Regulation of In Vivo Anti-Polysaccharide Responses to Intact Gram-Positive and Gram-Negative Extracellular Bacteria

Thesis

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

By

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2008PHXF014P

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN), INDIA

Completed at Uniformed Services University of the Health Sciences, Bethesda,

Maryland, USA under INDO-USUHS Collaboration

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN)

CERTIFICATE

This is to certify that the thesis entitled, "***Regulation of In Vivo Anti-Polysaccharide Responses to Intact Gram-Positive and Gram-Negative Extracellular Bacteria***" submitted by **Swadhinya Arjunaraja** ID: **2008PHXF014P** for the award of Ph.D degree of the Institute embodies the original work done by her under my supervision.

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DEDICATION

I dedicate this thesis to my amazing parents, *Mr. Arjunraja* and *Mrs. Dhanalakshmi* for their unconditional love, support and sacrifices. My academic and personal successes are credit to the values and morals that they instilled in me. I also dedicate this to my wonderful life partner who has been my motivating factor throughout my research career. Thank You from the bottom of my heart for always being there with me.

ACKNOWLEDGEMENTS

It's my pleasure to acknowledge the kind people around me who helped in the successful completion of my thesis.

First and foremost, I would like to express my heartfelt gratitude to my mentor and advisor *Dr. Clifford Snapper*, whose constant support and unwavering encouragement throughout my graduate tenure made my project successful and my doctoral training experience intellectually stimulating. His zeal and enthusiasm towards research was motivational for me even during the tough times of my PhD. He always inspires me by setting an excellent example of a successful scientist and an amazing human being.

I am indebted to *Dr. Radha Maheshwari*, who gave me this wonderful opportunity to pursue my graduate studies through the USUHS-BITS, Pilani collaborative program. His good advice and support have been invaluable at both the academic and personal level, for which I am extremely grateful. I would especially like to thank *Dr. Robert Friedman*, Chair, Department of Pathology, USUHS for his constant support to the BITS, Pilani graduate program.

I express my heartfelt appreciation to *Dr. K M Birla*, Chancellor, *Dr. B N Jain*, Vice chancellor, *Dr. G Raghurama*, Director, and *Dr. R N Saha*, Deputy Director, Administration, *Dr. S.K. Verma***,** Dean, Academic Research, BITS, Pilani and *Dr. Jitendra Panwar*, Head of the Department, Biological Sciences, BITS, Pilani for their constant support to the USUHS-BITS collaborative program. I am obliged to *Dr. Ashis K Das* who has initiated the fascination towards research studies. I am grateful to my Doctoral Advisory Committee members *Dr. Vishal Saxena* and *Dr. Sanjeev Kumar* for their helpful comments and valuable suggestions. I am thankful to *Academic Research Division*, BITS, Pilani for their kind help during my PhD tenure.

I owe my deepest gratitude to my lab members, who without their assistance this study would not have been successful. My special thanks to *Dr. Jesus Colino* for educating me on basic techniques and for his thoughtful inputs in helping me to solve technical problems. He never hesitated to step in for help at the time of need. I am most grateful to *Quanyi*, 'a superwoman' as we all call her, for her support, friendship and personal advice that boosted me for the successful completion of this work. I also appreciate our former lab member *Leah Duke* for helping me in breeding mice. My hearty thanks to *Saumyaa* who has been a wonderful colleague and an understanding friend to work along in the lab. We had great times together that I will cherish forever.

I express my heartfelt appreciation to *Dr. Edward Mitre, Dr. Brian Schaefer, Dr. David Scott, Dr. Joseph Mattapallil, Dr. Charles Via* and other immunology data club and journal club members at USUHS for their constructive criticism and valuable suggestions. I would like to thank *Marta Krzywucki, Dawn Patti, and Athena Bozak* in the Department of Pathology office, for taking care of the orders and other administrative work so that I could carry out my research work in a timely manner. I appreciate *Gus Parra* for helping me in autoclaving the media as needed. My special thanks to the Biomedical Instrumentation Center, USUHS, for having given me the opportunity to use their facility, and in particular to *Katherina Lund* for her enormous help in conducting the flow cytometry studies.

My time at USUHS was enjoyable in large part due to the many friends and groups that became a part of my life. I was privileged to have good friends like *Paridhi, Manish* and *Swagata* during my PhD tenure. The 'chai' and 'chat' sessions with friends were unforgettable. Many thanks to my senior friends *Anuj, Mathanraj, Rajesh* and *Sangeetha* who promoted a stimulating and welcoming academic and social environment. The warm support of all my

friends in the United States enabled me to complete this thesis and have a wonderful time along the way. My special thanks to *Anu, Jothy* and *Saravanan* for all their kind help and valuable advice. I wish I could mention each individually, but will throw a large party instead.

Words fail to express my deepest appreciation to my parents, who cheered and motivated me in all my pursuits, and helped me in all possible ways. Special thanks to my sister *Revathi*, *Saravanaraja* (brother in law) and my nephew *Rohith Pranav* who were very supportive during my research studies. My sincere gratitude is due to my in-laws, *Mr. Radhakrishnan* and *Mrs. Vasanthi*, who were very patient until my thesis completion. I am thankful to my cousins *Balaji, Hema, Meena, Satish, Suresh, Uma* and *Dheepa* who have been very encouraging and appreciative.

I am really blessed to have a loving and supporting husband *Vijaybalaji***.** He was traveling without hesitation for about ten hours every weekend for all these years to visit me which made my life much easier. His love and constant support encouraged me to successfully complete my PhD work. Thank you for being the man behind my success. Also, I owe special thanks to my little princess *Sriya* who made me a proud mother and gave me a new purpose in life.

I greatly acknowledge the laboratory mice that sacrificed their life without which this thesis would not exist.

Last but not the least; I would like to thank the *'One above'*, for giving me the perseverance and determination to carry out my research successfully.

ABSTRACT

Thesis Title : **Regulation of In Vivo Anti-Polysaccharide Responses to Intact Gram-Positive and Gram-Negative Extracellular Bacteria**

Supervisor : **Dr. Clifford M Snapper, MD**

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Abstract :

Proteins and polysaccharides (PS) are broadly categorized as T cell-dependent (TD) and T cell-independent (TI) antigens for the elicitation of immunoglobulin (Ig) responses. This dichotomy is derived mainly from studies using soluble protein and PS antigens. However, in any natural infection, the host samples these antigens in the context of the bacteria that cause invasive disease. Very little is known regarding the regulation of PS-specific Ig responses to the intact bacterium. Our lab has hypothesized that PS-specific Ig responses to intact bacteria may be regulated differently relative to that observed in response to isolated, soluble PS antigens. We believe that the basis for this is that intact bacteria coexpress their PS antigens in a particulate structure, along with various proteins and multiple adjuvanting moieties such as Toll-like receptor (TLR) ligands and scavenger receptor ligands. In particular, the association of PS with protein may convert the anti-PS response from TI to TD. Indeed, our previous studies using intact *Streptococcus pneumoniae* type 14 (Pn14), a Gram-positive extracellular bacterium demonstrated that the primary PS-specific IgG response was dependent on CD4+ T cells,

CD40/CD40L interactions and costimulation dependent on B7-CD28 interactions. However, similar to that observed using soluble, isolated PS, the PS-specific IgG responses to Pn14 were ICOS-independent and failed to induce any secondary boosting. These latter observations were in distinct contrast to the classic TD IgG anti-PS responses to a soluble covalent conjugate of PS and protein (i.e. conjugate vaccine). These studies demonstrated that PS expressed by intact Pn14 behaved as a unique immunogen, exhibiting both TD and TI properties. However, these studies left unexplained whether the immunologic properties of intact Pn14 could be generalized to other Gram-positive, as well as Gram-negative bacteria. Thus, distinct Gram-positive and Gram-negative bacteria express unique attachments of capsular PS to the underlying bacterial cell wall, different physical and chemical properties of the cell wall itself, and distinct chemical properties of the expressed capsular PS. Collectively, these differences could impact on the nature of the PS-specific Ig response to various intact bacteria.

To begin to address these questions, we used intact heat-killed *Neisseria meningitidis* type C (MenC), a Gram-negative bacterium to study the Meningococcal type C PS (MCPS)-specific Ig response, relative to the PS-specific Ig response elicited by intact Pn14. We found that the MCPS-specific Ig response to intact MenC was distinctly different from the PS-specific Ig response to intact Pn14, but similar to that of a MCPS-tetanus toxoid (TT) conjugate vaccine. Specifically, intact MenC induced an ICOS-dependent IgG response that was highly boosted following secondary immunization. In further contrast to Pn14, in which the primary PS-specific IgG response was TD and peaked rapidly, the primary response to MenC was TI and was relatively slower to develop. Thus, we demonstrate that the nature of an in vivo PS-specific IgG response to an intact bacterium is remarkably influenced by the structure and/or physicochemical composition of the underlying bacterial subcapsular domain.

These data suggested the possibility that PS-specific Ig responses to Gram-positive (e.g. Pn14) and Gram-negative (e.g. MenC) bacteria may differ in a general manner, or perhaps each bacterium may exhibit unique immunologic features, in addition to, or independent of their Gram-positive or Gram-negative classification. Therefore, we took advantage of the Gram-positive bacterium *Streptococcus agalactiae* type III (Group B *Streptococcus* [GBS-III]) that expresses desialylated capsular IIIPS that is similar to the PPS14 of Pn14 to compare the in vivo PS-specific Ig responses to these two Gram-positive bacteria. The IIIPS capsule differs from the PPS14 by the presence of terminal sialic acid and the antibodies generated in response to IIIPS crossreacted with PPS14. We found that the expression of PPS14 by GBS elicited a rapid TD primary PS-specific IgG response similar to Pn14, but in contrast also induced a boosted ICOS-dependent PPS14-specific IgG response following the secondary immunization. Of interest, we found that priming with Pn14, but boosting with GBS-III produced a similarly boosted PPS14-specific IgG response, indicating that Pn14 primed for memory, but was defective in eliciting it following secondary immunization. Thus, these data indicate that the structurally identical PS present on these two different bacteria could elicit distinct PS-specific Ig responses in vivo. Collectively, these data demonstrate that unique features of the subcapsular domain of a particular intact bacterium can markedly influence the Ig response to the expressed capsular PS.

LIST OF ABBREVIATIONS

Antibody	Ab
Antigen	Ag
Antigen presenting cells	APC
B cell activating Factor	BAFF
B cell receptor	BCR
Chemically defined media	CDM
Choline chloride	CC
Colony forming units	CFU
Dendritic cells	DC
Follicular B cells	FB
Follicular dendritic cells	FDC
Germinal center	GC
Gram-positive	GP
Gram-negative	GN
Group B *Streptococcus*	GBS
Immunoglobulin	Ig
Inducible costimulator	ICOS
Inner membrane	IM
Interferon	IFN
Interleukins	IL
Lipooligosaccharide	LOS
Lipopolysaccharide	LPS
Lipoteichoic acid	LTA
Major histocompatibility complex class II	MHC-II
Marginal zone	MZ
Membrane Immunoglobulin	mIg
Meningococcal polysaccharide type A	MAPS

Meningococcal polysaccharide type C	MCPS
Meningococcal Porin protein B	PorB
Meningococcus type A	MenA
Meningococcus type C	MenC
NOD-like receptor	NLR
Oligodeoxynucleotide	ODN
Outer membrane	OM
Outer membrane proteins	OMP
Periarteriolar lymphoid sheath	PALS
Phosphorylcholine	PC
Phosphate buffered saline	PBS
Platelet activating factor receptor	PAFR
Pneumococcus	Pn
Pneumococcal polysaccharide type 14	PPS14
Pneumococcal surface protein A	PspA
Poly N-acetyl glucosamine	PNAG
Polysaccharides	PS
T cell dependent	TD
T cell independent	TI
T cell receptor	TCR
T-follicular helper cells	T_{FH}
Teichoic acid	TA
Tetanus Toxoid	TT
Toll-like receptors	TLR
Tumor Necrosis Factor	TNF
Type III polysaccharide	IIIPS
Wild-type	WT

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INTRODUCTION

INTRODUCTION

Polysaccharide (PS)-encapsulated extracellular bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* are major sources of global morbidity and mortality among infants, elderly and the immunosuppressed, due to sepsis, pneumonia, and meningitis. Between 800,000 and 1 million children under 5 years of age die from pneumococcal disease annually (1). Hib and Meningococcus are thought to account for approximately 400,000 and 50,000 deaths respectively each year (2, 3). The huge global burden of disease caused by these extracellular Gram-positive and Gram-negative bacteria comes despite the availability of highly effective vaccines (4). Host protection against PS-encapsulated extracellular bacteria is initially conferred by a rapid innate immune response leading to phagocytosis and killing of bacteria (5). Early TLR-mediated signaling via the recognition of conserved microbial structure results in immune cell activation that drives the development of subsequent adaptive immunity mediated by B and T cells (6). Protein and PS-specific antibodies are known to protect the host from infection against lethal extracellular bacterial strains (5).

1.1 Differential regulation of protein and polysaccharide antigens

Proteins and PS are biochemically distinct entities and are processed differently by the immune system, leading to different mechanistic pathways for eventual elicitation of specific Ig isotypes (7) . Proteins are internalized by antigen presenting cells (APC) and are enzymatically degraded into peptide fragments that associate with major histocompatibility complex class II (MHC-II) molecules on the cell surface for presentation to specific CD4+ T helper cells by T cell receptor (TCR) crosslinking. Upon antigen exposure and initial T cell stimulation, APCs upregulate cell

surface co-stimulatory molecules like CD80 (B7-1) and CD86 (B7-2) that are critical for the activation of CD4+ T cell effector function including B cell proliferation, immunoglobulin (Ig) class switching, and differentiation into Ig secreting plasma cell and memory cells (8, 9). Thus, protein antigens are classified as T cell-dependent (TD) antigens.

In general, PS (10, 11), are not degraded within the APC for MHC-II association and presentation to T cells (12, 13) and therefore are classified as T cell- independent (TI) antigens (14). However, recent findings indicate that zwitterionic PS like PSA1, the capsular PS of *Bacteroides fragilis* are immunomodulatory and can be presented by MHC-II to elicit CD4+ T cell responses (15). Purified PS induces a rapid IgM response (Fig. 1a), with only a low IgG response (mostly IgG3 in mice, IgG2 in humans) with no appreciable affinity maturation (16). PS also typically fail to induce a state of memory associated with either absent or abortive germinal center formation (17, 18). PS antigens are composed of repeating, identical subunit sugars that effect multivalent crosslinking of specific membrane (m)Ig on the surface of B cells, resulting in B cell proliferation (19), and in the presence of cytokines or additional stimuli like TLR ligands and cytokines, induce Ig secretion and class switching (20). PS antigens are present on the surface of Gram-positive (GP) and Gram-negative (GN) bacteria in the form of a capsule, outer membrane lipopolysaccharide, cell wall peptidoglycan and teichoic acid, or cell membrane lipoteichoic acid. The overall immunogenicity of the PS depends on the variations in sugar composition, ring forms, linkage positions, anomeric-center configurations, isomer forms and conformation (21).

1.2 Polysaccharide Vaccines

PS vaccines are made of purified capsular PS. These vaccines are designed based on the fact that the capsular PS-specific antibodies protect against the disease by inducing complement-mediated

bactericidal antibodies (22) and opsonophagocytosis (23). Several PS-based vaccines have been in clinical use for extracellular bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitidis*. Pneumovax 23 is a pneumococcal PS vaccine that is effective against 23 different pneumococcal capsular types and covers approximately 90% of invasive pneumococcal isolates (24). Menomune (MPSV4), a quadrivalent PS vaccine containing capsular PS from meningococcal serogroups A, C, Y and W-135 is effective in protecting against meningococcal infection (25). These PS vaccines are efficacious in immune-competent adults, but are not effective in children <2 years of age (26). PS antigens activate B lymphocytes independently of CD4+ T cells by directly binding and crosslinking antigen receptors on the B cell surface and co-crosslinking CD21 (type 2 complement receptor) for costimulation. Neonatal B lymphocytes express low levels of CD21 and this could be one reason for the hyporesponsiveness of PS vaccines during infancy (27). BAFF (B cell activating factor) and APRIL (A Proliferation inducing ligand) have also been implicated in promoting PS-specific antibody responses and is dependent on the TACI (Transmembrane receptor protein) receptor to induce B cells to become Ig secreting plasma cells (28) This explains another possibility why newborns, who have low TACI expression, fail to respond to PS vaccines (29). Finally, neonatal B cells are poorly activated in response to membrane Ig crosslinking, which may further contribute to defective PS-specific antibody responses (30). Though PS vaccines induce protective immunity in adults, they do not produce immunological memory and are limited in their isotype switching. Even in healthy adults, the antibody levels start to decline after one year postvaccination (31). To overcome the problems associated with the TI nature of PS antigens, vaccine strategies against these organisms have focused on converting the TI immune response to that of a TD immune response.

1.3 Conjugate Vaccines

Avery and Goebel in the 1920s reported that the covalent linkage of PS to a protein carrier enhances the immunogenicity of the PS moiety (32). Conjugation of a protein carrier such as tetanus toxoid, diphtheria toxoid or crossreactive material 197 (CRM197) converts the PS into a TD antigen (4). The PS component of the conjugate interacts with the B cell receptor on B cells, which then internalize the conjugate, process the protein carrier by proteolytic degradation and present the peptide fragments in association with MHC-class II molecules to peptide-specific CD4+ T cells (33, 34) to induce Ig class switching and memory responses (35). Therefore, antibody production is achieved, and the consequent immunological memory results in antigen-specific immunity to both the PS and the protein (Fig.1b). This approach has been exploited to produce pathogen-specific vaccines that target bacterial PS, and which are effective in infants, in contrast to TI, purified PS vaccines (26, 36, 37).

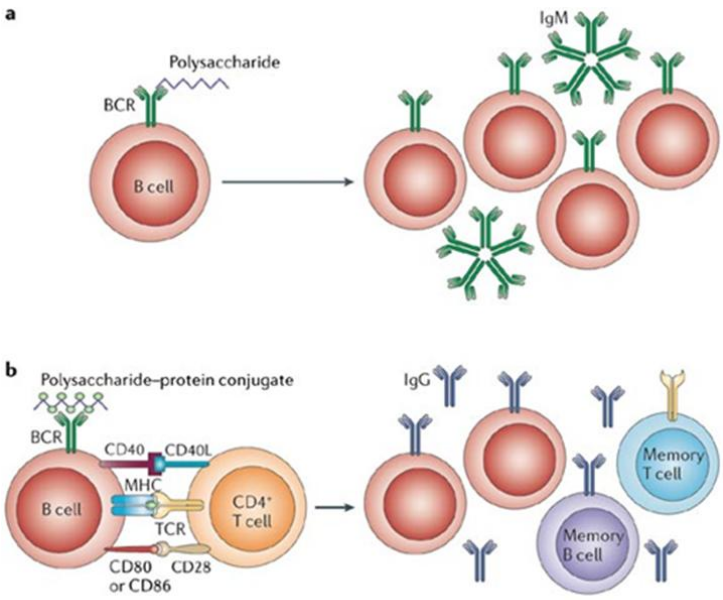


Fig.1 *Immune response to polysaccharide and protein-polysaccharide conjugate (21)*

The generation of the *Heamophilus influenzae* type B (Hib) conjugate vaccine has been one of the success stories in the history of biomedical sciences (38), which further encouraged the development of conjugate vaccines against pneumococcal and meningococcal infection. The 7-valent vaccine called Prevnar-7 consists of pneumococcal polysaccharide serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) conjugated to CRM_{197} and is known to confer protection against Pn infections in young children. Similarly, the meningococcal conjugate vaccine (MCV-4) containing the serotypes A/C/Y/W-135 conjugated to diphtheria toxoid is effective against meningococcal disease in children and adults. Such vaccines not only decrease the incidence of the disease but also confer herd immunity. However, the glycoconjugate is not without its own limitations. The immunogenicity of different conjugate vaccines varies as a result of differences in the chemical nature of the PS, the amount of unconjugated PS in the vaccine and the nature of the carrier protein (4). They are expensive to manufacture and are not feasible to use in developing countries. Hence, additional vaccination strategies are desired, necessitating the understanding of the mechanisms underlying anti-PS responses to intact bacteria as in the natural infection.

1.4 Intact bacteria, a more physiological model

Most studies aimed at the understanding of the parameters underlying PS-specific Ig responses have used soluble PS antigens (Ag). Very few studies are reported to use intact bacteria to study PS-specific responses (39). However, in any natural infection, the host does not encounter the PS Ag in isolation, but in the context of the intact bacterial organism. Such intact bacteria contain proteins and PS Ags that are intimately co-expressed within an organized particulate structure containing multiple adjuvanting TLR (Toll-like receptor) ligands, scavenger receptor ligands and NLR (NOD-like receptor) ligands (7). Thus, PS antigens containing

complex mixtures of proteins, lipids as well as PS are presented to the immune system within a particulate structure and may alter the PS-specific Ig responses in various ways, relative to that observed for purified PS. Our main hypothesis is that the bacterial subcapsular domain influences the nature of the PS-specific antibody response. Therefore, the use of intact bacteria to study the PS-specific responses represents a more physiological model and mimics the natural infection.

1.5 Structure of Gram-positive (GP) and Gram-negative (GN) bacteria

Based on the Gram stain, bacteria are broadly classified into GP and GN bacteria. The GP bacteria have a thick cell wall (15-80nm) composed of an outer layer of a repeating matrix of peptidoglycans as shown in Fig. 2a. Peptidoglycan is a thick rigid layer composed of an overlapping lattice of two sugars, N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) that are cross-linked by amino acid bridges into a dense three-dimensional network. In GP cells, the peptidoglycan is a heavily cross-linked woven structure that encircles the cell in many layers. The thick cell wall of GP bacteria allows them to do better in dry conditions because it reduces water loss. Bacterial surface proteins and other molecules such as teichoic acids are embedded within this peptidoglycan layer and lipoteichoic acids link the peptidoglycan layer to the bacterial cell membrane itself. The capsular PS in GP bacteria are covalently linked to the thick, underlying cell wall peptidoglycan to which a number of proteins are also covalently attached (40, 41).

The envelope of GN bacteria is composed of two distinct lipid membranes: an inner membrane (IM) and outer membrane (OM) as shown in Fig 2b (42). The IM is the bilayer composed of phospholipids and proteins. Between the IM and OM is the periplasm occupied by soluble proteins and the peptidoglycan layer. In GN bacteria the peptidoglycan is much thinner

with only 15-20% of the cell wall being peptidoglycan and it is only intermittently cross-linked. There are also proteins associated with the petidoglycan layer; for example, Lpp (Braun's lipoprotein) that covalently anchors the peptidoglycan layer to the OM (43). The OM is a unique asymmetrical lipid bilayer consisting of an inner face of phospholipids and an outer face of lipopolysaccharides (LPS) that can be toxic to animals. LPS is composed of lipid A, a core oligosaccharide and O-antigen PS of various lengths that extend out in to the environment (44, 45). Many bacterial pathogens vary the make-up of the O-antigen in an effort to avoid recognition by the host's immune system. Some GN species live in the gut of mammals and LPS repels fat-solubilizing molecules such as bile that the gall bladder secretes, thus enabling the bacteria to survive in such an environment. In addition to phospholipids and LPS, the OM also contains a unique set of transmembrane proteins such as porins that adopt a β-barrel architecture, which allows them to serve as channels for the passage of small hydrophilic molecules (46). Capsular PS expressed by GN bacteria is covalently attached to the acyl glycerol moiety of the outer membrane, which contains highly immunogenic proteins including porins (47). Shedding of the outer membrane/capsular PS complex to form vesicles is a unique property of GN bacteria that may have distinct immunologic consequences for the anti-PS response (48).

a) **b)**

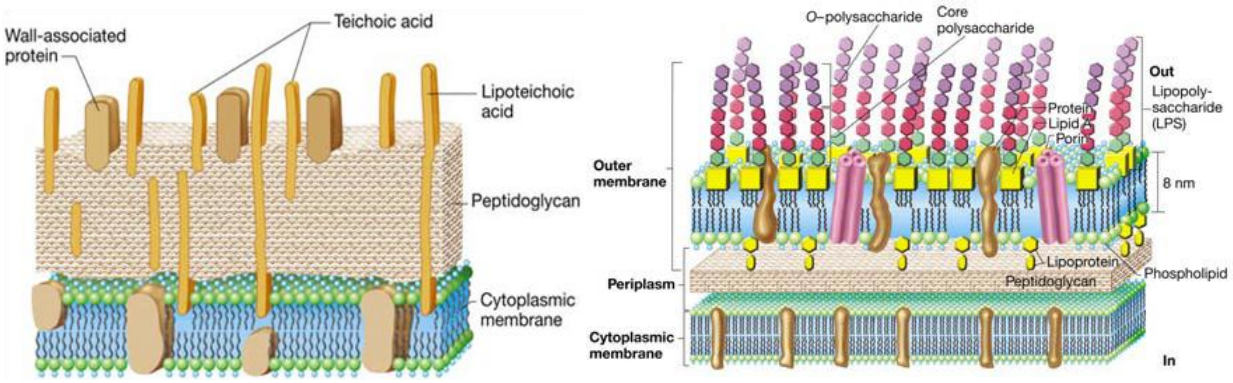


Fig.2 Structure of Gram positive (a) and Gram negative (b) bacterial outer surface (*Image courtesy*: "Difference between gram positive and gram negative bacteria", http://hawashpharma.blogspot.com/2011/07/difference-between-gram-positive-and.html)

1.5.1 Streptococcus pneumoniae

Streptococcus pneumoniae (Pn), a GP bacterium resides on the mucosal surface of the upper respiratory tract. The common manifestations of pneumococcal disease include acute otitis media, which involves infection of the middle-ear space and pneumonia, which affects the terminal airways. Bacteremia, as a complication of pneumonia, is associated with greater morbidity and mortality in adults and children (49). Capsular PS is 200-400 nm thick and forms the outermost layer of the bacterial cell wall that protects Pn by inhibiting phagocytosis (50). The virulence of a given capsular Pn type of which 90 have been defined to date, is determined both by the chemical composition of the capsular polymer and the amount synthesized (51). PS-specific antibodies confer protection against the bacterial infection by interacting with complement and Fc receptors on phagocytic cells and therefore promoting bacterial phagocytosis (52). Pneumoccoccal PS is known to bind to SIGN-R1, a C-type lectin expressed on the macrophages in the marginal zone (MZ) of the spleen to mediate the uptake of the bacteria (53). Pn are unique among GP bacteria because LTA and TA have identical chains which are substituted with phosphorylcholine (PC) residues (54). PC specifically binds to platelet activating factor receptor (PAFR) and facilitates adherence and invasion into endothelial and epithelial cells by virulent Pn (55). The other virulence factors of Pn are cell-surface proteins viz choline binding proteins (which includes PspA, PspC and LytA), lipoproteins and proteins that are covalently linked to the bacterial cell wall by a carboxy C-terminal sortase. Pneumolysin, a member of the family of cholesterol-dependent cytolysins is known for its TLR4-dependent activity and ability to directly mediate host cell lysis (56).

1.5.2 Streptococcus agalactiae

Streptococcus agalactiae are β-hemolytic GP bacteria with Lancefield's group B antigen. They are an important cause of serious neonatal infection characterized by sepsis and meningitis. Colonization of the maternal genital tract is associated with colonization of infants and risk of neonatal disease. Group B *Streptococcus* (GBS) infections of adults include bacteremia, endocarditis, skin and soft tissue infection, and osteomyelitis (57). Infants suffering from GBS sepsis exhibit reduced serum concentrations of antibody specific for GBS capsular PS. The adaptive immune system of infants is significantly impaired due to both decreased synthesis of IgG and constraints in the V_H gene repertoire (58). GBS expresses two distinct carbohydrate entities, i.e., type-specific and group-specific PS. Nine antigenically distinct capsular types of GBS are associated with human infection. Furthermore, type specific PS contributes to immune evasion by host structure mimicry via the sialic acid residue (59). In GBS, similar to other GP bacteria, cell wall proteins are linked to peptidoglycan by carboxy C-terminal sortases (60). Best studied classes of proteins in GBS are the C proteins alpha C, beta C and Rib. GBS can induce significant amounts of proinflammatory cytokines from macrophages including TNF-α (61).

1.5.3 Neisseria meningitidis

Neisseria meningitidis is a human-specific GN organism, recognized as the leading cause of bacterial meningitis globally (62). Acquisition of *N. meningitidis* in the upper respiratory tract may be asymptomatic or may infrequently result in local inflammation, invasion of mucosal surfaces, access to the blood stream and fulminant sepsis or focal infections such as meningitis (63). Capsule composed of sialic acid derivatives (except serogroup A) provides the organism with antiphagocytic properties, enhancing its survival in the bloodstream, or in the central nervous system. Thirteen structurally different capsular serogroups have been described, five of

which cause the majority of invasive disease (A, B, C, Y and W-135). Other major contributors of invasive meningococcal disease are lipooligosaccharide (LOS), pili and outer membrane proteins (OMP). Meningococcal endotoxin (LPS) plays a major role in eliciting an inflammatory response by inducing innate immunity via the TLR4 receptor (64). Pili and other outer membrane proteins (e.g., PorA, PorB, Opa, Opc) facilitate the adherence of the Meningococcus to endothelial surfaces (65). Porins can also activate innate immunity via TLR2 (66).

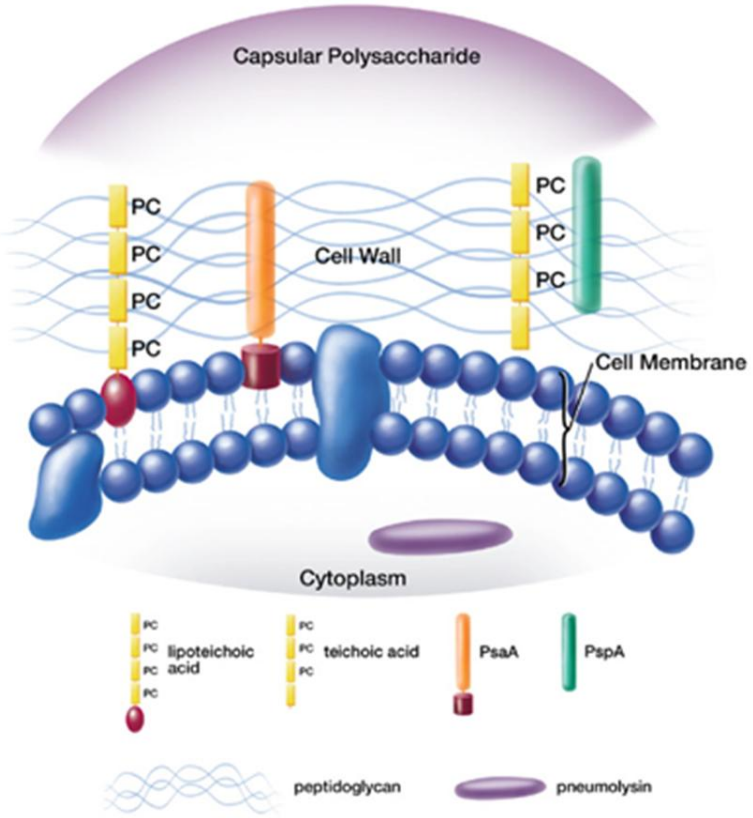


Fig 3. Structure of cell envelope of *Streptococcus pneumoniae*

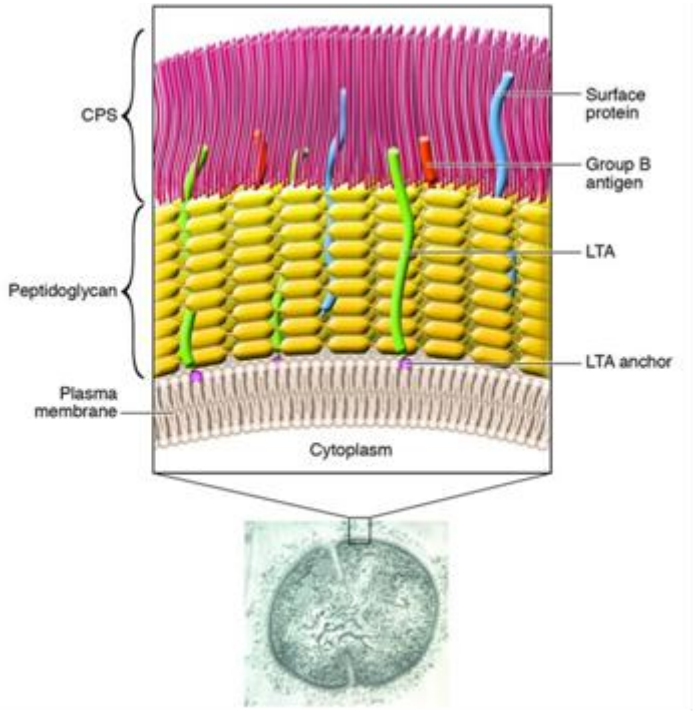


Fig 4. Structure of cell envelope of Group B *Streptococcus* (67)

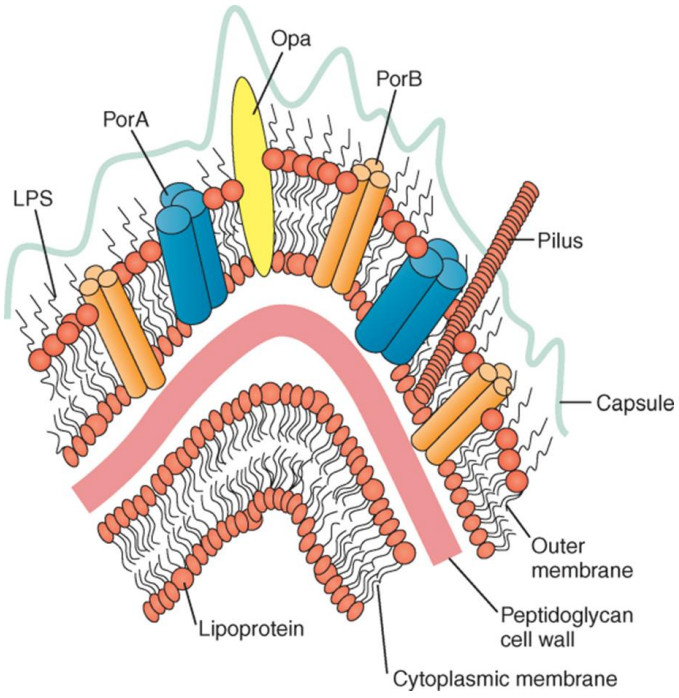


Fig 5. Structure of cell envelope of *Neisseria meningitidis (58)*

1.6 Structure of spleen

The spleen is the chief site for the induction of antibody responses to blood-borne antigens. It is composed of the red pulp and the white pulp. The red pulp consists of sinusoids through which the blood flows, and where senescent red blood cells, as well as blood-borne pathogens are removed by resident red pulp macrophages. The white pulp consists of the sheath of lymphocytes around an arteriole called the periarteriolar lymphoid sheath (PALS) made up of T cells and lymphoid follicles occurring at intervals along PALS that contain mainly B cells known as follicular B cells (FB). The outer limit of white pulp is separated from the red pulp by the marginal zone (MZ), that are rich in macrophages, dendritic cells (DC) and a resident, non-circulating population of B cells known as marginal zone B cells (MZB) (68).

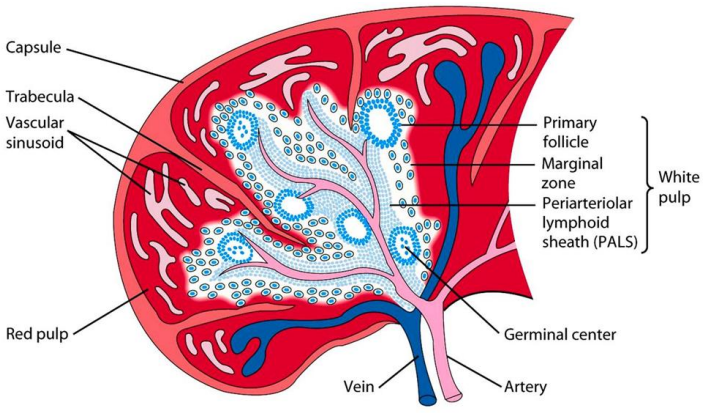


Fig 6. Structure of Spleen (*Image courtesy*: Dr. Jim Faix. http://www2.nau.edu/~fpm/immunology/spleen.html)

1.7 B cell subsets involved in immune responses

Naïve B cells are generally divided into three subsets: B-1 B cells, follicular B cells and marginal zone B cells. The location of MZB makes them easily accessible to sample and mount immune responses to blood borne pathogens and thereby along with DC might participate in the early stages of T cell activation (69). Splenic MZB cells can bind to immune complexes that

contain Ag through complement receptors CR1 and CR2 independent of BCR specificity and shuttle antigen into the follicle in a CXCR5-dependent manner, where the follicular dendritic cells (FDC) compete for binding to Ag that is presented by MZB cells (70). MZB cells then migrate back to the MZ, a process that is controlled mainly by $S1P_1$ and $S1P_3$ (71). LPS exposure and BCR engagement can also promote the redistribution of MZB cells from the MZ into the white pulp (72). The MZB differentiates rapidly to form plasma cells, making it the early antibody-secreting cells for both TI and TD Ags. Although MZB cells upregulate MHC-II, B7-1 and B7-2 to present antigen and activate T cells more efficiently than the FB cells (73), they appear to favor plasma cell differentiation over memory B cell generation (74). MZB cells contribute to TD responses to protein antigens by helping in the presentation of these antigens to FB cells. FB cells activated by TD Ags via the BCR in the context of CD40 and TLR signaling, differentiate into short-lived Ab-secreting cells in the extrafollicular region of the spleen, or enter into the germinal center for antigen-specific memory B cell generation and long-term Ab-secreting cells that migrate to and persist in the bone marrow for many years (75). B cell clones that are enriched for BCR specificity for TI antigens are concentrated in the MZ as well as in the B-1 compartment (69). B-1 B cells are present in low numbers in the spleen and lymph nodes (73) and are involved in the generation of IgM responses to TI antigens such as PC, an antigen commonly present on many pathogenic bacteria such as Pn, *Pseudomonas aeruginosa*, *N. meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenzae* on their LPS or pili (76-78). Cytokines such as IL-5 and IL-10 contribute to the activation and differentiation of B-1 B cells (75). B-1 cells can be induced to produce TI IgA responses at mucosal sites (79). B-1 B cells are further subdivided into B-1a and B-1b B cells. B-1a cells contribute to the production of natural antibody, whereas B-1b cells are involved in adaptive immune responses to PS antigens (80, 81).

1.8 CD4+ T cell activation

T cell activation is regulated by various receptor/ligand pairs that act in synergy with the T cell receptor (TCR) to enhance or inhibit immunity (82). TCR specifically recognizes the antigenic peptide-MHC-II complexes on the surface of APC. However, this signaling by itself is not sufficient to activate a naïve T cell. Costimulatory molecules present on APC interact with the costimulatory receptors on naïve T cell for their activation. CD28 costimulatory receptor on T cell is one of the best studied costimulatory receptors. The CD28 immunoglobulin superfamily includes coinhibitory molecules like PD-1 and CTLA-4 as well as costimulatory CD28 and inducible costimulator (ICOS) (83). CD28 is constitutively expressed on all naïve T cells and binds the costimulatory ligands B7.1 (CD80) and B7.2 (CD86) which are expressed mainly on APC such as DC (84, 85), whereas ICOS is induced on CD4+ T cells upon TCR crosslinking and CD28-mediated signaling. The respective cognate ligands ICOSL and B7.1/B7.2 are constitutively expressed on APC, but can be upregulated by inflammatory stimuli. Thus, CD28 is critical for the initiation of CD4+ T cell activation (86, 87), whereas ICOS plays a key role in the subsequent T cell effector response (88, 89). Genetic blockade of B7/CD28 and ICOS/ICOSL pathway inhibits both type 1 and type 2 CD4+ T cell-dependent humoral immune responses (90-92). ICOS is mainly expressed on the T cell subset (T_{FH}) that is present within the B cell follicles and plays a critical role in germinal center formation and production of isotype switched antibody responses following immunization (93, 94). ICOS signaling induces both higher IL-21 production and sustained CD40L expression on CD4+ T cells (83, 95). CD40L on CD4+ T cells interact with CD40 receptor present on B cells to further increase B cell maturation and survival (96). Thus, ICOS plays a central role in regulating immunologic memory.

1.9 Germinal center reaction

The key outcomes of humoral immune responses are the production of high affinity isotype switched Ab that is essential for the clearance of many infectious pathogens and the formation of memory B cells. Canonical memory B cells require direct contact between activated B cells and CD4+ T cells in a specialized structure called the germinal center (GC) (97). The recognition of peptide MHC-II complexes on activated APC is critical for effective CD4+ T cell selection, clonal expansion and development of effector T cell function. Localization to the B and T cell zones are dependent on the chemokine receptors CXCR5 and CCR7, respectively. Activated B cells increase their CCR7 expression and migrate to the T-B zone border where they encounter cognate T cells. The cognate interaction of T and B cells in the outer follicle results in further differentiation of B cells into extrafollicular plasmablasts to produce low affinity antibodies, short-lived early memory B cells or a return to the follicle to undergo rapid proliferation to form a GC, with subsequent generation of long-lived memory B cells and bone marrow plasma cells (98).

The GC reaction is the cascade of cellular and molecular events that regulates the Ag-specific evolution of immunologic events during the development of B cell memory and generation of long-lived bone marrow plasma cells. Mature GC is divided into dark and light zones. The dark zone contains dividing GC B cells known as centroblasts that undergo somatic hypermutation to enable clonal selection based on their antigen affinity. The light zone consists of a rich network of follicular dendritic cells (FDC) that bind the antigen in the form of immune complexes (9). The initial interaction of CD4+ T cells in the T cell zone with DC provides the stimulus for the upregulation of CXCR5, ICOS, PD-1, GL-7 and Bcl-6 and the entry of T cells into the follicle to become T_{FH} (T-follicular helper) cells (99-101). Centroblasts in the dark zone

differentiate into non-dividing centrocytes and then move into the light zone where they are selected according to their ability to bind Ag complexes on FDCs and to recruit T_{FH} cell help as shown in Fig 7. The centrocytes that have low affinity for the antigen are negatively selected by apoptosis. TCR crosslinking by T_{FH} with peptide MHC-class II complexes on the GC B cell is critical for the cognate interaction of T-B cells in the GC. Other molecular pairings that are critical for the T-B cell interaction are CD28-B7 family members, CD40 and its ligand CD40L, ICOS and its ligand ICOSL, PD-1 and its ligand PDL-1 and IL-21 and IL-21R (102) as shown in Fig 8. These interactions result in T cells secreting cytokines IL-4 and IL-21 that induce B cells to produce affinity-matured memory B cells and long lived plasma cells (103).

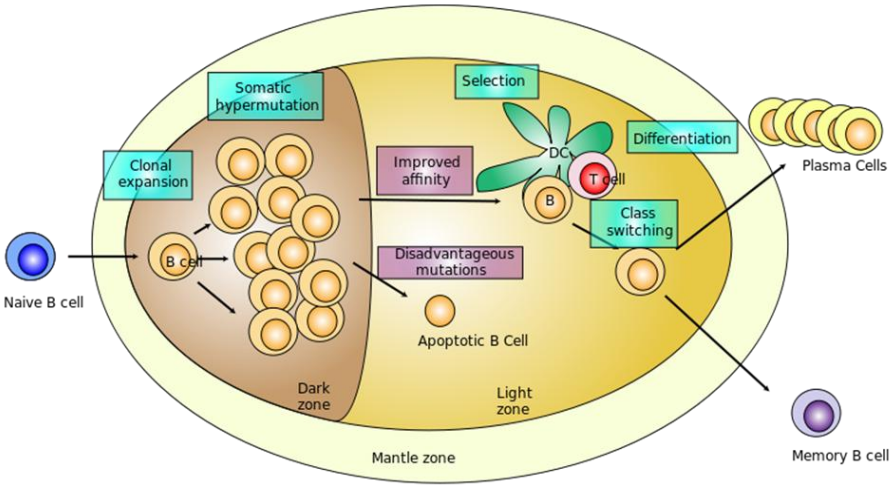


Fig 7. Germinal center showing proliferation and developmental stages of B cell (*Adapted from*: http://en.wikipedia.org/wiki/Germinal_center).

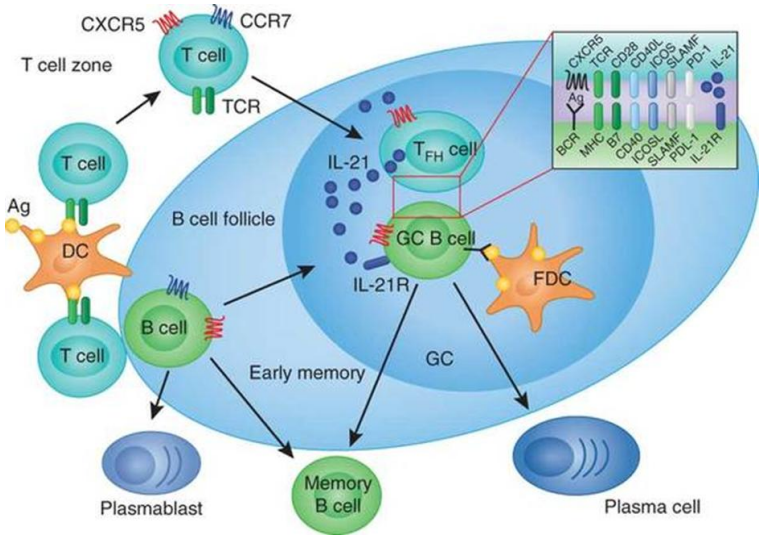


Fig 8. Signals controlling GC output (72).

1.10 Humoral immune responses to *Streptococcus pneumoniae*

Murine in vivo studies using intact heat-killed *Streptococcus pneumoniae* (Pn), capsular type 14 (Pn14) demonstrated that the pneumococcal polysaccharide type 14 (PPS14)-specific IgG responses were dependent on CD4+ T cells, CD28 and CD40L costimulation similar to the PS-specific IgG response to the conjugate vaccine, whereas the IgM PPS14-specific response to Pn14 was T cell-independent (39, 104). The PS-specific IgG response to intact Pn14 is comprised of all four IgG isotypes, while purified PS induces mainly IgG3. In contrast to the protein-specific IgG response to intact Pn14, the PS-specific IgG response peaked rapidly with a shorter period of T cell help (105), and was ICOS-independent (106) and failed to induce a boosted IgG response following the secondary immunization. In light of the critical role of ICOS in the generation of the GC reaction and memory (88, 89), the ICOS-independent nature of the PS-specific responses to intact Pn suggested an extrafollicular plasma cell response, unlike the conjugate vaccines that induces an ICOS-dependent PPS14-specific IgG response (107). The constitutive expression of Bcl-2 and Bcl-X_L, two anti-apoptotic proteins, selectively in B cells enhanced the PS-specific IgG response relative to the protein-specific IgG response to intact Pn with no effect on the generation of memory, suggesting an apoptosis-prone extrafollicular pathway for the PS-specific responses to intact Pn (108). Studies using Lsc-/- mice that exhibit a marked defect in MZB migration from MZ following immunization provide strong evidence that the PS-specific IgG response to intact Pn14 versus conjugate vaccine derive from MZB and FB cells, respectively (108). However, when the conjugate was adsorbed on the surface of bacteria, or attached covalently/noncovalently to 1 μm latex beads, it induced a complete or partial switch, respectively from FB to MZB usage for the PS-specific IgG response, associated with the generation of highly boosted PS-specific IgG titers following secondary immunization (109).

This highlights that the physiochemical context of the capsular PS within an intact bacterium can determine the nature of T cell-dependent PS-specific IgG responses in vivo. Coimmunization of mice with Pn and soluble PS-protein conjugate resulted in marked inhibition of conjugate induced PS-specific IgG memory, and primary and secondary protein-specific IgG responses, suggesting an inhibitory effect of Pn on the coimmunized antigen (110). The expression of PC by Pn may be critical for this inhibitory effect (111).

RESEARCH GAPS

Most studies on the regulation of anti-PS Ig responses utilize purified PS antigens, although very few studies are reported using intact bacteria that represent a more physiologic model system. Little is known regarding the regulation of PS-specific Ig responses to intact GP and GN bacteria. The distinct structure of GP and GN bacteria, in addition to the presence or absence of different TLR adjuvants may significantly impact on how anti-PS responses are elicited. The nature of the association of capsular PS with GP and GN bacteria is distinct and could impart different immunologic properties to PS antigens expressed by these two classes of pathogen. The information obtained will provide fundamental new insights into the manner by which these PS antigens elicit humoral immunity to an intact pathogen. These data will help to guide the rational development of new antibody-based anti-bacterial vaccines.

Previous studies using intact *Streptococcus pneumoniae* type 14 (Pn14) demonstrate that the PS-specific IgG responses are dependent on CD4+ T cells unlike the soluble isolated PS Ag (39). The PPS14-specific IgG responses to intact Pn require CD40/CD40L interaction and CD28 costimulation and comprise all four IgG isotypes (104) in contrast to the isolated PS Ag, which mainly elicits IgG1 and IgG3. However, in contrast to the TD conjugate vaccine, intact Pn14 exhibited an accelerated primary kinetics that are short-lived and upon secondary immunization failed to induce a boosted secondary response mediated by an ICOS-independent (106), apoptosis-prone extrafollicular pathway (108, 112). Thus, intact Pn14 combines the characteristics of a TD conjugate vaccine and isolated TI PS antigen. This emphasizes the fact that the PS in the context of the bacteria converts the TI characteristics of the humoral responses to TD. However, it is still unknown if the nature of PPS14-specific IgG responses to intact Pn14

is a general feature of all PS-specific IgG responses to intact bacteria, reflects the structural and functional attributes of the PS itself irrespective of the bacteria that expresses it, represents the structural duality of the underlying subcapsular domain of the GP and GN bacteria, or perhaps is unique for each bacterium.

MODELS USED FOR THE EXPERIMENTS

Mouse models have proven invaluable for the understanding of the parameters that govern an immune response to variety of pathogens. Due to defective BCR signaling, low surface expression of CD21, and poor responsiveness to BAFF and APRIL, in the neonatal B cell population, young mice do not respond well to PS Ag (113). Therefore we used adult mice 6-8 weeks of age to perform our studies to determine the PS-specific responses to intact bacteria. We used the intra-peritoneal (i.p.) route of immunization for most of our studies, as this route of immunization mainly triggers a systemic response that is relevant to sepsis and bacteremia, rather than a localized mucosal response.

Our interest is primarily focused on the intact bacteria as an immunogenic particle rather than as an infectious agent. Therefore, our lab generally uses intact heat-killed GP and GN bacteria to study the regulation of PS-specific responses in mice, precluding potential effects of bacterial physiology on the immune response. In some experiments, UV-inactivation of bacteria was also employed. Our lab has extensively used the GP Pn strain R614 expressing type 14 PS to study the PPS14-specific Ig response and the unencapsulated Pn strain R36A. We wished to determine the potential contribution of the bacterial subcapsular domain in the regulation of PS-specific antibody responses. Therefore, we utilized GN bacteria *Neisseria meningitidis* expressing type A (MenA) and type C PS (MenC). The encapsulated MenC strains M1883 (ATCC 53414), FAM18 C+ and unencapsulated FAM18 C- were used. To determine, if all GP bacteria behave similarly, we also made use of *Streptococcus agalactiae* type III (GBS-III) strain M781. We took advantage of the mutant strains of GBS-III expressing the desialylated IIIPS that is similar to PPS14 of Pn. Three different COH strains of GBS-III used are COH1 expressing

wild type IIIPS, COH1-11 expressing desialylated IIIPS and COH1-13 that lacks a capsule. We typically used 2 x 10^8 CFU/200µl of R614 and MenC and 1 x 10^9 CFU/200µl of GBS-III, unless otherwise specified, as these doses gave optimal Ig responses.

We additionally raised the question whether bacteria coexpressing proteins and PS antigens in a particulate structure along with multiple adjuvanting moieties behaved similar to that of a conjugate vaccine. Therefore, we compared the intact bacteria with the conjugate to study the PS-specific Ig responses in vivo. The conjugates TT-MenC, TT-MenA and IIIPS-rAlp3 were adsorbed on alum and CpG-containing oligodeoxynucleotide (ODN) as an adjuvant. The usual protocol is to immunize mice i.p. with either the bacteria in saline or the conjugate in alum + CpG and boost mice on D14 or later (D21 for MenC). Sera were collected every week post-immunization for determination of antigen-specific antibody titers by ELISA.

HYPOTHESES AND SPECIFIC AIMS

Previous studies in our lab have established the nature of PPS14-specific Ig responses to intact Pn14 in detail. However, the regulation of PS-specific Ig responses to different GP and GN bacteria, and the contribution of distinct bacterial subcapsular domains to the PS-specific responses are unknown. The goal of these studies was to acquire a better understanding of the regulation of in vivo PS-specific Ig responses to intact GP and GN bacteria and determine how the distinct bacterial structure contributes differently to PS-specific Ig responses. Determining the mechanisms underlying the distinct humoral responses to intact GP versus GN bacteria will provide insight into broader structure-function relationships for these two classes of bacteria.

Hypothesis 1: Nature of in vivo PS-specific Ig responses to intact bacteria is determined by the structural organization and composition of the underlying bacterial subcapsular domain.

Previous studies using intact Pn14 have demonstrated that the primary PPS14-specific IgG response peaked rapidly by D7 and are dependent on CD4+ T cells, CD40/CD40L costimulation and B7-dependent costimulation, similar to that observed with the conjugate vaccine. However, the PPS14-specific IgG response was ICOS-independent and failed to induce any boosting following the secondary immunization (39, 104, 106). Thus, the intact Pn combines the nature of a TD conjugate and TI isolated PS antigen in eliciting the PS-specific IgG response. This emphasizes the fact that the physiochemical context of the PS antigen plays an indispensible role in determining the nature of PS-specific IgG responses (109). Therefore, we hypothesized that the intact bacteria with distinct structural organization as in GP and GN bacteria would

contribute distinctly in triggering the immune response to the attached PS. The differential attachment of capsular PS to the underlying bacterial structure in GP and GN bacteria and the presence of different TLR ligands in GP and GN bacteria could influence the nature of the PS-specific IgG responses differently. We used intact heat-killed MenC, a GN bacterium to compare and contrast with isolated Meningococcal C PS (MCPS) and the conjugate vaccine in induction of PS-specific Ig responses. We also investigated the role of CD4+ T cells and CD40/CD40L interaction, B7- and ICOS-dependent costimulation in eliciting the PS-specific Ig responses. In addition, we wished to examine the role of TLR4 and TLR2 signaling in triggering the PS-specific responses to intact MenC.

Hypothesis 2:

Distinct GP bacteria with structurally similar PS elicit distinct PS-specific Ig responses in vivo.

Despite the similar structural organization shared by all GP bacteria, the nature of PS-specific Ig responses in vivo can potentially be altered based on the capacity of different bacteria to trigger the distinct innate immune signaling. Inactivated GBS is known to be a potent activator of macrophages and monocytes compared to inactivated Pn as evidenced by induction of higher amounts of TNF-α (57, 61). Therefore, we hypothesized that GBS would elicit boosted secondary PS-specific IgG response as opposed to that observed with Pn. We utilized the GBS strain, COH1-11 that expresses desialylated IIIPS, similar to PPS14 of Pn to study how two different bacteria with identical PS differentially alter the PS-specific Ig response. We also wished to determine, whether or not priming with one bacteria and boosting with the other bacteria, both expressing the same capsule, would elicit boosted PS-specific Ig responses. In

addition, we wished to examine if the coimmunization of Pn with GBS could enhance the innate immune signaling and help in inducing the secondary PS-specific Ig response.

CHAPTER 2

Regulation of In Vivo PS-specific Ig Responses to Intact Heat-Killed *Neisseria meningitidis*

2.1 Introduction

Polysaccharide (PS)-encapsulated extracellular bacteria like *Streptococcus pneumoniae* (Pn) [Gram-positive (GP)], and *Neisseria meningitidis* (Men) [Gram-negative (GN)] are major sources of global morbidity and mortality among infants, the elderly and the immunosuppressed (114). Adaptive immunity to extracellular bacteria is mediated largely by antibodies specific for both protein and PS Ags (115). Protein and PS Ags are biochemically distinct and are processed differently by cells of the immune system. Unlike proteins, non-zwitterionic PS fail to associate with MHC class-II molecules (13, 116) and are unable to recruit cognate $CD4^+$ T cell help for induction of anti-PS responses (14). However, PS in contrast to proteins can deliver strong and sustained signals to specific B cells through multivalent membrane Ig crosslinking via repeating, identical structural units (19), which critically impacts on the nature of the B cell response to various second signals (20). Thus, protein and PS Ags are classified as T cell-dependent (TD) and T cell-independent (TI) Ags, respectively.

This central dogma is derived mostly from studies using purified protein and PS (14). However, covalent linkage of protein and PS to create a soluble conjugate vaccine converts the PS into a TD Ag, including the ability to generate PS-specific memory (35). Intact bacteria are complex particulate immunogens in which multiple protein and PS Ags and bacterial adjuvants are co-expressed. This raises the question as to whether the PS expressed by intact bacteria also behave like TD Ags, similar to those in conjugate vaccines. It was previously demonstrated that the IgG anti-PS (PPS14) response to intact, heat-killed Pn, capsular type 14 (Pn14) a GP extracellular bacteria, is dependent on CD4+ T cells, B7-dependent costimulation and CD40/CD40L interactions and comprise all four isotypes of IgG (as opposed to predominantly

IgG3 and some IgG1 for isolated PS Ags) (106, 117), similar to that observed for the IgG anti-protein response. In contrast to the anti-protein response, the IgG anti-PPS14 response to intact Pn14 exhibits a rapid primary IgG response, dependent upon a shorter period of T cell help and B7-dependent costimulation, and fails to generate a boosted secondary response (7). Furthermore, the IgG anti-PPS14, in contrast to the IgG anti-protein response to Pn is ICOS-independent, extra-follicular (106) and more apoptosis prone (108). Thus, PPS14 in the context of intact Pn14 combines certain features of both an isolated PS Ag and a PS-protein conjugate vaccine (7).

Studies on the anti-PPS14 response to intact Pn14 indicate that the bacterium can markedly influence the immunobiology of the expressed PS Ag. These studies, however, left unresolved whether the nature of the PPS14-specific Ig response to intact Pn14 was characteristic of intact PS-expressing extracellular bacteria in general, or perhaps represented a characteristic feature of PPS14, the underlying structure and/or composition of intact Pn, or perhaps a more general dichotomy between GP and GN bacteria. Thus PPS14, among several other pathogen-derived substances, can bind to SIGN-R1, a scavenger receptor present on marginal zone macrophages (118). Capsular PS may additionally vary based on molecular weight (119), charge characteristics (11), sialic acid content (120), or unique immunomodulatory properties (121), which may influence the nature of the associated immune response. Further, bacteria may express components within the cell wall, such as PC, expressed by Pn as well as other pathogens, which may inhibit immunity (122).

In addition to the above considerations, the structure of intact GP and GN extracellular bacteria are significantly different, and these differences may influence the nature of the anti-PS response to the intact bacteria. Thus, capsular PS expressed by GP bacteria are covalently linked

to a thick, underlying cell wall peptidoglycan to which a number of proteins are also covalently attached (40, 41). Capsular PS expressed by GN bacteria, which express a thin peptidoglycan cell wall, is attached through a labile covalent linkage to the acyl glycerol moiety of the outer membrane. The outer membrane is known to have multiple immunomodulatory properties, in part due to the presence of porin proteins [TLR2 ligand] and LPS [TLR4 ligand] (66, 123). Notably, immunization of mice with either (i) crude outer membrane complexes from *Neisseria meningitidis* serogroup C [MenC] (containing both LPS and capsular PS [MCPS]), (ii) purified complexes (lacking LPS), and (iii) outer membrane complexes (lacking MCPS) all resulted in significant boosting of the IgG anti-MCPS response following secondary immunization (48). In addition, mice primed systemically with live MenC exhibited a highly boosted IgG anti-MCPS response following secondary infection (124). Collectively, these data suggest that the nature of the PS-specific Ig response to intact Pn and Men may differ.

In this study, we utilized intact, heat-killed MenC in order to determine how the biochemically complex bacterial particle influences the nature of the anti-MCPS response, relative to that elicited by isolated, soluble MCPS. In addition, these experiments were performed in a manner analogous to those previously conducted using intact heat-killed Pn14, in order to determine whether bacteria comprising different structures may influence the Ig anti-PS responses in distinct ways.

2.2 Materials and methods

Mice. CD28$^{-/-}$ mice (C57BL/6 background; B6.129S2-Cd28^{tm1Mak}/J, catalog no. 002666), ICOS$^{-/-}$ mice (C57BL/6 background; B6129P2-Icos^{tm1Mak}/J, catalog no. 004859), C3H/HeJ (catalog no. 000659) and C3H/HeOuJ mice (catalog no. 000635), TLR2$^{-/-}$ mice (C57BL/6 background; B6.129-Tlr2^{tm1Kir}/J, catalog no. 004650), and MyD88$^{-/-}$ mice (C57BL/6 background; B6.129P2(SJL)-Myd88$^{tm1.1Defr}$/J, catalog no. 009088) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6, BALB/c, and athymic nude (BALB/c background) mice were purchased from the National Cancer Institute (Frederick, MD). Female mice were used between 7 and 10 week 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 Care and Use Committee.

Bacterial strains. *Neisseria meningitidis* serogroup C (MenC) [strain M1883; ATCC 53414] and serogroups A (MenA) [strain M1027; ATCC 53417] was obtained from American Type Culture Collection [Manassas, VA]. The encapsulated MenC strain FAM18 C+ and its isogenic unencapsulated variant strain FAM18 C-was a kind gift from Dr. Mustafa Akkoyunlu (FDA, Bethesda, MD) (125). Lyophilized or frozen stocks of bacteria were grown overnight on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Brain Heart Infusion media (BD Biosciences, San Jose, CA) to mid-log phase, collected, and heat-killed by incubation at 65°C for 2 h or inactivated by overnight UV

irradiation. 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 -20°C until their use as immunogen for mouse immunizations. *Streptococcus pneumoniae*, capsular type 14 (strain R614) was grown similarly in Todd Hewitt broth media and heat-killed similarly by incubation at 65°C for 2 h. R614 bacteria were then aliquoted at 10^{10} CFU/ml and frozen at -20°C until their use as immunogen for mouse immunizations.

Reagents. A covalent conjugate of MCPS and tetanus toxoid (TT) [MCPS-TT] and MAPS (Meningococcal A PS) and TT (MAPS-TT) was prepared as previously described (117). Purified MCPS was obtained from the Serum Institute of India (Pune, India). Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from ATCC. Rat IgG2b anti-mouse CD4 mAb (clone GK1.5) was purified from ascites by ammonium sulfate precipitation and passage over a protein G column. Purified polyclonal rat IgG was purchased from Sigma (St. Louis, MO). Hamster IgG mouse anti-CD40L mAb (clone MR1), polyclonal hamster IgG, rat IgG2a anti-mouse CD275 mAb (HK5.3), and rat IgG2a isotype control (clone 2A3) were purchased from BioXcell (West Lebanon, NH). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Denmark). A stimulatory 30 mer CpG-ODN) was synthesized as previously described (126).

Purification of native PorB protein from *Neisseria meningitidis*. *N. meningitidis* serogroup B [strain H44/76] (127) was grown on GC-agar plates containing 1% (v/v) Isovitalex overnight at 37°C in a humidified incubator with 5% (v/v) CO_2. The next day, colonies were inoculated in liquid GC medium and grown to exponential phase. After overnight expansion of the cultures, the bacterial suspension was centrifuged at 3900 x g for 15 min at 4ºC and the total

protein content was extracted with the $CaCl_2$-Zwittergent method. PorB is purified using an AktaPrime protein chromatography machine as previously described (128). Briefly, the protein suspension was first subject to ion-exchange chromatography on two DEAE/carboxymethyl columns in tandem, followed by a gel filtration chromatography on a Sephacryl S-300 column and finally a Matrex Cellufine Sulfate column for removal of endotoxin traces.

Preparation of MenC whole protein extract. The MenC whole protein extract was prepared from the unencapsulated FAM18 C- bacteria using the B-PER bacterial protein extraction reagent from Pierce (Rockford, IL), as per the manufacturer's protocol. Bacteria were centrifuged at 3000 rpm for 20 min and the supernatant was discarded. The B-PER bacterial protein extraction reagent was added to the bacterial pellet (1:10 ratio), mixed well by shaking and incubated at RT for 15 min and again centrifuged at 3000 rpm for 10 min. The supernatant containing the soluble proteins was obtained and the protein concentration was determined by the BCA assay (Pierce, Rockford, IL), as per the manufacturer's protocol.

Production and purification of recombinant PspA. Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* BJ3505 (129). The supernatant was then passaged through a Q FF column (Amersham Pharmacia, Piscataway, NJ) and eluted with 0.2 M NaCl solution containing 20 mM Tris pH 9.0. Eluted PspA was then added to a Phenyl HP column (Amersham Pharmacia) equilibrated with 40 mM phosphate buffer containing 1.3 M ammonium sulfate, pH 7.0. PspA was then eluted from this column using a 1.3 M to 0.4 M ammonium sulfate gradient. Pooled PspA was dialyzed against 20 mM sodium acetate, pH 4.7 solution, and loaded into an S15 column (Amersham Pharmacia). PspA was then eluted with a 0.3 M NaCl solution containing 20 mM sodium acetate. Eluted PspA was dialyzed

and concentrated and found to be >95% pure by densitometric analysis of Coomassie blue stained gels.

Immunization. Depending on the experiment, groups of 7 mice were immunized with 2 x 10^8 CFU heat-killed or UV-inactivated MenC in saline (i.p. or i.v.), 5 x 10^6 CFU live MenC (i.p.), 10 µg of purified MCPS in saline (i.p), or 1 µg of TT-MCPS adsorbed on 13 µg of alum mixed with 25 µg of CpG-ODN (i.p.). Serum samples were prepared, at different time points, from blood obtained through the tail vein.

ELISA. For measurement of serum titers of MCPS-specific Ig, Immulon 4 ELISA plates were pre-coated with poly-L-lysine [Sigma] (5 µg/ml, 100 µl/well) in PBS for 1 h at 37ºC. The plates were then washed 3x with PBS + 0.1% Tween 20 and then coated overnight at 4ºC with purified MCPS (10 µg/ml, 100 µl/well) in PBS. To measure the serum titers of PorB- or whole protein-specific Ig, the plates were directly coated with PorB or whole protein extract (2 µg/ml, 50 µl/well). Plates were then washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1.0% BSA for 1 h at 37°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS + 1.0% BSA were incubated overnight at 4°C and plates were then washed 3x with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, IgG3, IgG1, IgG2b, IgG2a, or IgG2c Abs (200 ng/ml) [Southern Biotech, Alabama, USA] in PBS + 1.0% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were washed 3x with PBS + 0.1% Tween 20. Substrate (p-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in 1M Tris + 0.3 mM $MgCl_2$ (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). ELISA plates were coated with 0.5µg/ml of Biotin-MAPS to measure the serum titers of MAPS-

specific IgG. Serum titers of PPS14- and PspA-specific Ig were measured as described previously (106).

Flow Cytometric Analysis. Mice were injected with 0.5mg of control rat IgG or GK1.5 mAb. After 24 hr, the spleens were harvested and RBC lysed using 1ml of Ack lysing buffer for 5 min at RT and stained for the mouse specific antibodies CD3-PE and CD4-FITC (BD Biosciences) for 30 min at 4 $^{\circ}$C. The cells were then washed twice using PBS for 10 min at 5000 rpm and then analyzed using a BD LSR-II flow cytometer.

Statistics. Serum Ig isotype titer was expressed as the geometric means ± SEM of the individual serum Ig isotype titer. Significance was determined by the Student *t* test. *p*-values of ≤ 0.05 were considered statistically significant. All experiments were performed at least two times.

2.3 Results

The primary and secondary IgG anti-MCPS responses to intact MenC are similar to that elicited by a meningococcal conjugate vaccine.

Previous studies demonstrate that the anti-PPS14 response to intact heat-killed Pn14, a GP bacterium, is distinct from that elicited by a soluble covalent conjugate of PPS14 and pneumococcal surface protein A (PPS14-PspA), in that the former exhibited rapid primary kinetics (peak day 6) with no secondary boosting, despite the CD4+ T cell-dependence of the IgG anti-PPS14 responses to both Pn14 and PPS14-PspA (7). Based on the distinct subcapsular composition and/or structure of GP and GN bacteria, we hypothesized that the PPS14-specific Ig response to Pn14 might be immunologically different from a PS-specific Ig response induced by a GN bacterium. In this regard, we chose to study the nature of the anti-MCPS response to intact MenC, a GN bacterium, in comparison to that elicited by a meningococcal conjugate vaccine. We immunized BALB/c mice i.p. with either UV-inactivated MenC [strain M1883] (2 x 10^8 CFU/mouse) in saline or MCPS-TT conjugate (1 µg/mouse) in alum + CpG-ODN, and boosted the mice 28 days later in a similar fashion. As illustrated in Fig. 9A, there was minimal induction of serum IgM anti-MCPS in the primary response to M1883, with a ~4-fold significant boost in serum titers following secondary immunization. In contrast, MCPS-TT elicited a significant, though transient primary induction of IgM anti-MCPS, and no boosting following secondary immunization. The primary IgG anti-MCPS responses to both M1883 and MCPS-TT were relatively modest and peaked on day 21 (M1883) or day 14 (MCPS-TT) (Fig. 9A). In contrast to IgM, the secondary IgG anti-MCPS responses to both M1883 and MCPS-TT were highly and

rapidly boosted. The two MCPS-specific IgG responses were similar in kinetics and boosting to the associated anti-protein responses (PorB for M1883, TT for MCPS-TT).

The specificity of the MCPS-specific ELISA was established using a different strain of encapsulated MenC (FAM18 C+) versus its unencapsulated isogenic mutant (FAM18 C-) (125) (Fig. 9B). Thus, FAM18 C- failed to elicit a detectable IgG anti-MCPS response, in contrast to FAM18 C+, the latter exhibiting detectable primary and highly boosted secondary IgG anti-MCPS responses, whereas both strains induced comparable IgG anti-PorB responses. Similar primary and secondary IgG anti-MCPS responses were observed in response to 5 x 10^6 CFU/mouse of live M1883 (Fig. 10B) or 2 x 10^8 CFU/mouse of heat-killed M1883 (injected i.v.) [Fig. 10A]. Immunization via i.p. route with 1 x 10^9 CFU of heat-killed M1883 gave similar results (Fig. 10B). Collectively, these data demonstrate that the kinetics and boosting characteristics of the IgG anti-MCPS response to intact MenC is similar to that of a corresponding conjugate vaccine, but distinct from the previously reported IgG anti-PPS14 response intact Pn14. In addition, the weak IgM anti-MCPS response to M1883 contrasts with the previously reported high-titer IgM anti-PPS14 response to Pn14.

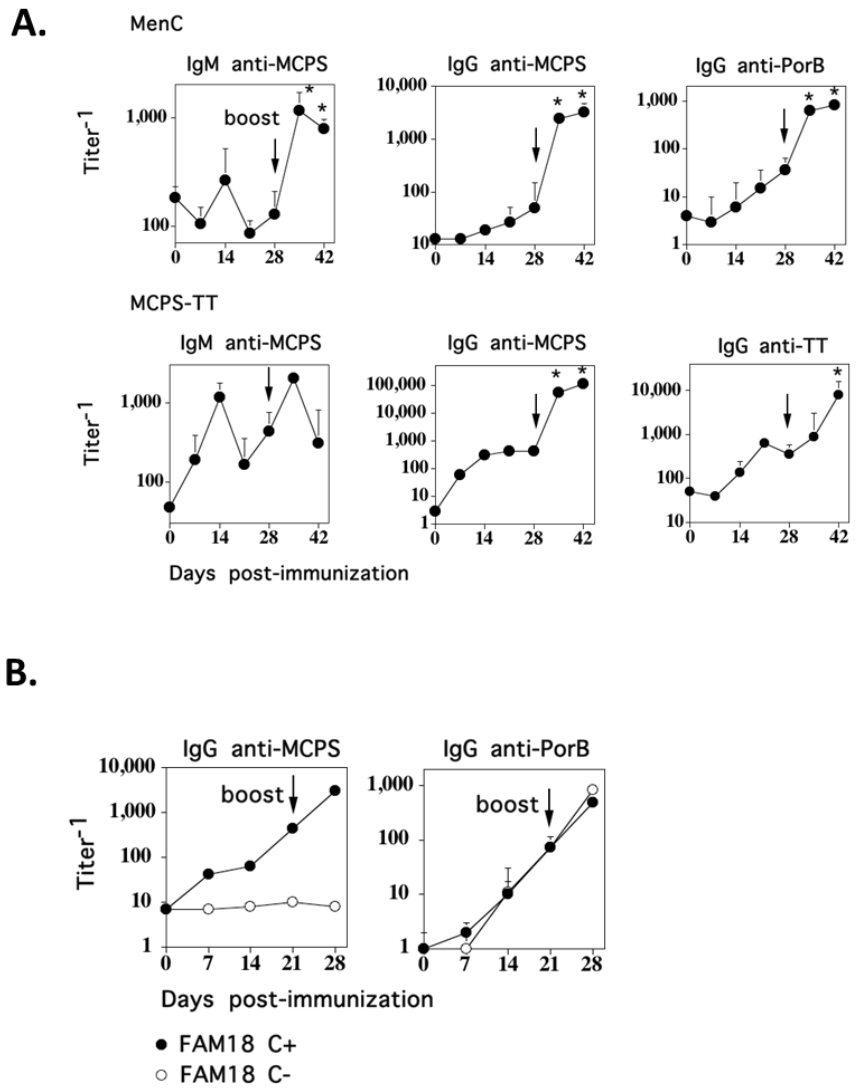


Fig 9. The primary and secondary IgG anti-MCPS responses to intact MenC are similar to that elicited by a meningococcal conjugate vaccine. (A). BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of U.V.-inactivated intact MenC (strain M1883) in saline or 1μg of MCPS-TT in alum + CpG-ODN, and boosted i.p. with the same dose on day 28. Serum titers of Ag-specific IgM and IgG were measured by ELISA. Significance (*) $p \leq 0.05$ between secondary titers relative to peak primary titers (**B)**. BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact FAM18 C+ (encapsulated MenC) or FAM18 C- (unencapsulated MenC), and boosted i.p. with the same dose on day 21. Serum titers of Ag-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between FAM18 C+ and FAM18C-.

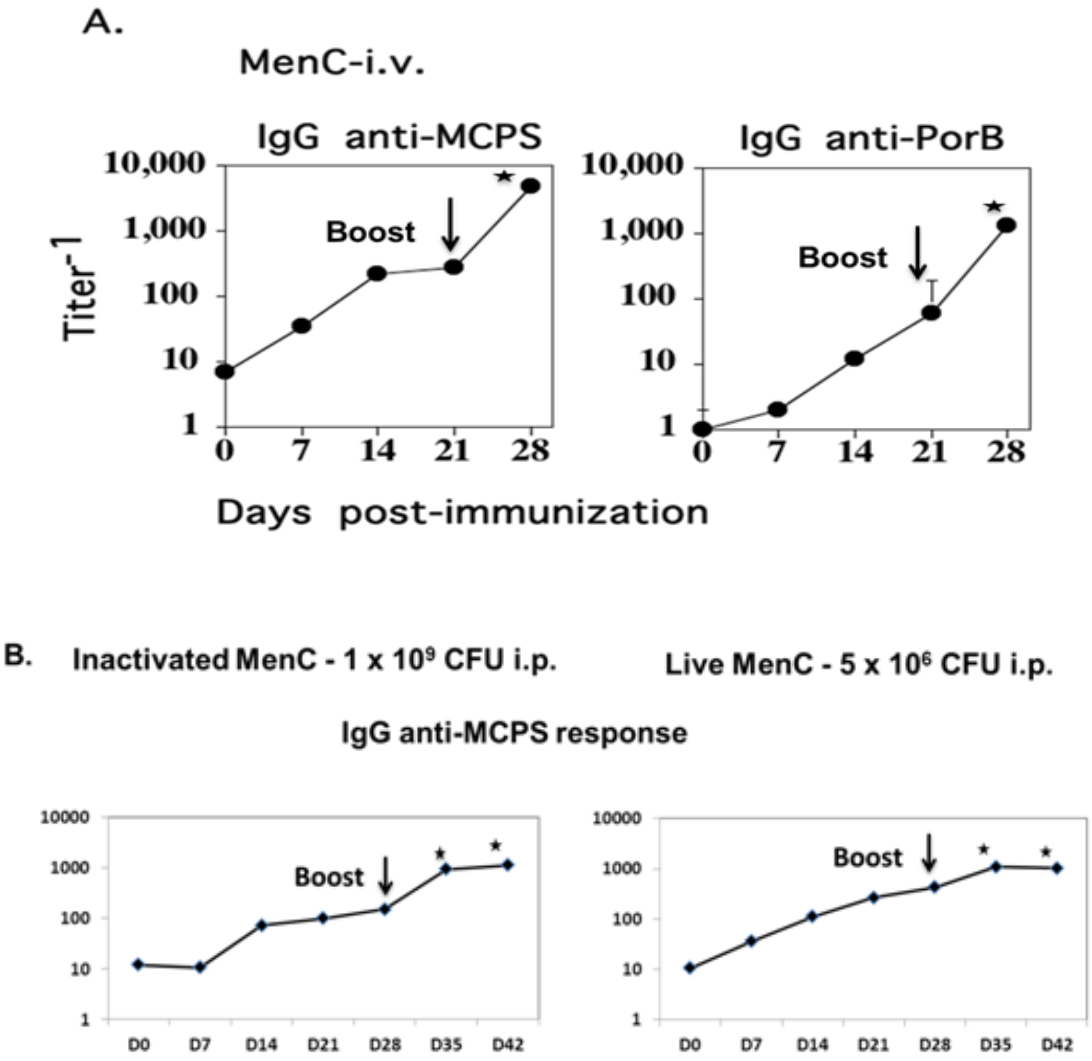


Fig.10. MCPS-specific IgG response to intact MenC. **A.** BALB/c mice (7 per group) were immunized i.v. with 2 x 10^8 CFU of heat killed intact MenC (strain M1883) in saline and boosted with the same dose on day 21. Serum titers of Ag-specific IgG were measured by ELISA. **B.** BALB/c mice (7 per group) were immunized with 1 x 10^9 CFU of heat killed MenC or 5 x 10^6 CFU of live MenC and boosted respectively with the same doses on day 28. Serum titers of Ag-specific IgG were measured by ELISA. Significance (*) indicates $p \leq 0.05$ between the primary and secondary titers.

The primary and secondary IgG anti-MAPS responses to intact MenA are similar to that elicited by a meningococcal conjugate vaccine.

We wished to determine whether or not the similar bacterial structure with distinct capsule produces a similar PS-specific Ig response. Therefore, we utilized intact heat killed *Neisseria meningitidis* serogroup A (MenA) to compare the PS-specific IgG response to that of intact MenC and the conjugate vaccine MAPS-TT. BALB/c mice were immunized i.p. with 2 x 10^8 CFU of MenA or MAPS-TT conjugate (1 μg/mouse) in alum + CpG-ODN, and boosted the mice 21 or 28 days later in a similar fashion. As shown in Fig. 11, the primary anti-MAPS IgG titers to both intact MenA and the conjugate MAPS-TT appeared gradually and peaked by D21 similar to the anti-protein IgG responses to MenA. The secondary IgG responses to both intact MenA and MAPS-TT produced a highly boosted secondary response similar to that of the anti-PorB IgG response to intact MenA. Thus, the primary and secondary anti-MAPS IgG responses to intact MenA are similar to that of the conjugate vaccine, as observed with MenC.

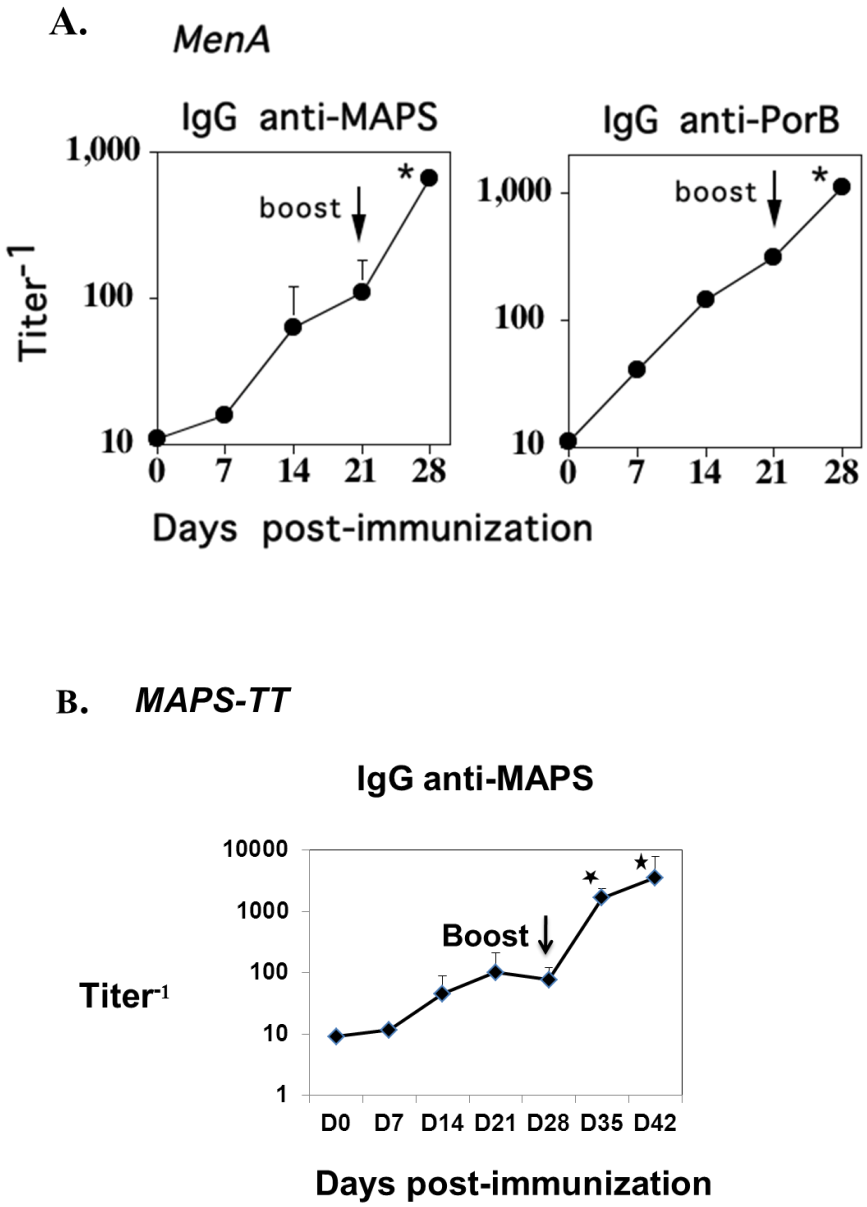


Fig 11. IgG anti-MAPS responses to intact MenA and conjugate MAPS-TT. BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat killed intact MenA in saline **(A)** and 1µg of conjugate MAPS-TT **(B)** and boosted with the same dose on days indicated. Serum titers of Ag-specific IgG were measured by ELISA. Significance (*) indicates $p \leq 0.05$ between the primary and secondary titers.

The MCPS-specific IgM and IgG isotype responses to isolated MCPS and intact M1883 are distinct.

Isolated PS are poorly immunogenic, elicit Ig responses largely of the IgM isotype, with smaller amounts of IgG3, and sometimes IgG1, and fail to show boosted IgG anti-PS responses following secondary immunization (14). To determine the effect of M1883 on the associated anti-MCPS response, we directly compared the MCPS-specific IgM and IgG isotype responses to isolated, soluble MCPS (10 μg/mouse in saline) versus heat-killed, intact M1883 (2×10^8 CFU/mouse) both injected i.p., followed by a similar secondary immunization on day 21. As illustrated in Fig. 12, the primary IgM anti-MCPS response to purified MCPS peaked by day 7 and failed to show a boosted secondary response, whereas the primary IgM anti-MCPS response to intact M1883 peaked by day 14 at somewhat lower peak titers than that observed for isolated MCPS, and also failed to show a boosted secondary response. The primary peak titers of MCPS-specific IgG were somewhat higher for mice immunized with M1883 versus MCPS (3-fold, $p<0.05$), although the latter response peaked earlier (Fig. 12). In contrast, M1883 induced a 15-fold boost in IgG anti-MCPS serum titers following secondary immunization relative to the primary, whereas MCPS failed to induce any significant boost. Thus, M1883 overall elicited 42-fold higher secondary serum titers of IgG anti-MCPS relative to secondary titers following MCPS immunization. The IgG isotypes elicited by isolated MCPS were IgG3 and IgG1, with no detectable IgG2b or IgG2a, whereas intact M1883 induced all 4 IgG isotypes (Fig. 12). Collectively, these data demonstrate that the intact bacterium markedly changes the nature of the associated MCPS-specific Ig response relative to that observed with isolated MCPS.

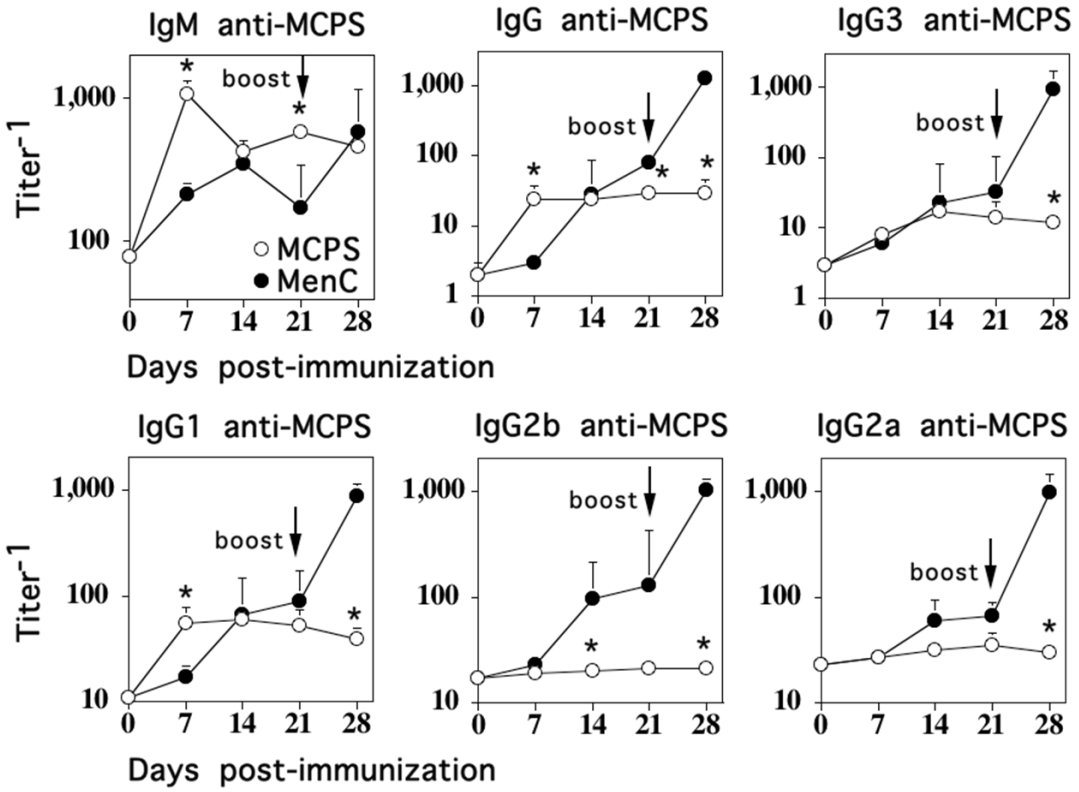


Fig 12. The MCPS-specific IgM and IgG isotype responses to isolated MCPS and intact M1883 are distinct. BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact M1883 or purified MCPS in saline, and boosted i.p. with the same dose on day 21. Serum titers of MCPS-specific IgM, IgG, and IgG subclasses (IgG3, IgG1, IgG2b and IgG2a) were determined. Significance (*) $p \leq 0.05$ between MenC and MCPS.

CD4+ T cells are required for the induction of the secondary, but not primary IgG anti-MCPS response to intact M1883.

Whereas the Ig responses to isolated PS are largely TI, our previous studies using intact Pn14 demonstrated that the rapid primary IgG anti-PPS14 response was dependent on CD4+ T cells (7). In light of the slower primary, and highly boosted secondary IgG anti-MCPS response to MenC (both M1883 and FAM18 C+), we were interested in determining their relative CD4+ T cell-dependence. To determine this, we injected M1883 into T cell-deficient athymic nude mice or BALB/c mice acutely depleted of CD4+ T cells by a single injection of a depleting rat IgG anti-mouse CD4 mAb (clone GK1.5) given 24h prior to primary immunization with M1883. M1883-immunized mice injected 24h earlier with polyclonal rat IgG were used as controls. Injection of anti-CD4 mAb depleted CD4+ T cells by >95%, as determined by flow cytometry 24 hr later, whereas rat IgG had no effect as shown in Fig. 13A. Mice were boosted with M1883 on day 21 in the absence of anti-CD4 mAb or polyclonal rat IgG. As illustrated in Fig. 13B, the primary IgG anti-MCPS IgG response to intact M1883 in athymic nude or anti-CD4 mAb-injected mice was similar to that observed in control mice, whereas the primary IgG response specific for either PorB or a total protein extract derived from unencapsulated FAM18 C-, was largely abrogated. In contrast, little or no boosting of serum titers of IgG anti-MCPS, anti-protein, or anti-PorB following secondary immunization was observed in the absence of T cells (Fig. 13B). Collectively, these data demonstrate that the primary and boosted secondary IgG anti-MCPS responses to M1883 are TI and TD respectively, in contrast to previous observations that the primary IgG anti-PPS14 response to Pn is TD, with no secondary boosting, even in the presence of T cells. However, both the primary and secondary anti-protein responses to either Pn14 or M1883 are TD.

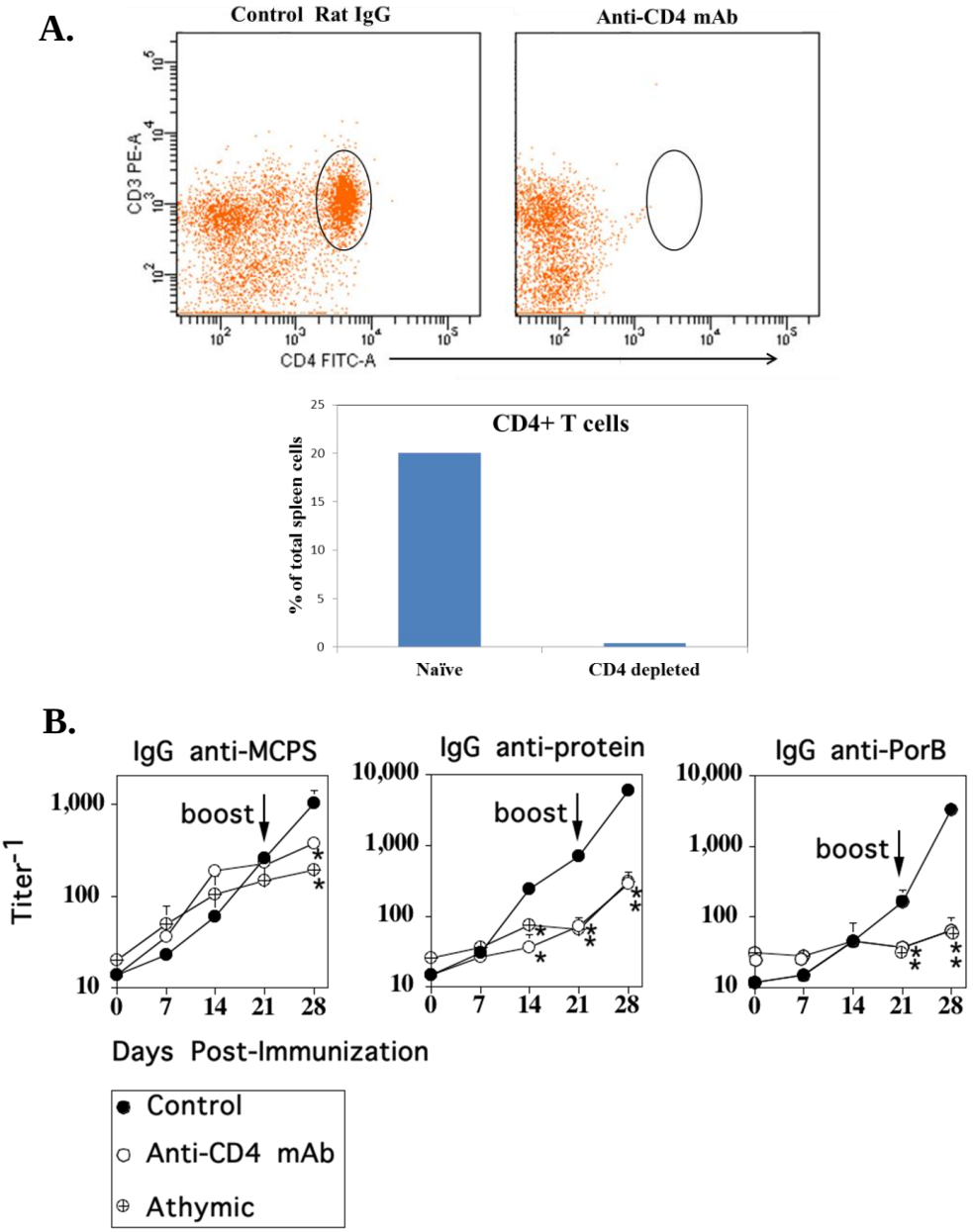


Fig 13. CD4+ T cells are required for the induction of the secondary, but not primary IgG anti-MCPS response to intact M1883. **A.** Flow cytometric analysis of spleen cells 24 hr post i.p. injection with either with a depleting anti-CD4+ mAb (clone GK.1.5) or control rat IgG. **B.** BALB/c mice were injected with polyclonal control Rat IgG or GK1.5 mAb one day prior to i.p immunization with 2 x 10^8 CFU of heat-inactivated intact M1883 in saline, and boosted 21 days later in the absence of Ab. Serum titers of Ag-specific IgG were measured by ELISA. Significance (*) $p \leq 0.05$ between "Control" versus and-CD4 mAb-injected mice or athymic nude mice.

CD28- ICOS-, and CD40L-dependent costimulation is critical for the induction of the secondary, but not primary, IgG anti-MCPS response to M1883.

Inducible Costimulator (ICOS) is a member of the CD28 family that is induced on $CD4^+$ T cells upon T cell receptor cross-linking and CD28-mediated signaling (84, 85). CD28, which is expressed constitutively on CD4+ T cells, is critical for initiation of $CD4^+$ T cell activation, whereas ICOS plays a role in the subsequent T cell effector response such as the germinal center reaction and the generation of memory (88, 89). We previously demonstrated that the CD4+ T cell-dependent primary IgG anti-PPS14 response to Pn14 is CD28/B7-2-dependent, but ICOS-independent (106). In light of the CD4+ T cell dependence of the boosted secondary IgG anti-MCPS response to M1883, we determined whether or not it was CD28- and/or ICOS-dependent. Thus, mice genetically-deficient in CD28 ($CD28^{-/-}$), ICOS ($ICOS^{-/-}$) and strain-matched C57BL/6 WT mice were immunized i.p. with M1883 and boosted 3 weeks later. As illustrated in Fig. 14, M1883-immunized WT mice elicited a detectable primary IgG anti-MCPS and anti-PorB response, and following secondary immunization, significant boosting in both MCPS- and PorB-specific IgG serum titers. The primary IgG anti-MCPS responses in $CD28^{-/-}$ and $ICOS^{-/-}$ mice were similar to that observed in WT control mice, consistent with the TI nature of the response (Fig. 14A), whereas the CD4+ T cell-dependent secondary IgG anti-MCPS responses were essentially abrogated in both mutant mouse strains. In contrast, both the primary and secondary IgG anti-PorB responses were markedly inhibited in both $CD28^{-/-}$ and $ICOS^{-/-}$ mice. Similar data were obtained using neutralizing anti-ICOSL mAb (HK5.3) versus control polyclonal hamster IgG, injected into mice one day before immunization with M1883 (Fig 14B).

The interaction of CD40 on APC with CD40L, induced on CD4+ T cells following TCR/CD28-mediated activation, is critical for the induction of the TD primary Ig response,

germinal center reaction, and the development of memory in response to immunization with protein Ags, or for the anti-PS response to conjugate vaccines (130, 131). A role for endogenous CD40L has also been reported in stimulating Ig responses to some, but not all PS Ags (132-135). We previously demonstrated that the primary CD4+ T cell-dependent IgG anti-PPS14 response to Pn was CD40L-, as well as CD28-dependent (106, 117). Thus, we tested whether CD40L-dependent costimulation was critical for the primary TI and/or TD IgG anti-MCPS response to M1883. Thus, we injected BALB/c mice i.p. with a single dose of blocking hamster IgG anti-mouse CD40L mAb (MR1) one day before M1883 immunization, using polyclonal hamster IgG as a control, with a secondary immunization, 21 days later, using M1883 alone. As shown in Fig. 15, there was no difference in the primary anti-MCPS IgG response between the control and the MR1-injected group, whereas the secondary IgG anti-MCPS response in the MR1-injected group was markedly inhibited compared to control mice. In contrast, both the primary and secondary IgG anti-PorB responses were significantly reduced in the MR1-treated mice compared to controls. Collectively, these data demonstrate that the TD secondary, though not TI primary, IgG anti-MCPS response to intact M1883 is dependent on CD28-, ICOS-, and CD40L-dependent costimulation, in contrast to the TD primary IgG anti-PPS14 response to Pn14 which is CD28- and CD40L-dependent, but ICOS-independent.

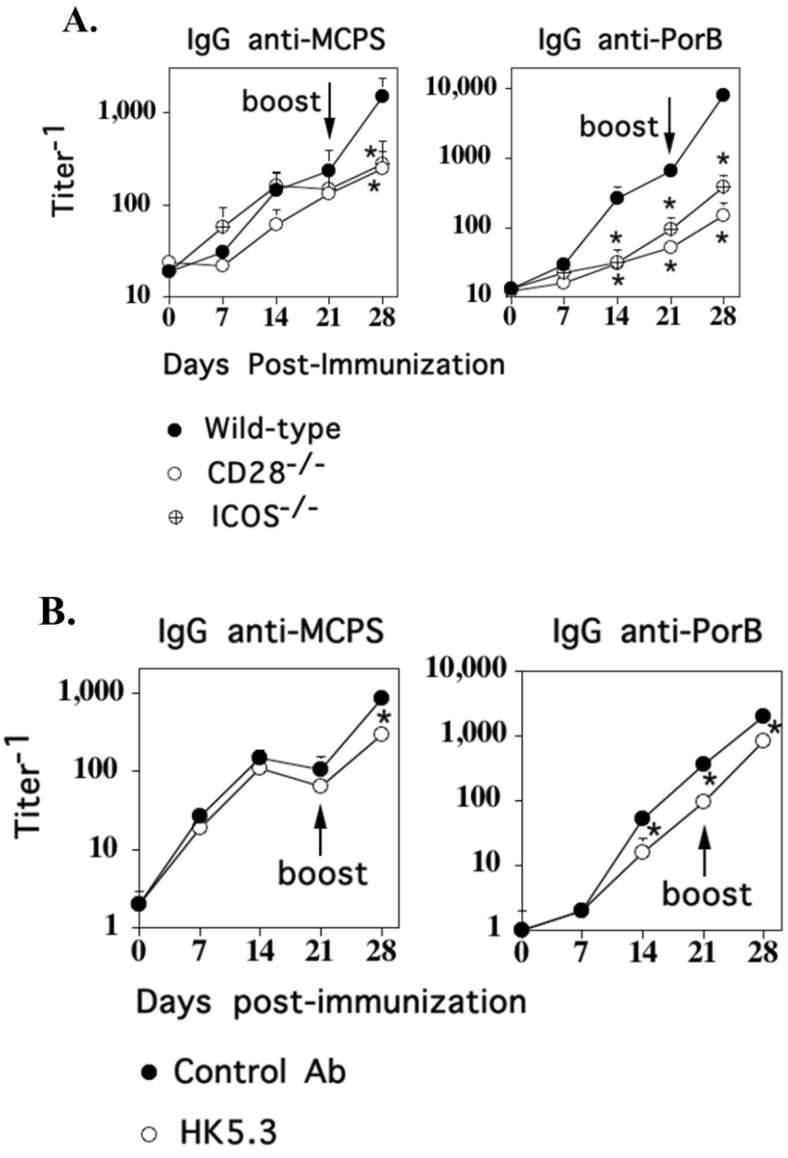


Fig 14. CD28- and ICOS-dependent costimulation is critical for the induction of the secondary, but not primary, IgG anti-MCPS response to M1883. A. C57BL/6 (WT), $CD28^{-/-}$ (C57BL/6 background), and $ICOS^{-/-}$ (C57BL/6 background) mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact M1883 in saline and boosted on day 21. Serum titers of Ag-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between "WT" versus $CD28^{-/-}$ or $ICOS^{-/-}$ mice. **B.** BALB/c mice (7 per group) were injected i.p. either with blocking anti-ICOS mAb (clone HK5.3) or control rat IgG2a mAb (clone 2A-3) (0.5 mg/mouse) 24 h i.p. immunization with 2 x 10^8 CFU of heat-inactivated intact M1883 in saline, and boosted 21 days later in the absence of Ab.

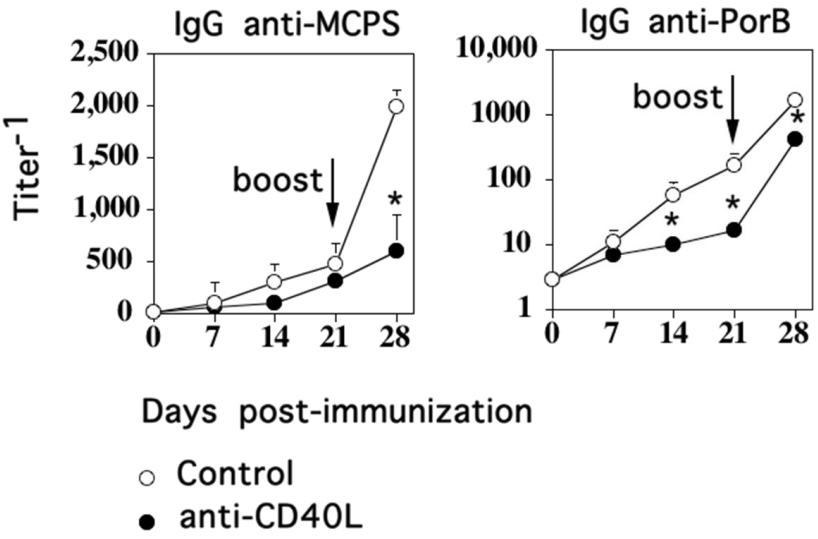


Fig 15. CD40 ligand-dependent costimulation is critical for the induction of the secondary, but not primary, IgG anti-MCPS response to M1883 BALB/c mice (7 per group) were injected i.p. with either anti-CD40L mAb (clone MR1) or polyclonal hamster IgG ("Control") [0.3 mg per mouse]. Mice were then immunized, 1 day later, i.p. with 2 x 10^8 CFU of heat-inactivated intact M1883 in saline and boosted 21 days later. Serum titers of Ag-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between "Control" versus anti-CD40L-injected mice.

Endogenous TLR4, but not TLR2 or MyD88, signaling is critical for induction of peak primary and secondary serum titers of MCPS-specific IgG in response to M1883, but is not critical for secondary boosting.

Lipopolysaccharide (LPS), a component of the outer membranes of GN bacteria is a ligand for TLR4, and a potent activator of the innate immune system (64). We thus wished to determine whether endogenous TLR4 signaling affected the humoral immune response to M1883 and might account for the differences observed with the IgG anti-PPS14 response to Pn14. We previously observed no role for TLR4 in the IgG anti-PPS14 response to Pn14, although genetic deficiency of TLR2 resulted in a significant reduction in IgG3, IgG2b, and IgG2a, but not IgG1 PPS14-specific serum titers (136). C3H/HeJ ($Tlr4^{Lps\text{-}d}$) mice that carry a spontaneous mutation in the TLR4 gene and C3H/HeOuJ control mice were immunized i.p. with M1883 and boosted on days 21 and 35. As illustrated in Fig. 16A, both the primary, secondary, and tertiary IgG anti-MCPS responses in C3H/HeJ mice were significantly reduced, though not eliminated relative to control mice. Of note, the absence of TLR4 signaling did not prevent a secondary boost in the IgG anti-MCPS response. Further analysis of serum titers of IgM and IgG isotypes following tertiary immunization indicated that TLR4 deficiency significantly reduced MCPS-specific IgM and IgG2a (Fig. 16A). Thus, endogenous TLR4 signaling plays a significant role in the magnitude of both the TI and TD components of the IgG anti-MCPS response, but is not critical for the generation of memory. Of note, in additional experiments utilizing $TLR2^{-/-}$ (Fig. 16B) and $MyD88^{-/-}$ (Fig. 16C) mice, no significant reductions in serum titers of MCPS-specific IgM or IgG were observed, although a significant 4-fold reduction in the IgG2b anti-MCPS titer was noted in $MyD88^{-/-}$ relative to control C57BL/6 mice. In contrast,

there were no significant differences in the primary and secondary PorB-specific IgG serum titers between the WT and TLR4 defective mice or $TLR2^{-/-}$ or $MyD88^{-/-}$ as shown in Fig. 17

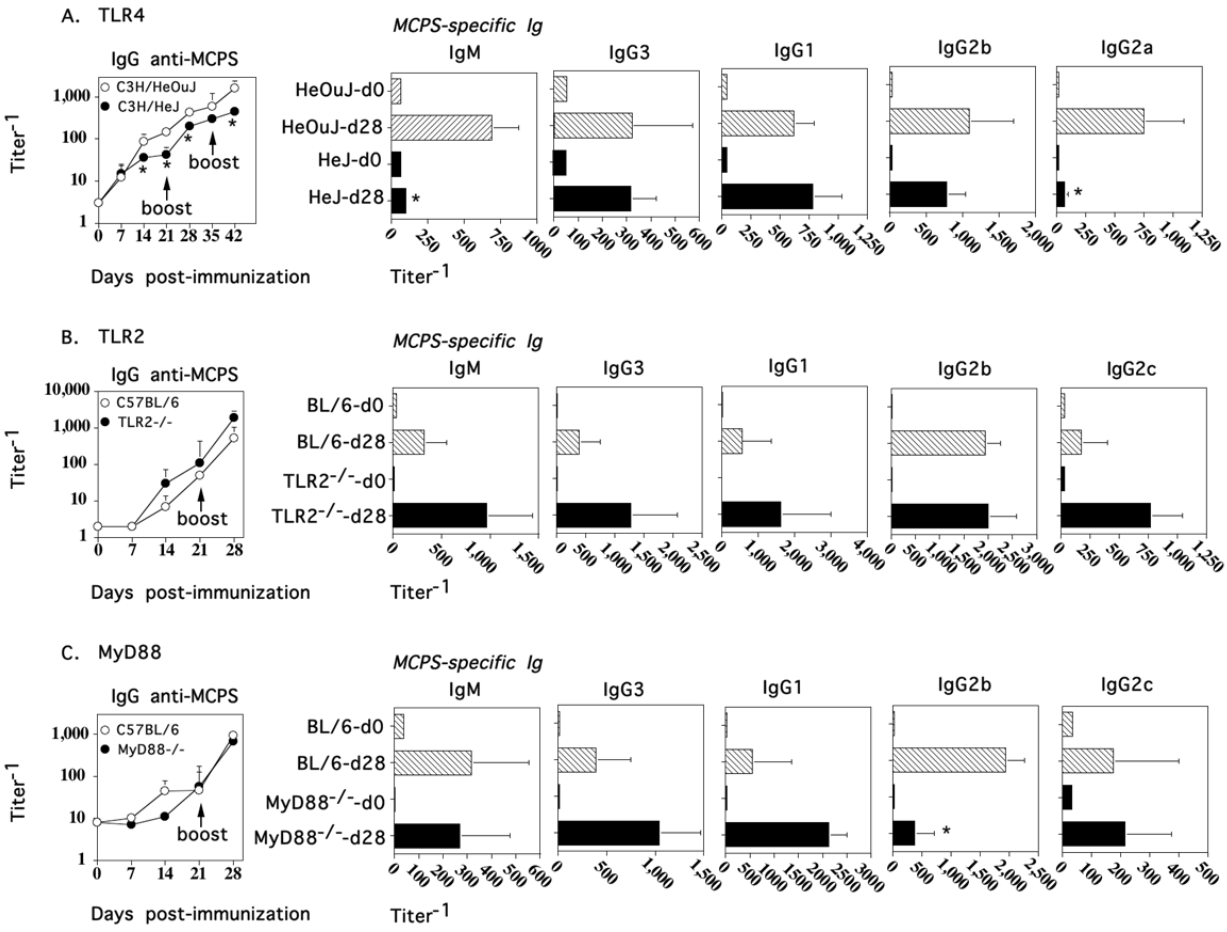


Fig 16. Endogenous TLR4, but not TLR2 or MyD88, signaling is critical for induction of peak primary and secondary serum titers of MCPS-specific IgG in response to M1883, but is not critical for secondary boosting. (A) C3H/HeJ(*Tlr4*$^{Lps\text{-}d}$) and C3H/HeOuJ (control mice), or **(B)** TLR2$^{-/-}$ or **(C)** MyD88$^{-/-}$ mice (same group of control C57BL//6 mice used for both "B" and "C") were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact M1883, boosted on days indicated. Serum titers of MCPS-specific IgM, IgG, and IgG isotypes were determined by ELISA with sera obtained on indicated days. Significance (*) $p \leq 0.05$ between "Control" versus C3H/HeJ or TLR2$^{-/-}$ or MyD88$^{-/-}$ mice

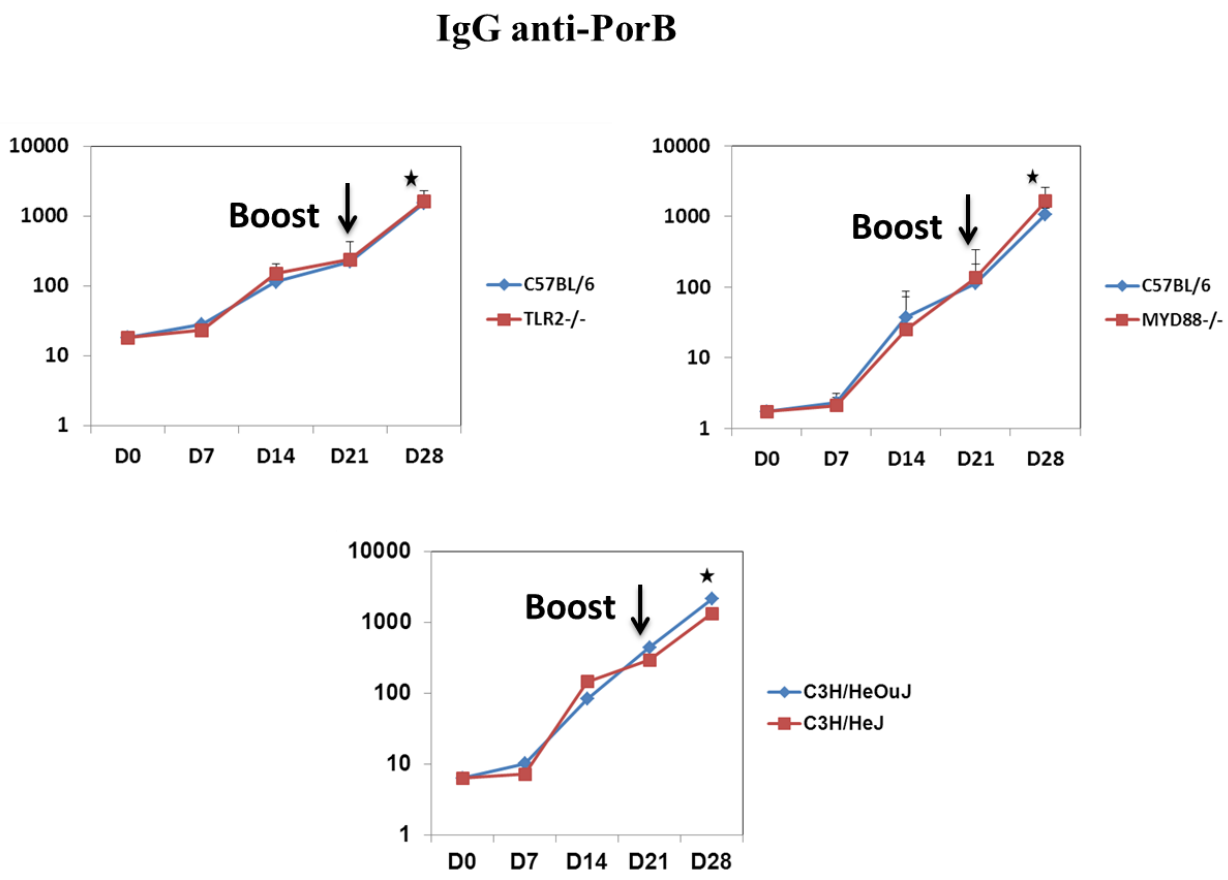


Fig 17. TLR signaling plays no role in eliciting PorB-specific IgG responses to intact MenC. TLR2$^{-/-}$ or MyD88$^{-/-}$ mice (same group of control C57BL//6 mice used for both) or C3H/HeJ(*Tlr4*$^{Lps\text{-}d}$) and C3H/HeOuJ (control mice) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact M1883, boosted on days indicated. Serum titers of PorB-specific IgG were determined by ELISA with sera obtained on indicated days. Significance (*) $p \leq 0.05$ between primary and secondary titers.

Co-immunization of MenC and Pn14 promotes secondary boosting of the IgG anti-PPS14 response to Pn14.

The inability of Pn14 to induce a boosted secondary IgG anti-PPS14 response remains unexplained. One possibility is that Pn14 fails to provide adequate innate signaling and/or inhibits the generation and/or elicitation of PPS14-specific IgG memory following secondary immunization. We therefore wished to determine whether signals provided by MenC could promote secondary boosting to the IgG anti-PPS response to Pn. Therefore, we immunized mice i.p. with Pn14 (strain R614) or M1883 alone or the two together. In all groups, 2×10^8 CFU/mouse of each bacterial strain was used. Mice were similarly boosted on day 21. As shown in Fig. 18A, the primary IgG anti-PPS14 response to R614 alone peaked on day 7, with no boosting in titers following secondary immunization. In the presence of M1883, the primary IgG anti-PPS14 response to R614 was modestly reduced at day 14, but more importantly, showed significant secondary boosting (3-fold increase). Of note, priming and secondary immunization of mice with as high as 1×10^9 CFU R614/mouse failed to elicit a boosted secondary IgG anti-PPS response (data not shown). M1883 had no effect on the IgG anti-pneumococcal surface protein A (PspA) response to R614 (Fig. 18). Conversely, with the exception of a modest increase in the primary IgG anti-MCPS response to M1883 on day 14, co-immunization with R614 had no other significant effects on either the IgG anti-MCPS or IgG anti-PorB response. Isolated MCPS has been reported to increase expression of CD86 and MHC-II on murine B cells (121), suggesting a possible role in augmenting adaptive immunity. In this regard, in an additional experiment, co-immunization of R614 with either FAM18 C+ or FAM18 C- similarly boosted the secondary IgG anti-PPS14 response (Fig. 18B) indicating that the boosting effect of MenC is not secondary to the presence of the MCPS capsule. Thus, these data suggest that the

failure of Pn to induce a boosted secondary IgG response may reflect either sub-critical innate signaling by Pn or an inhibitory effect that can be overcome by MenC.

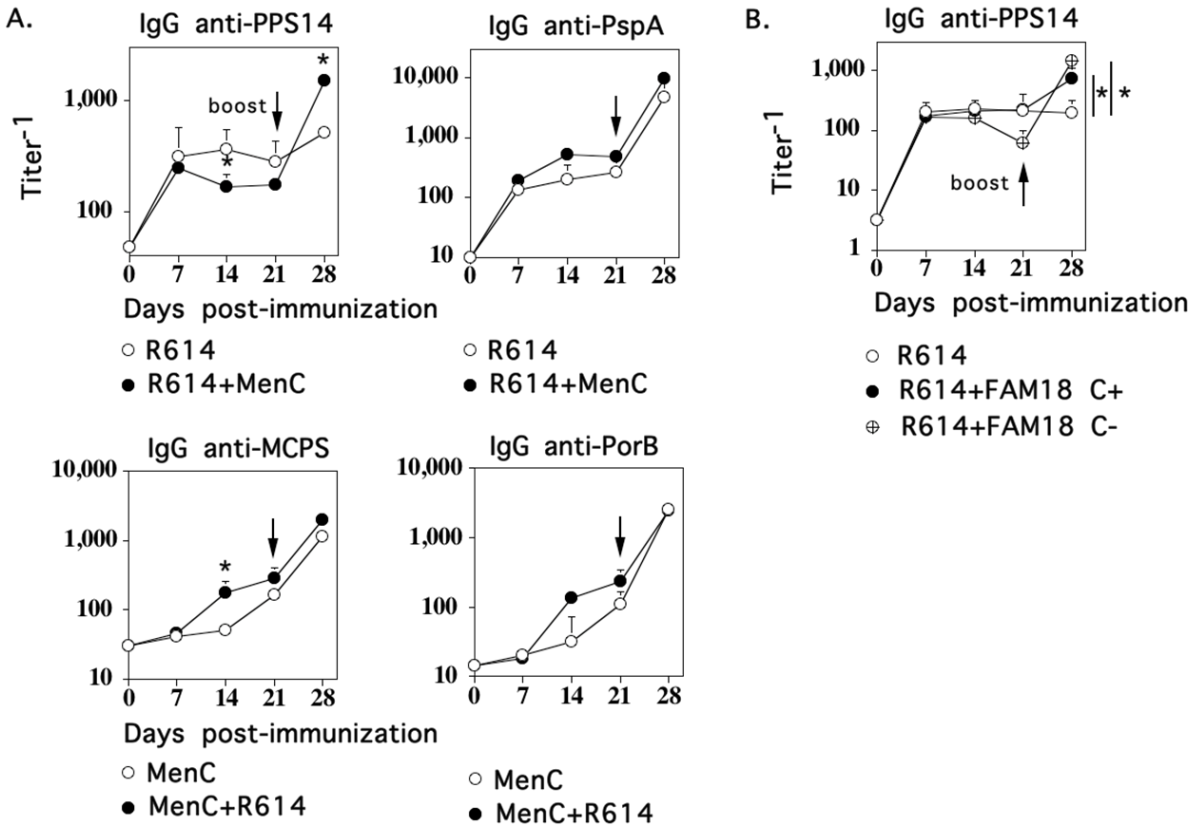


Fig 18. Co-immunization of MenC and Pn14 promotes secondary boosting of the IgG anti-PPS14 response to Pn14. A. BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact MenC strain M1883 or 2 x 10^8 CFU of heat-inactivated intact Pn14 (strain R614) or a combination of MenC and R614 (2 x 10^8 CFU each), and boosted in a similar fashion on day 21. **B.** BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU of heat-inactivated intact R614 or a combination of R614 with FAM18 C+ or FAM18 C-. Serum titers of Ag-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between mice immunized with single bacteria versus combination.

2.4 Discussion

The inability of non-zwitterionic capsular PS to associate with MHC-II molecules for presentation to CD4+ T cells (13, 116), results in a limited capacity of these Ags to elicit PS-specific IgG isotypes, GC formation, and IgG-specific memory (14). However, isolated PS have been reported to induce TI IgM-specific memory that is down-regulated by PS-specific IgG (137). Covalent attachment of an immunogenic protein to an isolated PS, results in the induction of a TD PS-specific IgG memory response (138). This likely occurs through specific uptake of PS-protein conjugate by PS-specific B cells with subsequent presentation of protein peptide/MHC-II to CD4+ T cells (131), although CD4+ T cells specific for the PS and combined PS-protein components are also elicited (139). Capsular PS expressed by extracellular bacteria is associated indirectly with immunogenic proteins, through covalent attachment to cell wall peptidoglycan (GP bacteria) (40, 41) or the acyl glycerol moiety of the outer membrane (47). This suggests the possibility that PS-specific Ig responses to intact bacteria might behave in a manner similar to that elicited by conjugate vaccines. Indeed, somatically mutated PS-specific IgG is elicited in response to encapsulated bacteria, suggesting a role for T cells in this process (140, 141). However, BCR-mediated, Ag uptake by B cells involves endocytic vesicles that are only 50-150 nm in diameter, and thus would be predicted to exclude intact bacteria (142). Nevertheless, a single study demonstrated BCR-mediated uptake of intact *Salmonella typhimurium* by human B cells with subsequent display of MHC-II/peptide complexes and primary CD4+ T cell activation (143).

Previous studies on the in vivo IgG anti-PPS14 response to intact, heat-killed Pn14 indicated that the bacteria significantly altered the nature of the associated PPS14-specific IgG response relative to that elicited by isolated PPS14. The IgG anti-PPS14 response to intact Pn14 was similar to that elicited by a TD conjugate vaccine, in that both responses were dependent on CD4+ T cells, B7-dependent costimulation, and CD40/CD40L interactions, with elicitation of all 4 IgG isotypes (106, 117). However, in contrast to conjugate vaccines, the IgG anti-PPS14 response to Pn14 was relatively rapid, failed to induce a boosted secondary response, and was ICOS-independent, similar to that elicited by TI isolated PS Ags. Nevertheless, Pn14 elicited a protein-specific (i.e. PspA) IgG response that was classically TD (106, 117). The basis for this unique immunologic behavior of intact Pn14 is a matter of ongoing investigation (109).

In the present study we analyzed the murine anti-capsular PS (MCPS) Ig response to i.p.-injected intact, heat-killed *Neisseria meningitidis* serogroup C (MenC), a GN bacterium. In contrast to Pn, the IgG anti-MCPS response to MenC exhibited delayed primary kinetics and a highly boosted secondary IgG anti-MCPS response, whereas the IgG anti-MCPS response to isolated MCPS was rapid, failed to show secondary boosting, and consisted of only IgG1 and IgG3, as opposed to all four IgG isotypes in response to intact MenC. The secondary, but not primary, IgG anti-MCPS response to MenC was dependent on CD4+ T cells, CD40L, CD28 and ICOS. The primary and secondary IgG anti-MCPS responses were lower in TLR4-defective (C3H/HeJ), although not $TLR2^{-/-}$ or $MyD88^{-/-}$ mice, but secondary boosting was still observed. Of interest, co-immunization of Pn and MenC resulted in a boosted secondary IgG anti-PPS response to Pn that was not dependent on expression of MCPS by MenC.

As previously reported with Pn14, the presence of intact bacteria markedly changes the nature of anti-PS IgG responses and induced all four isotypes of IgG. However this contrasts to

the previous observation made with formalin fixed MenC which exhibited a more restricted IgG3 and IgM serum profiles (144, 145). Different doses of heat-killed bacteria or live MenC showed a highly boosted secondary PS-specific IgG responses consistent with the previous studies by Colino et al (146). Immunization of MenC via i.p. or i.v route of immunization essentially generated similarly boosted anti-MCPS specific IgG responses.

These data, and our previously published reports, demonstrate distinct differences in the nature of the PS-specific Ig responses to intact Pn14 versus MenC. Although it is clear from these data that the intact bacterium significantly alters the nature of the PS-specific Ig response to the associated PS Ag, it remains unresolved whether the differences between intact Pn14 and MenC reflect a more general dichotomy between GP and GN bacteria, or unique features in the composition and/or structure of one or both of these bacteria, not manifested by other bacterial species. Therefore, we also tested whether the structurally similar GN bacteria with a different PS would elicit similar PS-specific IgG responses. Therefore, heat killed MenA was used to compare with the conjugate vaccine MAPS-TT in inducing the MAPS-specific IgG responses. Our data suggest that the primary and secondary anti-MAPS IgG responses to intact MenA are similar to that of the conjugate vaccine, as observed with MenC. This could be due to the covalent linkage of the capsular PS in GN bacteria to the acyl glycerol moiety of the outer membrane (47), which is known to have immunostimulatory components like LPS and porin proteins that are TLR4 (44) and TLR2 ligands respectively (66).

The biochemical nature of the specific expressed PS Ag and its interaction with the immune system could affect the nature of PS-specific responses. In this regard, isolated MCPS and type V capsular PS from *Streptococcus agalactiae*, but not NP-Ficoll were shown to inhibit a number of B cell functions mediated by BAFF and APRIL (121). Additionally, MCPS enhanced

the expression of costimulatory molecules like CD80 and CD86 and MHC-II on involved in Ag presentation machinery. Other bacterial PS, such as C-polysaccharide and PPS1 of Pn, *Staphylococcus aureus* capsular polysaccharides types 5 and 8, and PS-A of *Bacteroides fragilis*, are zwitterionic on the basis of expression of both positive and negative charges, and can stimulate CD4+ T cells through association with MHC-II (11). Additional differences between capsular PS such as the ability to bind to SIGN-R1, a scavenger receptor on splenic marginal zone macrophages (118, 147), capacity to fix complement (148), or expression of terminal sialic acid residues that interact with the inhibitory CD22 receptor on B cells (120), may also have potential effects on PS-specific Ig responses to intact bacteria. Thus, biochemically identical PS capsules on two structurally distinct bacteria would determine how differences in the bacterial composition and structure could potentially impact the PS-specific Ig response.

The boosting of the IgG anti-MCPS response following secondary immunization with intact MenC and its dependence on CD4+ T cells, CD40L, CD28 and especially ICOS (84) strongly suggests that MCPS-specific B cells enter into a germinal center reaction in which MCPS-specific memory B cells are generated. This further suggests a possibility that MCPS-specific B cells engage in cognate interactions with CD4+ T cells that are specific for bacterial protein, that are indirectly linked to the PS or to the T cells specific for the PS itself that are not MHC restricted (139) or to the T cells specific for the PS-peptide generated similar to that observed with the glycoconjugate vaccine (149). Although, the ICOS dependence of secondary MCPS-specific IgG responses suggest the generation of GC reaction, there are also reports demonstrating a role of ICOS in extrafollicular immune responses (90, 150). Thus, further studies are required to confirm that GC formation by MCPS-specific B cells eventually undergoes affinity maturation to produce high affinity antibodies.

Lsc-/- mice that are genetically deficient in *lsc* have MZ B cells that are defective in detaching from the MZ for transport to the T cell region, following activation, to recruit CD4+ T cell help to mount TD Ig responses (151). Our previous studies using Lsc-/- mice demonstrated an important role of MZ B cells in eliciting the anti-PPS14 specific IgG responses to intact Pn14 unlike the anti-PspA IgG response that arises from the FB cells. However, the anti-PPS14 IgG responses to conjugate vaccine are also known to be originating from the FB cells (108). TLR adjuvants like LPS induce the mass migration of MZ B cells in Lsc-/- mice and therefore activate T cells for TD immune responses (152). In light of the boosted MCPS-specific memory response to MenC, FB cells could possibly be involved in such MCPS-specific Ig responses. However, the generation of memory response is independent of the B cell subsets involved (109). Therefore, the differential role of MZ B cells and FB in inducing the anti-PS Ig responses to intact MenC is to be determined.

It remains to be determined whether PS-specific B cells can directly internalize intact bacteria for presentation of bacterial protein to CD4+ T cells or perhaps obtain bacterial protein indirectly following uptake, transport and/or processing of intact bacteria by other immune cells, including dendritic cells and macrophages (153). Of note, the primary TI IgG anti-MCPS response to MenC was independent of CD40L. Previous reports have indicated a role for CD40L-CD40 interactions in the PPS-specific IgM and IgG response to isolated PPS (132), although not to TNP-Ficoll (135). The reasons for these differences remain unexplained.

Previous studies using an *in vitro* polyclonal model for multivalent membrane (m)Ig crosslinking in response to PS Ags (i.e. multiple anti-IgD antibodies conjugated to dextran [αδ-

dex]) indicated that multivalent mIg crosslinking alone, or in concert with CD40-mediated activation, induced vigorous proliferation but no Ig secretion or isotype switching by highly purified B cells (20). However, addition of a number of cytokines or various TLR ligands such as bacterial lipoproteins (TLR2), Neisserial porins (TLR2), unmethylated CpG-containing oligodeoxynucleotides (TLR9), or LPS (TLR4) to αδ-dex-activated B cell cultures induced substantial Ig secretion and isotype switching (20). On this basis we proposed that during natural infections with PS-encapsulated bacteria, the associated bacterial TLR ligands will play an especially critical role in eliciting the TI component of the anti-PS response, either through direct activation of B cells and/or via cytokine induction by through second signals provided by NK cells or macrophage stimulation (20). The significant reduction in the primary TI anti-MCPS response to intact MenC in TLR4 defective mice (C3H/HeJ mice) as shown in Fig. 16 demonstrate demonstrating an important role for TLR4 during the TI primary responses. TLR4 signaling in response to MenC is likely mediated by lipooligosaccharide (LOS) (154). In this regard, our study using purified MCPS induced a rapid primary IgM response and a modest IgG response without any secondary boosting. However, previous studies using pneumococcal PS vaccines demonstrate that the PS antigen in itself is ineffective at inducing detectable IgG in vivo in the absence of a distinct second signal provided by the unappreciated TLR ligands in the PS preparations. The capacity of isolated MCPS to induce IgG secretory responses in vivo may reflect the presence of contaminating TLR ligands remaining after PS purification from the intact bacteria (155).

TLR4-defective mice also elicited a reduced secondary TD MCPS-specific IgG response to MenC, with the reduction in IgG due to selective abrogation of the IgG2a response. The capacity of LPS to potently induce IL-12-dependent IFN-γ (156), a switch factor for the IgG2a

subclass (157), likely accounts for this effect. In addition to LPS, IFN-γ has also been shown to stimulate maturation of B cells into IgM-secreting cells (158), perhaps accounting for the reduction in the IgM anti-MCPS as well. In this regard, a recent report demonstrated the ability of isolated MCPS to inhibit IFN-γ-mediated DC release of BAFF (121), a mediator of B cell maturation and class switching to IgG. Perhaps the stimulating effects of intact MenC override this inhibitory property of isolated MCPS. Nevertheless, TLR4-defective mice exhibited a boosted MCPS-specific secondary IgG response to MenC, despite reduced serum titers relative to WT controls, and no changes in the more prolonged kinetics of primary MCPS-specific IgG induction. The comparable IgM and IgG anti-MCPS responses to MenC, observed in MyD88$^{-/-}$ versus WT mice, despite reduced responses in TLR4-defective mice, suggest a possible role for TLR4-dependent TRIF signaling (159). Also, the significant inhibition of IgG2a and IgG2b in C3H/HeJ and MyD88$^{-/-}$ mice respectively suggests the probable Th1 skewing of humoral immune responses in these mice. Finally, although MenC expresses porins that are TLR2 ligands and signal in a MyD88-dependent manner (66), no significant differences in Ig responses to MenC were observed in TLR2$^{-/-}$ versus WT mice as shown in Fig. 17.

Thus, differences in TLR signaling between Pn14 and MenC per se are unlikely to account for the distinct PS-specific IgG responses between the two bacteria. The absence of a boosted secondary response to intact Pn could be due to inefficient immune signaling. Studies show that the GN bacteria induce higher levels of cytokine induction than GP bacteria at the same concentration (160). In this regard, the demonstration that co-immunization of Pn14 with MenC results in a boosted secondary IgG anti-PPS14 response to Pn14, suggests the possibility that non-viable Pn14 exerts an inhibitory influence on the PPS14-specific secondary IgG response that is at least partially overcome by innate signaling in response to MenC. However,

the presence of Pn14 did not result in any inhibition of the primary or secondary MCPS-specific IgG responses.

CHAPTER 3

Structurally Identical PS Expressed by Intact Heat-Killed *Streptococcus pneumoniae* versus. *Streptococcus agalactiae* Elicits Distinct PS-specific Ig responses

3.1 Introduction

Extracellular bacteria expressing polysaccharide (PS) capsules such as *Streptococcus pneumoniae* (Pn), *Streptococcus agalactiae* (group B *Streptococcus* [GBS]), and *Neisseria meningitidis* are leading causes of morbidity and mortality due to sepsis, pneumonia and meningitis (1, 3, 161). The PS capsule is a major virulence factor and vaccines targeting these pathogens are thus designed to elicit protective PS-specific IgG (115). In distinct contrast to proteins, non-zwitterionic PS are not enzymatically processed within the APC for association with MHC-II molecules (12, 13) and therefore do not recruit CD4+ T cells. Thus, isolated PS are T cell-independent (TI) antigens that fail to elicit robust germinal center formation and immunologic memory (14). However, PS are comprised of repeating sugar units that effect multivalent BCR crosslinking resulting in efficient and robust B cell proliferation (19), and in the presence of second signals, such as TLRs and/or cytokines, the induction of Ig secretion and class switching (20).

The view of proteins and PS as T cell dependent (TD) and TI antigens, respectively have largely come from studies based on immunization with isolated proteins and haptenated or non-haptenated PS. However during natural infections, the host encounters intact PS-encapsulated bacteria, in which proteins and PS are co-expressed in a single particulate structure along with multiple moieties like TLR, NLR, and scavenger receptor ligands that engage the innate immune system (7). Thus, the non-covalent co-expression of PS and proteins by the intact bacterium could potentially elicit bacterial peptide-specific CD4+ T cell help for PS-specific B cells, leading to the generation of enhanced PS-specific primary IgG responses and induction of

memory. In this regard, our previous studies using intact *Streptococcus pneumoniae*, capsular type 14 (Pn14), a Gram-positive (GP) bacterium, provide strong evidence that intact PS-encapsulated extracellular bacteria may represent unique immunogens, exhibiting both TI and TD characteristics of the associated PS-specific IgG response. Thus the primary PPS14 (pneumococcal PS type 14) -specific IgG response to intact Pn14 is dependent on CD4+ T cells, B7-dependent costimulation and CD40/CD40L interaction, and comprise all 4 IgG isotypes (TD-like). However, the primary PPS14-specific IgG response develops rapidly as an apoptosis-prone extrafollicular response that is more dependent on BCR signaling than the protein-specific IgG response. In addition, the PPS14-specific IgG response is ICOS-independent and fails to generate a boosted IgG response after secondary immunization (TI-like) (104, 106, 108, 112, 162).

In distinct contrast, our more recent studies using the Gram negative (GN) extracellular bacterium, *Neisseria meningitidis* type C (MenC), have shown that the primary MCPS (Meningococcal type C PS)-specific IgG response develops more slowly, with a significant boosting following secondary immunization. The primary MCPS-specific IgG response is TI, whereas the boosted MCPS-specific IgG response is dependent on CD4+ T cells, B7-dependent co-stimulation, CD40/CD40L and ICOS/ICOSL interactions (163). Collectively, these studies utilizing intact Pn14 and MenC demonstrate that the bacterial sub-capsular domain markedly influences the Ig response to the associated PS, relative to that observed using isolated PS (111). However, they leave unresolved whether differences observed in PS-specific Ig responses between distinct intact extracellular bacteria reflect intrinsic differences in their biochemically unique capsular PS, and/or the nature of the PS attachment to, or composition of, the underlying sub-capsular bacterial domain.

In this regard, capsular PS expressed by GP bacteria are covalently attached to an underlying thick peptidoglycan cell wall to which a number of immunogenic proteins are also covalently linked (40, 41), whereas in GN bacteria, PS are covalently attached to the acyl glycerol moiety of the outer membrane that contains LPS and immunogenic proteins (66, 164). PS may also exhibit intrinsic features that potentially impact on the elicited immune response. Thus, PPS14 can bind to SIGN-R1, a scavenger receptor present on marginal zone macrophages (118), while MCPS has unique immunomodulatory properties that can impact on the MCPS-specific Ig response (121). In addition, the physical and chemical nature of the capsular PS such as the molecular weight (119), charge (11) or sialic acid content (120) could also influence the induction of PS-specific Ig. In light of these potentially unique contributions of biochemically distinct capsular PS and/or unique bacterial attachments, to the PS-specific Ig response to an intact bacterium, it remains of interest whether different bacterial sub-capsular domains themselves can exert differential effects on PS-specific Ig responses between distinct bacteria.

In the present study, we used intact heat-killed group B *Streptococcus* type III (GBS-III), a GP bacterium, to initially determine the nature of the murine *in vivo* IIIPS-specific Ig response relative to that observed using isolated IIIPS antigen. Further, to directly assess the potential contribution of the sub-capsular bacterial domain on the associated PS-specific responses between two distinct intact bacteria, we took advantage of the previous demonstration that the core IIIPS antigen of the IIIPS capsule expressed by GBS-III is biochemically identical to PPS14 expressed by Pn14, differing from the native IIIPS in lacking the terminal sialic acid (165). Thus, immunization with either conjugated or unconjugated IIIPS vaccines produce IgG that cross-react with PPS14 (166). In particular, we utilized a mutant GBS-III strain (COH1-11) that expresses the desialylated IIIPS that is identical to PPS14. In this manner, we were able to

directly determine whether two distinct GP bacteria (Pn14 and GBS-III) expressing identical PS with similar attachments to the underlying sub-capsular bacterial domain, potentially elicit distinct PS-specific IgG responses *in vivo*.

3.2 Materials and methods

Mice. BALB/c mice and athymic nude (BALB/c background) mice were purchased from the National Cancer Institute (Frederick, MD). Female mice were used between 7 and 10 weeks 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 Care and Use Committee.

Bacterial strains. Group B *Streptococcus* (*Streptococcus agalactiae*) type III (GBS-III) [strain M781] was obtained from American Type Culture Collection [Manassas, VA] (ATCC BAA-22). Another GBS strain COH1 expressing the same capsular PS type III and two isogenic COH1 mutants designated as COH1-13 lacking a capsule and COH1-11 expressing the desialylated type III capsule that is similar to the PPS14 capsule of Pn14, were also used in this study (167). All three COH strains were a kind gift of Dr. Craig Rubens of Children's Orthopedic Hospital (Seattle, WA). *Streptococcus pneumoniae*, capsular type 14 (strain R614) was prepared as described previously (109). The isogenic non-encapsulated mutant of D39 (Pn, capsular type 2), [strain R36A]) was provided by Dr. David Briles (University of Alabama at Birmingham, Birmingham, AL). Lyophilized or frozen stocks of bacteria were grown overnight on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies on blood agar were grown in Todd Hewitt Broth media (BD Biosciences, San Jose, CA) to mid-log phase, collected, and heat-killed by incubation at 65°C for 2 h or inactivated by overnight U.V. irradiation. 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 -20°C until their use as immunogens for mouse immunizations.

Reagents. Purified capsular PS from GBS-III **(**IIIPS) was isolated and purified from strain M781 (168). The conjugates IIIPS-HSA and III-rAlp3 were made respectively, as previously described (166, 169). Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from ATCC. Rat IgG2b anti-mouse CD4 mAb (clone GK1.5) was purified from ascites by ammonium sulfate precipitation and passage over a protein G column. Purified polyclonal rat IgG was purchased from Sigma (St. Louis, MO). Hamster IgG anti-mouse CD40L mAb (clone MR1), polyclonal hamster IgG, rat IgG2a anti-mouse CD275 mAb (clone HK5.3), rat IgG2a isotype control (clone 2A3), hamster IgG2 anti-mouse CD80 (B7-1) mAb [clone 16-10A1], and rat IgG2a anti-mouse CD86 (B7-2) mAb [clone GL-1] were purchased from BioXcell (West Lebanon, NH). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Frederikssund, Denmark). A stimulatory 30mer CpG-ODN was synthesized as previously described (126).

Preparation of GBS whole protein extract. Fifty ml of live unencapsulated GBS-III (strain COH1-13) or unencapsulated Pn14 (strain R36A) bacteria were centrifuged at 3000 rpm for 20 min and the supernatant fluid was discarded. About 5 ml of B-PER bacterial protein extraction reagent from Pierce (Rockford, IL), was added to the bacterial pellet (1:10 ratio), mixed well by shaking and incubated at RT for 15 min. The treated bacterial culture was again centrifuged at 3000 rpm for 10 min. The supernatant fluid containing the soluble proteins was obtained and the concentration was determined by the BCA assay from Pierce (Rockford, IL) as per the manufacturer's protocol.

Growing of R614PC- and R614Ch- bacteria. Chemically defined media (CDM) was prepared as described previously (170). Actively growing R614 (O.D. of 0.45 at 600nm) in Todd-Hewitt broth media was diluted 1:1 ratio in CDM containing 0.1% choline chloride (CC), 0.03% ethanolamine and 0.073% Cysteine hydrochloride. The bacteria were subcultured atleast four times to adapt them in CDM. Thereafter, the amount of CC added was titered from 0.1% to 0.01%, then down to 0.001% and sequentially diluted until 0.000,001% CC was reached. The amount of ethanolamine was kept constant as 0.03% in all the steps. Live bacteria were frozen after every step. Thus, the R614 bacteria were slowly adapted to grow in the ethanolamine media with little or without choline chloride. The bacteria were then heat killed at 60ºC in the water bath for 2 hr and aliquoted and stored at -20ºC. The absence of PC in R614 grown in CDM-ethanolamine media (R614PC-) was confirmed by ELISA.

Live R614 growing in Todd-Hewitt broth media in the log phase was centrifuged at 3000 rpm for 30 min and washed with PBS twice. The bacterial pellet was resuspended in 200 ml of PBS solution containing 0.25M Sodium chloride (NaCl) + 2% CC and incubated at RT for 20 min. The choline binding proteins in the bacteria competitively bind with the choline in the media. The culture was then washed with PBS twice. The bacteria were then heat killed at 60ºC in the water bath for 2 hr and aliquoted and stored at -20ºC. The bacterial stock obtained (R614Ch-) did not contain any choline binding proteins, although PC was held intact.

Immunizations. Mice (n=7/group) were immunized i.p. with 1 x 10^9 colony forming units (CFU) of heat-killed or UV-inactivated GBS-III in saline (2 x 10^8 CFU) or 10 µg of purified IIIPS in saline, or 1 µg of IIIPS-rAlp3 adsorbed on 13 µg of alum mixed with 25 µg of

CpG-ODN. Serum samples for measurement of anti-protein and anti-PS Ig isotype titers by ELISA were prepared from blood obtained through the tail vein.

ELISA. For measurement of serum titers of IIIPS-specific Ig, Immulon 4 ELISA plates were coated with IIIPS-HSA (1 μg/ml, 100 μl/well) in PBS overnight at 4°C. To measure the serum titers of whole GBS-protein-specific Ig, the plates were coated with GBS protein extract (30 μg/ml, 50 μl/well). Plates were then washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 2 h at 37°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS + 1% BSA were incubated overnight at 4°C and plates were then washed 3x with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM, IgG, IgG3, IgG1, IgG2b, or IgG2a Abs (200 ng/ml final concentration) in PBS + 1% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were washed 3x 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 $MgCl_2$ [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). Serum titers of PPS14-specific Ig were measured as described previously (106).

Flow cytometry Flow cytometric studies were performed as described in Chapter 2.

Inhibition ELISA Immulon 4 ELISA plates were coated with IIIPS-HSA (1 μg/ml, 100 μl/well) in PBS overnight at 4°C. Plates were then washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 2 h at 37°C. Serum samples in PBS + 1% BSA were preincubated overnight at 4°C with 10 μg/ml of PPS14 before adding them to the IIIPS-HSA coated plates. Plates were then washed thrice with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG (200 ng/ml final concentration) in PBS + 1% BSA

were then added, and incubated at 37°C for 1 h. Plates were washed thrice 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 $MgCl_2$ [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).

ELISA to check the presence of PC and PspA in R614PC- and R614Ch- To ensure the presence or absence of PC and PspA in the R614PC- and R614Ch- bacteria, the ELISA plates were coated with 1/5 dilution of heat killed $1*10^9$ CFU/ml stock of R614 or R614PC- or R614Ch- bacteria in the first well and then serially diluted 5 times in the subsequent wells. Plates were incubated ON at 4º C. Plates were then washed 3x with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 2 h at 37°C. About 1µg/ml of anti-PC IgG2a mAb or anti-PspA IgG mAb (clone DC-10) or anti-PPS14 IgG mAb (clone 44.1) were added and incubated overnight at 4°C and plates were then washed 3x with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG Ab (200 ng/ml final concentration) in PBS + 1% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were washed 3x 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 $MgCl_2$ [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).

Statistics. Serum Ig isotype titers were expressed as geometric means ± SEM of the individual serum Ig isotype titers. Significance was determined by the Student's *t* test. P-values of ≤ 0.05 were considered statistically significant. All experiments were performed at least twice.

3.3 Results

Intact GBS-III elicits a boosted secondary IIIPS-specific IgG response similar to a IIIPS conjugate vaccine.

Earlier studies demonstrated that the PPS14-specific IgG response to intact Pn14 is distinct from that of a PPS14 conjugate vaccine, in that Pn14 elicits more rapid primary kinetics, and fails to boost serum titers of PPS14-specific IgG following secondary immunization, despite the primary response being CD4+ T cell dependent (39, 104). As discussed above, we wanted to compare the PS-specific Ig response to Pn14 with that elicited by another intact GP bacterium, GBS-III. We began by comparing the *in vivo* IIIPS-specific Ig response to intact GBS-III relative to that induced by a IIIPS-rAlp3 conjugate vaccine (169). Alps (alpha-like proteins) are a family of related surface-anchored proteins with large numbers of repeat units that are expressed by most GBS strains, with Alp3 expressed on most isolates of GBS-V and GBS-VIII (171). BALB/c mice were immunized i.p. with either U.V.-inactivated GBS-III (strain M781) in saline or IIIPS-rAlp3 conjugate (1 μg/mouse) in alum + CpG-ODN, and boosted 28 days later in a similar manner. The primary IIIPS-specific IgG response to intact M781 exhibits relatively rapid kinetics of induction with peak titers observed by day 7, similar to that elicited by IIIPS-rAlp3, but distinct from the much slower rate of induction of the rAlp3-specific IgG response to the conjugate (Fig. 19). Further, M781, in addition to IIIPS-rAlp3, elicits a secondary IIIPS-specific IgG response that is ~10-fold higher than the primary, similar to the level of boosting of the secondary IgG anti-rAlp3 response to the conjugate. The primary IIIPS-specific IgM response to M781 also peaked early (day 7) with modest, but transient, secondary boosting. Thus, intact

GBS-III, like Pn14 generates a relatively rapid primary PS-specific IgG response, but in contrast to that previously reported for Pn14, elicits a highly boosted secondary PS-specific IgG response, similar to that observed for a conjugate vaccine.

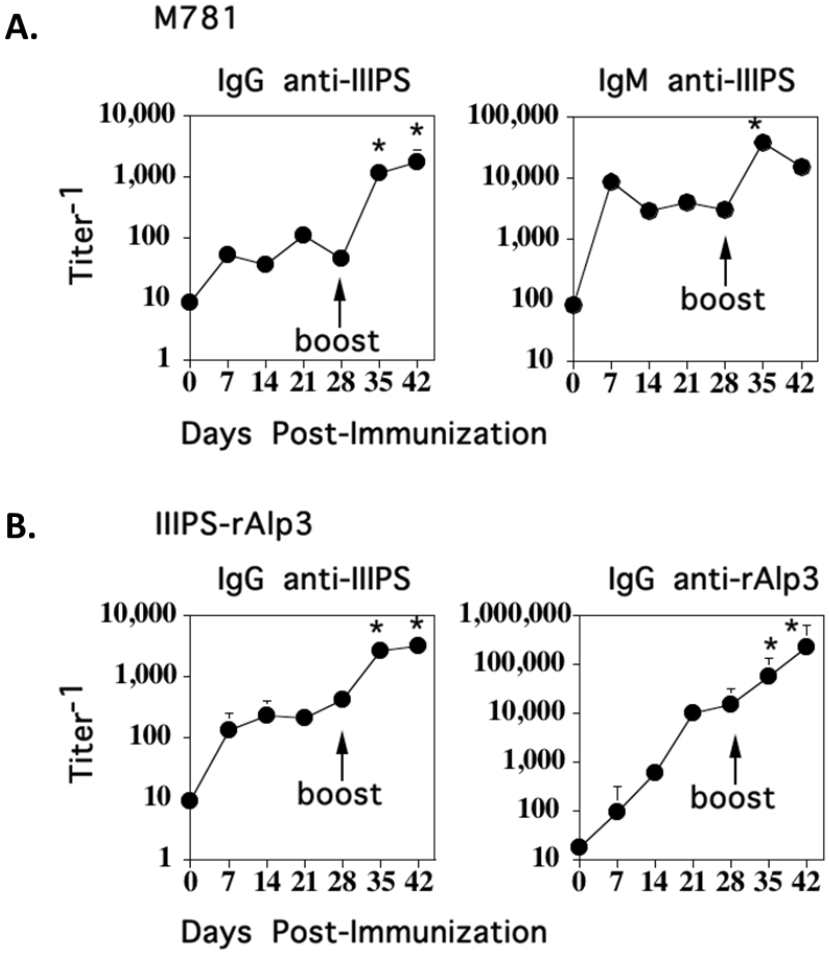
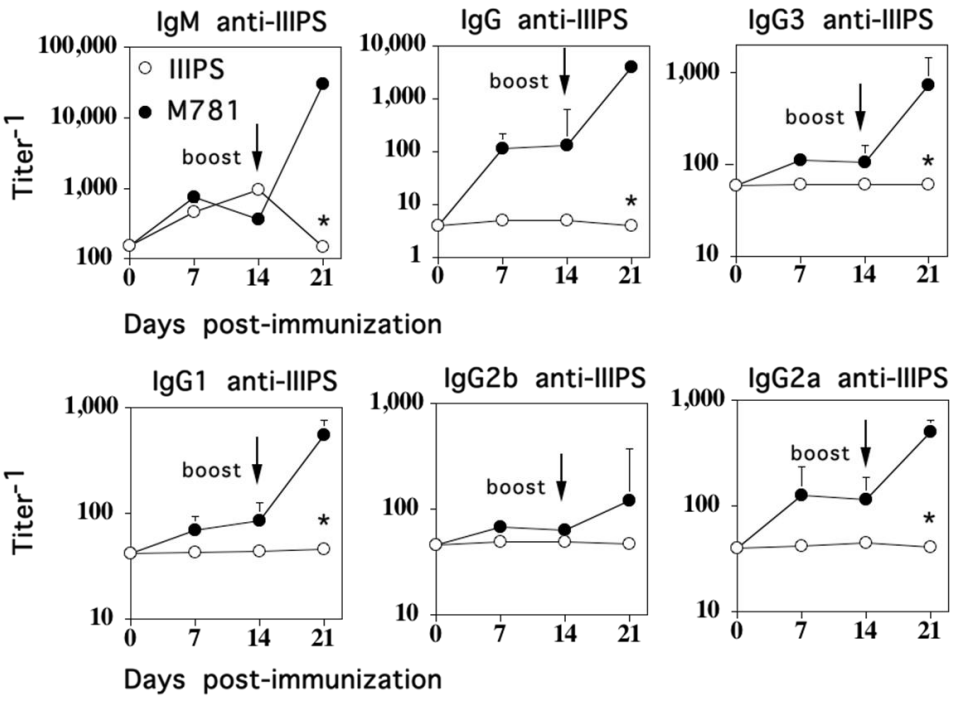


Fig 19. Intact GBS-III elicits a boosted secondary IIIPS-specific IgG response similar to a IIIPS conjugate vaccine. BALB/c mice (7 per group) were immunized i.p. with 2 x 10^8 CFU/ml of U.V.-inactivated intact GBS-III (strain M781) in saline **(A)** or 1 μg of IIIPS-rAlp3 in alum + CpG-ODN **(B)**, and boosted i.p. with the same dose on day 28. Serum titers of Ag-specific IgM and IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between secondary titers relative to peak primary titers.

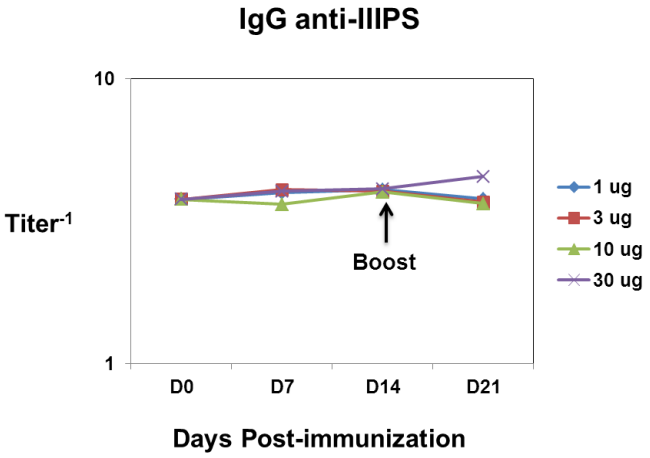
The IIIPS-specific IgG responses to intact GBS-III versus isolated IIIPS are distinct.

Isolated non-zwitterionic PS are unable to recruit CD4+ T cell help and thus typically induce primarily IgM, with relatively minor IgG responses following primary immunization. These PS also fail to induce IgG memory upon secondary challenge (14), although IgM memory responses to these antigens have been observed (137). In this regard, we directly compare the IIIPS-specific Ig response to isolated IIIPS versus intact GBS-III to determine the impact of the bacterial sub-capsular domain on the associated PS-specific Ig response. Mice were immunized with either isolated IIIPS or heat-inactivated M781 in saline and boosted on day 14 in a similar fashion. As illustrated in Fig. 20A, the primary IgM responses to both isolated IIIPS and M781 were similar in titer and peaked by day 7. Whereas secondary immunization with isolated IIIPS produced no further increases in serum titers of IIIPS-specific IgM, M781 induced a boosted secondary response. No detectable primary or secondary IIIPS-specific IgG or IgG isotype response to isolated IIIPS was observed. In contrast, M781 induced a significant primary IIIPS-specific IgG response, peaking by day 7, with ~30 fold increase in titers following secondary immunization. Secondary IIIPS-specific IgG in response to M781 comprised boosted serum titers of IgG3, IgG1 and IgG2a, with little if any IgG2b. Immunization of mice with 1, 3, 10, or 30 μg of isolated IIIPS in saline essentially generated similar results as shown in Fig. 20B. Thus, the IIIPS-specific Ig response to intact GBS-III is distinct from that elicited by purified IIIPS, demonstrating the significant impact of the bacterial sub-capsular domain on the associated PS-specific IgG response, in agreement with our previous studies utilizing intact Pn14 and MenC .

A.



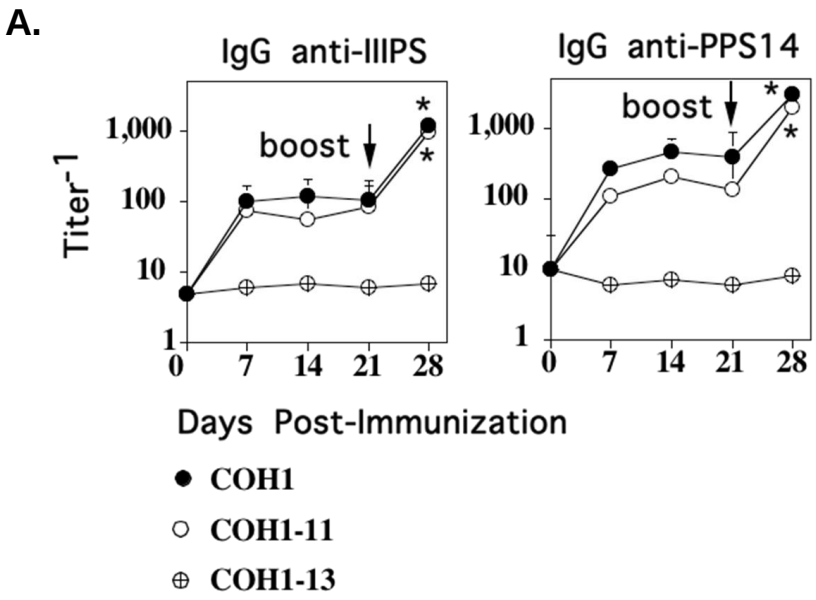
B.



<u>Fig 20. The IIIPS-specific IgG responses to intact GBS-III versus isolated IIIPS are distinct</u>. A. BALB/c mice were immunized i.p. with 1 x 10^9 CFU/ml of heat- inactivated intact GBS-III (strain M781) or purified IIIPS in saline, and boosted i.p. with the same dose on day 14. Serum titers of IIIPS-specific IgM, IgG, and IgG subclasses (IgG3, IgG1, IgG2b and IgG2a) were determined by ELISA. Significance (*) $p \leq 0.05$ between M781 and IIIPS. **B.** BALB/c mice immunized with 1, 3, 10 or 30 µg of IIIPS and boosted on D21. Serum titers of IIIPS-specific IgG was determined by ELISA

The IIIPS-specific IgG elicited in response to intact GBS-III cross-reacts with PPS14.

It has been reported that individuals immunized with either unconjugated or conjugated IIIPS vaccines produce antibodies that cross-react with PPS14 (166). In the present experiment, we wished to determine this potential cross-reactivity of the IIIPS-specific Ig response using three different strains of GBS-III: 1) COH1 that expresses the native type III capsule, 2) COH1-11, an isogenic mutant of COH1 that expresses the desialylated type IIIPS which is identical to the PPS14 capsule of Pn14, and 3) COH1-13, an isogenic mutant of COH1 that lacks the capsule. Mice were immunized with heat-killed COH1, COH1-11 or COH1-13 and boosted on day 21 in a similar fashion. As illustrated in Fig. 21, the primary IIIPS-specific IgG response to intact COH1 and COH1-11 peaked by day 7 with >10-fold enhancements following secondary immunization, similar to that observed in Figs. 19 and 20A. In addition, coating of ELISA plates with purified PPS14, instead of IIIPS demonstrates a primary and secondary PPS14-specific IgG response to both intact COH1 and COH1-11, which is similar to that observed for IIIPS-specific IgG. Indeed, pre-incubating COH1- and COH1-11-induced immune sera with PPS14 largely abrogates the detection of IIIPS-specific IgG using the IIIPS-specific ELISA as shown in Fig. 21B. Thus, these data indicate that the majority of the IIIPS-specific IgG are directed towards the type III core antigen that is identical to PPS14. The COH1-13 bacterial strain that lacks capsular PS fails to induce any detectable serum titers of IIIPS-specific or PPS14-specific IgG, thus establishing the specificity of the ELISA assay for the capsular PS.



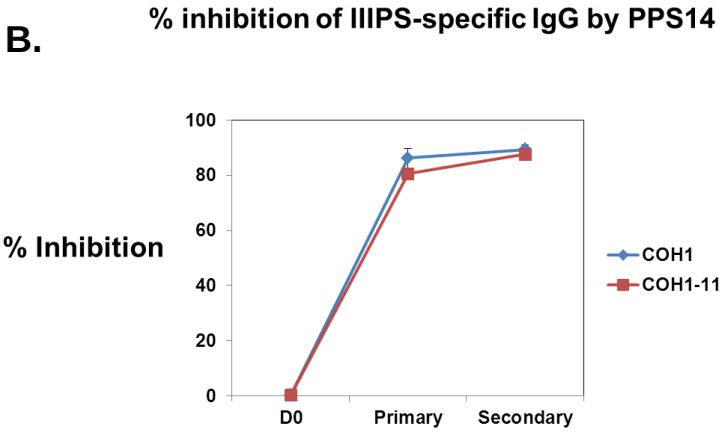


Fig 21. The IIIPS-specific IgG elicited in response to intact GBS-III cross-reacts with PPS14. A. BALB/c mice were immunized i.p. with 1 x 10^9 CFU/ml of heat- inactivated intact GBS-III (strains COH1, COH1-11 or COH1-13) in saline and boosted i.p. with the same dose on day 14. Serum titers of IIIPS-specific and PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between secondary titers relative to peak primary titers. **B.** The percent inhibition of IIIPS-specific IgG binding of sera from GBS-III (strains COH1 or COH1-11) immunized mice onto the IIIPS-HSA coated plates in the presence of the inhibitor PPS14 were determined by inhibition ELISA.

The primary and secondary IIIPS- and PPS14-specific IgG responses to GBS-III are dependent upon CD4+ T cell help and CD40L-dependent co-stimulation.

While the PS-specific Ig responses to most isolated PS are TI, studies using intact Pn14 demonstrated dependence on CD4+ T cells, as well as CD40L, for the primary PPS14-specific IgG response (39, 104). Further, studies using intact MenC have shown that the secondary, but not primary, MCPS-specific IgG response is also dependent on CD4+ T cells and CD40L (163) . In this regard, we wished to determine the role of CD4+ T cells and CD40L in the induction of the IIIPS-specific and PPS14-specific IgG responses to intact GBS-III. To determine this, BALB/c mice were injected with an anti-CD4+ T cell-depleting mAb (clone GK1.5) or control polyclonal rat IgG 24 h before immunization with heat-inactivated GBS-III. Flow cytometric analysis confirmed that 95% of the CD4+ T cells were specifically depleted after 24 h, in the anti-CD4 mAb-injected group as shown in Fig. 13A. Mice were boosted on day 14 in the absence of anti-CD4 mAb or control polyclonal rat IgG. In addition, athymic nude mice (BALB/c background), which are markedly deficient in T cells, were also immunized at the same time with GBS-III and boosted similarly on day 14. As illustrated in Fig. 22A, the primary IIIPS-specific and PPS14-specific IgG responses to M781 in mice injected with control rat IgG peaked by day 7 with a >10-fold boost following secondary immunization. In contrast, both the anti-CD4 mAb-injected mice and athymic nude mice, showed a nearly complete abrogation of the primary and secondary IIIPS- specific and PPS14-specific IgG responses compared to the control group, establishing their critical dependence on CD4+ T cells.

The induction of CD40L on activated CD4+ T cells is critical in mediating TD humoral immune responses, including induction of isotype switched antibodies, germinal center formation and the generation of memory, through delivery of co-stimulatory signals via CD40 on

APC (130). Preventing CD40/CD40L interactions *in vivo* through the use of blocking antibodies largely abolishes TD humoral immunity (172). To determine the role for CD40L co-stimulation on the IIIPS- and PPS14-specific IgG responses to intact GBS-III, BALB/c mice were injected with a blocking anti-CD40L mAb (clone MR1) or polyclonal hamster IgG as a control, 24 h before immunization with intact heat-killed GBS-III. Mice were boosted in a similar fashion on day 14 in the absence of blocking anti-CD40L mAb or control IgG. As shown in Fig. 22B, both the primary and secondary IIIPS-specific and PPS14-specific IgG responses were significantly inhibited compared to the control (primary: 4.6-7.0-fold reduction; secondary: 3.8-7.9-fold reduction). Of interest, blocking of CD40L did not prevent boosting of the IgG responses to either IIIPS or PPS14 following secondary immunization, although the serum titers were significantly reduced compared to the control. Collectively these data demonstrate that the primary and boosted secondary PS-specific IgG responses to intact GBS-III require CD4+ T cell help as well as CD40L-dependent co-stimulation.

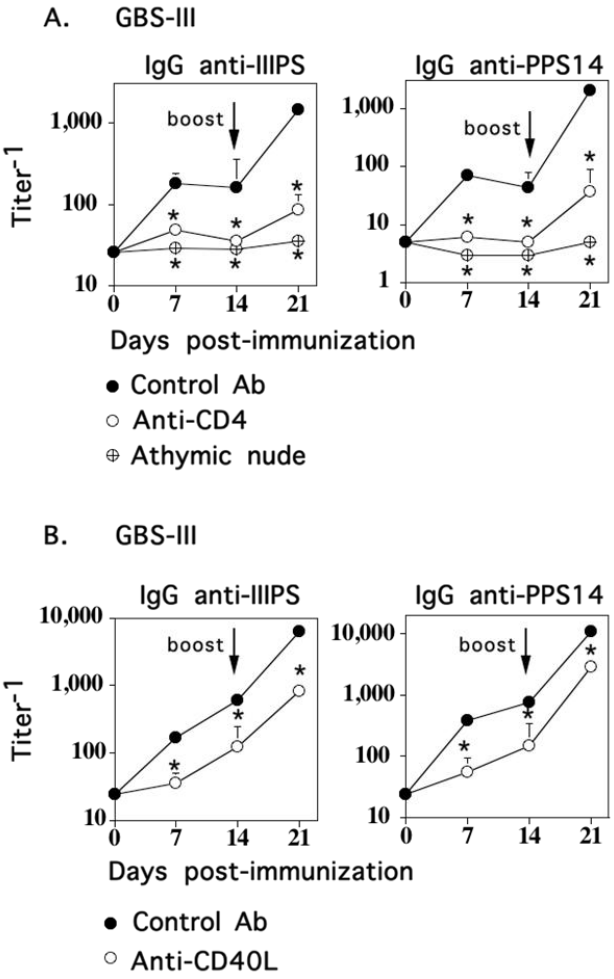


Fig 22. The primary and secondary IIIPS- and PPS14-specific IgG responses to GBS-III are dependent upon CD4+ T cell help and CD40L-dependent co-stimulation. A. BALB/c mice (7 per group) were injected i.p. with either depleting anti-CD4 mAb (clone GK1.5) or control polyclonal rat IgG (0.5 mg/mouse) 24 h before immunization. Both athymic nude mice and Ab-injected mice were immunized i.p. with 1 x 10^9 CFU of GBS-III and boosted on day 14 in the absence of depleting antibodies. **B.** BALB/c mice were injected i.p. with either blocking anti-CD40L mAb (clone MR1) or control polyclonal hamster IgG (0.5 mg/mouse) 24 h before i.p. immunization with 1 x 10^9 CFU of GBS-III and boosted similarly on day 14 in the absence of antibodies. Serum titers of IIIPS- and PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between control versus Ab-injected mice or athymic nude mice.

B7- and ICOS-dependent co-stimulation is required for optimal induction of PS-specific IgG responses to intact GBS-III.

CD28 constitutively expressed on CD4+ T cells binds to CD80/CD86 (B7-1/B7-2) expressed on APCs and this interaction is critical for the initiation of CD4+ T cell activation (84, 173). We previously demonstrated that the primary PPS14-specific IgG response to intact Pn14, and the secondary, but not primary, MCPS-specific IgG response to MenC, is dependent on CD28 (106). In this regard, we examined the role of CD28-mediated signaling in the PS-specific response to intact GBS-III. Therefore, mice were injected with blocking anti-CD80 (clone 16-10A1) and anti-CD86 (clone GL-1) mAbs or polyclonal hamster and rat IgG as a control, 24 h before immunization with intact GBS-III, with secondary immunization on day 14. As illustrated in Fig. 23A, the primary and secondary IIIPS- and PPS14-specific IgG responses to GBS-III were markedly inhibited in the presence of blocking mAbs compared to the control (primary: 8.2-17.3-fold reduction; secondary: 14.9-133-fold reduction).

Inducible costimulator (ICOS), a member of the CD28 family, induced on CD4+ T cells upon TCR crosslinking and CD28-mediated signaling, binds to ICOSL expressed on APC (84, 85). ICOS is the key regulator of germinal center formation and immunological memory (88, 89). Our earlier studies using Pn14 demonstrated that the PPS14-specific IgG response is ICOS-independent, while the secondary, but not primary, MCPS-specific IgG response to MenC required ICOS-co-stimulation. In light of the boosted secondary PS-specific IgG responses to GBS-III, we determined whether or not it was ICOS-dependent. Therefore, we injected BALB/c mice with anti-ICOSL mAb (clone HK5.3) or control polyclonal rat IgG, 24 h before immunization with intact GBS-III. Mice were boosted on day 14 in the absence of anti-ICOSL mAb or control Ab. As shown in Fig. 23B, the secondary, but not primary, IIIPS-specific and

PPS14-specific IgG responses to GBS-III were significantly, although not completely, inhibited in anti-ICOSL mAb-injected mice (3.2-3.3-fold reduction), whereas a near-complete abrogation of the secondary protein-specific IgG response was observed. Thus, these data demonstrate that B7-dependent, T cell co-stimulation is critical for induction of primary and boosted secondary IIIPS-specific and PPS14-specific IgG responses to intact GBS-III. Further, the importance of ICOSL-dependent co-stimulation for the boosted PS-specific IgG response to GBS-III supports the notion that GBS-III induces PS-specific IgG memory, likely dependent on GC formation.

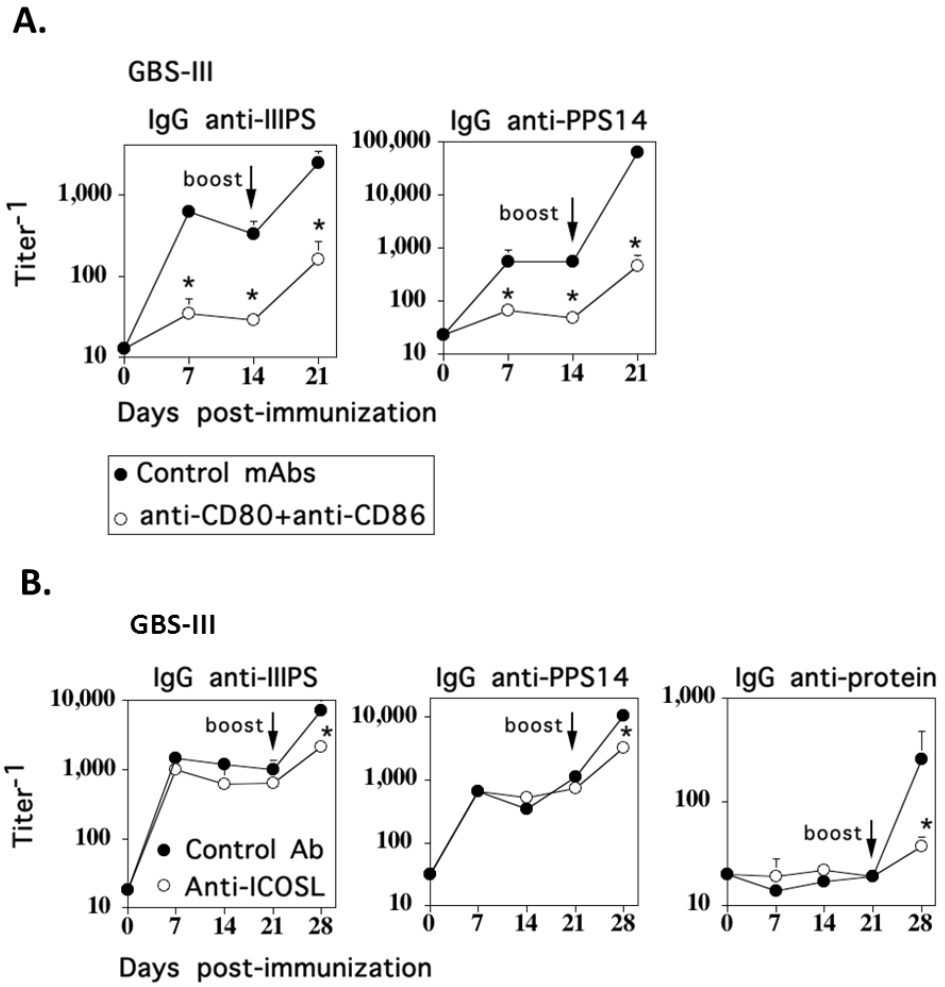


Fig 23. B7- and ICOS-dependent co-stimulation is required for optimal induction of PS-specific IgG responses to intact GBS-III. A. BALB/c mice were injected i.p. with either blocking hamster anti-CD80 mAb (B7-1) (clone 16-10A1) and rat anti-CD86 mAb (B7-2) (clone GL-1) or control polyclonal hamster and rat IgG 24 h before i.p. immunization with 1 x 10^9 CFU of GBS-III. Mice were boosted similarly on day 14 in the absence of antibodies. Serum titers of IIIPS- and PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between control versus anti-CD80- and anti-CD86 mAb-injected mice. **B.** BALB/c mice were injected i.p. with either blocking anti-ICOSL mAb (clone HK5.3) or control rat IgG2a mAb (clone 2A-3) 24 h before i.p. immunization with 1 x 10^9 CFU of GBS-III. Mice were boosted similarly on day 14 in the absence of antibodies. Serum titers of Ag-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between control versus anti-ICOSL mAb-injected mice.

A boosted secondary PPS14-specific IgG response is elicited by GBS-III in Pn14-primed mice.

Previous studies using intact Pn14 show that Pn fails to generate a boosted PPS14-specific IgG response upon secondary immunization, despite the dependence of the primary response on CD4+ T cells(39). Although these data suggested that Pn14 failed to induce memory for PPS14-specific IgG, an alternative possibility was that Pn14 induced a state of memory, but was unable to elicit a boosted memory response upon secondary immunization, in contrast to GBS-III. Therefore, we sought to determine whether priming of mice with Pn14 (strain R614) or GBS-III (strain COH1-11) followed alternatively by secondary immunization on day 14 with COH1-11 or R614, respectively, would elicit a boosted PPS14-specific IgG response. We utilized strain COH1-11 since it expresses the identical PPS14 capsule as R614. As illustrated in Fig. 24A, primary and secondary immunization with R614, in contrast to COH1-11, failed to elicit a boosted PPS14-specific IgG secondary response, as previously observed. Of note, primary immunization with R614 followed by secondary immunization with COH1-11 resulted in a boosted PPS14-specific IgG response similar to that observed using COH1-11 for both immunizations ($p<.001$). These data thus indicate that R614 can induce memory for PPS14-specific IgG, but fails to elicit a boosted secondary response in R614-primed mice. Of interest, mice primed with COH1-11 followed by secondary immunization with R614 showed a more modest boost in the secondary PPS14-specific IgG response in two identical experiments, relative to COH1-11/COH1-11 and R614/COH1-11 immunized mice. In light of this cross-priming with two different bacteria, we wanted to test the presence of cross reactive B cell epitopes on these two bacterial cell surfaces. Therefore, sera from R614 and COH1-11 immunized mice were used to evaluate the induction of Anti-Pn whole protein specific IgG and

anti-GBS whole protein specific IgG antibodies. Of interest, as shown in Fig. 25 there were no detectable levels of Pn-protein specific IgG in GBS immunized mice or GBS-protein specific IgG in R614 immunized mice.

We previously demonstrated that primary, followed by secondary, co-immunization of mice with R614 and MenC results in secondary boosting of the PPS14-specific IgG response, suggesting that provision of sufficient MenC-induced innate stimulation during the secondary response might compensate for inadequate signaling or an inhibitory effect of Pn14 (163). Therefore, we wished to determine whether GBS-III could provide a similar compensatory effect. To accomplish this, mice were co-immunized (primary and secondary) with R614 and the unencapsulated GBS-III strain COH1-13, to preclude a PPS14-specific contribution of GBS-III to the PPS14-specific IgG response. As shown in Fig. 24B, co-immunization with R614 and COH1-13 resulted in a significant boost in the secondary PPS14-specific IgG response, although significantly lower than in mice immunized only with the encapsulated COH1-11 strain. Mice immunized with COH1-11 in the presence of unencapsulated Pn14 (strain R36A) also elicited a significantly boosted secondary PPS14-specific IgG response similar to mice co-immunized with R614 + COH1-13, but significantly lower than mice immunized with COH1-11 alone. Collectively, these data suggest that the absence of a boosted secondary PPS14-specific IgG response to Pn14 may reflect an inhibitory effect mediated by this bacterium during secondary immunization of Pn14-primed mice, and that this might be overcome by enhanced innate immune stimulation.

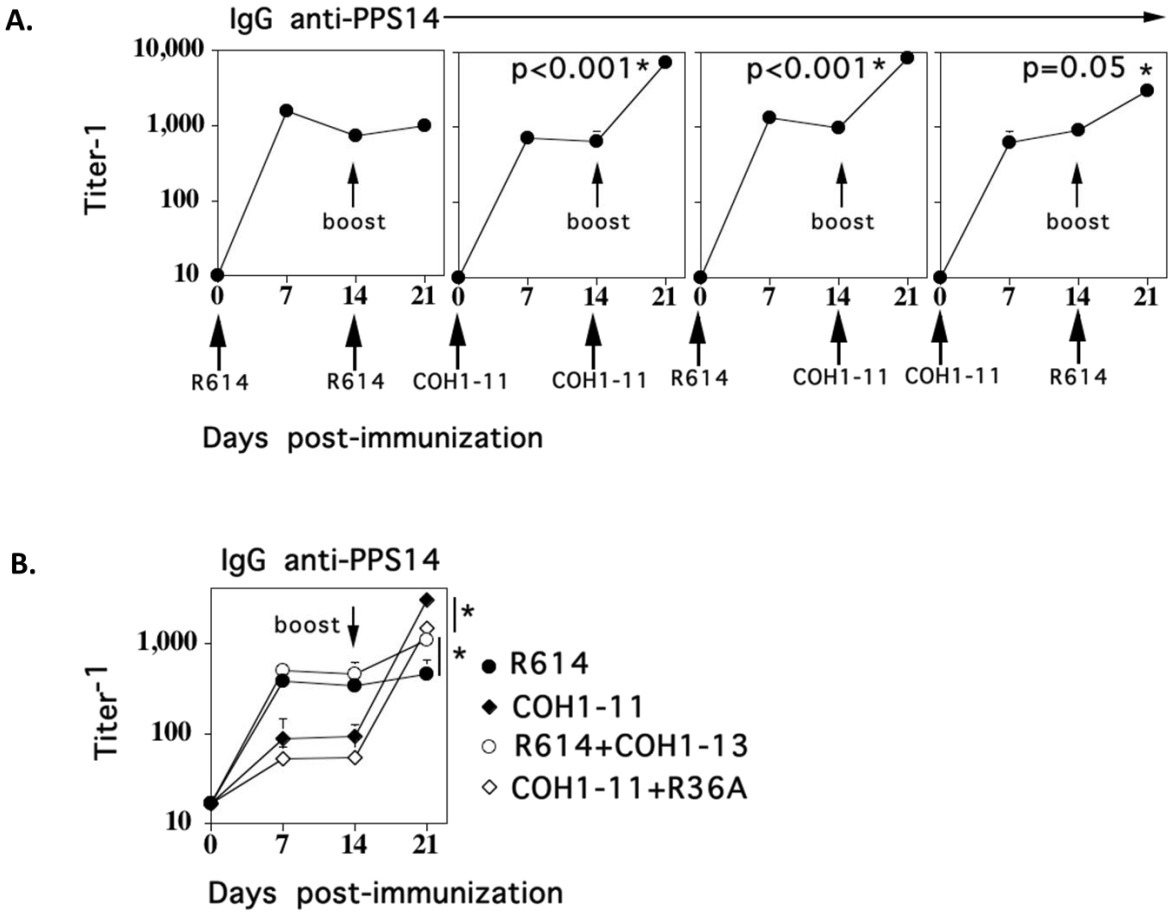


Fig 24. A boosted secondary PPS14-specific IgG response is elicited by GBS-III in Pn14-primed mice. A. BALB/c mice (7 per group) were immunized i.p. on D0 and D14 with 1 x 10^9 CFU of either Pn14 (strain R614) or GBS-III (strain COH1-11) in various combinations as indicated. Serum titers of PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between secondary titers relative to peak primary titers. **B.** BALB/c mice (7 per group) were primed with 5 x 10^8 CFU of heat inactivated Pn14 (strain R614) or 5 x 10^8 CFU of heat inactivated GBS-III (strain COH1-11) or R614+ GBS-III (strain COH1-13) (5 x 10^8 CFU/mouse each) or COH1-11 + Pn2 (strain R36A) (5 x 10^8 CFU each). Mice were boosted in a similar fashion on d14. Serum titers of PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between secondary titers of R614- and R614+COH1-13-immunized groups and secondary titers of COH1-11- and COH1-11+R36A-immunized groups.

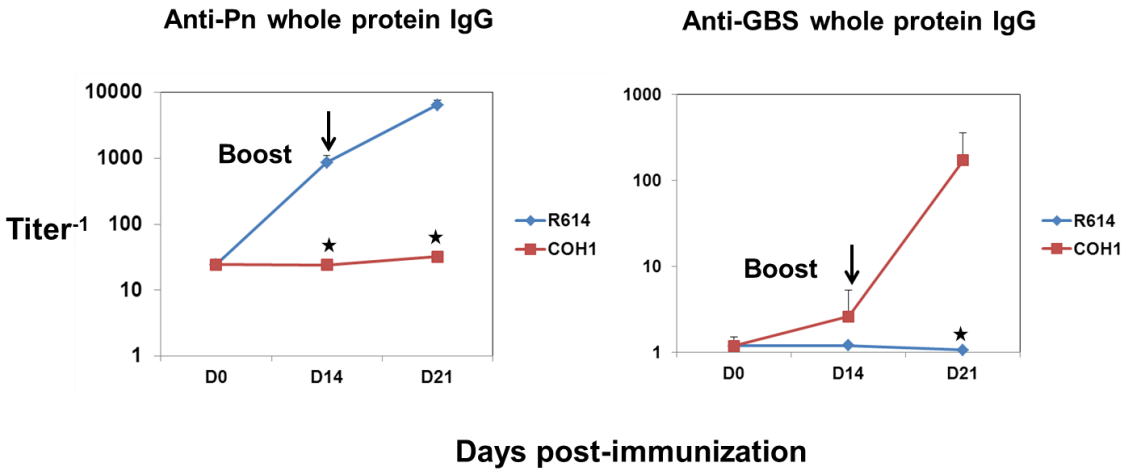


Fig 25. The absence of a detectable anti-Pn whole protein IgG response in GBS-III immunized mice and vice versa BALB/c mice immunized i.p. with 1 x 10^9 CFU of either Pn14 (strain R614) or GBS-III (strain COH1) on D0 and boosted with the same dose respectively on D14. Serum titers of anti-Pn whole protein IgG and anti-GBS whole protein IgG for both Pn14 and GBS-III immunized mice were determined by ELISA using the whole bacterial (Pn and GBS) protein extract as coating antigens.

The role of phosphorylcholine (PC) in the PPS14-specific IgG response to intact Pn.

PC is covalently attached to the cell wall teichoic acid and lipoteichoic acid in Pn (174). The nutritional requirement of Pn for choline can be substituted with ethanolamine (175) and thus Pn strain R614 was depleted of PC (R614PC-) by growing in medium containing ethanolamine. PC-epitopes present on various organisms such as prokaryotic bacteria or eukaryotic parasites are known to have anti-inflammatory properties and immune inhibitory effects (122). In light of the absence of a boosted secondary PS-specific IgG response to Pn, in contrast to GBS-III which did not express PC, we wished to determine the immunomodulatory role of PC in eliciting the PS-specific IgG response. Therefore, we made R614PC- bacteria that lacked PC. Since R614PC- also lacks choline-binding proteins we also prepared control R614 bacteria (naturally containing PC) that selectively lacked choline-binding proteins, through incubation of bacteria with high concentrations of choline as a competitor, which strips choline-binding proteins from the cell wall PC (R614Ch-). The presence or absence of PC and choline binding proteins such as PspA was tested by ELISA. As shown in Fig. 26, R614 was used as a control to measure the levels of PspA, PC and PPS14. R614PC- had very little PC and PspA as compared with the control R614. R614Ch- bacteria had PC levels comparable with the control R614, but the levels of PspA were significantly reduced. This confirms the absence of choline binding proteins like PspA in both R614PC- and R614Ch- bacteria and the absence of PC in R614PC- bacteria. The presence of capsular PPS14 in all these bacteria was confirmed by ELISA.

BALB/c mice (7 per group) were immunized with 1 x 10^9 CFU of R614 or R614PC- or R614Ch- bacteria and boosted with the same dose on D14. As shown in Fig. 27A, R614 induced a peaked primary response by D7 without any secondary boosted PPS14-specific IgG response.

Similarly, the R614PC- also elicited a peaked primary PPS14-specific IgG response by D7, but did not elicit any secondary response, following the boost. R614 and R614Ch- both having PC on their cell wall induced a rapidly peaked anti-PC IgG response without any secondary boosting. However, the R614PC- bacteria that lacked PC induced a markedly lower anti-PC IgG response relative to controls, as expected. We wished to consider if a lower dose of the R614PC- bacteria could result in an induction of the secondary boosted PPS14-specific IgG response. Therefore, BALB/c mice were immunized with 2×10^8 or 5×10^7 CFU of heat killed R614 and R614PC- and boosted with the same dose on D14. R614 as described in previous figures did not induce any boosted secondary PPS14-specific IgG response. However, the R614PC- bacteria induced a peak primary response by D7, and showed only a modest increase in secondary titers following the boost, although this was not statistically signficant (Fig. 27B). The lower doses of R614PC- also induced markedly reduced, but still detectable anti-PC IgG titers. This suggests that the R614PC- bacteria still contained minor amounts of PC that could mediate its inhibitory responses during the secondary immunization in Pn-primed mice. However, the absence of choline binding proteins did not affect the anti-PS Ig responses to intact Pn.

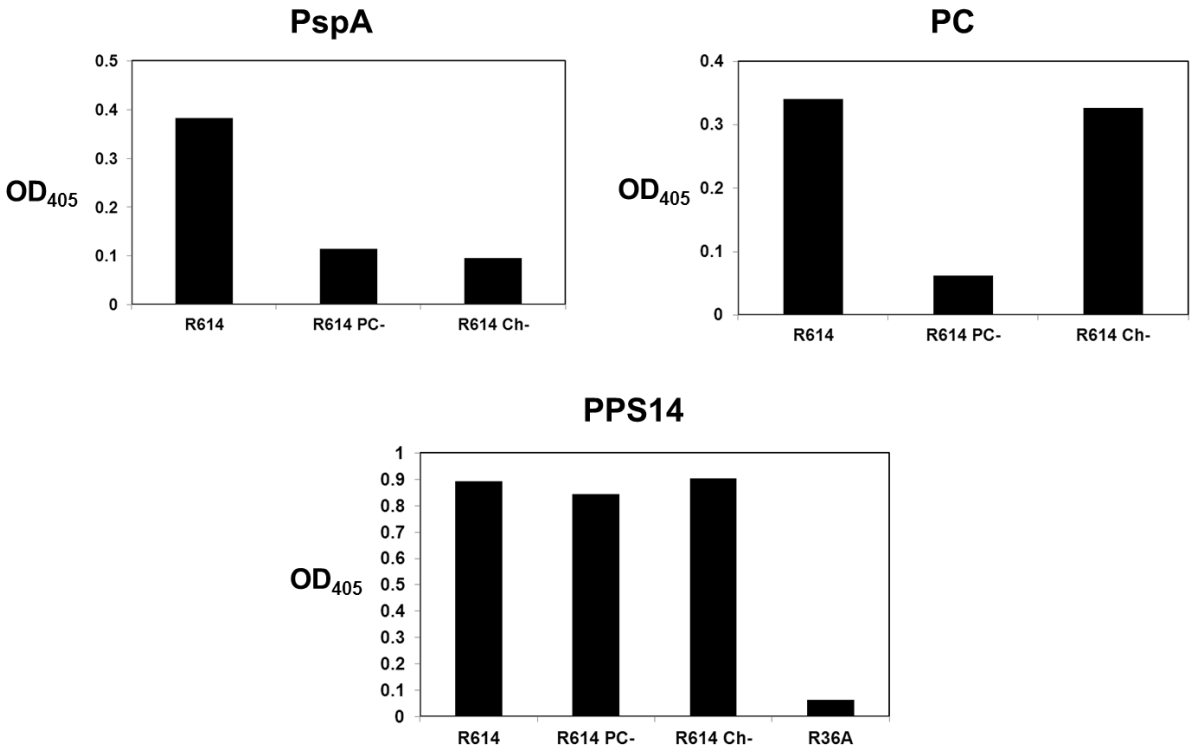


Fig 26. Presence or absence of PC and PspA in R614, R614PC- and R614Ch- bacteria. R614 or R614PC-or R614Ch- bacteria were coated onto the ELISA plates and anti-PspA mAb or anti-PC mAb or anti-PPS14mAb were added to test the presence or absence of PspA, PC and PPS14.

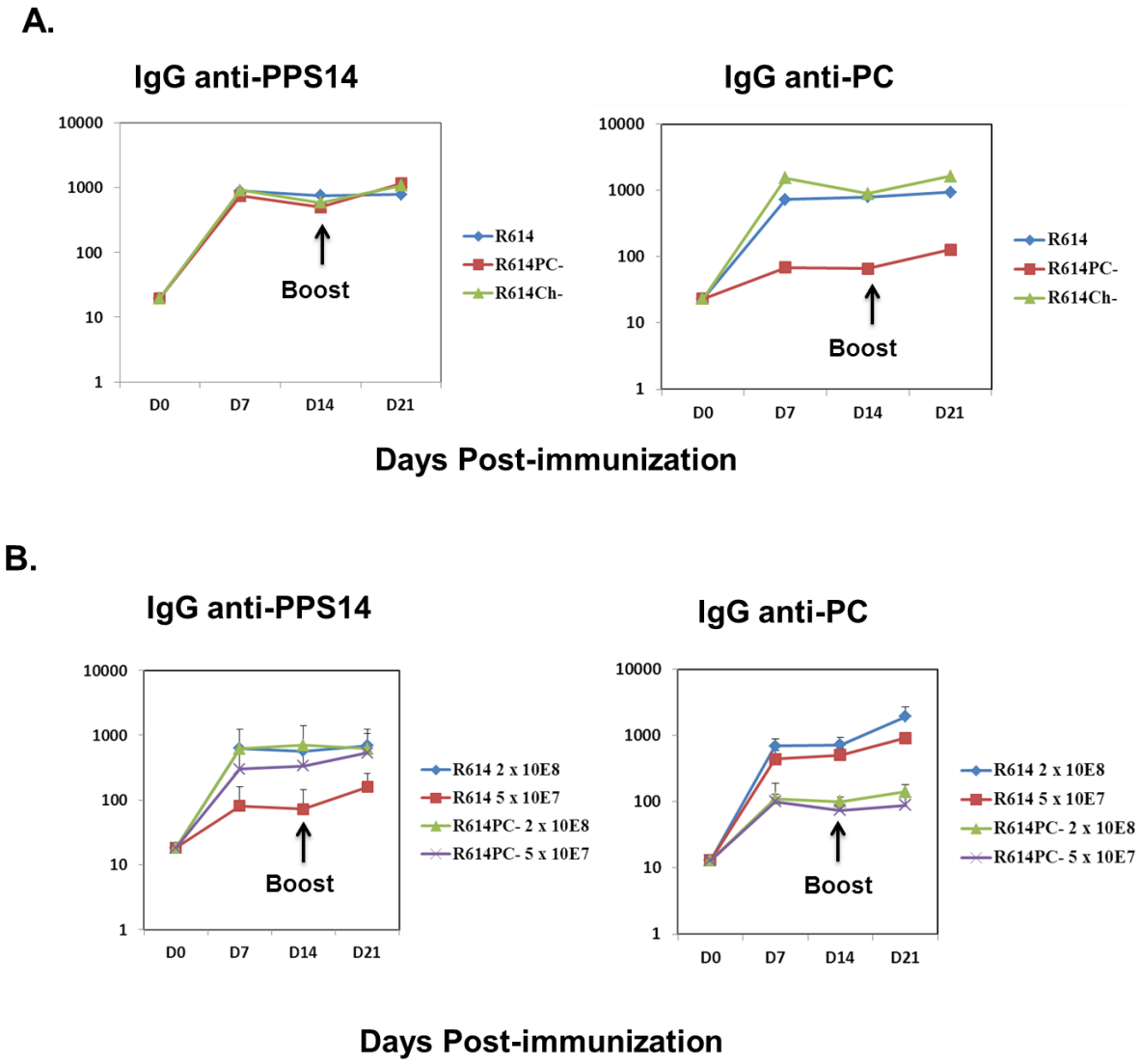


Fig 27. Role of PC in eliciting the PPS14-specific IgG responses to Pn. **A.** BALB/c mice (7 per group) were immunized with 1 x 10^9 CFU of heat killed R614 or R614PC- or R614Ch- bacteria and boosted with the same dose on D14. Serum titers of PPS14-specific IgG and PC-specific IgG were determined by ELISA. **B.** BALB/c mice (7 per group) were immunized with 2 x 10^8 and 5 x 10^7 CFU of heat killed R614 or R614PC- or R614Ch- bacteria and boosted with the same dose on D14. Serum titers of PPS14-specific IgG and PC-specific IgG were determined by ELISA.

The boosted secondary PPS14-specific IgG response to GBS-III requires GBS-III-associated PPS14 and CD4+ T cells during secondary immunization.

The ability of COH1-11 to boost the secondary PPS14-specific IgG response in COH1-11- or R614-primed mice left unresolved whether boosting during secondary immunization simply required BCR crosslinking of PPS14-specific B cells in the absence of a putative inhibitory influence of R614, appropriate non-specific innate stimulation, or both, and whether this effect required CD4+ T cells during secondary immunization. To test this we immunized mice with heat-inactivated COH1-11 or R614 followed by secondary immunization with isolated PPS14 on day 14. As a control group, mice were immunized with isolated PPS14 for both the primary and secondary. In addition, to determine the BCR specificity of the secondary PPS14-specific IgG response, mice were primed with COH1-11 or R614 and boosted with their unencapsulated variants, COH1-13 and R36A respectively. Neither isolated PPS14 nor non-specific stimulation by unencapsulated bacteria was able to elicit a boosted PPS14-specific IgG response in either COH1-11- or R614-primed mice (Fig. 28A). Additionally, as illustrated in Fig. 28B, mice primed with COH1-11 alone followed by secondary immunization with COH1-11 in the presence of depleting anti-CD4 mAb, failed to elicit a boosted PPS14-specific IgG response relative to mice receiving control antibody. Collectively, these data suggest that boosting of the secondary PPS14-specific IgG response in GBS-III-immunized mice requires both PPS14-specific BCR engagement and CD4+ T cells, the latter likely triggered via recognition of bacterial-derived peptides in association with MHC-II. These data also suggest that APC processing of intact Pn14 and GBS-III might generate cross-reactive peptides for the generation of CD4+ T cell help.

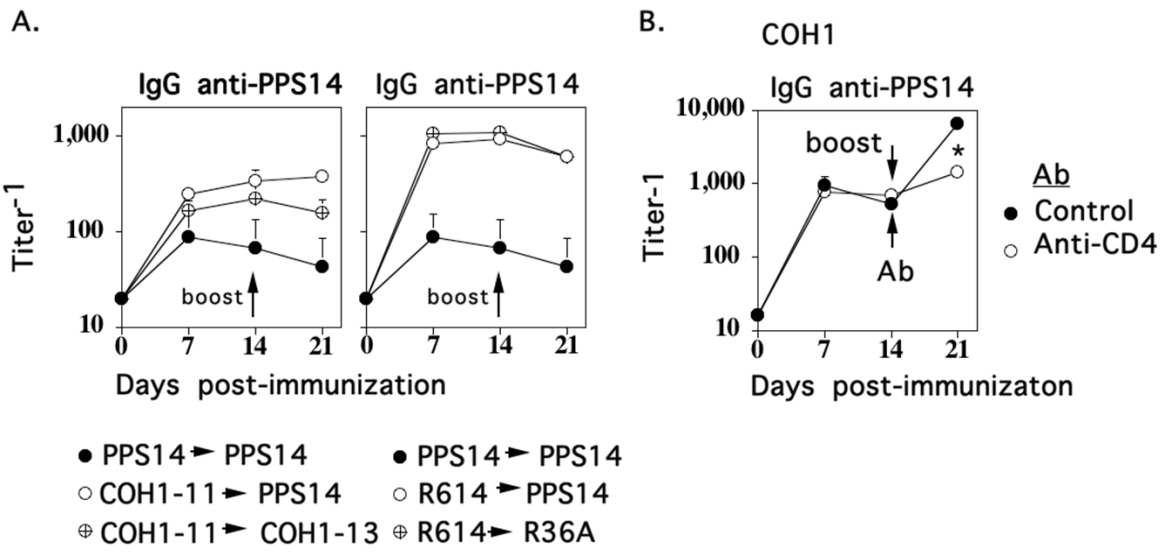


Fig 28. The boosted secondary PPS14-specific IgG response to GBS-III requires GBS-III-associated PPS14 and CD4+ T cells during secondary immunization. A. BALB/c mice (7 per group) were immunized and boosted i.p. on D0 and D14 with either 1 µg of PPS14 or 1 x 10^9 CFU of either Pn 14 (strain R614) or GBS-III (strain COH1-11) as indicated. Serum titers of PPS14-specific IgG were determined by ELISA. **B.** BALB/c mice (7 per group) were immunized i.p. with 1 x 10^9 CFU of heat inactivated GBS-III (strain COH1) on d0 and d14. 0.5 mg of depleting anti-CD4 mAb (clone GK1.5) or control rat IgG was injected on d13. Serum titers of PPS14-specific IgG were determined by ELISA. Significance (*) $p \leq 0.05$ between secondary titers of control group versus anti-CD4-mAb-injected group.

3.4 Discussion

To determine how differences in bacterial composition and structure may potentially impact the nature of PS-specific Ig response to an expressed capsular PS, it was required to study two distinct bacteria with biochemically identical PS capsules having similar bacterial attachments.In this regard, we made use of inactivated intact type III GBS, a GP extracellular bacterium that expresses a capsule containing a core PS (i.e. GBS-III capsular PS without the terminal sialic acid) identical to that of the capsular PS of *S. pneumoniae* type 14. We also employed an isogenic mutant of GBS-III that expresses the core PS alone. *In vivo* IIIPS- and PPS14-specific IgM and IgG responses to intact inactivated GBS-III were compared to those elicited by isolated IIIPS, as well as to intact inactivated Pn14, another GP extracellular bacterium, using the i.p. route of immunization. The use of inactivated, as opposed to viable, intact bacteria was to focus selectively on the properties of these pathogens as complex particulate immunogens, with the understanding that bacterial metabolism and alternate routes of immunization will add additional layers of complexity to the conclusions being drawn.

These data, combined with our previous observations using intact Pn14 and MenC (176) lend further support to the proposal that the bacterial sub-capsular domain converts a normally TI PS-specific Ig response to one that is dependent on CD4+ T cells, including a requirement for B7-, and potentially ICOS-dependent, co-stimulation, and CD40/CD40L interactions. CD4+ T cell help during the primary response to the GP bacteria, GBS-III and Pn14, induces a relatively rapid and robust PS-specific IgG response comprising multiple IgG isotypes (136) (Fig. 19). In contrast, the primary PS-specific IgG response to the GN bacterium, MenC, develops more

gradually and is TI (163). Whether this phenomena represents a more general dichotomy between GP and GN bacteria, remains to be determined. However, the induction of PS-specific IgG memory, a process dependent on CD4+ T cells, can occur following primary immunization with all three bacteria although only MenC and GBS-III elicit a boosted PS-specific IgG response following secondary immunization (163) (Fig. 23). Thus, recruitment of CD4+ T cells may potentially enhance PS-specific adaptive immunity to these pathogens, although as discussed below, the sub-capsular domain of Pn also appears to contain a structure that is immunosuppressive.

The immunologic properties of PS expressed by intact bacteria are thus distinct from those observed using classical TI antigens (14), which in isolation are divorced from the more complex context likely observed during natural infections. The capsular PPS14 by itself may exhibit distinct immunological properties irrespective of the bacteria on which they are expressed. Studies by Koppel et al reported that the interaction of SIGN-R1 expressed by marginal zone macrophages with MZ B cells is essential for the early IgM responses against the encapsulated Pn (53, 177) and is known to be protective against lethal infection in mice and is protective against lethal infection in mice (118).

In particular, the inability of isolated IIIPS to induce a detectable IIIPS-specific IgG response *in vivo* (Fig. 19), is consistent with our earlier *in vitro* studies demonstrating a requirement for a second signal in addition to multivalent BCR cross-linking, for induction of Ig secretion and class switching in response to PS (20). The requisite second signal may comprise a TLR ligand, various inflammatory cytokines, and/or BAFF/TACI, collectively recruited into the PS-specific Ig response by the sub-capsular bacterial domain in the presence of innate immune cells (20, 178, 179). The degree to which natural purified bacterial PS induce IgG response *in*

vivo may depend on the degree of contamination of the PS preparation with activating bacterial cell wall structures. In this report isolated PPS14 in contrast to IIIPS, induced a detectable, though relatively modest, PPS14-specific IgG response. However, we previously demonstrated that this PPS14-specific IgG response was critically dependent upon a contaminating TLR2 ligand, likely derived from the Pn cell wall (155).

The IIIPS of GBS-III is structurally similar to PPS14 except for the sialic acid in the side chain of the repeating pentasaccharide of IIIPS. The potential presence of terminal crossreactivity between the antibodies generated against the capsule of GBS-III to the PPS14 of Pn has been demonstrated (166, 180). In this regard, we confirmed this crossreactivity by using three different mutants of GBS: strain COH1 that has IIIPS capsule, strain COH1-11 that has PPS14 and strain COH1-13 that lacks the capsule. COH1-13 did not elicit any primary or secondary PS-specific IgG response. Both COH1 and COH1-11 showed a rapidly peaked primary and a secondary boosted anti-PPS14 IgG similar to the anti-IIIPS IgG response to intact GBS (Fig. 21). Inhibition studies using PPS14 confirms that most of the IIIPS-specific antibodies generated in response to intact GBS are targeted against the core PS that is similar to PPS14. Fischer et al postulated that this structural and immunochemical similarity between IIIPS and PPS14 could result in a phenomenon where the type-specific antibodies induced by one of these organisms would protect against the disease caused by the other (181, 182).

The dependence of the boosted secondary IIIPS- and PPS14-specific IgG responses to GBS-III on CD4+ T cells, CD40/CD40L interaction, and B7- and ICOS-dependent co-stimulation (Figs. 22 and 23) suggests that PS-specific memory B cells and/or bacterial peptide-specific memory CD4+ T cells may be generated within a germinal center reaction in response to intact GBS-III. The key outcome of the GC reaction is to produce high affinity memory B cells.

Studies by Colino et al in our lab illustrate a lack of affinity maturation despite the boosted PPS14-specific secondary IgG response to COH1-11 as opposed to the conjugate vaccine, PPS14-PspA that produces a high affinity PPS14-specific IgG after the boost. It is possible that in response to intact GP bacteria, the memory B cells are generated by the extrafollicular pathway independent of the GC and affinity maturation (150, 183). The potential role of NKT cell help to B cells as described by Chang et al in such PS-specific Ig responses needs to be explored (184). The stronger reduction of the PPS14- and IIIPS-specific IgG responses to GBS that occurred in the absence of T cells or in the presence of blocking anti-CD80/86 mAbs, in contrast to that resulting from blockade of CD40L or ICOSL, may reflect either incomplete blockade by the mAbs used in the latter responses, or additional helper activities of CD4+ T cells independent of CD40 or ICOS costimulation. However, a more absolute requirement may exist for B7-dependent costimulation for the early activation of T cells.

To determine the differential effect of the subcapsular domain on the PS-specific Ig responses during the primary and secondary immunization, we primed and boosted the mice with GBS and Pn both having the PPS14 capsule in various combinations. The priming of mice with Pn and boosting with GBS induced significantly enhanced secondary PPS14-specific IgG titers compared to the mice group immunized with Pn14 alone (Fig. 24). This suggests that the Pn is able to prime for PS-specific memory, but unable to elicit it. The ability of GBS-III to trigger a boosted PPS14-specific IgG response in Pn14-primed mice, that is dependent on CD4+ T cells (Figs. 24A and 28B), may indicate that these two different pathogens share cross-reactive CD4+ T cell epitopes. This observation is not unprecedented in nature as memory T cells that are specific for one virus can become activated during infection with an unrelated heterologous virus (185). Of interest, we observed no evidence for cross-reactive protein-specific B cell epitopes

between GBS-III and Pn14, in that detectable serum titers of protein-specific IgG, using whole protein extract from the corresponding unencapsulated bacteria for ELISA, are only observed for the homologous bacteria (Fig. 25). Alternatively, it is possible that CD4+ T cells that mediate help for PS-specific IgG responses to intact bacteria are largely specific for PS, as recently illustrated using a conjugate vaccine of IIIPS and carrier protein (149). Nevertheless, the attachment of PS to MHC-II was via carrier-derived peptide, covalently linked to the PS. Thus, PS-specific IgG memory responses to conjugate vaccines appear to require the covalent attachment of PS and protein (35, 131, 139, 149).

However, in bacteria, it does not appear that capsular PS is directly linked to protein via a covalent bond (40, 41). In this regard, in a previous study using intact vaccinia virus, CD4+ T cell help for an *in vivo* IgG response to a specific viral protein was induced only by CD4+ T cells with the same protein specificity as the B cell (142). This requirement for intra-molecular help would thus be satisfied by a soluble PS-protein conjugate vaccine, but not by an intact bacterium. In addition, BCR-mediated, Ag uptake by B cells involves endocytic vesicles that are only 50-150 nm in diameter, and thus would be predicted to exclude intact bacteria (142). However, a single study demonstrated BCR-mediated uptake of intact *Salmonella typhimurium* by human B cells with subsequent display of MHC-II/peptide complexes and primary CD4+ T cell activation (143). Alternatively, PS-specific B cells could potentially acquire bacterial protein subsequent to processing by macrophages and/or DC (186, 187). Given these considerations, it is possible that intact bacteria elicit a TD response that produces a stable expansion of un-mutated PS-specific B cells, lacking properties of bona-fide memory B cells. The requirement for CD4+ T cell help could be cognate at the level of initial DC priming, but non-cognate for B cells, or perhaps entirely non-cognate in nature. In any event, our data indicate that BCR cross-linking is

necessary, but not sufficient for triggering B cells from GBS-III-primed mice for a boosted secondary PS-specific IgG response, as neither secondary immunization with isolated PPS14 or unencapsulated GBS-III can mediate this effect (Fig. 28A). In addition, CD4+ T cells are also required during secondary immunization to affect the PS-specific IgG booster response (Fig. 28B).

Although the majority of PPS14-specific IgG elicited in response to either Pn14 or GBS-III shares the same dominant idiotype (188), Pn14 fails to elicit a boosted secondary PPS14-specific IgG response in Pn14-primed mice, and induces a blunted booster response in GBS-III-primed mice (Fig. 24A). Further, co-immunization with GBS-III and unencapsulated Pn also leads to a lower boosted PPS14-specific IgG response, relative to immunization with GBS-III alone (Fig. 24B). In light of our previous report that Pn inhibits TD IgG responses to co-immunized soluble antigens independent of capsule expression (110), these data suggest that Pn expresses an immunosuppressive structure in its sub-capsular domain, that is not present in the GBS-III strains used in this study. This does not appear to be secondary to the generation of CD25+ regulatory T cells (189). One possible candidate is phosphorylcholine (PC), a haptenic moiety covalently attached to the Pn cell wall teichoic acid and membrane lipoteichoic acid (115). PC has been characterized as an immunomodulatory moiety utilized by a number of pathogens and could be responsible for the anti-inflammation in arthritis (190). It has been reported that the PC-bearing components interfere with key proliferative signaling pathways in B and T cells (191), development of dendritic cells (192) and macrophages (193) and mast cell degranulation and contribute to low antibody and cytokine levels (194).

To study the effect of PC on PPS14-specific IgG responses to intact Pn14, we made R614 bacteria that lack PC by growing them in a special ethanolamine-containing media, in which the

PC was replaced by phosphorylethanolamine. Studies using R614 PC- bacteria did not result in significantly boosted PPS14-specific IgG response. Nevertheless, we found a modest increase in the secondary PPS14-specific IgG titers though not statistically significant, compared to the control R614 (Fig. 26). R614 and R614PC- bacteria grown in THB and ethanolamine media respectively could immunologically behave differently. To overcome this, both R614 and R614PC- bacteria must be grown in similar media for direct comparison of PS-specific IgG responses to these bacteria. Ongoing studies in our lab identified the unique role of PC in inhibition of the primary and secondary anti-cOva IgG responses after the coimmunization of cOva with unencapsulated Pn. The induction of anti-PC IgG titers by the R614PC- bacteria indicates the presence of minor amounts of PC on the surface of R614 that could potentially cause the failure to induce boosted PS-specific Ig response to intact R614PC- bacteria (Fig. 26). Alternative approaches may consist of utilizing a mutant Pn that lacks the PC on its cell wall as described by Zhang et al (174). PC is known to induce IL-10 production in B1-b B cells which further suppresses proinflammatory cytokines like TNF-α, IL-6 and IL-12 (122, 195). Previous studies demonstrated the ability of a secreted filarial protein (ES-62) to mediate immunosuppression through a mechanism that is dependent on ES-62 expression of PC and the triggering of IL-10 secretion (196). Earlier studies in our lab using IL-10-/- mice showed a significant elevation in the anti-PspA IgG titers in response to the unencapsulated Pn. A marked enhancement in the proinflammatory cytokines was observed in the absence of IL-10 (197). Extracellular bacteria, in addition to Pn, such as *Pseudomonas aeruginosa*, *N. meningitidis*, *N. gonorrhoeae*, and *Haemophilus influenzae* can also express PC on their LPS or pili in a regulated manner (76-78), but the immunologic consequences of this remain largely unexplored. Further

investigation is required to understand how the expression of PC by Pn could affect the elicitation of secondary anti-PPS14 IgG responses in vivo.

Early innate immune responses mediated by proinflammatory cytokines are critical for adaptive immune responses to extracellular bacteria (197). Inactivated GBS stimulates macrophages and monocytes with high efficiency compared to Pn (57). GBS is known to induce elevated levels of TNF-α compared to Pn at the same bacterial densities (61) and the existence of capsule on the bacterial surface played no significant role in cytokine induction (198). The ability of unencapsulated GBS-III to partially restore the boosted IgG anti-PPS14 response when co-immunized with Pn14 (Fig. 24B) suggests that innate immune activation mediated by the GBS-III sub-capsular domain can overcome this potential Pn-mediated suppressive effect. Coimmunization of mice with Pn and unencapsulated GBS resulted in the boosted secondary PPS14-specific IgG responses (Fig. 24). Thus the failure of Pn to elicit the anti-PPS14 secondary IgG response is compensated by the presence of a powerful stimulant, GBS. However, the presence of unencapsulated Pn did not affect the elicitation of anti-PS memory responses to intact GBS, but significantly reduced the secondary anti-PS IgG titers compared to the control. Thus, this study demonstrates that differences in underlying sub-capsular bacterial domains can indeed differentially regulate PS-specific Ig responses *in vivo*.

CHAPTER 4

CONCLUSIONS

4.1 CONCLUSIONS

Project#1: Regulation of In vivo PS-specific Ig Responses to Intact Heat-Killed *Neisseria meningitidis.*

In this chapter, we determined whether the nature of the PPS14-specific Ig response to intact Pn14 was characteristic of intact PS-expressing extracellular bacteria in general, or perhaps represented a characteristic feature of PPS14, or the underlying structure and/or composition of intact Pn, or perhaps a more general dichotomy between GP and GN bacteria. To begin to address this question, we studied the nature of the MCPS-specific Ig response to intact MenC, a GN bacterium, and compared this response to that elicited by an MCPS-TT conjugate or isolated MCPS, as well as to our previously published data on Pn14. In this report we demonstrate the following: 1) Intact MenC elicits an IgG anti-MCPS response that, similar to the MCPS-TT conjugate, shows prolonged primary kinetics of development and significant boosting upon secondary immunization. This is in distinct contrast to the IgG anti-PPS14 response to intact Pn14 that showed rapid primary kinetics of induction and no secondary boosting. The IgG anti-PorB and total IgG anti-MenC protein response to MenC shows similar kinetics and boosting to that of the IgG anti-MCPS response. 2) In contrast to MenC, the IgG anti-MCPS response to isolated MCPS shows rapid primary kinetics of induction, no boosting upon secondary immunization, and expression of IgG3 and IgG1 in contrast to all 4 IgG isotypes elicited by MenC, indicating a marked effect of the intact bacterium on the associated MCPS-specific IgG response. The absence of secondary boosting observed using isolated MCPS is consistent with early studies using other soluble PS Ags (199, 200). 3) Surprisingly, the primary IgG anti-MCPS response to intact MenC is TI, although the boosted secondary response, similar

to both the primary and secondary IgG anti-PorB response, is dependent on CD4+ T cells, and requires CD28, ICOS, and CD40L. This is in contrast to the primary IgG anti-PPS14 response to Pn14 that was dependent on CD4+ T cells, CD28, and CD40L, although not on ICOS. 4) Both the TI and TD IgG anti-MCPS response to MenC is significantly reduced in TLR4-defective (C3H/HeJ), but not $TLR2^{-/-}$ or $MyD88^{-/-}$ mice, although boosting of the IgG anti-MCPS response is still observed after secondary MenC immunization. 5) Co-immunization of MenC and Pn14 results in a boosting of the IgG anti-PPS14 response to Pn14, independent of expression by MenC of MCPS.

These studies establish that the subcapsular domains of intact GP and GN bacteria significantly change the biology of the immune response to the expressed PS antigens. However, the distinct structure of the bacteria and/or the differential attachment of the PS to the underlying subcapsular domain in GP and GN bacteria contribute differently to the PS-specific Ig responses in vivo as observed with Pn versus MenC. Irrespective of the structure of the bacteria, the PS itself could immunologically be different. However, the boosting of PPS14-specific IgG responses to Pn14 in the presence of MenC suggests the likely contribution of the innate stimulation by MenC in eliciting the boosted PPS14-specific memory responses.

Collectively, this study contributes to an understanding of the regulation of in vivo PS-specific Ig responses to intact GN MenC and their potential dichotomy with the GP Pn14. Thus, the nature of in vivo PS-specific Ig responses to intact bacteria is dependent on the bacterial structure and composition of the underlying subcapsular domain.

Project# 2: Structurally Identical PS Expressed by Intact Heat-Killed *Streptococcus pneumoniae* versus *Streptococcus agalactiae* Elicits Distinct PS-specific Ig Responses

Previous studies in our lab highlighted the fact that the presence of intact bacteria radically changes the immunobiology of the PS-specific Ig response (39, 104, 163). In addition to the distinct structure of the bacterium, the underlying subcapsular bacterial domain adds an additional layer of complexity to the humoral immune response to intact bacteria (111). To establish the effect of bacterial non-capsular composition on the regulation of PS-specific responses, it was required to study two distinct bacteria with biochemically identical PS. Therefore, we utilized a mutant strain of group B *Streptococcus* (*S. agalactiae*) type III (GBS-III) that expresses desialylated IIIPS, biochemically identical to type 14 capsular PS (PPS14) of *Streptococcus pneumoniae* (Pn14), in order to directly compare the *in vivo* PPS14-specific IgG responses to two distinct Gram-positive bacteria. We found that intact GBS produced a rapid anti-PS IgG primary response that peaked by D7 similar to that observed with intact Pn14. However, unlike Pn, GBS produced a significantly boosted PS-specific IgG secondary response similar to that of the conjugate vaccine (Fig 19). We confirmed that the presence of intact GBS significantly changes the nature of the IIIPS-specific Ig response, in eliciting all four isotypes of IgG as opposed to little or no IgG observed with isolated IIIPS (Fig. 20A).

Although both GBS-III and Pn14 elicited relatively rapid primary PPS14-specific IgG responses that were dependent on CD4+ T cells, B7-dependent costimulation, and CD40/CD40L interactions, only GBS-III induced a highly boosted ICOS-dependent PPS14-specific IgG response following secondary immunization. Of note, priming with Pn14 and boosting with GBS-III elicited a similar boosted PPS14-specific IgG response after secondary immunization,

indicating that Pn14 primes for memory but, unlike GBS-III, fails to elicit it. The PPS14-specific memory IgG responses to intact bacteria such as Pn or GBS could not be elicited by isolated PPS14 given during the secondary immunization suggesting the need for PS in the context of bacteria to elicit the PS-specific memory responses. Of interest, the elicitation of PS-specific memory responses required the presence of CD4+ T cell help during the secondary immunization. However, the requirement of cognate T-B cell interaction for PS-specific responses is yet to be determined.

As reported in the previous chapter with MenC, the inability of Pn14 to elicit a boosted PPS14-specific IgG response was overcome by co-immunization with unencapsulated GBS-III. Thus, GBS and Pn, both being GP bacteria containing similar PS behave differently in eliciting the anti-PS Ig responses. This difference exhibited by these two bacteria indicates the potential role of certain immunomodulatory components like PC in Pn but not in GBS. However, our studies using the Pn that lacks PC did not result in boosting of the PS-specific Ig responses.

Collectively, these data demonstrate the distinct differences between the two GP bacteria GBS and Pn in eliciting the anti-PPS14 IgG responses. Thus, the structurally similar PS present on two distinct GP bacteria elicit distinct PS-specific IgG responses. The detailed anti-PS Ig responses to intact GP and GN bacteria have been summarized in Table 1.

Table 1. Anti-PS IgG responses to intact Pn14, GBS-III and MenC

Anti-PS Ig responses	**Pn14**	**GBS-III**	**MenC**
Primary	Peaks on D7	Peaks on D7	Peaks on D21
Secondary	No	Yes	Yes
IgG	All 4 IgG isotypes	All 4 IgG isotypes	All 4 IgG isotypes
CD4+ T cells	Yes, primary only	Yes (Both)	Primary-No Secondary-Yes
CD40/CD40L	Yes, primary only	Yes (Both)	Primary-No Secondary-Yes
CD28 costimulation	Yes, primary only	Yes (Both)	Primary-No Secondary-Yes
ICOS costimulation	No	Primary-No Secondary-Yes	Primary-No Secondary-Yes

4.2 LIMITATIONS OF WORK

- Although the present work has studied the regulation of intact GP and GN bacteria in eliciting the anti-PS IgG responses, the detailed mechanisms underlying these responses are yet to be elucidated.
- The present study is limited in addressing how the differences in the structure of bacteria contribute to the difference in the kinetics of the primary PS-specific IgG responses.
- The question whether the PS-specific B cells enter into a GC reaction to undergo affinity maturation to produce high affinity antibodies after the boost still remains unanswered.
- The present study did not determine the potential role of immunoinhibitory components in *S. pneumoniae* in regulating the secondary PS-specific IgG responses in vivo.
- These studies utilized heat-killed intact bacteria. Live bacteria, as will be encountered during infections, likely will contribute additional layers of complexity to the elicitation of PS-specific antibody responses.

4.3 FUTURE PERSPECTIVES

Our present studies provide an initial understanding of the regulation of anti-PS IgG responses to intact GP and GN bacteria. However, this study gave rise to many questions that are currently under investigation: 1) Are both memory B cells and T cells produced after the secondary immunization with intact heat-killed MenC and GBS? 2) Do PS-specific B cells engage in cognate interaction with CD4+ T cells to elicit the boosted PS-specific IgG responses to intact bacteria? 3) Why is the kinetics of primary PS-specific IgG response by GN bacteria slow compared to the rapid primary kinetics by GP Pn or GBS? 4) Have the CPS-specific B cells undergone avidity maturation to produce high affinity secondary antibodies after the boost? 5) What are the B cell subsets that are involved in the MCPS-specific IgG responses? 6) Do PS-specific Ig responses to GP versus GN extracellular bacteria differ when studying the same capsular PS expressed on these structurally different organisms? It is currently under investigation whether or not the two structurally different GP and GN bacteria viz. *Staphylococcus aureus* and *Acinetobacter baumannii* both expressing the same PS poly-N-acetyl glucosamine (PNAG) would elicit distinct PS-specific Ig responses.

Other possible future experiments would include A) determining the role of anti-PC IgG and IgM antibodies during the secondary immunization with R614 in eliciting the anti-PS IgG responses. B) Using alternative methods to create the mutant R614 that lacks the PC in its cell wall to study the effect of PC on the humoral PS-specific immune responses. C) Elucidating the mechanism of inhibition of PC in eliciting the PS-specific Ig responses to intact R614. D) Investigating the role of ICOS-dependent extrafollicular pathway as opposed to the germinal

center response in inducing the boosted anti-PS IgG titers in response to intact GBS. E) Establishing the role of NK T cells in regulating the anti-PS IgG response to intact bacteria.

4.4 SPECIFIC CONTRIBUTIONS OF THE RESEARCH

1) This thesis has attempted to redefine the traditional view of the PS as TI antigens by studying their immunologic behavior in the context of the intact bacterium. The presence of PS on the surface of bacteria identifies them as unique antigens different from the protein antigens expressed by the same bacterium, isolated PS, and the PS that are covalently linked to the protein in a conjugate vaccine.

2) Our present study provides an initial understanding of the nature of the PS-specific Ig responses to intact bacteria that were not studied in detail before.

3) The present work emphasizes the importance of the physiochemical context of the PS and the differential attachment to the underlying cell wall in regulating the nature of the anti-PS Ig response.

4) The differential contribution of distinct bacterial subcapsular domains as in GP and GN bacteria on the PS-specific Ig responses has been determined.

5) It has been described that the same PS on two distinct, but structurally similar bacteria can function differently based on the differential cell wall composition of the bacteria.

6) Thus, the nature of in vivo anti-PS Ig responses to intact bacteria is dependent on the structure, architecture and composition of the underlying subcapsular bacterial domain.

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LIST OF PUBLICATIONS

Journal Publications

1) **Swadhinya Arjunaraja**, Paola Massari, Lee M. Wetzler, Andrew Lees, Jesus Colino, and Clifford M. Snapper. 2012. The nature of an in vivo anti-capsular polysaccharide response is markedly influenced by the composition and/or architecture of the bacterial subcapsular domain. *J Immunol* 188:569-577

2) **Swadhinya Arjunaraja**, Lawrence C Paoletti, Clifford M. Snapper. 2012 Structurally identical capsular polysaccharide expressed by intact Group B *Streptococcus* versus *Streptococcus pneumoniae* elicits distinct murine polysaccharide-specific IgG responses in vivo. *J Immunol* 188 :5238-5246

3) Jesus Colino, Leah Duke, **Swadhinya Arjunaraja**, Quanyi Chen, Leyu Liu, Alexander H. Lucas and Clifford M. Snapper. 2012. Differential idiotype utilization for the in vivo type 14 capsular polysaccharide-specific immunoglobulin responses to intact *Streptococcus pneumoniae* versus a pneumococcal conjugate vaccine as a result of the engagement of distinct B cell subsets. *J Immunol* 189: 575-586.

Abstract publications

1) **Swadhinya Arjunaraja**, Paola Massari, Andrew Lees, Jesus Colino and Clifford M Snapper. The nature of the murine *in vivo* polysaccharide-specific IgG response to intact *Neisseria meningitidis* is distinct from that elicited by intact *Streptococcus pneumoniae.*

Research Week 2011 at Uniformed Services University of the Health Sciences, Bethesda, MD, USA

2) Saumyaa, **Swadhinya Arjunaraja** and Clifford M Snapper**.** The regulation of T cell dependent IgG response to soluble protein antigens in the presence and absence of intact bacteria. *Research Week 2011* at Uniformed Services University of the Health Sciences, Bethesda, MD, USA.
3) **Swadhinya Arjunaraja,** Paola Massari and Clifford M. Snapper. Structurally identical capsular polysaccharide expressed by intact Group B *Streptococcus* versus *Streptococcus pneumoniae* elicits distinct murine polysaccharide-specific IgG responses in vivo. *American Association of Immunologists Meeting 2012* at Boston, MA, USA.
4) Saumyaa Saumyaa, **Swadhinya Arjunaraja**, Raul M. Torres, Clifford M Snapper. Regulation of T cell dependent IgG responses to soluble protein antigens in the presence and absence of intact bacteria. *American Association of Immunologists Meeting 2012* at Boston, MA, USA.
5) **Swadhinya Arjunaraja,** Paola Massari and Clifford M. Snapper. Structurally identical capsular polysaccharide expressed by intact Group B *Streptococcus* versus *Streptococcus pneumoniae* elicits distinct murine polysaccharide-specific IgG responses in vivo. *Research week 2012* at Uniformed Services University of the Health Sciences, Bethesda, MD, USA
6) Saumyaa Saumyaa, **Swadhinya Arjunaraja**, Raul M Torres and Clifford M Snapper. *Streptococcus pneumoniae* selectively inhibits the humoral immune response to a soluble protein through a phosphorylcholine dependent mechanism. *Research week 2012* at Uniformed Services University of the Health Sciences, Bethesda, MD, USA

7) **Swadhinya Arjunaraja,** Paola Massari and Clifford M. Snapper. Structurally identical capsular polysaccharide expressed by intact Group B *Streptococcus* versus *Streptococcus pneumoniae* elicits distinct murine polysaccharide-specific IgG responses in vivo. *Frontiers in Immunology 2012* at National Institute of Health, Bethesda, MD, USA

BRIEF BIOGRAPHY OF THE CANDIDATE

Swadhinya Arjunaraja obtained her B. Tech. degree in Biotechnology from School of Engineering and Technology, Bharathidasan University, Tiruchirapalli, TN, India. She received the ‘Best outstanding student award’ for her academic excellence during the first degree education. She did her M. E., in Biotechnology from Birla Institute of Technology and Science (BITS), Pilani, India. She was working as a Teaching Assistant in the Biological Sciences Group at BITS, Pilani from Aug 2005-Dec 2007. She taught various courses like General Biology, Biochemistry and handled practical classes like Measurement Techniques-I and Instrumental Methods of Analysis for first degree students at BITS, Pilani. She pursued research in the field of Immunology in 2008 at USUHS, Bethesda, MD, USA through BITS-USUHS collaborative program under the guidance of Dr. Clifford M Snapper MD.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. Clifford M. Snapper obtained his M.D., from Albany Medical College, Albany, NY, USA, his residency training in Pathology at Cornell-New York Hospital, NY, NY, USA, and his scientific training in the field of immunology as a Medical Staff Fellow at the National Institutes of Health, Bethesda, MD, USA. He has been doing research in the field of Immunology for the past 25 years. Currently, he serves as a Professor in the Department of Pathology, and Professor, Molecular and Cell biology and Emerging Infectious Diseases graduate programs at USUHS, Bethesda, USA. He is also Director, Institute of Vaccine Research at USUHS that is tasked to conduct basic and translational immunologic studies that have relevance for the rational design of new or improved vaccines against infectious agents. He has received number of honorary awards including the Burroughs Wellcome Developing Investigator Award in Immunopharmacology of Allergic Diseases, the Jeffrey Modell Foundation Lifetime Achievement Award, Outstanding Biomedical Graduate Educator Award (USUHS) and the Henry Wu Award (USUHS) for his excellence in basic research. He served as an Associate Editor for the journal Infection and Immunity and is currently serving as a Section Editor for the Journal of Immunology. He has published 94 original research articles in the peer reviewed journals including Science, The Journal of Experimental Medicine, The Journal of Immunology and Infection and Immunity, and has written 23 invited reviews and chapters. He is actively engaged in teaching Pathology to USUHS medical students, teaching Immunology to, and serving as a Ph.D. advisor for graduate students in the BITS-USUHS collaborative program, and USUHS Molecular Cell Biology, and Emerging Infectious Diseases programs.