 M L-lysine (pH 7.4), 2 mg/ml NaIO₄, and 10 mg/ml paraformaldehyde). The fixed samples were washed in PBS, and dehydrated in 30% sucrose in PBS. Tissues were snap-frozen in Tissue-Tek (VWR, West Chester, PA). Twenty to thirty μ m thick frozen sections were cut and stained with rat anti-mouse B220-PE (clone RA3-6B2, BD Bioscience, San Jose, CA) and rat anti-mouse CD169-FITC (clone MOMA-1, AbD Serotec, Raleigh, NC) for 45 min followed by washing 3x in Tris buffer. Sections were mounted with ProLong Antifade Kit (Molecular Probes, Carlsbad, CA). Immunofluorescence imaging was performed with a Zeiss Pascal Laser Scanning Confocal Microscope. Separate images were collected for each fluorochrome and overlaid to obtain a multicolor image. Final image processing was performed with ImageJ software (National Institutes of Health, Bethesda, MD) and Adobe Photoshop.

Statistics. Serum Ig isotype titers were expressed as geometric means $\pm$ standard errors of the means (SEM) of the individual serum Ig isotype titers. Significance was determined by the Student *t* test. *P* values of <0.05 were considered statistically significant. Each experiment was performed at least twice to ascertain reproducibility.

Results

Pn14 fails to induce memory and inhibits memory generation by pneumococcal conjugate. We previously reported that mice immunized i.p. with intact, heat-inactivated Pn14 fail to elicit an enhanced PPS14-specific IgG secondary response upon boosting with additional Pn14, in distinct contrast to priming and boosting with a pneumococcal conjugate consisting of PPS14-PspA adsorbed to alum + CpG-ODN [a TLR9 agonist (Hemmi, Takeuchi et al. 2000)] (Khan, Sen et al. 2006); (Chen, Sen et al. 2006). To better define the nature of this dichotomy, we wished to determine whether Pn14-primed mice could be effectively boosted by secondary immunization with conjugate or, conversely, whether conjugate-primed mice could be boosted with Pn14. As illustrated in Figure 17A, an enhanced secondary PPS14-specific IgG response was elicited only when mice were both primed *and* boosted with conjugate. In contrast, enhanced secondary PspA-specific IgG responses were observed both when Pn14-primed mice were boosted with either conjugate or Pn14, or when primary conjugate immunization was followed by secondary challenge with Pn14 or conjugate. These data were thus consistent with our recent observation that the Pn14- and conjugate-induced PPS14-specific IgG responses derived from different B cell subsets (i.e. MZB and FB cells, respectively), whereas the PspA-specific IgG responses to either Pn14 or conjugate appeared to arise from FB cells (Chattopadhyay, Khan et al. 2007).

In light of the failure of Pn14 to elicit memory for the PPS14-specific IgG response, we next asked whether it could prevent induction of memory when co-

immunized with conjugate. Mice were thus co-immunized with Pn14 and conjugate adsorbed to alum/CpG-ODN at two different i.p. sites and boosted with conjugate alone, or first primed with conjugate alone and boosted with Pn14 and conjugate, again at two different i.p. sites (Figure 17B). As controls, mice were both primed and boosted with conjugate alone or Pn14 alone. In contrast to mice primed with conjugate alone, primary co-immunization with Pn14 and conjugate resulted in the complete abrogation of the enhanced PPS14-specific IgG response following secondary immunization with conjugate. A partial, though significant reduction in the secondary response was also observed in conjugate-primed mice boosted with Pn14 and conjugate. In contrast, secondary PspA-specific IgG responses were essentially equivalent in all 4 immunization groups (Figure 17B). Thus, Pn14 appeared to prevent the generation of PPS14-specific IgG memory in response to conjugate.

Inhibition of conjugate-induced PS-specific memory is not dependent upon Pn capsular PS or particulation per se. Pn typically expresses over 90 different non-cross-reacting capsular serotypes (AlonsoDeVelasco, Verheul et al. 1995). Previous studies indicated that free PPS could inhibit the IgG anti-PPS response to conjugate, in a PPS serotype-specific manner (Peeters, Tenbergen-Meekes et al. 1992; Rodriguez, van den Dobbelsteen et al. 1998). In this regard, we wished to determine whether the inhibitory effect of Pn14 on the induction of conjugate-induced PPS14-specific IgG memory was dependent on co-immunization of the same PPS serotype, and/or whether Pn was even required to express any PPS capsule. We thus utilized PPS2 (strain D39) and PPS3 (strain WU-2) encapsulated Pn strains and their respective non-encapsulated

isogenic mutants (strains R36A and JD11) in co-immunization studies with the PPS14-PspA conjugate. As illustrated in Figure A co-immunization of conjugate with either of the two encapsulated Pn or their unencapsulated mutants significantly inhibited the enhanced conjugate induced PPS14-specific IgG response following secondary immunization with conjugate alone. D39 and R36A were more effective inhibitors than WU-2 or JD11, although in each case the same dose of Pn was used. In light of these data, we utilized R36A to effect inhibition in all subsequent studies.

The IgM, like the IgG, anti-PS response to conjugate is also TD (Guttormsen, Wetzler et al. 1998; Guttormsen, Sharpe et al. 1999), in contrast to the IgM anti-PS response to Pn14 that is TI (Wu, Vos et al. 1999; Khan, Lees et al. 2004). In Figure 18B we further demonstrate that co-immunization of conjugate with R36A also inhibits the enhanced IgM anti-PPS14 response to secondary immunization with conjugate alone. Collectively, these data demonstrate that the mechanism of inhibition of the PPS14-specific Ig response to conjugate is neither strain nor PPS serotype-specific and indeed occurs in the complete absence of PPS capsule. Since particulate and soluble antigens exhibit distinct immunologic features (Vidard, Kovacsovics-Bankowski et al. 1996; Nayak, Hokey et al. 2006), we next wished to determine whether the particulate nature of Pn itself was mediating the inhibitory effect on the soluble conjugate. Thus, we co-immunized mice with 1.1 μ m biodegradable latex beads in alum + CpG-ODN with PPS14-PspA conjugate, also in alum + CpG-ODN. As illustrated in Figure 18C, we observed no significant inhibitory effects of the beads or alum/CpG-ODN adjuvant on the IgG anti-PPS14 response to conjugate.

Pn inhibits maintenance of primary IgG anti-PPS14 response to conjugate and induces long-lasting abrogation of memory. A primary TD Ig response to systemic immunization with a protein antigen is typically characterized by an early and transient extrafollicular plasma cell response in the spleen followed by a germinal center reaction that produces long-lived BM plasma cells and memory B cells (MacLennan, Toellner et al. 2003; Shapiro-Shelef and Calame 2005). In light of our observation that Pn inhibits the conjugate-induced PPS14-specific IgG memory response, we wished to determine whether it also inhibited the maintenance of primary serum Ig titers, both processes being dependent upon the GC reaction. Additionally, we wished to determine whether the Pn-mediated inhibition of conjugate-induced memory was relatively long-lasting. Thus, we co-immunized mice with R36A and conjugate and instead of boosting with conjugate on day 14 (see Figures 17 and 18), we delayed secondary immunization until day 42. As illustrated in Figure 19, primary immunization with conjugate alone, resulted in peak IgG anti-PPS14 serum titers at day 14 that were maintained at an equivalent level until day 42. In contrast, co-immunization with R36A, while having no significant effect on conjugate-induced PPS14-specific IgG serum titers at day 14, as we demonstrated in Figures 17 and 18, resulted in a steady decline in serum titers until day 42 resulting in >10-fold lower titers relative to mice immunized with conjugate alone. In addition, whereas mice primed and boosted with conjugate alone elicited >30-fold greater secondary versus primary IgG anti-PPS14 titers, mice co-immunized with R36A and conjugate, elicited secondary titers no greater than that observed for mice initially injected with either PBS or R36A alone followed by secondary immunization with

conjugate (Figure 19). Thus, Pn inhibits both the maintenance, although not early induction, of primary serum titers and inhibits the generation of memory in a relatively sustained manner, suggesting a possible inhibitory effect on initiation or maintenance of the GC reaction.

Potency of the carrier protein for PS-specific IgG induction can modulate inhibitory effect of Pn. Conjugate vaccines can exhibit varying potencies in their ability to elicit a given anti-PS response depending upon the immunogenicity of the associated carrier protein (Jakobsen, Adarna et al. 2001), although this correlation is not always observed (McCool, Harding et al. 1999). We thus wished to determine whether PPS14-containing conjugates associated with different carrier proteins and exhibiting differing potencies for induction of the IgG anti-PPS14 response, had different levels of sensitivity to inhibition with R36A. Further, in light of reports that priming with a specific protein might lead to inhibition of a subsequent anti-PS response to a conjugate containing the same protein as a carrier (Schutze, Leclerc et al. 1985), we also wished to know whether the R36A-mediated inhibition of conjugate-induced memory depended upon the presence, in the PPS14-PspA conjugate, of PspA (an immunogenic protein expressed by all Pn strains) (Briles, King et al. 1996). Thus, we co-immunized mice with R36A and either PPS14-PspA, PPS14-gp350, or PPS14-TT. As mentioned in the “Methods” section, the conjugates were made with the same conjugation chemistry and were designed to generate conjugates with similar molar ratios of protein to PPS14. In each case, mice were injected with conjugate containing 1 µg of PPS14. As demonstrated by either more rapid and/or higher primary IgG anti-PPS14 serum titers and/or greater

secondary titers following boosting, the order of carrier protein potency was TT>gp350>PspA (Figure 20). Of interest, whereas R36A markedly inhibited the secondary IgG anti-PPS14 response to PPS14-PspA, it exerted only partial, but significant, inhibition of the response to PPS14-gp350, and failed to inhibit PPS14-TT. Thus, at the doses used, different conjugates exhibit varying sensitivity to R36A-mediated inhibition that is directly correlated with their relative potency in inducing the anti-PPS14 response.

Pn also inhibits induction of protein-specific IgG memory in response to conjugate. Conjugate induces both an anti-PS and anti-protein response that is TD and that leads to the generation of PS- and protein-specific memory, respectively. We thus wished to determine whether R36A also inhibits the induction of protein-specific memory in response to conjugate. To accomplish this we took two separate approaches. In the first, since PspA is a choline-binding protein, we depleted R36A of PspA (“R36A^{-PspA}”) by using choline chloride as a competitor (Briles, King et al. 1996), and co-immunized R36A^{-PspA} with PPS14-PspA conjugate, followed by boosting with conjugate alone. As illustrated in Figure 21A (right panel), primary immunization with R36A alone induced a robust IgG anti-PspA response, whereas essentially no induction was observed using R36A^{-PspA}, confirming that PspA was largely depleted from the bacteria. Boosting of R36A^{-PspA}-primed mice with conjugate resulted in only a modest enhancement in the secondary IgG anti-PspA response, 7 days later, compared to what was observed 7 days following primary immunization with conjugate alone (compare right and left panels). Of interest, co-immunization of mice with R36A^{-PspA} and conjugate resulted in a nearly

complete inhibition in the conjugate-induced primary anti-IgG anti-PspA response and in a partial, though significant, inhibition (10-fold reduction) of the secondary IgG anti-PspA response following boosting with conjugate alone (Figure 21A, compare left and center panels).

In a second set of studies we co-immunized mice with R36A and a conjugate consisting of PPS14 and gp350 (“PS-gp350”) followed by boosting with PS-gp350, and measurement of serum IgG anti-gp350 titers. Gp350 is an EBV-derived protein (Nemerow, Mold et al. 1987) not expressed by any Pn strain. Co-immunization of PS-gp350 with R36A resulted in a marked inhibition of both the primary IgG anti-gp350 response, and in the enhanced secondary response following boosting with PS-gp350 alone (Figure 21B). These two sets of experiments demonstrate that co-immunization of mice with R36A and conjugate results in inhibition of not only conjugate-induced PS-specific memory, but also the elicitation of both the primary and secondary conjugate-induced IgG anti-protein response.

In Fig.21C we demonstrated that conjugate, in contrast, is unable to inhibit protein-specific immune response to intact bacteria. So this inhibition is largely unidirectional.

Pn acts largely within the first 24 h following conjugate immunization to inhibit memory. To better understand the mechanism underlying the R36A-mediated inhibition of conjugate-induced Ig production, we determined during what time period following co-immunization, R36A is exerting its inhibitory effect. Separate sets of mice were immunized with PPS14-PspA, and R36A was injected on either day 0 (co-

immunization), 1, 2, or 3. All mice were boosted with PPS14-PspA alone on day 14. R36A had no significant effect on the conjugate-induced primary IgG anti-PPS14 response in any of the groups (Figure 22). R36A co-injected with PPS14-PspA (“R36A d0”) resulted in a complete inhibition of the conjugate-induced secondary response. Delay of addition of R36A by 1 day resulted in only a partial, though significant, inhibitory effect, whereas no effect was observed if R36A was injected either 2 or 3 days following immunization with conjugate. Thus, R36A acts relatively early (largely within the first 24 h) during the immune response to conjugate, to inhibit the subsequent generation of conjugate-induced PPS14-specific IgG memory.

Pn inhibits trafficking of conjugate from splenic marginal zone into white pulp. Our observation that R36A exerts its inhibitory effect on the conjugate-induced Ig response largely within the first 24 h following immunization with conjugate, suggests the possibility that R36A is interfering with transport and/or processing of conjugate upon its entry into the spleen, the primary site where Ig responses are elicited in response to systemic immunization. Mice were thus immunized with Alexa Fluor 405-labelled PPS14-PspA in the absence or presence of R36A, and spleens were removed 4 h later, for confocal fluorescence microscopic analysis. Sections were stained with PE-labeled anti-B220 (B cells) and FITC-anti-CD169 (MOMA-1, marginal metallophilic macrophages). As illustrated in Figure 23, mice immunized with PPS14-PspA alone, exhibited large amounts of conjugate within both B cell follicles and the T cell compartment of the splenic white pulp, as well as conjugate within the marginal zone. In contrast, conjugate

was localized almost entirely within the marginal zone in mice co-immunized with PPS14-PspA and R36A, with minimal amounts present within the white pulp. Thus, the observation that R36A inhibits conjugate translocation into the white pulp could account for its ability to inhibit the conjugate-induced Ig response.

Discussion

During systemic bacterial infections both soluble antigens released from the pathogen, in addition to antigens associated with the intact bacteria, likely enter the spleen concomitantly. The cellular events that mediate a primary humoral immune response and/or the generation of immunologic memory upon encounter with soluble antigens versus antigens expressed by an intact bacterial pathogen, and between proteins versus polysaccharides, exhibit distinct features. In this regard, the binding, uptake, transport, processing, and immune stimulation in response to antigen associated with an intact pathogen likely differs from its soluble counterpart on the basis of its repetitive expression within a particulate structure, its physical association with innate activating and scavenger receptor ligands, and its linkage to other types of antigens. In particular, studies from our laboratory indicate that the polysaccharide (PS)-specific IgG responses to intact *Streptococcus pneumoniae* (Pn) and soluble conjugate vaccine are both dependent on CD4⁺ T cell help and B7/CD28, and CD40/CD40L interactions, and elicited IgG anti-PS antibodies of all 4 IgG subclasses. However, the response to the former immunogen differs in displaying a shorter period of primary induction, T cell help and B7-dependent costimulation, and the absence of PS-specific memory (Guttormsen, Wetzler et al. 1998; Guttormsen, Sharpe et al. 1999; Wu, Vos et al. 1999; Wu, Khan et al. 2000; Khan, Lees et al. 2004; Khan, Sen et al. 2006) More recently, we obtained evidence that the PS-specific IgG response to intact Pn versus conjugate is largely ICOS-independent and extrafollicular versus ICOS-dependent and follicular, respectively (unpublished). In contrast the IgG anti-protein responses to both intact Pn and soluble

conjugate, in addition to isolated proteins, appear to be follicular in nature and generate protein-specific memory (Wu, Vos et al. 1999; Khan, Sen et al. 2006). Thus, these models appeared ideal for studying the immunologic consequences of systemic bacterial infections, when these two pathways are likely to be engaged simultaneously.

In this report we demonstrate that the induction of PS-specific IgG memory, but not the primary anti-PS response to conjugate is inhibited upon co-administration of conjugate and intact Pn (Fig. 17B). In addition, Pn inhibits both primary and secondary IgG anti-protein responses to either conjugate or soluble protein (Fig. 21A, 21B). Pn-mediated inhibition of the anti-protein response is observed both when Pn is suspended in saline, and conjugate is adsorbed to alum + CpG-ODN adjuvant, and each is injected at separate i.p. sites, or when soluble protein is mixed with Pn in saline and injected together at the same i.p. site. Of note, intact Pn itself expresses its own adjuvanting moieties including ligands for TLR2, 4, and 9 (Lee, Scanga et al. 2007). The mechanism of this inhibition appears to be antigen-non-specific, independent of Pn expression of PS capsule, mediated by several Pn strains (Fig. 18A), and not related to the particulation of the antigen per se (Fig. 18C), but to the ability of intact Pn to prevent conjugate transport from the marginal zone to the white pulp, where it presumably is required to initiate a follicular response. This notion is supported by the requirement for the relatively concomitant delivery of conjugate and Pn, as evidenced by a loss of most of the inhibitory effect when injection of Pn is delayed by 24 h following conjugate immunization (Fig. 22). That the effect of Pn is dominant to that of soluble antigen is evidenced by the use of optimal Ig-inducing dosages of each immunogen, and the

inability of the soluble conjugate or isolated protein to inhibit protein-specific Ig induction in response to intact Pn (Fig.21C).

Infections with extracellular bacteria develop in an acute, life-threatening manner, and thus require not only a robust innate immune response, but also the rapid elicitation of specific antibody to enhance opsonophagocytosis (AlonsoDeVelasco, Verheul et al. 1995). Upon entry into the blood, these bacteria quickly travel to the spleen where they are initially delivered into the marginal sinus and red pulp. In this location they are exposed to several distinct macrophage subsets that mediate phagocytosis and pathogen killing. The marginal zone also contains a specialized B cell subset, the marginal zone B cell (MZB) that is programmed to differentiate rapidly into short-lived PS-specific Ig-secreting plasma cells in an extra-follicular immune response that can be both TI (IgM) and TD (IgG) (Song and Cerny 2003; Phan, Gardam et al. 2005). MZB, along with macrophages and DC, may also play an important role in transporting both particulate and soluble antigens from the marginal zone into the white pulp, which contains follicular B cells (FB), T cells, and DC. In this location antigen can trigger a germinal center reaction by FB cells critical for the generation of immunologic memory and sustained Ig secretion from long-lived bone marrow plasma cells (Allen, Okada et al. 2007). Indeed, we recently provided evidence that MZB and FB cells mediate the PS-specific IgG response to intact Pn and soluble conjugate, respectively, whereas FB cells mediate the anti-protein response to both immunogens (Chattopadhyay, Khan et al. 2007). We observed (Fig.23) that the ability of Pn to block soluble antigen entry into the white pulp, without its own entry being inhibited might allow for both a rapid primary MZB-

mediated, PS-specific and more prolonged FB-mediated, protein-specific memory Ig response that is focused on pathogen-associated, but not pathogen-released antigen, thus optimizing antibody-mediated pathogen recognition and subsequent opsonization for effective phagocyte killing.

Alternatively, an effective high affinity and high titer IgG response to a soluble bacterial toxin, involving a GC reaction and immunologic memory, would be desirable to the host. Of interest, toxins appear to be robust immunogens by virtue of expressing either strong T cell epitopes (e.g. tetanus toxin [TT] (Valmori, Pessi et al. 1992)) or TLR stimulating activity (e.g. pneumolysin, a TLR4 ligand (Malley, Henneke et al. 2003)). These characteristics, which make these proteins effective carriers for PS conjugate vaccines, could potentially override the dominant inhibitory effect of intact bacteria on a soluble antigen-induced Ig response. Indeed, co-injection of Pn with PPS14 conjugated to TT failed to inhibit the conjugate-induced anti-PPS14 response, in contrast to the marked inhibition observed for the Ig response to the weaker PPS14-PspA conjugate (Fig.20). The anti-PPS14 response to PPS14-gp350, which exhibited an immunologic potency intermediate between these two conjugates, was partially inhibited by Pn, suggesting that the immunogenicity, and perhaps the physiologic significance, of the soluble bacterial protein is inversely correlated with its sensitivity to inhibition by the intact pathogen. This may reflect the ability of small amounts of soluble antigen to enter into the white pulp in the presence of intact Pn, and elicit an Ig response that is directly correlated with antigen potency.

Both soluble conjugate and intact Pn have relatively large molecular weights that preclude their passive diffusion through the conduit system present within the spleen (Gretz, Norbury et al. 2000; Nolte, Belien et al. 2003; Sixt, Kanazawa et al. 2005). The capacity of both immunogens to elicit an ICOS-dependent, protein-specific IgG memory response dependent upon FB cells strongly suggests that both conjugate and Pn are transported from the marginal zone to the B cell follicle via one or more immune cell types. One cellular candidate for transport of conjugate and/or Pn into the follicle is the marginal zone B cell (MZB). MZB can bind antigen-associated complement components of C3 via CD21 and CD35 and therefore capture antigen in a BCR-independent manner (Nossal, Austin et al. 1966; Mitchell and Abbot 1971; Brown, Harris et al. 1973). Upon migration to the follicle, MZB are able to deposit antigen onto FDC that can then serve as a platform for FB cell capture of antigen in a BCR-dependent manner and subsequent initiation of a GC reaction (Gray, Kumararatne et al. 1984; Groeneveld, Erich et al. 1986; Ferguson, Youd et al. 2004). Conjugate and Pn likely activate complement in vivo via the classical pathway, as evidenced in part by the ability of natural IgM to enhance immune responses to both soluble and particulate antigens in a CD21/CD35-dependent manner. The ability of repetitive surfaces, as manifested by intact pathogens, to efficiently activate complement (Matsushita and Fujita 2001) suggests that Pn might bind MZB via CD21 more efficiently than conjugate and thus directly block the latter's binding to and transport by MZB. This could in turn result in enhanced trapping of conjugate by macrophages within the marginal zone (Youd, Ferguson et al. 2002; Ferguson, Youd et al. 2004). Arguing against this scenario however is a report, that although the pneumococcal conjugate vaccine, Prevnar, is indeed transported from the marginal zone

to the follicle, it does not bind to MZB cells (Breukels, Zandvoort et al. 2005). Both macrophages (Groeneveld, Erich et al. 1986; Martinez-Pomares, Kosco-Vilbois et al. 1996; Mueller, Cremer et al. 2001; Karlsson, Guinamard et al. 2003), and DC (Bjorck, Flores-Romo et al. 1997; Kushnir, Liu et al. 1998; Wykes, Pombo et al. 1998; Bergtold, Desai et al. 2005; Huang, Han et al. 2005) are also known to bind and transport intact antigen for delivery to FDC and/or FB cells. Thus, direct Pn-mediated inhibition of conjugate binding and transport via these latter cells are other potential mechanisms for Pn-mediated inhibition of the conjugate-induced Ig response. The possibility also exists that Pn rapidly induces rapid migration of antigen transport cells to the follicle before conjugate has sufficient time to bind, again potentially resulting in conjugate trapping by macrophages in the marginal zone.

Footnotes

1. This study was supported by N.I.H. grants 1R01 AI49192 and the U.S.U.H.S. Dean's Research and Education Endowment Fund.

- 2) Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

- 3) Abbreviations used in this paper: PS, polysaccharide; Pn, intact *Streptococcus pneumoniae*; Pn14, intact *Streptococcus pneumoniae*, capsular type 14; PPS14, capsular polysaccharide, serotype 14; PspA, pneumococcal surface protein A; TT, tetanus toxoid; gp350, EBV envelope protein.

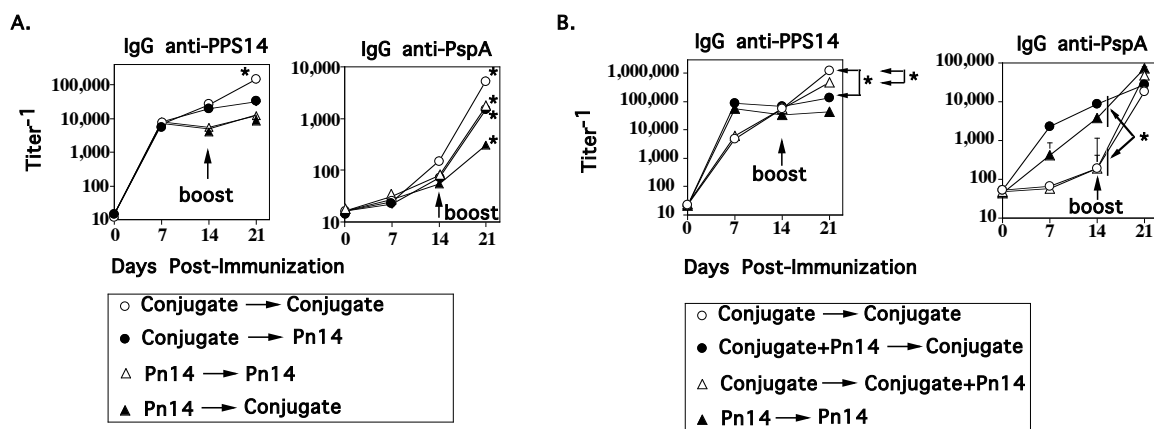
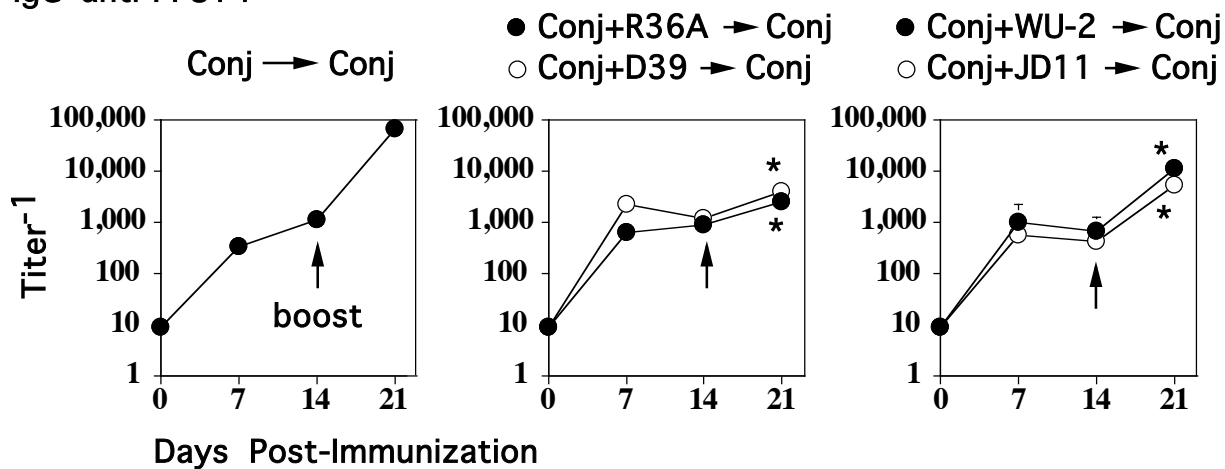


Figure 17.

Figure17. Pn14 fails to induce memory and inhibits memory generation by pneumococcal conjugate. (A) Mice were initially immunized i.p. with either PPS14-PspA (conjugate) in alum + CpG-ODN or Pn14, and boosted on day 14 with either conjugate or Pn14. Serum titers of IgG anti-PPS14 and IgG anti-PspA were determined by ELISA. *significance ($p < 0.05$) between primary (day 14) and secondary (day 21) titers. (B) Mice were initially immunized i.p. and boosted on day 14 as indicated. “Conjugate”=PPS14-PspA in alum + CpG-ODN. Serum titers of IgG anti-PPS14 and IgG anti-PspA were determined by ELISA. *significance ($p < 0.05$) between groups indicated by arrows.

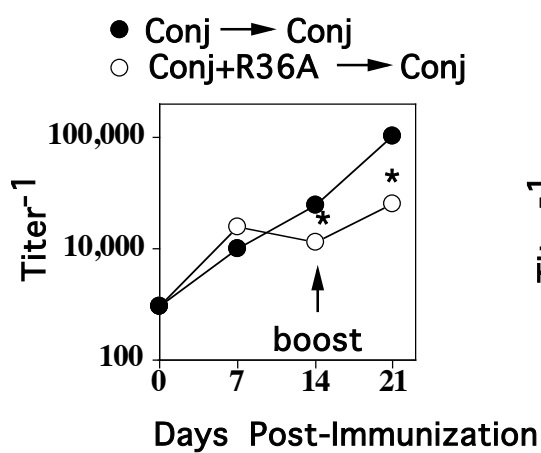
A.

IgG anti-PPS14



B.

IgM anti-PPS14



C.

IgG anti-PPS14

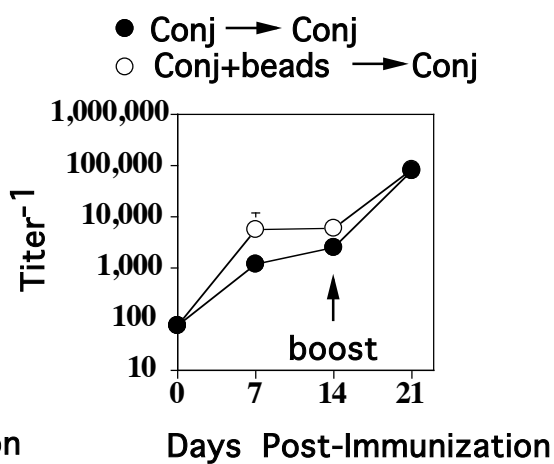


Figure 18.

Figure 18. Inhibition of conjugate-induced PS-specific memory is not dependent upon Pn capsular PS or particulation per se. (A) Mice were initially immunized i.p. with PPS14-PspA in alum + CpG-ODN (conjugate) without or with co-immunization with one of the following Pn strains: D39, R36A, WU-2, or JD11. All groups of mice were boosted with conjugate alone on day 14. Serum titers of IgG anti-PPS14 were measured by ELISA. *significance ($p < 0.05$) between mice initially injected with conjugate alone (left panel) versus mice co-injected with conjugate and a Pn strain (center and right panels). (B) Serum samples from “A” (left and center panels) for measurement of IgM anti-PPS14 titers by ELISA. * significance ($p < 0.05$) between two groups of mice. (C) Mice were initially immunized i.p. with PPS14-PspA in alum + CpD-ODN (conjugate) without or with co-immunization with 1.1 μm polystyrene latex beads in alum + CpG-ODN. Both groups of mice were boosted on day 14 with conjugate alone. Serum titers of IgG anti-PPS14 were measured by ELISA. * significance ($p < 0.05$) between two groups of mice.

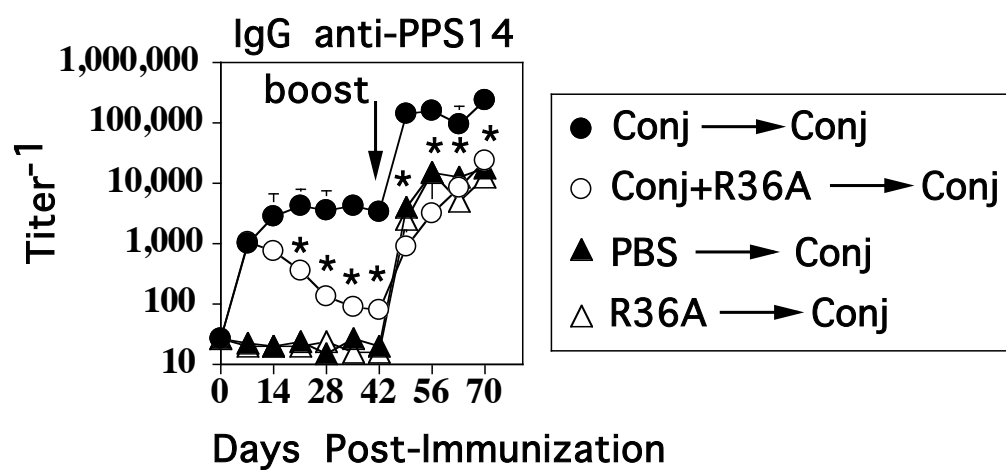


Figure 19.

Figure 19. Pn inhibits maintenance of primary IgG anti-PPS14 response to conjugate and induces long-lasting abrogation of memory. (A) Mice were initially immunized as indicated and then boosted on day 42 with conjugate alone.

“Conjugate”=PPS14-PspA in alum + CpG-ODN. Serum titers of IgG anti-PPS14 were measured by ELISA. *significance ($p < 0.05$) between mice initially immunized with conjugate versus conjugate + R36A.

IgG anti-PPS14

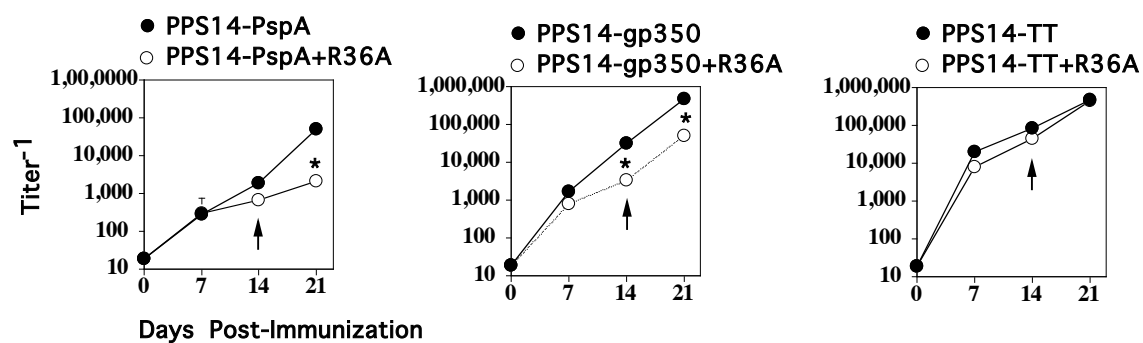
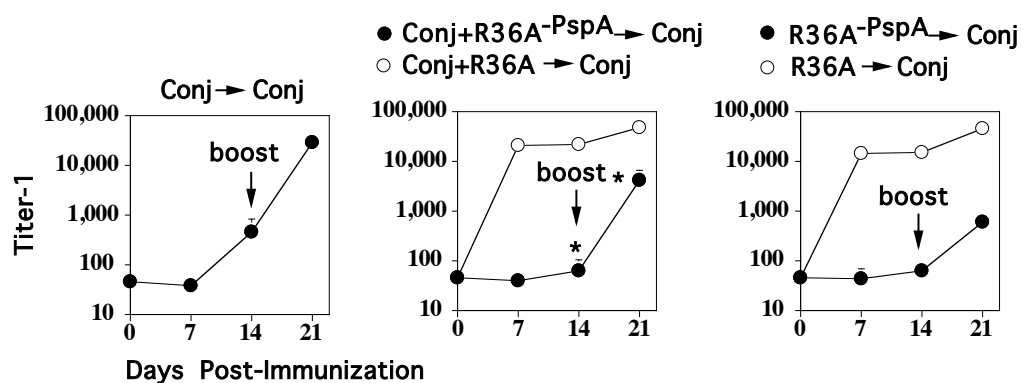


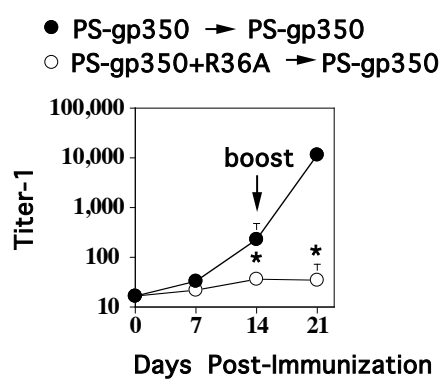
Figure20.

Figure 20. Potency of the carrier protein for PS-specific IgG induction can modulate inhibitory effect of Pn. Separate groups of mice were initially immunized i.p. with the following conjugates (1 µg per mouse [weight of PPS14]) in alum + CpG-ODN: PPS14-PspA, PPS14-gp350 or PPS14-TT without or with co-immunization with R36A. Each group was boosted on day 14 with the corresponding conjugate alone. Serum titers of IgG anti-PPS14 were measured by ELISA. *significance ($p < 0.05$) between the two groups of mice in each panel.

A. IgG anti-PspA



B. IgG anti-gp350



C. IgG anti-PspA

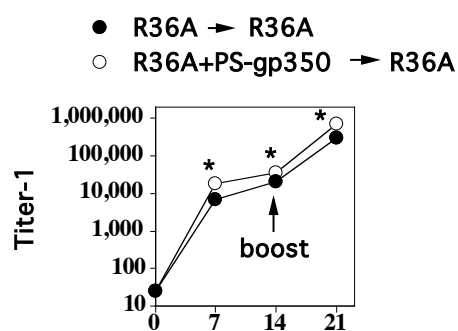


Figure 21.

Figure 21. Pn also inhibits induction of protein-specific IgG memory in response to conjugate. (A) Mice were initially immunized i.p. as indicated and boosted on day 14 with conjugate alone. “Conjugate”=PPS14-PspA in alum + CpG-ODN; “R36A^{-PspA}”=choline chloride-treated R36A to remove PspA. Serum titers of IgG anti-PspA were measured by ELISA. *Significance ($p<0.05$) between mice initially immunized with conjugate alone (left panel) versus mice initially immunized with Conjugate + R36A^{-PspA} (center panel, filled circle). (B) Mice were initially immunized i.p. with PPS14 (PS)-gp350 in alum + CpG-ODN without or with co-immunization with R36A. Both groups were boosted on day 14 with PS-gp350 alone. Serum titers of IgG anti-gp350 were measured by ELISA. *Significance ($p<0.05$). (C) Mice were initially immunized with R36A in the presence or absence of PPS-gp350 in alum + CpG-ODN. Both groups were boosted on day 14 with R36A alone. Serum titers of IgG anti-PspA were measured by ELISA. *Significance ($p<0.05$).

PPS14-PspA

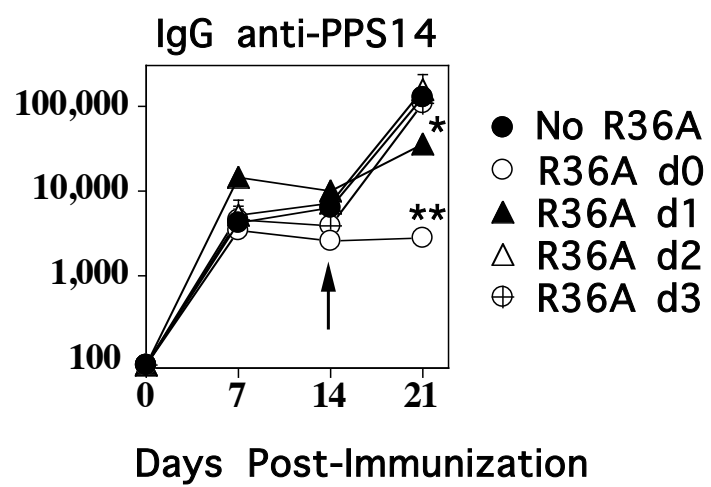


Figure 22.

Figure 22. Pn acts only within the first 24 h following conjugate immunization to inhibit memory. Separate groups of mice were immunized i.p. with PPS14-PspA in alum + CpG-ODN. On different days following immunization, mice were co-immunized (R36A d0) or subsequently immunized (R36A d1, d2, or d3) with R36A or not given R36A at all (No R36A). Serum titers of IgG anti-PPS14 were measured by ELISA. *Significance ($p < 0.05$) between mice not co-immunized with R36A (No R36A) versus other groups (R36A d0, 1, 2, or 3); *Significance ($p < 0.05$) between “R36A d0” and “R36A d1”.

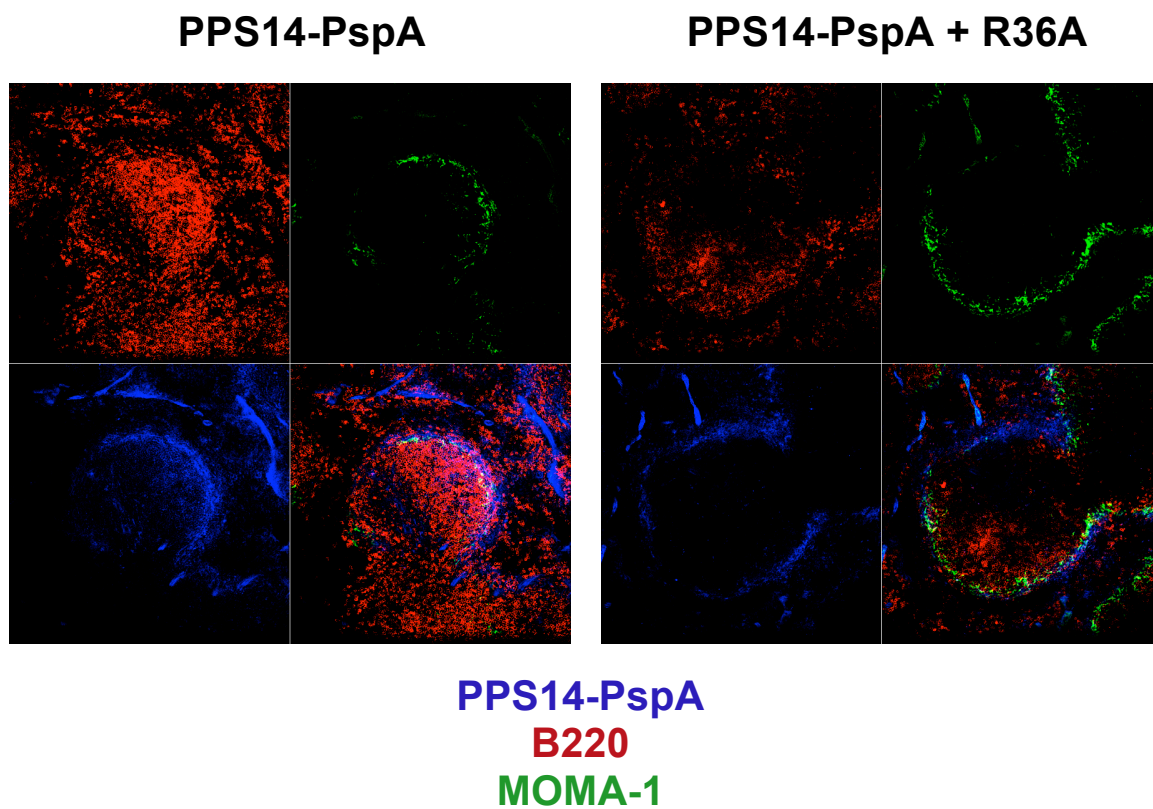


Figure 23.

Figure 23. Pn inhibits trafficking of conjugate from splenic marginal zone into white pulp. Mice were immunized with Alexa Fluor 405-labelled PPS14-PspA in the absence or presence of R36A, and spleens were removed 4 h later, for confocal fluorescence microscopic analysis. Sections were stained with PE-labeled anti-B220 (B cells) and FITC-anti-CD169 (MOMA-1, marginal metallophilic macrophages).

Chapter Four

Discussion

Project #1: Transgenic expression of Bcl-x_L or Bcl-2 by murine B cells enhances the in vivo anti-polysaccharide, but not anti-protein, response to intact *Streptococcus pneumoniae*

Previously, we demonstrated that unlike purified soluble PS, PS in the context of intact bacteria (Pn) behaves partially like a TD Ag. Thus, optimal IgG ant-PS responses to intact Pn requires CD4⁺T cell help (Khan, Lees et al. 2004), B-7 dependent costimulation (Wu, Khan et al. 2000) and CD40-CD40L interaction and comprise all four IgG isotypes (Wu, Vos et al. 1999). However, in contrast to classical TD-response, the primary PS-specific immune responses to intact Pn are relatively short-lived and exhibit no PS-specific memory after secondary boosting (Khan, Sen et al. 2006). Anti-PS responses to soluble pneumococcal conjugate are largely T cell-dependent and gives rise to a memory response after secondary boosting (Sen, Chen et al. 2006). Thus, the differences observed in the PS-specific humoral immune response against intact Pn versus soluble conjugate is not based simply on the ability to recruit CD4⁺T cell help but instead may depend upon differences in the physical/biochemical context of the PS Ag, the strength and/or duration of T-cell help and/or the subset of B cell responsible for the antibody response. We tested the hypothesis that the preferential induction of apoptosis in PS-specific versus protein-specific B cells in response to Pn, or perhaps in the anti-PS response to intact Pn versus pneumococcal conjugate, might be a critical parameter in determining the induction of a memory. Towards this end, we utilized mice that constitutively expressed the anti-apoptotic proteins, Bcl-x_L or Bcl-2, selectively on B cells on the basis of the transgene being linked the B cell-specific Ig enhancer, with a SV40 promoter. Our data strongly suggests that apoptosis preferentially limits the mIg-dependent clonal expansion of PS-

specific B cell subsets and primary PS-specific IgM and IgG response against intact Pn, although not conjugate, but has no effect on the generation of memory (Chattopadhyay, Khan et al. 2007). As demonstrated in Fig.9, both Tg mice exhibited a significant increase in peak primary titer in Tg mice in contrast to wild type (WT). Although upon secondary immunization both PS-specific IgM and IgG titer were higher than that observed in WT mice, there was no indication of PS-specific memory in Tg mice (Fig.9). This high primary IgM and IgG titer in Tg mice, correlates with the more sustained in vitro proliferation of purified Tg B cells after mIg crosslinking compared to WT counterparts. Additionally, Tg B cells exhibited more clonal expansion (CFDA-SE staining; Fig.13) and less apoptosis (propidium iodide staining; Fig.14) than WT B cells activated via mIg cross-linking. Thus, the higher PS-specific Ig titer in Tg relative to WT mice are presumably a consequence of enhanced extrafollicular growth and survival of plasma cell precursors with a resultant increase in overall Ab synthesis. After encountering a pathogen splenic MZB cells, follicular B cells, GC-cells or memory B-cells can differentiate into plasma cells (Shapiro-Shelef and Calame 2005). The nature, form, dose and the location of encounter dictates which population of B-cell will terminally differentiate into plasma cells. MZB cells are non-circulating, have a distinct cell surface phenotype, are slightly larger than FB cells. MZB nuclei are more-irregular, have less condensed chromatin, and the cell has a higher expression of the T cell costimulatory molecules, CD80/CD86 suggesting a pre-activated state (Spencer, Perry et al. 1998; MacLennan, Toellner et al. 2003). Indeed, when stimulated with LPS, MZB cells respond more quickly and vigorously than FB cells for proliferation and Ig secretion (Oliver, Martin et al. 1997). After TD Ag encounter, a portion of the specific B cells

undergo rapid proliferation and plasmacytic differentiation to form extrafollicular foci of plasmablast and plasma cells (Shapiro-Shelef and Calame 2005). Interestingly, plasma cells in the foci do not have somatically-mutated immunoglobulin genes and are short-lived due to their extensive apoptosis (Smith, Hewitson et al. 1996). Using cytoplasmic staining for IgG1 and counterstaining by TUNEL (apoptosis) assay Smith et al (Smith, Weiss et al. 1994) detected apoptosis in splenic foci, whereas B cells expressing Bcl-2 transgene demonstrate prolonged survival in antibody forming cells (AFCs) (Smith, Hewitson et al. 1996). Additionally, Vinuesa et al (Garcia De Vinuesa, Gulbranson-Judge et al. 1999) showed that CD11c^{high} DC supports plasmablast survival in extrafollicular foci. They demonstrated that these DC co-localized with plasmablasts in the extrafollicular foci of the splenic junction zone adjacent to the T-zone (Garcia de Vinuesa, O'Leary et al. 1999). Failure of plasmablast to associate with DC lead to plasmablast apoptosis. Thus, the high primary PS-specific Ig titer that we observed expressing Bcl-2 or Bclx_L Tg mice following immunization with intact Pn could be a result decreased apoptosis within the extrafollicular plasmablast response. Although the extrafollicular response to intact Pn is TD, an extrafollicular response to purified PS Ags is TI, although similar cellular events may otherwise occur in both types of response. Using NP-Ficoll, a classical purified TI-2 Ag, MacLennan et al showed in several studies that after Ag encounter NP-specific B cells translocate into T zone from marginal zone within 8h (de Vinuesa, Cook et al. 2000) and induced Blimp-1 (B-lymphocyte-induced maturation protein-1) within 18h, a transcription factor which is critical for differentiation into plasmablast (MacLennan, Toellner et al. 2003). For 48h B cells dwell in the T zone after primary activation and then upregulate syndecan-1 (CD138) which is an important

marker for plasma cells. From this T zone they relocate to the red pulp around the outer surface of marginal zone near the T zone (MacLennan, Toellner et al. 2003). When Ag-activated B cells relocate from T zone to red pulp, Btk signaling is crucial (Vinuesa, Sunners et al. 2001). Previously Khan et al (Khan, Sen et al. 2006) demonstrated that Btk signaling is critically important in generating immune response against intact Pn14.

Commitment to the extrafollicular pathway is correlated to the up-regulation of Blimp-1 whereas taking follicular route is directly correlated with the induction of a distinct transcription factor Bcl-6 (Fearon, Manders et al. 2001). The choice between extrafollicular versus follicular route is also determined by the strength of Ag recognition and relative multivalency of the antigenic epitopes (Paus, Phan et al. 2006). We failed to see PS-specific memory enhancement in Tg as well as wild type mice after secondary immunization in contrast to anti-protein (anti-PspA) response, which likely reflects the inability of PS-specific B cells to enter a GC-reaction in response to intact Pn, despite a decreased level of apoptosis. Presumably, this is correlated with the shorter period of T-cell help which may be insufficient for a sustained GC-reaction. GC-reaction is a dynamic microenvironment where Ag-specific B cells, CD4⁺ helper T-cell and follicular DC interact to produce long-lived, high affinity plasma cell that eventually translocate to the bone marrow, and memory cells that recirculate through the blood. In order for the GC reaction to progress, B cell must receive co-stimulatory signals from CD4⁺ helper T-cell which include induction of CD40-Ligand (CD40L) on the T cell and subsequent CD40-mediated activation of the B cell, and costimulation of the T cell via CD28 and then later ICOS (Inducible T-cell co-stimulator, CD278). CD40L costimulation and cytokines from cognate T helper cells is critical for the expansion of highly Ag-specific B

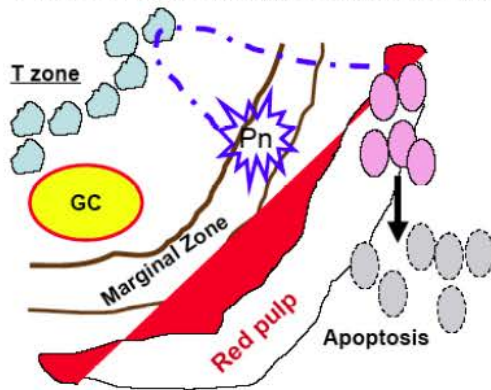
cells in GC (Foy, Laman et al. 1994; Wolniak, Shinall et al. 2004). Since purified PS Ags are TI, it is not surprising that there is no difference in PS-specific immune response between CD40 or CD40L knockout mice compared to wild type against purified PS Ag (Renshaw, Fanslow et al. 1994). However, Wu et al in our laboratory (Wu, Vos et al. 1999) demonstrated that TD IgG-PS but not TI anti-IgM response to intact Pn was reduced in CD40L^{-/-} or CD28^{-/-} versus WT mice. In contrast to involvement of CD40/CD40L and B7/CD28 interactions in PS-specific IgG responses against intact Pn, ICOS^{-/-} mice did not show any significant difference in IgG anti-PS response as compared to wild type mice (unpublished data), whereas ICOS^{-/-} might had a marked defect in the anti-protein response. As mentioned ICOS is important for sustained CD4⁺T cell activation, whereas CD28 is important for the initiation of the T cell response. These data are consistent with the shortened period of T cell help in the anti-PS versus anti-protein response to Pn. Thus, PS-specific B cell activation following immunization with intact Pn does not lead to a GC reaction, and hence after receiving T cell help from T zone, these B cells may migrate directly to red pulp to become a short-term plasmablast, which can secrete class-switched Ab during primary response. In contrast, mice immunized with soluble conjugate showed significant reduction in both the primary and memory PS-specific, as well as protein-specific, Ig response response in ICOS^{-/-} mice as compared to wild type mice, which probably consistent with the involvement of GC-reaction in the PS- and protein-specific Ig response to conjugate.

We found a substantial increase in both percentage wise and absolute number in the peritoneal B-1b and B-2 population in Tg mice in contrast to wild type mice. Also in Tg mice, splenic B-1 population was increase in number. Peritoneal B-1b, B-2 and

splenic B-1 (Hastings, Tumang et al. 2006) cells are implicated as key mediators of the Ig response to PS (TI-2) Ags in addition to marginal zone B cells. We failed to see any enhancement in the subset of MZB-cell population in Tg mice. However, Tg mice exhibited a reduction in the splenic MZB cell subset. Haas et al (Haas, Poe et al. 2005) showed that B-1a cells produce high level of natural Abs and plays an important role in innate immune response. By contrast, peritoneal B-1b cells elicit Ig responses against purified TI-2 Ags. Hsu et al (Hsu, Toellner et al. 2006) demonstrated that B-1b cells give rise persistent extrafollicular responses to NP-Ficoll, a purified TI-2 antigen. In our study the higher serum titers of IgM and IgG anti-PS in Bcl-x_L or Bcl-2 Tg mice in response to intact Pn, might be a result of increased Ig production from the expanded B1-b population. However, at least the IgG anti-PS response was shown to derive largely from MZB cells that were not increased in Tg mice. We show that MZB cells from Tg mice proliferate to much higher levels in vitro following Ig crosslinking, which likely results in an expanded extrafollicular response in vivo and higher serum titers of anti-PS Ig.

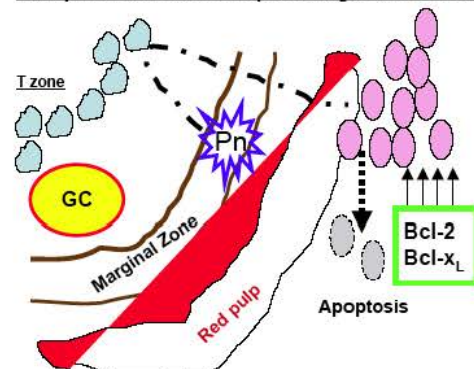
Collectively, our study suggests that apoptosis plays a critical role by limiting the mIg-mediated clonal expansion of PS-specific B cells during the primary immune response against intact Pn, with consequent loss of the MZB-derived plasmablasts and loss of ongoing Ig production during the TD extrafollicular response (Fig.24). Of interest, transgenic expression of Bcl-x_L or Bcl-2 apparently plays no major role in the generation or enhancement of PS-specific, or indeed protein-specific, memory responses against intact Pn or conjugate. We conclude that intact Pn and soluble protein-PS conjugate initiates distinct cellular events that are not appreciably affected by a forced diminution in the propensity to undergo apoptosis.

PS-specific immune response against Pn in Wild Type



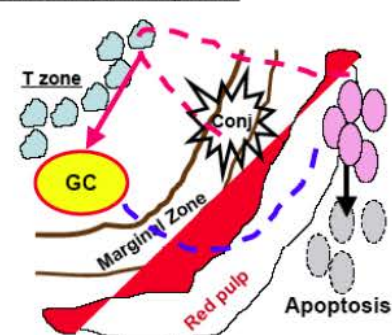
- Intact Pn enters into the white pulp and gets T-cell help.
- Then it comes back into the red-pulp and involves in the plasmablast generation.
- Presumably, in wild type mice, PS-specific B cells undergo extensive apoptosis.

PS-specific immune response against Pn in Tg mice



- In Tg mice also intact Pn enters into the white pulp to get T-cell help.
- B cell specific overexpression of Bcl-2/Bcl-x_L in Tg mice helps PS-specific plasmablast to rescue from extensive apoptosis and thus increase sustained antibody production.

PS-specific immune response against conjugate in Wild Type & Tg mice



- Soluble conjugate enter into the white pulp and get T-cell help.
- For primary antibody secretion PS specific B cells involves in the plasmablast generation within white pulp.
- PS-specific immune response also participate in the germinal center reaction for the generation of PS-specific memory.
- There is no difference in PS-specific immune response in both wild type and Tg mice, as the secondary antibody production is probably due to germinal center reaction.

Figure 24. Postulated model of humoral immune response against intact Pn and soluble conjugate in wild type versus Tg mice.

Project # 2. Concomitant systemic delivery of soluble and intact bacteria-associated antigens results in marked inhibition of the soluble antigen-induced Ig response. A model for humoral immunity during blood-borne bacterial infections

Data from our laboratory indicates that both the anti-protein and anti-PS Ig response to soluble conjugate is primarily a classical TD response, as evidenced by prolonged primary kinetics of induction, elicitation of all 4 IgG subclasses, and the generation of immunologic memory (Sen, Chen et al. 2006). Whereas the anti-protein response to intact Pn is also classically TD, being largely follicular in nature, and resulting in a memory response after secondary boosting (Khan, Lees et al. 2004). The anti-PS response to Pn, is partially TD, is primarily extrafollicular and fails to generate a memory response. Recently, our lab has demonstrated that systemic (i.p.) injection of soluble conjugate results in localization of antigen largely with DCs in the spleen, whereas intact Pn preferentially localizes within marginal zone (MZM) and metallophilic (MMM) macrophages. In addition, data from our laboratory also indicates that conjugate is rapidly transported, in relatively large amounts, into the splenic white pulp (both T cell areas and B cell follicles) from the marginal zone of the spleen, whereas much smaller amounts of intact Pn is transported to the white pulp, and only at a relatively slower rate (Snapper et al unpublished). Others have also shown that soluble Ag and particulate Ags are delivered to the spleen by separate pathways (Berney, Herren et al. 1999; Sixt, Kanazawa et al. 2005; Carrasco and Batista 2007).

The parameters that regulate the choice between the extrafollicular routes versus GC route of immune response to immunogen is complex and not fully understood. In addition to different localization patterns of soluble and particulate antigens within the

secondary lymphoid organ, differential recognition specificities of Ag by B cell receptors expressed on individual B cells can influence their subsequent commitment to either an extrafollicular or GC reaction (Dal Porto, Haberman et al. 1998; Blink, Light et al. 2005). A recent report by Paus et al (Paus, Phan et al. 2006) demonstrated that TD Ag specific B-cells undergo an extrafollicular response if the BCR affinity is high for the Ag or the Ag contains abundant repeating antigenic epitopes. In contrast, Ags binding with weaker BCR strength tend to direct B cells towards a GC reaction and with resultant memory generation, affinity maturation of Ig, and development of long-term bone marrow plasma cells (Paus, Phan et al. 2006).

In light of the likely release of soluble bacterial antigens during bacterial infections, and simultaneous transport of both soluble antigen and particulate bacteria into the secondary lymphoid organ, we were interested in determining whether these two distinct pathways were cross-regulatory and influence antigen-specific Ig responses they induced. As a model for this study we thus co-immunized intact Pn with either several different soluble PS-protein conjugate vaccines or with a soluble protein alone (i.e. chicken ovalbumin [cOVA]). Of note, intact bacteria inhibited both IgG anti-PS (Fig.17) and IgG anti-protein memory generation (Fig.21) and the PS-specific IgM response (Fig.18B) to conjugate. However, soluble conjugate had no inhibitory effect on the humoral immune response against intact Pn. Intact Pn differentially inhibited the different conjugate vaccines, inversely correlated with the immunogenic potency of the carrier protein, and thus the kinetics and titer of the IgG anti-PS response, perhaps a reflection of differential recruitment of T cell help (Fig.20). In contrast, intact Pn had little affect on the early primary anti-PS response to conjugate, although it inhibited the maintenance of

the anti-PS response over time (Fig.17), likely reflecting inhibition of the ensuing GC reaction and development of long-lived bone marrow plasma cells. Intact Pn inhibited both the early and late phase of the primary anti-protein response (Fig.21).

In situ confocal microscopy demonstrates that intact Pn blocks transport of soluble conjugate into the white pulp and thus prevents localization of Ag within the compartment necessary to generate a GC reaction (Fig.23). Thus, it appears that intact Pn effectively competes for the same transport mechanism as that utilized by conjugate for transport from the marginal zone to the white pulp. The nature of this transport system is not yet determined but could involve binding to MZB via complement receptor CD21, with subsequent migration of the MZB from the marginal zone into the B cell follicle where it can deliver antigen to follicular dendritic cells (FDC) and thus indirectly to FB cells, or directly to FB cells themselves.

Our data demonstrated that the Pn-induced inhibition of the conjugate response is long-lasting likely due to complete abrogation of memory induction at an early point in the immune response. Thus, after boosting with conjugate at day 42, the inhibitory effect of Pn is still apparent up to day 70 following secondary immunization with conjugate (Fig.19). This is likely due to the brief period during which conjugate can move into the white pulp before it is degraded by marginal zone macrophages. Indeed, if immunization with intact Pn is delayed by as little as 24 h most of the inhibitory effect is eliminated (Fig. 22).

This negative regulatory interaction is not due solely to the particulate nature of Pn because when we co-immunized bacteria sized biodegradable beads adsorbed to CpG-ODN + alum adjuvant with conjugate, no inhibition of the conjugate-induced memory

response was observed (Fig.18). This likely reflects a requirement for certain bacterial cell wall components that perhaps activate complement thus enhancing the binding to MZB cells that express CD21. Additionally intact Pn may express other cell wall ligands that facilitate direct binding to MZB or other transport cells, such as ligands for TLR2 that is highly expressed on MZB cells (Kadioglu, Weiser et al. 2008). That cell wall structures may be important for Pn-induced inhibition is reflected by the fact that not only bacteria-sized inert beads had any effect, but also the Pn-induced inhibition is not capsular PS serotype –specific or require any capsular PS at all (Fig.18A), as Pn strains expressing serotypes not found in the PS of the conjugate vaccine were equally inhibitory (Fig.20). That capsular PS doesn't play a role in inhibiting the conjugate-specific memory response, is evidenced by the inhibitory effects of the unencapsulated isogenic mutants of Pn type 2(R36A) or Pn type 3 (JD11) used for co-immunization with soluble conjugate (Fig.18). Thus, this inhibition is a unique property of the bacteria itself.

Our observation from this concomitant immunization of intact Pn and soluble bacterial conjugate could be visualized as a model of systemic bacterial infection where both the bacteria and its soluble mediators are present within the host at the same time. During extracellular bacterial infection, in addition to an innate immune response, a rapid production of specific antibody generation is critical to enhance opsonophagocytosis (AlonsoDeVelasco, Verheul et al. 1995). In the splenic location bacteria and their soluble products are detected by distinct cell types including macrophage subsets in the marginal sinus and also MZB cells in the marginal zone. MZB cell along with macrophages and DC, play distinct role in both follicular and extrafollicular immune response including transporting both particulate and soluble antigens from the marginal zone into the white

pulp, which contains follicular B cells (FB), T cells, and DC. In follicular location antigen can trigger a germinal center reaction by FB cells critical for the generation of immunologic memory and sustained Ig secretion from long-lived bone marrow plasma cells. Indeed, we recently provided evidence that MZB and FB cells mediate the PS-specific IgG response to intact Pn and soluble conjugate, respectively, whereas FB cells mediate the anti-protein response to both immunogens (Chattopadhyay, Khan et al. 2007). We speculate that the ability of Pn to block soluble antigen entry into the white pulp, without its own entry being inhibited might allow for both a rapid primary MZB-mediated, PS-specific and more prolonged FB-mediated, protein-specific memory Ig response that is focused on pathogen-associated, but not pathogen-released antigen, thus optimizing antibody-mediated pathogen recognition and subsequent opsonization for effective phagocyte killing. Accumulating evidence from our laboratory demonstrate that intact bacteria do not induce a complete PS-specific follicular response, while initiating an anti-protein specific GC response after bacterial challenge. This report shows that Pn likely inhibits the generation of the memory response against soluble bacterial substances and could be a mechanism whereby the host focuses the antibody response upon the intact bacteria for optimal clearance.

Toxin are soluble bacterial substances that can be robust immunogen by expressing either strong T cell epitopes (e.g. tetanus toxin [TT] and diphtheria toxin [DT]) or TLR stimulating activity (e.g. pneumolysin, a TLR4 ligand). It is advantageous for the host to elicit an antibody to these particular proteins. Our data demonstrate that toxins could potentially override the dominant inhibitory effect of intact bacteria on a soluble antigen-induced Ig response. Indeed, co-injection of Pn with PPS14 conjugated to

TT failed to inhibit the conjugate-induced anti-PPS14 response, in contrast to the marked inhibition observed for the Ig response to the weaker PPS14-PspA conjugate. The anti-PPS14 response to PPS14-gp350, which exhibited an immunologic potency intermediate between these two conjugates, was partially inhibited by Pn, suggesting that the immunogenicity, and perhaps the physiologic significance, of the soluble bacterial protein is inversely correlated with its sensitivity to inhibition by the intact pathogen.

In conclusion, our results demonstrate a novel mechanism of interaction between two distinct immunologic pathways. These studies raise interesting questions regarding the involvement of cell surface molecules on MZB cells including CR1/CR2, or antigen presenting cells in the marginal sinus that bind these two types of Ag and transport them into the splenic follicle. These questions can be approached using differential fluorochrome-labeled conjugate and Pn and following antigen trafficking in mice following immunization. Thus, it can be predicted that following immunization with conjugate alone, MZB cells will be found by flow cytometry to have bound conjugate. However, with co-immunization of Pn, MZB cells will bind Pn but not conjugate. Additionally, we predict that forced migration of MZB from the marginal zone into the follicle by pharmacologic means [treatment with FTY720 (Cinamon, Zachariah et al. 2008)] will result in inhibition of both the conjugate- and Pn-induced anti-protein memory response to each of the immunogens injected separately, as well as the conjugate-induced PS-specific memory response. Additionally, several other questions could arise from these studies such as: 1) Why are bacteria so efficient in blocking conjugate transport? 2) How do these two pathways operate functionally during systemic infections? 3) Could blocking of the immune response to soluble antigens by “artificial

bacteria” (i.e. constructed to mimic the Pn-mediate inhibitory effect) play a therapeutic role in allogeneic transplantation or autoimmunity, where circulating antigens trigger pathogenic humoral responses? 4) Can intact Pn also inhibit a cell-mediated response to a systemic immunogen?

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