

*“Regulation of polysaccharide- and protein-specific antibody responses to intact extracellular bacteria”*

*By*

*Swagata Kar*

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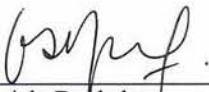
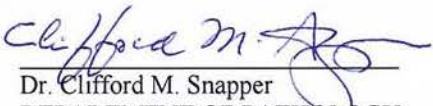
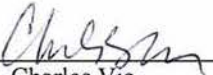
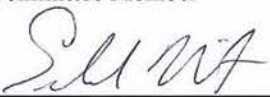
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Swagata Kar  
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# *Abstract*

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Title of Dissertation: **“Regulation of polysaccharide- and protein- specific antibody responses to intact extracellular bacteria”**

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Many studies have been published detailing the mechanisms of antibody production in response to bacterial polysaccharides (PS) and proteins, either in soluble, isolated form and/or as conjugate vaccines composed of an immunogenic carrier protein covalently linked to PS. However, much less is known regarding antibody responses to PS and protein expressed naturally by intact extracellular bacteria. We describe two major projects in mice that investigated the mechanism of PS- and/or protein-specific anti-IgG responses to intact, heat-killed Gram-positive and/or Gram-negative bacteria, in comparison to PS- and/or protein-specific IgG responses elicited by isolated PS or protein, and/or conjugate vaccine.

In the first project we determined the identity of the antigen-presenting cells (APC) involved in the in vivo uptake and presentation of protein expressed by intact heat-killed *Streptococcus pneumoniae* (Pn) versus a soluble, isolated protein or conjugate vaccine, introduced into mice via the systemic route where the spleen plays a dominant role in immunity. We further wished to determine the effect of selectively depleting particular APC in vivo, using clodronate-containing liposomes (CL), on the subsequent PS- and protein-specific IgG responses to these immunogens. Specifically, we hypothesized that particular APC

that are more efficient in phagocytosis and hence selectively depleted by CL, would play a more dominant role in responses to the particulate intact bacteria, in contrast to soluble immunogens that are internalized by APC via endocytosis, and thus unaffected by CL treatment. We demonstrate that surprisingly, injection of CL markedly inhibited protein-specific IgG responses to both intact, heat-killed Pn as well as a soluble OVA-PS conjugate or OVA alone. PS-specific IgG responses to bacteria and conjugate were also reduced, but more modestly. In both instances, CL-mediated inhibition was associated with a significant reduction in induced germinal centers (GC) and CD4<sup>+</sup> GC T follicular helper cells. However, CL injection which largely abrogated the proliferative response of adoptively transferred OVA peptide-specific transgenic CD4<sup>+</sup> T cells in response to immunization with Pn expressing OVA peptide, did not inhibit T cell proliferation in response to OVA-PS or OVA. In this regard, monocyte-derived cells (MoC), depleted by CL, internalized Pn in vivo, whereas CD11c<sup>low</sup> dendritic cells, unaffected by CL injection, internalized soluble OVA. Ex vivo isolation and co-culture of these respective APCs from Pn- or OVA-immunized mice with OVA-specific T cells, in the absence of exogenous antigen, demonstrated their selective ability to induce T cell activation. These data suggested that while distinct APCs initiate CD4<sup>+</sup> T cell activation in response to antigen expressed by intact bacteria versus antigen in soluble form, CL-sensitive cells appear necessary for the subsequent IgG responses to both forms of antigen.

The second project was based on previous work from our laboratory that priming of mice with intact, heat-killed cells of Gram-negative *Neisseria meningitidis*, (MenC) or Gram-positive *group B Streptococcus* (GBS-III) bacteria resulted in augmented serum PS-specific IgG titers following booster immunization. Induction of memory required CD4<sup>+</sup> T cells during primary immunization. In the current study we determined whether PS-specific memory for IgG production was contained within the B cell and/or T cell populations, and whether augmented IgG responses following booster immunization were also dependent

on CD4<sup>+</sup> T cells. Adoptive transfer of purified B cells from MenC- or GBS-III-primed, but not naïve mice resulted in augmented PS-specific IgG responses following booster immunization, with similar responses observed whether co-transferred CD4<sup>+</sup> T cells were from primed or naïve mice. Similarly, primary immunization with unencapsulated MenC or GBS-III, to potentially prime CD4<sup>+</sup> T cells, failed to enhance PS-specific IgG responses following booster immunization with their encapsulated isogenic partners. Further, in contrast to GBS-III, depletion of CD4<sup>+</sup> T cells during secondary immunization with MenC or another Gram-negative bacteria, *Acinetobacter baumannii*, did not inhibit augmented PS-specific IgG booster responses of mice primed with heat-killed cells. Also, in contrast with GBS-III, booster immunization of MenC-primed mice with isolated MenC-PS, a TI antigen, or a conjugate of MenC-PS and tetanus toxoid elicited an augmented PS-specific IgG response similar to booster immunization with intact MenC. These data demonstrated that memory for augmented PS-specific IgG booster responses to Gram-negative and Gram-positive bacteria are contained solely within the B cell compartment, with a differential requirement for CD4<sup>+</sup> T cells for augmented IgG responses following booster immunization.

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# *Dedication*

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I dedicate my work to my parents and my husband who have always been there through thick and thin and encouraged and inspired me to follow my dreams and aspirations.

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## *List of abbreviations*

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- Ab: antibody
- AcB: *Acinetobacter baumannii*
- Ag: antigen
- APC: antigen-presenting cell
- APRIL: A Proliferation inducing ligand
- BAFF: B cell activating factor
- BCR: B cell receptor
- CFU: colony forming unit
- DC: dendritic cell
- FB: follicular B cell
- FDC: follicular dendritic cell
- GBS: Group B Streptococcus (*Streptococcus agalactiae*)
- GC: germinal center
- GN: Gram-negative
- GP: Gram-positive
- ICOS: inducible costimulator
- Ig: immunoglobulin
- IL: interleukin
- IM: inner membrane
- LOS: lipooligosaccharide
- LPS: lipopolysaccharide

- LTA: lipoteichoic acid
- MCPS: Meningococcal polysaccharide type C
- MenC: *Neisseria meningitidis*, capsular type C
- MHC-II: Major histocompatibility complex class II
- mIg: membrane immunoglobulin
- MZB: marginal zone B cell
- NLR: Nod-like receptor
- ODN: oligodeoxynucleotide
- OM: outer membrane
- OMP: outer membrane protein
- PALS: periaarteriolar lymphoid sheath
- PBS: phosphate buffered saline
- PC: phosphorylcholine
- Pn: *Streptococcus pneumoniae*
- PNAG: Poly N-acetyl glucosamine
- PorB: Meningococcal porin protein B
- PPS14: Pneumococcal capsular polysaccharide, serotype 14
- PS: polysaccharide
- PspA: Pneumococcal surface protein A
- TA: teichoic acid
- TACI: Transmembrane activator and calcium-modulator and cyclophilin ligand interactor
- TCR: T cell receptor
- TD: T cell-dependent

- T<sub>FH</sub>: T follicular helper cell
- TI: T cell-independent
- TLR: Toll-like receptor
- TT: tetanus toxoid
- WT: wild-type

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# Chapter 1

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## INTRODUCTION

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# Introduction

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## 1.1. Clinical significance of extracellular bacterial infections.

Infections with extracellular polysaccharide (PS)-encapsulated bacteria such as *Streptococcus pneumoniae* (Pn, Gram-positive [GP]), *Neisseria meningitidis* (Men, Gram-negative [GN]) and *Hemophilus influenzae* type b (Hib, GN) are major sources of global morbidity and mortality among infants, the elderly and the immunosuppressed, due to sepsis, pneumonia and meningitis (1-4). Globally, Hib and Men are estimated to account for ~200,000 and ~50,000 deaths, respectively each year (3, 5, 6). The Centers for Disease Control estimated that in the United States alone, ~900,000 people are infected with Pn with 400,000 hospitalizations, with a 5-7% mortality rate, whereas globally Pn infections are responsible for 11% of all deaths in children 1 month to 5 years. Despite effective vaccines against these bacteria, there remains a huge global burden of these diseases due to economic and logistical limitations in resource-poor countries. *Streptococcus agalactiae* (Group B Streptococcus [GBS], GP) colonizes the vagina of 15-30% of healthy women, and is a leading cause of neonatal pneumonia, septicemia, and meningitis (7). GBS colonization during pregnancy increases the preterm rupture of membranes and premature delivery (8). GBS infections are also seen in the elderly population. There is currently no licensed prophylactic vaccine in clinical use for GBS. Infections with *Acinetobacter baumannii* (AcB, GN) may produce pneumonia, bacteremia, meningitis, skin and soft tissue infections (9). Although infections with AcB are primarily nosocomial, there have also been reports of community-acquired AcB pneumonia. Like GBS, there is no licensed prophylactic vaccine in clinical use against AcB.

## **1.2. Immune protection against extracellular bacteria.**

PS capsules of extracellular bacteria are the major virulence factors, and hence, are the targets of antibody-mediated protection (10). Induction of IgM, IgG, or IgA specific for PS antigens is a major adaptive mechanism for the clearance of extracellular bacteria from the blood and tissues (10). Both isolated PS and PS-protein conjugate vaccines, confer protection by eliciting antibodies to the capsular polysaccharide (11, 12). Ig binding to the bacterial surface facilitates Fc- and complement-mediated opsono-phagocytosis of bacteria by neutrophils and macrophages expressing Fc and complement receptors (10, 13). Ig specific for bacterial protein has also been shown to confer host protection (14). In addition to adaptive immunity, cell-mediated immunity, in particular CD4<sup>+</sup> T cells secreting IL-17 (Th17 cells) can confer antibody-independent protection against bacteria such as Pn (15) or *Staphylococcus aureus* (16).

## **1.3 Mechanisms of PS-specific Ig induction-defining the scientific questions.**

Much of our current knowledge of PS-specific antibody responses has come from studies using isolated PS, which demonstrated that these antigens, with the exception of zwitterionic PS (11), are T cell independent (TI), eliciting weak PS-specific IgG responses with no associated memory, poor if any germinal center (GC) responses, and limited somatic hypermutation (17). These properties are based on the inability of non-zwitterionic PS to associate with MHC-II molecules (18, 19), a process required for recruitment of CD4<sup>+</sup> T cell help, as observed for isolated proteins (20). However, less is known regarding the immunologic properties of PS and protein naturally expressed by intact extracellular bacteria. The goal of these current studies was to further elucidate the mechanism by which induction of PS- and protein-specific Ig responses to extracellular bacteria occur in vivo using the mouse as the model system. The cellular processes underlying PS- and protein-specific Ig responses to GP and GN bacteria were compared,

and further related to the mechanisms governing Ig responses to isolated PS and proteins, and PS-protein conjugate vaccines.

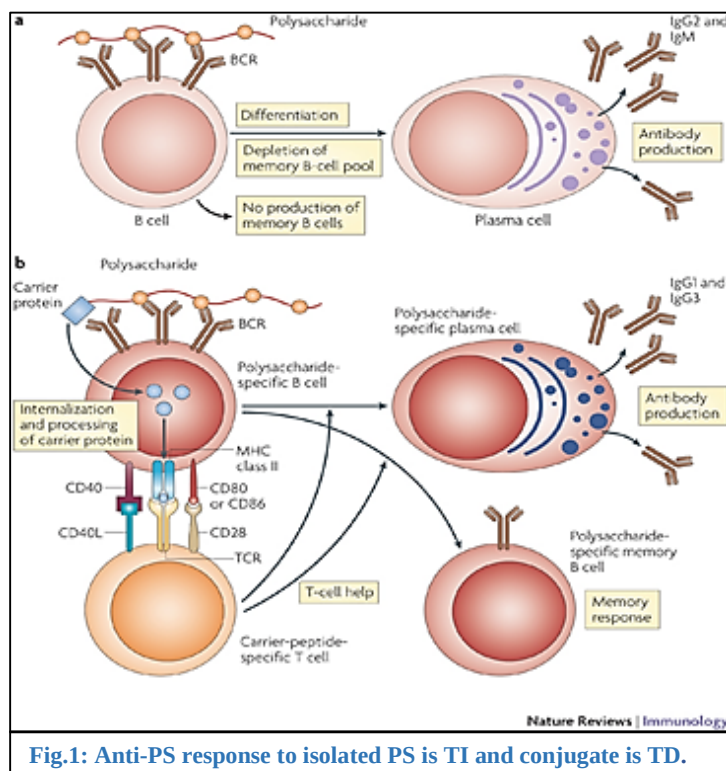
#### **1.4 Isolated PS vaccines:**

Bacterial PS capsules are one of the major targets of antibody-mediated protection (21, 22). Hence, for many decades, isolated PSs were used as vaccines to prevent bacterial infections caused by PS-encapsulated bacteria such as *Streptococcus Pneumonia*, *Neisseria meningitidis* and *Haemophilus influenzae* type b. Pneumovax 23, a pneumococcal PS vaccine that contains 23 different pneumococcal capsular serotypes, confers protection against ~90% of invasive pneumococcal isolates found in the developed world (23). Another PS vaccine providing protection against meningococcal infection, Menomune, is a quadrivalent PS vaccine containing meningococcal capsular PS from serogroups A, C, Y and W-135 (24). Though, these PS vaccines are effective in adults, they are not effective in children less than 2 years of age (25). Isolated PS activate B cells to proliferate in the absence of MHC class-II-restricted T-cell help by cross-linking the antigen-specific B cell receptor (BCR) in a multivalent fashion. The co-cross-linking of BCR and CD21 (complement receptor type 2) on the B cell surface, through complement bound to PS, markedly lowers the dose of antigen required for B cell activation (26). Many TI antigens either activate complement directly via the alternative pathway (27) or indirectly through the classical pathway by interaction with low affinity, polyreactive natural serum antibody (28). Subsequent induction of Ig secretion and class switching requires second signals including cytokines, Toll-like receptors (TLR) and BAFF/APRIL (29). In particular, B cell activating factor (BAFF) and A Proliferation inducing ligand (APRIL) act on Transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) receptor, for induction of B cell differentiation into Ig-secreting plasma cells (30). TACI-deficient mice exhibit a selective reduction in Ig responses to TI antigens such as haptenated-Ficoll and

pneumococcal polysaccharide (31, 32). In light of these collective observations, defective PS-specific Ig responses in neonates could be due in part to low expression of CD21 on neonatal B cells (33). In addition, neonatal B cells are poorly activated in response to membrane Ig crosslinking (34). Finally, B cells from neonate have low TAC1 expression which thus limits Ig responses to PS vaccines (35). Although isolated PS vaccines confer protective immunity in adults, the PS-specific Ig levels start declining within a year post- vaccination (36). The PS vaccines fail to generate immunological memory and are limited in their isotype switching. Hence, to overcome the limitations of PS vaccines, including non-responsiveness in neonates, PS-protein conjugate vaccines (PCV) were developed in order to recruit CD4<sup>+</sup> T cell help for the PS-specific Ig response (37).

## 1.5 Conjugate vaccines

In 1920 Avery and Goebel reported that covalent linkage between an isolated PS with an immunogenic



protein carrier could enhance the immunogenicity of the associated PS (38). Conjugation of protein to PS converts the anti-PS response from TI to TD. In contrast to a protein, PS is a TI antigen (17). For most PS, in contrast to proteins, the APC is unable to enzymatically process it within endosomes to generate a MHC class II-PS complex on its surface for presentation to CD4<sup>+</sup> T cells (19, 39). Hence, PS fail to recruit classical cognate CD4<sup>+</sup> T cell help. Covalent attachment of

immunogenic protein and PS to create a soluble conjugate vaccine converts the anti-PS response into a classic TD response with generation of PS-specific memory (40, 41). This is due to the ability of the APC to internalize the protein and enzymatically processes it, and present MHC class II-peptide complexes on its surface to CD4<sup>+</sup> T cells, recruiting classical cognate CD4<sup>+</sup> T cell help (Fig.1) (42). T cell clones harvested from mice immunized with a conjugate vaccine were mostly MHC-II-restricted CD4<sup>+</sup> T cells specific for the protein carrier (43). However, a recent report demonstrated that the CD4<sup>+</sup> T cell help elicited in response to conjugate vaccines is specific for the polysaccharide, which is bound to MHC-II via attached peptide (44). Conjugate vaccines, in contrast to isolated PS, have shown clinical effectiveness in infants and elicit memory for PS-specific IgG responses (42).

The success associated with the generation of the Hib conjugate vaccine (45), encouraged the development of conjugate vaccines against pneumococcal and meningococcal infections. Prevnar-7, is a 7-valent vaccine, consisting of pneumococcal polysaccharide serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), conjugated to CRM<sub>197</sub>, a protein toxoid derived from the bacterium, *Corynebacterium diphtheriae* (46). It has been shown to confer protection against Pn infections in young children. Similarly, MCV-4, the meningococcal conjugate vaccine, containing the serotypes A/C/Y/W-135 was conjugated to diphtheria toxoid and has been demonstrated to be effective against meningococcal disease in children and adults (47). Although glycoconjugate vaccines are highly effective, they are also associated with certain limitations. The chemical nature of the PS, the amount of unconjugated PS in the vaccine and the nature of the carrier protein determines the immunogenicity of different conjugate vaccines, thus adding complexity to their proper formulation (42). Peumococcal conjugate vaccines with more serotypes are being manufactured, which make these vaccines more expensive and complex. Prevnar13 is licensed and vaccines with more serotypes are being tested. Hence these pose limitations to their widespread use in

developing countries. Thus, there is a need for improvements in design of vaccines particularly for use in adults, in order to make them more cost-effective. In this regard, a better understanding of the mechanisms underlying PS-specific Ig responses to intact extracellular bacteria may help inform new vaccine strategies.

### **1.6. Intact PS-encapsulated extracellular bacteria as unique immunogens.**

The great majority of immunological studies pertaining to mechanisms underline PS-specific Ig responses utilized isolated PS, which were demonstrated in most cases to be T cell-independent (TI) antigens (Fig.1) (17). In contrast to protein, PS internalized by APC are not processed in a manner that leads to an MHC class II-PS complex on its surface for presentation to CD4<sup>+</sup> T cells (19, 39). Hence, these PS fail to recruit classical cognate CD4<sup>+</sup> T cell help. However, the concept that PSs are TI Ags is divorced from the physiologic context of the intact bacterium that co-expresses these Ags. As discussed earlier, covalent attachment of immunogenic protein and PS to create a soluble conjugate vaccine converts the anti-PS response into a classic TD response with generation of PS-specific memory. In a somewhat analogous fashion, during infections with intact bacteria, the immune system encounters the PS *non-covalently* associated with proteins, as well as innate immune stimulating moieties such as TLR and Node Like Receptor (NLR) (48) and scavenger receptor ligands (49) within the particulate sub-capsular domain. The expression of PS in this context may confer unique immunologic properties for elicitation of the PS-specific Ig response (50). Evidence suggesting that PS-specific Ig responses to bacteria are TD came from earlier studies which demonstrated significant degrees of somatic hypermutation (SH) (51, 52) in the variable regions of natural human PS-specific Ig, suggesting that the host interaction with colonizing encapsulated bacteria might have induced TD germinal center (GC) reactions where SH is known to occur. Similar studies were carried out analyzing sequences of PS-specific Abs, obtained from rabbits immunized

with formaline treated encapsulated Pn, which also demonstrated a high degree of somatic hypermutation (53). Previous studies from our laboratory demonstrated that the PS-specific IgG response to Pn was dependent on CD4<sup>+</sup> T cells, B7-dependent co-stimulation, and CD40/CD40L interactions, which was further confirmed using intact MenC and GBS (54-56). The mechanism by which CD4<sup>+</sup> T cell help is recruited for PS-specific B cells, in response to intact bacteria *in vivo*, requires further study, but recent evidence suggests that CD4<sup>+</sup> T cells recognizing bacterial protein may play a key role (57).

### **1.6.1 Humoral immune responses to intact encapsulated extracellular bacteria**

PS are non-covalently co-expressed with protein by intact extracellular bacteria within a particulate framework, associated with multiple TLR, NLR, and scavenger receptor ligands (56). Thus, PS-specific B cells responding to intact bacteria could potentially recruit CD4<sup>+</sup> T cell help for PS-specific Ig responses through uptake and presentation of associated bacterial protein. *In vivo* studies in mouse models have demonstrated that intact, heat-inactivated Pn, capsular type 14 (Pn14) induces TD PS-specific (PPS14) IgG responses as well TD IgG responses specific for several pneumococcal proteins, including PspA. Both PPS14- and protein-specific IgG responses to Pn14 required CD28- and CD40L-mediated co-stimulation similar to the PS-specific IgG responses to conjugate vaccine (58). In contrast to isolated PS, a TI antigen that elicited mostly IgG3 and some IgG1, the TD PS-specific IgG response to Pn14 consisted of all four IgG isotypes. Also, in contrast to the protein-specific IgG response to Pn14, the PPS14-specific IgG response exhibited a more rapid primary response characterized by a shorter period of T cell help and B7-dependent co-stimulation. Moreover, response to Pn14 was ICOS-independent, and failed to generate an augmented booster response (59, 60). Thus, although both the PPS14- and protein-specific IgG responses to Pn14 were TD, they appeared to differ mechanistically.

GBS-III expresses a biochemically similar capsular PS as Pn14, where PPS14 is only lacking the terminal

sialic acid. The primary PS-specific IgG response to GBS-III is also dependent on CD4<sup>+</sup> T cells, B7-dependent co-stimulation, and CD40–CD40L interactions, but unlike Pn14, GBS-III induced a highly augmented, ICOS-dependent PPS14-specific IgG response after booster immunization (54). In contrast, studies using the GN extracellular bacterium MenC demonstrated a slow, TI primary PS (MCPS)-specific IgG response with a highly augmented TD MCPS-specific IgG response after booster immunization. The augmented MCPS-specific response was dependent on CD4<sup>+</sup> T cells, B7-dependent co-stimulation, and CD40–CD40L and ICOS–ICOSL interactions (55). Collectively, these studies demonstrated that the subcapsular domain of the intact bacterium plays a major role in determining the nature of the PS-specific IgG response and is likely involved in transforming the TI Ig response to one that is dependent on CD4<sup>+</sup> T cells, including a requirement for B7-dependent, and potentially ICOS-dependent, co-stimulation, and CD40–CD40L interactions. However, although these studies demonstrated the relative role of CD4<sup>+</sup> T cells for eliciting the primary PS-specific IgG response and induction of memory, they left unresolved whether naïve or memory CD4<sup>+</sup> T cells, generated after the primary responses, are relevant for elicitation of the secondary response, and whether memory for PS-specific IgG responses to intact bacteria was contained within the B cell and/or T cell compartments.

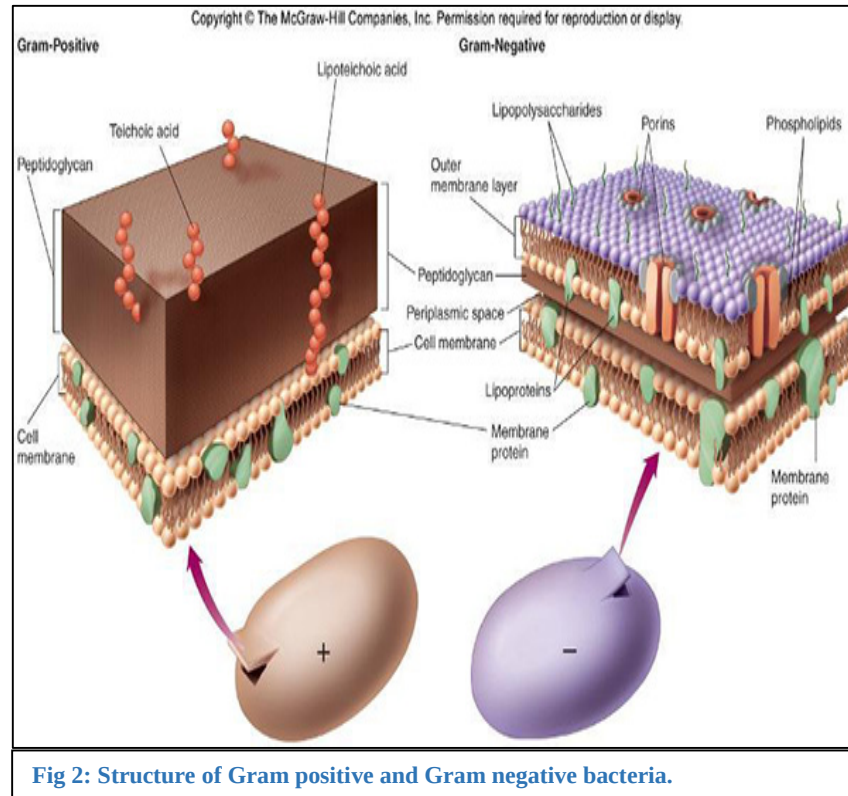
#### 1.6.2 **Distinct characteristics of particulate Ags relative to their soluble counterparts:**

PS-specific IgG responses to intact Pn and conjugate vaccines are dependent on CD4<sup>+</sup> T cells, CD40L and CD28-dependent co-stimulation (58, 60, 61). However, we previously demonstrated specific features in the mechanisms underlying PS-specific IgG responses to these two distinct immunogens: 1) Primary PS-specific IgG responses to Pn peaked earlier than that to conjugate (61), 2) expressed different idiotypes (62), and 3) PS-specific IgG responses to intact Pn and conjugate are mediated by MZB and FB cells, respectively (61, 63).

Although numerous studies have been published on the uptake and presentation of soluble proteins by B cells and subsequent presentation to T cells (64, 65), little is known regarding the mechanism by which B cells capture and present bacterial Ag following host exposure to intact bacteria (66, 67). A recent study demonstrated that B1 cells from the peritoneum efficiently internalize particulate bacteria in BCR-dependent fashion (68) and prime CD4<sup>+</sup>T cells (69). Our unpublished studies also demonstrated peritoneal B cell uptake of intact Pn, whereas splenic B cells were unable to internalize bacteria. This suggested that there might be other potential ways of antigen acquisition by splenic B cells for inducing CD4<sup>+</sup>T cell-dependent PS-specific IgG responses to intact bacteria. One study has demonstrated that MZB cells may capture complement-bound antigen on their surface via binding to CD21, either directly or from MZ macrophages (MZM). MZB can then migrate and transport Ag to the follicles, where it is picked up by FDC (70). Specific B cells bind Ag on the FDC surface via the BCR, leading to BCR crosslinking and Ag internalization for eventual presentation of MHC-II peptide complexes to CD4<sup>+</sup> T cells (71). We have observed that MZB cells play a major role in the PS-specific TD IgG response to intact Pn, but FB are largely responsible for the same TD PS-specific IgG response to a soluble conjugate vaccine. Of interest, FB cells appear responsible for protein-specific IgG responses to either immunogens. These data suggest the existence of two distinct mechanistic pathways for elicitation of TD PS-specific IgG responses in vivo. However, the cellular events that lead to these two different outcomes are largely unknown.

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

Bacterial species are classified as Gram-positive (GP) or Gram-negative (GN) based on a staining method/system that was introduced by Hans Christian Gram known as **Gram staining**, also

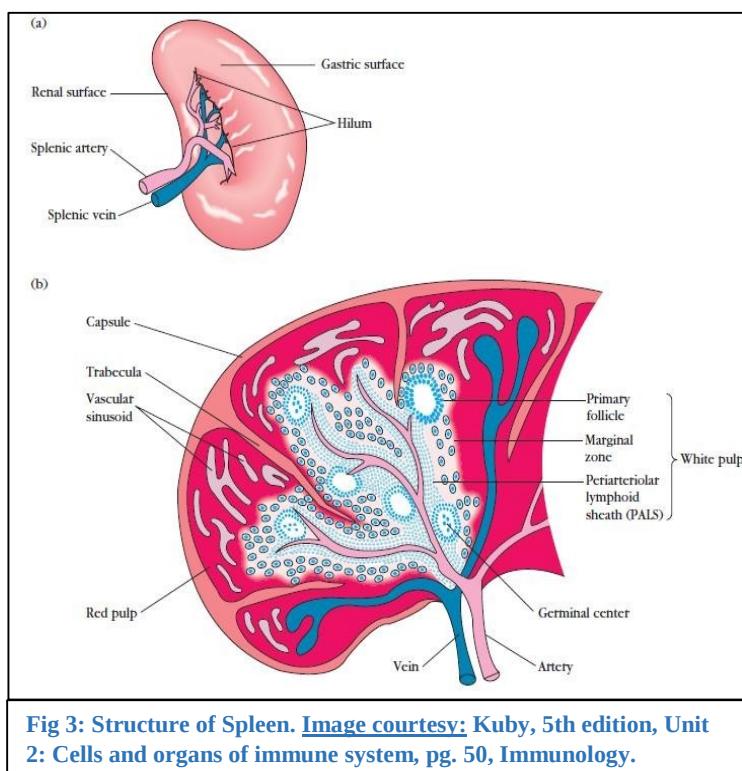


called **Gram's method**. This differentiation is based on the distinct structural and compositional differences between GP and GN bacteria. GP bacteria, in contrast to GN bacteria have a thick cell wall (15-80 nm), overlying an inner cell membrane, composed of repeating units of peptidoglycans as shown in Fig. 2. Peptidoglycan forms a 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 bacteria, the peptidoglycan is a heavily cross-linked woven structure that encircles the cell in many layers. The capsular PS is covalently linked to the underlying cell wall peptidoglycan to which a number of bacterial proteins and other molecules, such as teichoic acids, are embedded (50, 72). GN bacteria express two distinct lipid membranes: an inner cell membrane (IM), composed of phospholipids and proteins, and an outer membrane (OM), consisting of an inner face of phospholipids and an outer face of lipopolysaccharides (LPS), as shown in Fig 2 The periplasm lies between the IM and OM that is occupied by soluble proteins and a thin peptidoglycan layer that is only intermittently cross-linked. PS is covalently attached to the

acylglycerol moiety of the outer membrane consisting of LPS, a potent stimulator of the innate immune system via TLR4 (73, 74). The OM also contains highly immunogenic protein, including porins (75). Porins are a unique set of transmembrane proteins that adopt a  $\beta$ -barrel architecture, which allows them to serve as channels for the passage of small hydrophilic molecules (73). Shedding of the outer membrane/PS complex, to form vesicles, is a unique property of GN bacteria that may have distinct immunologic consequences for the PS-specific IgG response (76, 77). How these structural and biochemical differences between GP and GN bacteria lead to differences in the cellular regulation of PS-specific IgG responses remains to be determined.

### 1.7 Structure of the spleen



The spleen is the body's largest blood filtering organ, which is located in the abdomen, just beneath the diaphragm connected to the stomach (78). Blood enters the spleen through branching arterial vessels, and is subsequently delivered into a sluggish venous sinusoidal system where blood elements come into prolonged contact with splenic immune cells (Fig 3). The spleen is composed of the red pulp and the white pulp. Senescent red blood cells and any blood borne pathogens flow

enter the venous sinusoids contained in the red pulp, facilitating their removal by the resident red pulp macrophages. It is also the major site for antibody-secreting plasma cells, induced in response to incoming

blood-borne antigens. The white pulp consists of the periarteriolar lymphoid sheath (PALS) which contain T cells and CD8 $\alpha$ <sup>+</sup> dendritic (DC), and adjacent lymphoid follicles containing mainly circulating B cells, known as follicular B cells (FB). The outer boundary of the white pulp, adjacent to the red pulp, constitutes the marginal zone (MZ), which contains an inner ring of marginal metallophilic macrophages (MMM, MOMA-1<sup>+</sup>), CD11b<sup>+</sup> DC, a non-circulating B cell subset called the marginal zone B cell (MZB), and marginal zone macrophages (MZM, ERTR-9<sup>+</sup>) (78). The sluggish blood flow in the MZ that facilitates prolonged contact of blood-borne pathogens with these immune cells, makes the spleen a crucial site in the initial host response to infectious agents, including PS-encapsulated extracellular bacteria (79).

### 1.7.1 Antigen-presenting cells (APC) and antigen presentation in the spleen

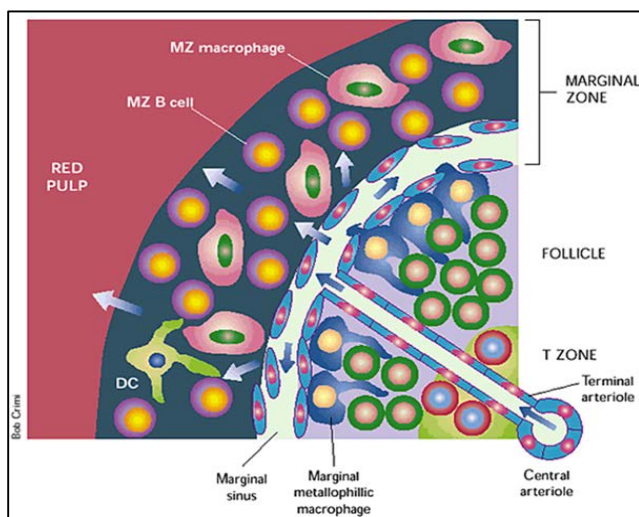


**Fig 4: Schematic view of the mouse splenic marginal zone.**  
**Image courtesy: Nature Immunology 1, 9 - 10 (2000).**

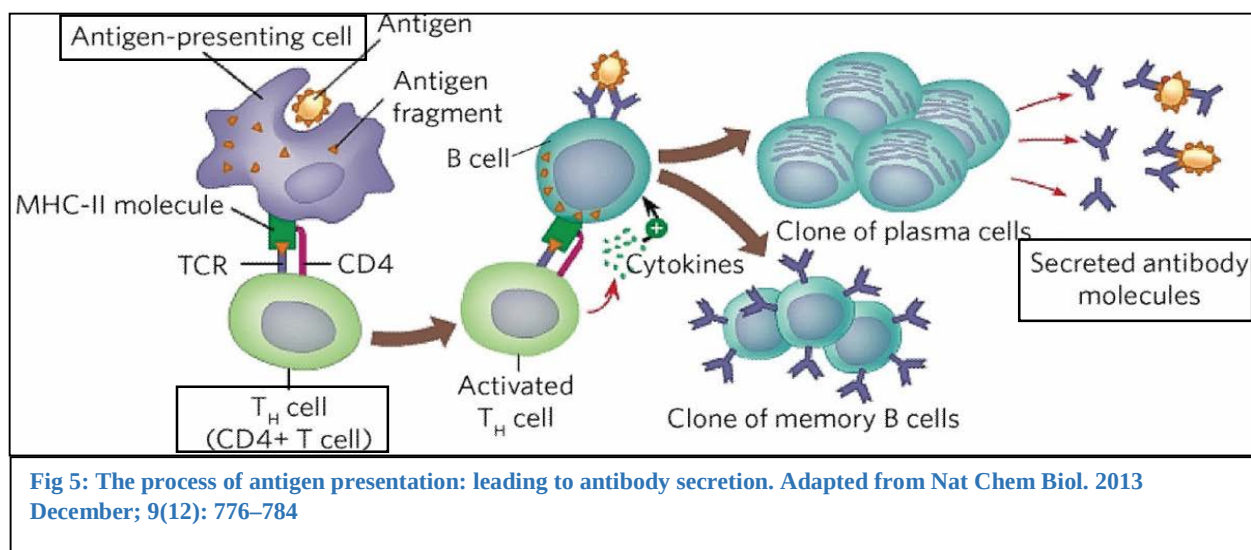
DC, B cells, macrophages and, monocytes/monocyte-derived cells are considered the classical APCs which are responsible for capturing antigens (Ag), and further processing and presenting the derived peptides in non-covalent association with major histocompatibility complex (MHC) molecules, to naïve T cells, resulting in their activation and differentiation into effector T cells. These APC, excluding B cells, are considered part of the

mononuclear phagocyte system (MPS) (80). In addition to their dominant roles as APC for CD4<sup>+</sup> T cells, they also play an important role in tissue homeostasis. Cells of the MPS originate from distinct cell lineages at different stages of embryonic development, and can be distinguished phenotypically (80, 81). Though macrophages are capable of acting as APC to activate T cells, they are also highly efficient

phagocytic cells, involved in clearing and digesting senescent and apoptotic cells, cellular debris and pathogens (82). In mouse spleen, macrophages are further categorized into red pulp (RP) macrophages (F4/80<sup>high</sup>CD11b<sup>low</sup>Ly6C<sup>-</sup>) (83), the marginal zone (MZ) macrophages (MZM) expressing ER-TR9 and marginal metallophilic macrophages (MMM) expressing MOMA-1; the latter two are located within the MZ of the spleen (84) (Fig 4). Mouse DC within the spleen are divided into classical/conventional (c)DC (MHC-II+CD11c<sup>high</sup> [either CD8α<sup>+</sup> or CD11b<sup>+</sup>]) and plasmacytoid (p)DC (PDCA-1+CD11c<sup>low</sup>) (85-87). DC are cells with stellate morphology and can potentially present antigens on MHC molecules to activate naïve T cells (88, 89). Although DC are efficient in uptake of soluble antigens, they also exhibit phagocytic activity. Mouse monocytes are classified as Ly6C<sup>hi</sup> classical monocytes and Ly6C<sup>low</sup> non-classical monocytes (80, 90). Recent studies have demonstrated that Ly6C<sup>hi</sup> monocytes can capture and transport antigens to secondary lymphoid organs including spleen and lymph nodes where they mature into APC capable of activating naïve T cells, without differentiating into, but acquiring characteristics of cDC or macrophages. They are then referred to, generally as monocyte-derived cells (MoC) characterized as Ly6C<sup>hi</sup>CD11b<sup>hi</sup>F4/80<sup>low</sup> (80, 91, 92). MoC appear to be especially efficient in capturing particulate antigens (93).

Antigen uptake and presentation by APC (Fig 5) is an early crucial event in the initiation of a protective antigen-specific antibody response (94, 95). APC internalize antigen, either via phagocytosis, pinocytosis or receptor mediated endocytosis, based on the property of the antigen (20, 96). Antigen is then processed and presented to naïve CD4<sup>+</sup> T cells as peptide-MHC-II complexes, for initial priming within the T cell zones of secondary lymphoid organs followed by migration of T cells to the T cell-B cell border. B cells initially coming in contact with Ag either through direct Ag binding in the MZ, or following transfer from DC, macrophages, or B cells to FDC which then display the intact Ag to B cells, are also activated to migrate to the T cell-B cell border where they come into contact with primed T cells (97, 98). Activation

of CD4<sup>+</sup> T cells by antigen-presenting B cells, leads to the differentiation of T cells into T follicular helper (T<sub>FH</sub>) cells that then enter the follicle to initiate a germinal center (GC) reaction (99). During the first week of the immune response, sustained B cell-T cell interactions result in the transition of T<sub>FH</sub> into GC T<sub>FH</sub> cells, critical for induction of a GC reaction (99), during which the activated B cells undergo iterative cycles of targeted mutagenesis and affinity-based selection resulting in B cell proliferation and differentiation into either long term plasma cells, that secrete antigen-specific antibody, and somatically mutated high-affinity memory B cells, that ensure sustained immune protection along with rapid recall responses against previously encountered foreign antigens (100-102). An early T cell-independent or T cell dependent extra-follicular reaction can also occur leading to antibody production by short-lived plasma cells (103, 104).



### 1.7.2 Splenic B cell subsets and CD4<sup>+</sup> T cells involved in immune responses.

Although numerous studies have been published on the uptake and presentation of soluble proteins by B cells and subsequent presentation to T cells (64, 65), little is known regarding the mechanism by which B cells capture and present bacterial Ag following host exposure to intact bacteria (66, 67). In mice, naïve

B cells are classified as B-1 B cells, FB cells, and MZB cells. MZB cells are strategically located within the marginal zone, where blood first enters the spleen, and hence, can act as initial responders to blood-borne bacteria and viruses (105-107), where they can participate in the early stages of T cell activation (106). Splenic MZB can non-specifically bind to immune complexes, independent of the BCR, through complement receptors CR1 and CR2 and also capture antigen from MZM, and then migrate and transport Ag to the follicles, in a CXCR5-dependent manner, where follicular dendritic cells (FDC) compete for binding to the Ag (70, 71). As suggested in Project #1, the depletion of select APCs involved in antigen transport might lead to a reduction in the subsequent generation of TFH, GC, and antibody responses. Following this process of Ag transport, MZB migrate back to the MZ, a chemotactic process controlled mainly by S1P1 and S1P3 (108). MZB show heightened responses to various stimuli and can differentiate rapidly to form plasma cells, making it the initial antibody-secreting cells for both T-independent (TI), as well as T-dependent (TD) Ag (109-111). MZB expresses the highest levels of TACI, which is mainly responsible for the development of Abs against PS antigens. Although MZB are strongly implicated in PS-specific Ig responses, they can also elicit rapid protein-specific Ig responses, where they are programmed to favor plasma cell differentiation over memory B cell generation (112). The accelerated immune response mediated by MZB may reflect their higher levels of MHC-II, B7-1 and B7-2 relative to FB, making them more efficient at activating T cells (113). FB are considered the major B cell subset eliciting TD Ig responses to proteins. FB are activated by TD Ag of microbial origin through delivery of synergistic signals by BCR, CD40 and TLR, resulting either in their differentiation into short-lived Ab-secreting cells in the extrafollicular region of the spleen, or their entry into the GC for differentiation into long term antigen-specific memory B cells or Ab-secreting plasma cells, that migrate to the bone marrow and persist there for many years (114). TI Ig responses are largely mediated by MZ and B-1 B cells (106). B-1 B cells make up a high percentage of B cells at serosal sites such as the peritoneum and pleura, and

although present in much lower percentages in the spleen, are still quantitatively more numerous at this site relative to the serosa. (113). B-1 cells play a major role in Ig responses to TI antigens like phosphorylcholine (PC) and antigens expressed by intact encapsulated pathogenic bacteria like Pn, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae* and *Haemophilus influenza* (115-117). B1 cells from the peritoneum efficiently internalize particulate bacteria in a BCR-mediated fashion (68) and prime CD4<sup>+</sup>T cells (69). B-1 B cells are further divided into B-1a and B1b cells. B-1a B cells are involved in production of natural antibody whereas B-1b B cells are associated with induction of adaptive immunes to PS antigens (118, 119).

CD4<sup>+</sup>T cell activation is initiated following engagement of the T cell antigen-specific receptor (TCR) with peptide-MHC-II complexes on the surface of APC, in concert with secondary co-stimulatory signals provided by a number of APC surface proteins, including B7-1 (CD80) and B7-2 (CD86), which bind to CD28 on the T cell, and ICOSL which binds to ICOS on the T cell (120). Other members of the CD28 immunoglobulin superfamily consist of co-inhibitory molecules like PD1 and CTLA-4 on T cell which bind to PDL1/PDL2, and B7-1/B7-2, respectively on the APC (121). CD28 is constitutively expressed on the surface of naïve T cells and thus binds rapidly to CD80 and CD86 constitutively expressed on the surface of APC (122, 123), including B cells. ICOS, in contrast, is induced only upon CD4<sup>+</sup> T cell activation through TCR crosslinking and CD28-mediated signaling, which then binds to its co-stimulatory ligand ICOSL. CD28 plays an important role in initial activation of CD4<sup>+</sup> T cells (124, 125), whereas ICOS is critical in subsequent T cell effector functions (126, 127). The B7/CD28 and ICOS/ICOSL signaling pathway is critical for both, type 1 and type 2 CD4<sup>+</sup> T cell-dependent humoral immune responses (128-130). ICOS, which is expressed on T<sub>FH</sub>, is critical for promoting GC formation, and also production of isotype-switched antibody secretion, following immunization (131, 132). ICOS signaling

induces expression of CD40L expression on CD4<sup>+</sup> T cells (121), which is required for interacting with CD40 receptor on B cell to further increase B cell maturation, survival and Ig isotype switch (133), thereby playing a major role in regulating immunological memory.

## **1.8 Current Projects**

In the first study, we were interested in identifying the Antigen Presenting Cells involved in the immune responses to intact bacteria versus soluble conjugate vaccine or protein antigen. In spite of the similarities shared between intact Pn and conjugate vaccine, they differ in several important ways (61-63). Moreover, it has been already established previously that soluble antigens are internalized through pinocytosis/endocytosis and particulate antigens like intact extracellular bacteria are internalized by phagocytosis. Dendritic cells and macrophages MC are both phagocytic, taking up both large and small particles, but ingestion varies with particle size. DC preferentially ingest smaller particles in the viral size range, while macrophages take up more of the larger, bacterial size particles (134). Based on this rationale we hypothesized that *Distinct APCs are involved in activating CD4<sup>+</sup> T cells in response to the same protein expressed by intact heat-killed bacteria versus soluble antigen.*

The second project was a continuation of a previous work from the laboratory that was involved in studying the mechanism of capsular polysaccharide specific IgG responses to heat-killed Gram-negative *Neisseria meningitidis*, (MenC) and Gram-positive *group B Streptococcus* (GBS-III) bacteria. In this current study, we were involved in determining the role of memory B and/ T cells in elicitation of augmented antibody responses to Gram positive and Gram negative bacteria.

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## Chapter 2

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# MANUSCRIPT 1

## **Title**

Distinct cellular pathways for induction of CD4+ T cell-dependent antibody responses to antigen  
expressed by intact bacteria versus isolated soluble antigen

## **Running title**

Distinct APCs for TD responses to bacteria versus soluble Ag

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## **Abstract:**

Uptake of intact bacteria and soluble antigens by APCs are mediated by phagocytosis and endocytosis or pinocytosis, respectively. Thus, we predicted that injection of clodronate-containing liposomes (CL), which selectively deplete cells efficient in phagocytosis, would inhibit murine CD4<sup>+</sup> T cell-dependent IgG responses to antigens expressed by intact bacteria but not isolated soluble antigens. Surprisingly, injection of CL markedly inhibited protein-specific IgG responses to both intact, heat-killed *Streptococcus pneumoniae* (Pn) as well as a soluble OVA-polysaccharide conjugate or OVA alone. IgG anti-polysaccharide responses to bacteria and conjugate were also reduced, but more modestly. In both instances, CL-mediated inhibition was associated with a significant reduction in induced germinal centers (GC) and CD4<sup>+</sup> GC T follicular helper cells. However, CL injection which largely abrogated the proliferative response of adoptively transferred OVA peptide-specific transgenic CD4<sup>+</sup> T cells in response to immunization with Pn expressing OVA peptide, did not inhibit T cell proliferation in response to OVA-PS or OVA. In this regard, monocyte-derived cells (MoC), depleted by CL, internalized Pn in vivo, whereas CD11c<sup>low</sup> dendritic cells, unaffected by CL injection, internalized soluble OVA. Ex vivo isolation and co-culture of these respective APCs from Pn- or OVA-immunized mice with OVA-specific T cells, in the absence of exogenous antigen, demonstrated their selective ability to induce T cell activation. These data suggest that while distinct APCs initiate CD4<sup>+</sup> T cell activation in response to antigen expressed by intact bacteria versus antigen in soluble form, CL-sensitive cells appear necessary for the subsequent IgG responses to both forms of antigen.

## **Introduction:**

Dendritic cells (DC), monocytes (and monocyte-derived cells [MoC]), and macrophages are members of the mononuclear phagocyte system (MPS) that originate from distinct cell lineages at different stages of embryonic development, and can be distinguished phenotypically (80, 81). Collectively, they play dominant roles as antigen-presenting cells (APCs) for CD4<sup>+</sup> T cells (80). Mouse DC within the spleen are further divided into conventional/classical (c)DC (MHC-II<sup>+</sup>CD11c<sup>high</sup> [either CD8α<sup>+</sup> or CD11b<sup>+</sup>]) and plasmacytoid (p)DC (PDCA-1<sup>+</sup>CD11c<sup>low</sup>) (80, 135-137). Although DC are efficient in uptake of soluble antigens, they also exhibit phagocytic activity. Mouse monocytes are classified as Ly6C<sup>hi</sup> classical monocytes and Ly6C<sup>low</sup> non-classical monocytes (80, 90). Ly6C<sup>hi</sup> monocytes, in particular can internalize and transport antigen to secondary lymphoid organs such as the spleen, where they mature into APCs capable of activating naïve T cells. They are then referred to, generally as monocyte-derived cells (MoC) characterized as Ly6C<sup>high</sup>CD11b<sup>high</sup>F4/80<sup>low</sup> (80, 91, 92). MoC appear to be especially efficient in capturing intact bacteria (93). Macrophages are highly efficient at phagocytosis and play a major role in clearing senescent and apoptotic cells, cellular debris and pathogens, but are also capable of acting as APC to activate T cells (82). In mouse spleen, macrophages are further divided into red pulp (RP) macrophages (F4/80<sup>high</sup>CD11b<sup>low</sup>Ly6C<sup>-</sup>) (83), marginal zone macrophages (MZM) expressing ER-TR9, and marginal metallophilic macrophages (MMM) expressing MOMA-1, the latter two located within the MZ (84).

Within the first 24 hours of the initiation of an immune response, APCs capture, internalize, and process antigen into peptides that are then presented, via MHC-II to naïve CD4<sup>+</sup> T cells for priming within the T cell zones of secondary lymphoid organs followed by migration to the T cell-B cell border (138, 139). During this time antigen-specific B cells also capture antigen, via their B cell receptor (BCR), obtained, either directly or via transfer from other B cells, macrophages, or DC, internalize and process the antigen

into peptides, migrate to the T cell-B cell border and present peptide-MHC-II complexes to APC-primed T cells (97, 98). Over the ensuing week, sustained B cell-T cell interactions result in the induction of germinal center T follicular helper (GC T<sub>FH</sub>) cells, critical for induction of a GC reaction (99). The GC serves as the critical microenvironment for the generation of both memory B cells and long-term plasma cells. An early T cell-independent (TI) or T cell-dependent (TD) extrafollicular reaction can also occur leading to antibody production by short-lived plasma cells (103, 104).

Little is known regarding the specific APCs that initiate T cell activation during TD antibody responses to intact extracellular bacteria versus soluble antigens. We previously demonstrated that both polysaccharide (PS)- and protein-specific IgG responses to intact extracellular bacteria (140), as well as soluble conjugate vaccines (63) are dependent on CD4<sup>+</sup> T cells. However, the PS-specific IgG response to an intact bacterium arose from MZ B cells expressing a distinct and dominant idiotype in contrast to the same PS-specific IgG response to a soluble conjugate vaccine, which arose from follicular B cells expressing a different idiotype(s) (61-63). This dichotomy was specifically dependent on whether the PS was co-expressed with protein in a particulate or soluble form (57). Of note, although alum adjuvant is particulate, antigen adsorbed to alum did not behave as a particulate antigen (61). In this regard, DC exposed to alum-adsorbed antigen exhibited facilitated antigen uptake, but did not internalize the alum particles (141). These data suggested that soluble antigens, even those adjuvanted with alum, and antigens expressed by intact bacteria are processed through distinct cellular pathways that might utilize different APCs.

In this study we determined the role of distinct APCs in initiating TD IgG responses to PS and protein expressed by intact, heat-inactivated *Streptococcus pneumoniae* (Pn) versus a soluble PS-protein conjugate vaccine, or soluble protein alone. Since intact bacteria, in contrast to soluble antigens, are internalized by APCs via phagocytosis, we utilized clodronate liposomes to selectively deplete cells highly efficient in phagocytic uptake, to determine a potential differential effect on the immune response to these different forms of antigen. Specifically, we observed that MoC selectively internalized intact Pn in vivo and presented Pn-derived peptide to specific CD4<sup>+</sup> T cells, whereas DC that are CD11c<sup>low</sup>, internalized and presented peptide from conjugate vaccine or isolated protein, to the same specific CD4<sup>+</sup> T cells. However, we also observed a possible common pathway for optimal induction of the GC T<sub>FH</sub> and GC response, as well as the subsequent IgG response to both forms of antigen, mediated by clodronate liposome-sensitive, phagocytic cells.

## **Materials and methods**

**Mice.** BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Homozygous DO11.10 x RAG-2<sup>-/-</sup> mice (C.Cg-Rag2tm1Fwa Tg(DO11.10)10Dlo) (BALB/c background) [from here on referred to as “DO11.10 mice”], in which all CD4<sup>+</sup> T cells express a transgenic T cell receptor that encodes for an OVA peptide (amino acids 323-339), presented by MHC-II I-A<sup>d</sup>, were purchased from Taconic Farms (Hudson, NY). They were thereafter bred in our facility. 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.

**Pneumococcal strain R614-OVA<sup>323-339</sup>.** The *S. pneumonia* (Pn) strain R614JPspA, a variant of the strain R614 (Pn expressing capsular polysaccharide type 14) in which the *pspA* (Pn protein) locus is substituted by a Janus cassette, was transformed (142) with the PCR amplification product of *pspA*, using purified genomic DNA of the strain TIGR4-PspA<sup>OVA</sup> as template. Primers used are those corresponding to positions 910-888 upstream (TTM053) and 941-923 downstream (TTM069) of the *pspA* in strain TIGR4 (143). The strain TIGR4-PspA<sup>OVA</sup> has been extensively described, and expresses a fusion protein of PspA and the OVA<sup>323-339</sup> immunodominant peptide at the proline-rich domain of the PspA (143). Expression of the truncated PspA<sup>OVA</sup> by the R614-PspA<sup>OVA</sup> (R614-OVA<sup>323-339</sup>) as transformant colonies was confirmed by reactivity with rabbit polyclonal anti-OVA (SIGMA), and mouse mAbs anti-PPS14 (clone 44.1).

**Bacteria, reagents, and immunizations.** A frozen stock of R614-OVA<sup>323-339</sup> was thawed and sub-cultured on BBL premade blood agar plates (VWR International) and processed for immunization as described earlier (60). OVA (“Imject OVA”) were purchased from Thermo Scientific (Rockford, IL). Purified Pn capsular polysaccharide type 14 (PPS14) was purchased from ATCC. A covalent conjugate of PPS14 and OVA [PPS14-OVA] was prepared as previously described (144). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Denmark). A stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN) was synthesized as previously described (145). Mice were immunized i.p. at day 0 and boosted on day 14. Depending on the experiment, groups of 7 mice were immunized with  $2 \times 10^8$  CFU heat-killed R614-OVA<sup>323-339</sup> or pHrodo labeled R614-OVA<sup>323-339</sup> in saline (i.p.), 5 µg of purified PPS14-OVA in saline (i.p), or 50 µg of OVA or pHrodo labeled OVA, adsorbed on 13 µg of alum mixed with 25 µg of CpG-ODN (i.p). Serum samples were prepared, at different time points (day 0, 7, 14 and 21), from blood obtained through the tail vein.

**Labeling R614-OVA<sup>323-339</sup> and OVA with pHrodo.** R614-OVA<sup>323-339</sup> was labeled as described earlier (146). In brief, bacteria were grown in Todd Hewitt broth (BD Biosciences) and heat-killed, as mentioned above. Immediately before labeling, cells were washed with PBS, followed by suspending 20 mg (wet weight)/ml of the cells in freshly prepared 100 mM sodium bicarbonate solution, pH 8.5 (Sigma) and divided into 750  $\mu$ l aliquots. A 10 mM stock solution of pHrodo<sup>TM</sup> Red Succinimidyl Ester (Molecular Probes®) in dimethyl sulfoxide (DMSO) (Sigma) was added to the bacterial suspension to adjust the final concentration to 0.1 mM. Samples were then incubated for 45 min at room temperature in the dark. 750  $\mu$ l of Hank's Balanced Salt Solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> (Gibco) was then added, and the mixture was centrifuged for 60 s at 14,000 x g. The supernatant was discarded and the pellet suspended in PBS and stored at 4°C in the dark. The maximal fluorescence emission of pHrodo<sup>TM</sup> red-labeled R614-OVA<sup>323-339</sup> was 585 nm and the property was used to evaluate the labeling, by FACS. To estimate the concentration/count of the labeled bacteria, TruCOUNT tubes (BD pharmingen) were used, according to manufactures protocol. pHrodo® Red microscale protein labeling kit (Molecular Probes®) was used to label OVA, according to the manufacture's protocol. In brief, 10  $\mu$ l of sodium bicarbonate was added to 100  $\mu$ g of OVA. 10  $\mu$ l DMSO was added to a vial of pHrodo. 1  $\mu$ l of the dye in DMSO was added to the protein and gently mixed by pipetting. The mixture was incubated for 15 min at room temperature and purified as recommended. The protein concentration was estimated by using NanoDrop.

**Clodronate-containing liposomes.** Phosphate-buffered saline encapsulated liposomes (PBSL) or clodronate-encapsulated liposomes (CL) (5 mg/ml) were obtained from ClodronateLiposomes.org (Amsterdam, The Netherlands). To deplete macrophages and monocytes, mice were injected i.v. with 200  $\mu$ l of CL (147). PBSL was injected as a control. The dimensions of CL vary between 150 nm and 3  $\mu$ m.

**Flow cytometric analysis.** Individual samples of RBC-lysed spleen cells from 3-5 mice/group were stained using the mouse-specific mAbs listed in Table I, after incubating them with *FcR-blocking antibody* (2.4G2, BioXcell) at a concentration of 1  $\mu\text{g}$ /million cells in 100  $\mu\text{L}$ . Cells were analyzed using a LSR-II flow cytometer (BD Biosciences) and results were generated using the software FlowJo (Tree Star, Ashland, OR) and FACSDiva (BD Biosciences).

**Measurement of T-cell proliferation by CFSE dilution.** The process was carried out as described earlier (148). Briefly, RBC-lysed spleen cells from DO11.10 mice ( $2.5 \times 10^7$  cells) were incubated in 1 ml of 5  $\mu\text{M}$  CFSE (Vybrant CFDA-SE; 20 Molecular Probes, Grand Island, NY) in PBS for 10 min at 37°C. Cells were then washed 1x, re-suspended in pre-warmed PBS, and incubated at 37°C for 30 min. Cells were then washed 2x with PBS and transferred i.v. into WT BALB/c mice ( $2.5 \times 10^6$  spleen cells/mouse containing  $\sim 5 \times 10^5$  DO11.10 Tg T cells). One day later, mice were injected i.v. with CL or PBSL. The following day, they were immunized with R614-OVA<sup>323-339</sup> or PPS14-OVA (+ alum + CpG-ODN) or OVA (+ alum + CpG-ODN). At 2.5 or 3 d post-immunization, spleen cells were obtained, and gated DO11.10 Tg T cells (CD4<sup>+</sup> DO11.10 TCR<sup>+</sup>) were analyzed for CFSE dilution using an LSR-II flow cytometer (BD Biosciences) and ModFit LT software (Verity Software House, Topsham, ME). The ModFit LT software was further used for calculating the proliferation index (PI) which determines the fold-expansion of the overall culture (149).

**ELISA.** For measurement of serum titers of PPS14-specific, OVA-specific, or PspA-specific IgG, Immulon ELISA plates were coated overnight at 4°C with purified PPS14, OVA or recombinant PspA, respectively (5  $\mu\text{g}/\text{ml}$ , 100  $\mu\text{l}/\text{well}$ ) in PBS. 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 IgG Abs

(200 ng/ml) 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 1 M Tris + 0.3 mM MgCl<sub>2</sub> (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 antigen-specific IgG were calculated as described previously (55, 60).

**Antigen uptake and presentation assays.** For study of antigen uptake, 3-5 BALB/c mice were immunized i.p. with pHrodo labeled OVA + alum + CpG-ODN or pHrodo-labeled R614-OVA<sup>323-339</sup> or PBS. Mice were euthanized after 5 h and spleen was harvested and processed for flow cytometry as described above. For study of antigen presentation, 3 BALB/c were immunized with OVA + alum + CpG-ODN or R614-OVA<sup>323-339</sup>. After 5h, mice were euthanized, spleens were harvested and processed together/group, to obtain RBC-lysed single cell suspensions. The cells were counted and incubated with *FcR-blocking antibody* (2.4G2, BioXcell) at a concentration of 1-μg/million cells in 100 μl. APCs from the cell suspension were then enriched by carrying out a negative selection, by combining CD19 + CD90.2 microbeads (Miltenyl Biotec), according to manufactures protocol. The magnetically-sorted cells were stained using the mouse-specific mAbs listed in Table I and prepared for FACS sorting. Red pulp macrophages, MoC, monocytes, CD11c<sup>low</sup> DC and CD11c<sup>high</sup> DC were sorted in individual tubes, washed and counted. Spleens from DO11.10 Tg mice were harvested, processed and negatively selected using the Pan T Cell Isolation Kit II, mouse (Miltenyl Biotec), for obtaining OVA specific CD4<sup>+</sup> Tg T cells. APC and DO11.10 Tg T cells were co-cultured in 96 well plates overnight, at a ratio of 4:1, which was determined to be optimal for T cell activation in comparison to other ratios (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:8), employed in the initial experiments. The following day, plates were washed 2x and the cells in each well was stained with mouse-specific mAbs: PE-eFluor 610- anti CD4, FITC- D011.10 TCR, PE-anti-CD69, and stain for dead cells, SYTOX® Blue (Molecular Probes®). Cells were analyzed using

a LSR-II flow cytometer (BD Biosciences) and results were generated using the software FlowJo (Tree Star, Ashland, OR) and FACSDiva (BD Biosciences).

**Immunohistochemistry and immunofluorescence.** Tissues were fixed and processed as described earlier (60). Tissue sections of 8  $\mu\text{m}$  were obtained from Histoserv Inc (Germantown, MD). Tissue sections were stained with AF647-anti-B220 and FITC-MOMA or PE-ERTR9 for staining of MMM and MZM, respectively. For GC staining the sections were stained with AF488-GL7 and AF647-anti-IgD. Immunofluorescence imaging was performed at room temperature with a Zeiss Pascal Laser Scanning Confocal Microscope (original magnification  $\times 10$ ; type: Plan Apochromat; numerical aperture: 0.75 NA; acquisition software: AIM; Carl Zeiss).

**Statistics.** Serum IgG titers, GC  $T_{\text{FH}}$ , CFSE-based generation analyses, and PI indices were expressed as the geometric means  $\pm$  SEM of the individual values. Significance was determined by the Student *t* test. *p*-values of  $\leq 0.05$  were considered statistically significant. All experiments were performed at least two times.

## **Results:**

**Splenic macrophages and monocytes (and monocyte-derived cells [MoC]), but not conventional dendritic cells (cDC) or neutrophils, were depleted by clodronate liposomes (CL).**

To determine the potential role of macrophages and monocytes/MoC in mediating antibody production in response to systemic immunization with antigen expressed by an intact bacterium versus an isolated, soluble antigen, we tested the ability of CL (150, 151), injected i.v., to selectively deplete the former cell populations in the spleen, the major site of antibody production. Thus, we combined flow cytometric analysis and confocal microscopy to determine the effect of i.v. CL on splenic subpopulations using

multiple markers as listed in Table II (152-154). Mice (n=3) were injected with CL or PBSL and spleen cells were obtained 18-24h later. Cells were gated to remove CD19<sup>+</sup> (B cells) and CD90<sup>+</sup> (T cells) from the analyses. As shown in Fig. 1A, the percentage of red pulp macrophages (P1), MoC (P2), and monocytes (P3), as determined by flow cytometry, were markedly reduced following CL relative to PBSL injection, whereas the percentage of neutrophils (P4) was unaffected (Fig 1A). Further, flow cytometric analysis of CD11c<sup>high</sup> cDC (P1), CD11c<sup>low</sup> DC (P2), and pDC (P3) (Fig 1B) demonstrated no significant depletion by CL. Finally, confocal microscopy confirmed a marked CL-mediated depletion of MZM and MMM (Fig 1C). These data thus demonstrated the feasibility of using i.v. CL to selectively deplete splenic macrophages and monocytes/MoC while leaving DC and neutrophil numbers essentially unaffected.

**Injection of CL prior to immunization with intact R614-OVA<sup>323-339</sup>, conjugate vaccine (PPS14-OVA) or OVA resulted in inhibition of both PspA/OVA- and PPS14-specific IgG responses in vivo.**

We previously demonstrated that intact, heat-inactivated *Streptococcus pneumoniae*, capsular polysaccharide type 14 (PPS14) induces CD4<sup>+</sup> TD IgG responses specific for PPS14 and for several pneumococcal proteins, including pneumococcal surface protein (PspA) (140). IgG responses to both the protein and polysaccharide components of conjugate vaccines are also dependent on CD4<sup>+</sup> T cells (140). We produced a genetic variant of a strain of type 14 *S. pneumoniae* (R614) in which the gene for wild-type PspA was replaced by a gene encoding a fusion of PspA and an OVA peptide (OVA<sup>323-339</sup>) to which transgenic (DO11.10) CD4<sup>+</sup> T cells were specific, in the context of Ia<sup>d</sup> (BALB/c background). This variant of R614 (termed R614-OVA<sup>323-339</sup>) expressed PspA-OVA<sup>323-339</sup> naturally in the bacterial cell wall (data not shown) and was used in all subsequent experiments. To evaluate a possible selective role of macrophages and/or monocytes/MoC in the IgG response to intact R614-OVA<sup>323-339</sup> versus conjugate vaccine or isolated protein, mice were first injected i.v. with CL or PBSL, and then immunized 18-24h

later i.p. with either intact heat-killed R614-OVA<sup>323-339</sup>, PPS14-OVA, or OVA, the latter two in alum + CpG-ODN adjuvant. Mice were boosted in a similar fashion 14 d later, in the absence of additional CL. The doses of antigen used were shown previously to elicit optimal IgG responses. Injection of CL resulted in significantly reduced primary and secondary PspA-specific (6-8 fold) and PPS14-specific IgG (3-4 fold) responses to R614-OVA<sup>323-339</sup> relative to immunized mice treated with PBSL (Fig. 2A). In addition, the primary and secondary PPS14-specific (3 fold) and OVA-specific IgG (8-74 fold) responses to PPS14-OVA conjugate (Fig 2B) and the primary and secondary OVA-specific IgG (5-14 fold) response to OVA alone (Fig 2C) were also significantly reduced in CL-treated mice. The PspA/OVA-specific IgG responses were consistently reduced to a significantly greater degree than that observed for PPS14, in response to intact R614-OVA<sup>323-339</sup>, PPS14-OVA, and OVA alone.

**CL-mediated inhibition of the IgG responses to R614-OVA<sup>323-339</sup> and OVA were both associated with a marked reduction in the generation of germinal center (GC) T follicular helper (GC T<sub>FH</sub>) cells and GC formation.**

To better define the basis for the reduced PspA/OVA- and PPS14-specific IgG responses following treatment with CL, we determined if this reduction was associated with an inhibition in the generation of splenic GC T<sub>FH</sub> and GC, cellular events that are critical for induction of humoral immune responses (155). GC T<sub>FH</sub> cells are identified by flow cytometry as CD4<sup>+</sup> GL7<sup>+</sup>PD-1<sup>hi</sup> cells, a population that also expresses CXCR5 (156, 157). 5 x 10<sup>5</sup> DO11.10 Tg T cells were transferred into BALB/c mice, a day prior to either CL or PBSL injection, followed by an additional 24 hours with R614-OVA<sup>323-339</sup> or OVA + alum + CpG-ODN. Spleen cells were obtained 8 days post-immunization, a time point shown previously to exhibit peak numbers of GC T<sub>FH</sub> (157). The percentages of GC T<sub>FH</sub> cells among OVA-specific (DO11.10 Tg+) cells were determined by flow cytometry. As illustrated in Fig. 3A, the percentages of GC T<sub>FH</sub> were

significantly reduced in CL relative to PBSL-treated mice following immunization with either R614-OVA<sup>323-339</sup> (6% versus 2%) or OVA + alum + CpG-ODN (21% versus 6%). To determine the effect of CL injection on GC formation, BALB/c mice were immunized with R614-OVA<sup>323-339</sup> or OVA + alum + CpG-ODN 24 hours after injection with either CL or PBSL, and spleens were obtained 10 days post-immunization to visualize GCs (GL7+) (158). CL- relative to PBSL-treated mice exhibited a marked reduction in the GC response following immunization with either R614-OVA<sup>323-339</sup> or OVA + alum + CpG-ODN (Fig. 3B).

**Injection of mice with CL inhibited proliferation of OVA-specific T cells in response to intact R614-OVA<sup>323-339</sup> but not in response to soluble antigens (PPS14-OVA or OVA).**

We next determined whether the CL-mediated reduction in OVA-specific GC T<sub>FH</sub> (day 8) and total GC (day 10) reflected an earlier inhibitory effect on OVA-specific T cell proliferation. We adoptively transferred 5 x 10<sup>5</sup> CFSE-labeled OVA-specific (DO11.10 Tg+) T cells into BALB/c mice, 24 hours prior to either CL or PBSL injection. This was then followed 24 hours later by immunization with R614-OVA<sup>323-339</sup>, PPS14-OVA conjugate + alum + CpG-ODN, or OVA + alum + CpG-ODN. Flow cytometry confirmed depletion of CL-sensitive cells, similar to that demonstrated in Fig. 1 (data not shown). CFSE dilution, including the proliferative index (PI), of OVA-specific (Tg+) cells, 3d post-immunization, was determined by flow cytometry (Fig 4). Mice receiving only Tg+ cells in the absence of liposomes or immunization were used as controls. CL-treated mice immunized with R614-OVA<sup>323-339</sup> exhibited a significant reduction in the proliferation of the Tg+ T cells relative to PBSL-treated mice (PI index=8.7 +/- 3.7 versus 27 +/- 2.4, respectively), with a significantly diminished percentage of cells per generation (Fig. 4A). In contrast, CL-treated mice immunized with PPS14-OVA exhibited a significantly higher PI of 75 +/- 6.1 versus a PI of 26 +/- 1.3 for PBSL-treated mice (Fig 4B). CL- and PBSL-treated mice

immunized with OVA exhibited similar PIs (CL: PI=6.0 +/- 0.2, PBSL: PI=9.3 +/- 0.2) (Fig. 4C). These data indicated that the CL-mediated inhibition of GC T<sub>FH</sub>, GC, and specific IgG secretion in response to PPS14-OVA or OVA could not be explained by the early inhibition of specific CD4<sup>+</sup> T cell proliferation.

**CD11c<sup>low</sup> DC and MoC preferentially internalized, processed and presented OVA peptide ex vivo to DO11.10 Tg<sup>+</sup> T cells in response to OVA and intact R614-OVA<sup>323-339</sup>, respectively.**

The differential effects of CL on the proliferation, at day 3, of OVA<sup>323-339</sup>-specific CD4<sup>+</sup> Tg T cells in response to R614-OVA<sup>323-339</sup> versus PPS-14-OVA or OVA suggested that intact R614-OVA<sup>323-339</sup> may preferentially use CL-sensitive macrophages or monocytes/MoC as APC, whereas DC which are not depleted by CL, may preferentially present soluble OVA to CD4<sup>+</sup> T cells. To identify the APCs involved in uptake of R614-OVA<sup>323-339</sup> or OVA, BALB/c mice (3 per group) were immunized i.p. with pHrodo-labeled OVA (+ alum/CpG-ODN), pHrodo-labeled R614-OVA<sup>323-339</sup>, or PBS alone. Of note, fluorescence by pHrodo occurs only within the acidic endosome of the APC. Five hours post-immunization, spleen cell suspensions were prepared to measure uptake of pHrodo-labeled Ag by specific sub-populations of cells using multi-parameter flow cytometry (see Fig. 1 and Table II). As illustrated in Fig. 5A, intact R614-OVA<sup>323-339</sup> was internalized selectively by MoC (33 %+) and monocytes (16 %+) with little or no detectable uptake by macrophages, CD11c<sup>low</sup> DC or cDC. In contrast, uptake of soluble OVA was observed in CD11c<sup>low</sup> DC (16 %+) but not in cDC, macrophages, monocytes or MoC (Fig. 5B).

In light of the above data, we next determined if APCs uptake of R614-OVA<sup>323-339</sup> or OVA in vivo was associated with the ability of the APCs to activate OVA<sup>323-339</sup>-specific Tg<sup>+</sup> T cells ex vivo, in the absence of additional, exogenous antigen. Thus, BALB/c mice (3 per group) were immunized with soluble OVA

(+ alum + CpG-ODN) or R614-OVA<sup>323-339</sup> and spleen cell suspensions were prepared 5 hours later. Magnetic sorting followed by electronic cell sorting was then utilized to purify macrophages, monocytes, MoC, CD11c<sup>low</sup> DC and cDC. Sorted cells were co-cultured with DO11.10 CD4<sup>+</sup> Tg T cells for 16 hours and the percentage of Tg<sup>+</sup> cells expressing the activation marker CD69 was measured by flow cytometry. As illustrated in Fig. 6, only MoC were able to activate Tg<sup>+</sup> cells following isolation from R614-OVA<sup>323-339</sup>-immunized mice. In contrast, only CD11c<sup>low</sup> DC activated Tg<sup>+</sup> following immunization with OVA. Thus, selective APCs uptake of antigen in vivo was correlated with the ability of the APC to specifically activate Tg<sup>+</sup> cells ex vivo. Collectively, these data demonstrate CL-sensitive phagocytic cells are critical for uptake and early antigen presentation to CD4<sup>+</sup> T cells in response to an intact bacterium (R614-OVA<sup>323-339</sup>), but not in response to soluble antigens (PPS-OVA or OVA). Further, CL-sensitive cells are critical for induction of GC T<sub>FH</sub>, GC, and antibody production in response to soluble antigens. A potential role for CL-sensitive cells in events downstream of early CD4<sup>+</sup> T cell activation in response to intact bacteria requires further study.

## **Discussion:**

The nature and location of the APCs that initiate antigen presentation to CD4<sup>+</sup> T cells may significantly influence the quantity and/or quality of the subsequent humoral immune response. CL has been extensively used to study the role of highly phagocytic cells, including APCs in experimental animal models (159). In our previous studies on the induction of antibody responses to intact bacteria and soluble antigens we employed the systemic route of immunization, with the spleen as the primary site of the immune response. Thus, we injected CL by the systemic route, which effectively depletes splenic monocytes/MoC, and red pulp, marginal zone, and marginal metallophilic macrophages, with no

significant reductions in CD11c<sup>+</sup> DC (154, 160, 161), in order to determine its effect on antibody responses to both forms of antigen.

In the current study, we demonstrate that distinct APCs are responsible for the induction of CD4<sup>+</sup> T cell activation and proliferation in response to protein antigens expressed by an intact extracellular bacterium versus an isolated, soluble, protein. Thus, MoC, which were sensitive to CL-mediated inhibition, selectively internalized the intact bacterium, R614-OVA<sup>323-339</sup>, and presented the expressed OVA peptide to DO11.10, OVA-specific Tg T cells, which led to their activation. Although 16% of the monocytes also internalized bacteria, they were not efficient in activating DO11.10 Tg T cells (Fig 5A and Fig. 6). Although for technical reasons we were not able to study the potential ability of CL-sensitive, splenic MMM and MZM, for antigen uptake and presentation to CD4<sup>+</sup> T cells, a previous study demonstrated that they were not critical for Ag presentation, despite their ability to internalize antigen (162). Further, based on the distance of red pulp macrophages from the splenic marginal sinus (82) and our data that they do not internalize intact R614-OVA<sup>323-339</sup>, they are unlikely to play a crucial role in early CD4<sup>+</sup> T cell activation. Our current data complement an earlier study by Balázs et. al. that demonstrated the ability of blood MoC (CD11b<sup>+</sup>CD11c<sup>low</sup>) to capture blood-borne, intact *S. pneumoniae* and migrate to the spleen, where they promoted a TI antibody response (93).

In contrast, the CD11c<sup>low</sup> DC, but not the cDC, selectively internalized soluble OVA, followed by processing and activation of OVA-specific DO11.10 Tg T cells. These cells were resistant to CL-mediated depletion. This differential utilization of APCs could explain the CL-mediated inhibition of proliferation of OVA-specific DO11.10 Tg T cells in vivo in response to R614-OVA<sup>323-339</sup> but not in response to soluble

OVA or PPS14-OVA. Surprisingly, CL inhibited the induction of GC and GC T<sub>FH</sub> in response to both R614-OVA<sup>323-339</sup> and soluble OVA, including the subsequent antigen-specific IgG responses. Although APCs such as DC or MoC play a dominant role in CD4<sup>+</sup> T cell activation in the first 24-48 h post-immunization, the generation of GC T<sub>FH</sub> requires sustained interactions between antigen-specific B cells and CD4<sup>+</sup> T cells (97, 155, 163). This process further depends on continued uptake by B cells of antigen within the GC, typically on the surface of follicular DC (FDC) (164). The transfer of antigen to GC B cells and FDC in lymph node and spleen has been shown to be mediated by initial transfer from other immune cells at the site of antigen entry, including marginal zone B cells (108, 165), macrophages (166-169), or dendritic cells (170-174). B cell contact with antigen might occur via direct transfer by antigen-transporting cells, or by initial transfer of antigen from these latter cells to FDCs in the B cell follicle, followed by B cell binding to the FDC-bound antigen (175, 176). Thus, one potential mechanism of CL-mediated inhibition of IgG responses to soluble antigens could be a disruption in antigen transfer by macrophages to B cells for sustained GC B cell-CD4<sup>+</sup> T cell interactions. In addition to potentially mediating antigen transfer to B cells, macrophages are also a source of complement that may play a role in promoting humoral immunity (177, 178) and their production of multiple cytokines and chemokines post-immunization, may also have multiple downstream effects on the humoral immune response. In this regard, a recent study demonstrated that i.p. injection of CL into mice transgenic for Ars (*p*-azophenylarsonate)-specific B cells, followed by immunization i.p. with Ars-KLH in aluminum potassium sulfate (alum) inhibited Ars-specific B cell migration to, and proliferation at the B cell-T cell border, associated with an abrogation of the GC response (154).

Our data demonstrating CL-mediated inhibition of the IgG anti-PspA response to intact R614-OVA<sup>323-339</sup> is consistent with earlier studies demonstrating a significant CL-mediated reduction in TNP-specific IgG

antibody-forming cells following i.v. immunization with other micron-sized, particulate antigens including TNP-SRBC (179) or TNP-Lactobacillus acidophilus (180), or reduction in serum titers of IgG anti-human serum albumin (HSA) in response to liposome-associated HSA (160). However, these studies provided no mechanistic basis for these observations. Similarly, i.p. injection of CL resulted in a marked inhibition in priming of CD4<sup>+</sup> T cells, including IFN- $\gamma$ <sup>+</sup> T cells, following i.p. infection with live *Salmonella typhimurium* (STm) that was associated with a reduced accumulation of MoCs in the spleen (161). However, in contrast to our findings, CL had no effect on the STm-induced IgG2a plasma blast response and both MoC and cDC from STm-infected mice could activate STm-specific CD4<sup>+</sup> T cells ex vivo, in the absence of exogenous antigen (161). The use of a live Gram-negative bacterium in this former study, as opposed to a Gram-positive, heat-killed bacterium used in this study, may potentially underlie the observed differences. Of note, i.v. injection of CL failed to inhibit humoral immune responses to smaller, nanometer-sized particles (i.e. inactivated rabies virus or immune-stimulating complexes containing rabies virus antigens) immunized via the i.v. route (181). In contrast to our observations demonstrating CL-mediated inhibition of OVA-specific IgG responses to i.p. injection of soluble PPS14-OVA or OVA alone, s.c. injection of CL to eliminate phagocytic cells in peripheral lymph nodes, had no effect on serum titers of anti-TNP in response to soluble TNP-KLH injected in the same footpad (182), nor did i.v. administration of CL affect the anti-TNP response of TNP-KLH injected via the i.v. route (183). However, CL injected i.v. both before primary and secondary immunization with soluble bovine serum albumin (BSA) or OVA, markedly reduced the secondary antigen-specific IgG2a response, presumably due to an inhibition in IFN- $\gamma$  production, although CL enhanced the IgG1 response (184). Neither of the three latter studies using soluble antigens included adjuvant, whereas we utilized alum + CpG-ODN, one study used 20 times the dose of antigen (182), and another of the studies administered CL

at both the primary and secondary immunization, and determined only secondary antibody production (184).

Our current data expands upon previous observations that antigens in particulate form have distinct immunologic properties relative to soluble antigens. Thus, relative to soluble antigens, antigens in particulate form are selectively internalized through APC phagocytosis, with greater efficiency (61, 185) but with longer processing time (186), exhibit quantitative and qualitative differences in the antigenic epitopes generated (67), concentrate for extended periods within the marginal zone of the spleen (187), and are presented poorly, if at all, by splenic B cells (67), although efficiently internalized by peritoneal B1b cells (69). Of note, signaling responses of APC can differ markedly in response to microbe-associated molecular patterns (MAMPs) that are expressed in particulate versus soluble form (188). In this regard, we previously demonstrated that anti-PPS14 IgG elicited in response to a covalent conjugate of PPS14 and protein presented on the surface of 1  $\mu$ m latex particles, exhibits a distinct idiotype (44.1-Id) (57, 62) that is secreted by marginal zone B cells (61, 63), whereas the soluble conjugate fails to elicit the 44.1-Id and instead induces an anti-PPS14 IgG response from follicular B cells. The potential relationship between these latter observations and the utilization of distinct APCs for inducing CD4<sup>+</sup> T cell activation in response to these different forms of Ag remains to be determined.

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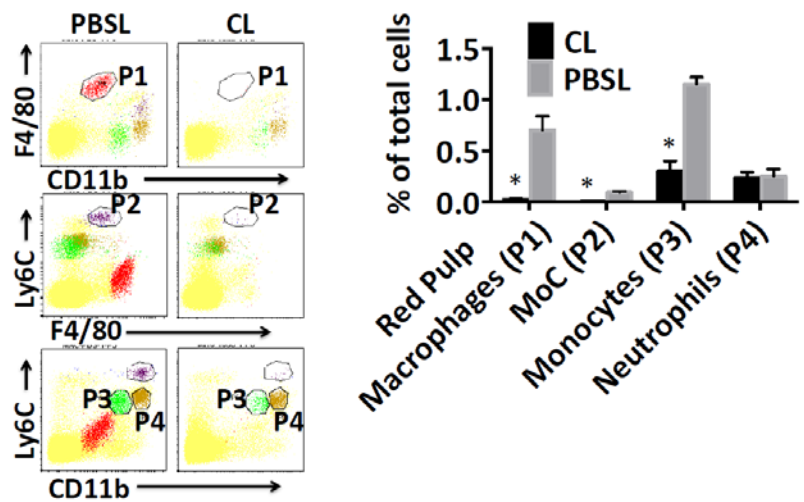
## **Footnotes**

1. Mandatory disclaimer: The opinions expressed herein are those of the authors and are not necessarily representative of those of the Uniformed Services University of the Health Sciences (USUHS), the Department of Defense (DOD), or the United States Army, Navy or Air Force.
2. Supported by: USUHS Dean's Research and Education Endowment Fund (CMS).
3. Correspondence: Clifford M. Snapper, M.D., Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814, Tel: 301-295-3490, FAX: 301-295-1640, E-mail: [clifford.snapper@usuhs.edu](mailto:clifford.snapper@usuhs.edu)
4. Abbreviations: CL, clodronate-containing liposomes; Pn, *Streptococcus pneumonia* strain R614JPspA, a variant of the strain R614 (Pn; expressing capsular polysaccharide type 14); PspA (Pn protein); R614-OVA<sup>323-339</sup>, R614-PspA<sup>OVA</sup> expressing the truncated PspA<sup>OVA</sup> (the OVA<sup>323-339</sup> immuno-dominant peptide at the proline-rich domain of the PspA); PS, polysaccharide; PPS14, Purified Pn capsular polysaccharide type 14; PPS14-OVA, a covalent conjugate of PPS14 and OVA; MoC, monocyte-derived cells; DO11.10 mice, Homozygous DO11.10 x RAG-2<sup>-/-</sup> mice (BALB/c background).
5. Keywords: Rodent, APC, B cells, T cells, macrophages, dendritic cells, monocytes, clodronate liposome, soluble antigen, particulate antigen, antigen presentation, bacterial, antibodies, T<sub>FH</sub>, germinal center, humoral immune response, T cell activation and proliferation.

2.1. Figures and figure Legends

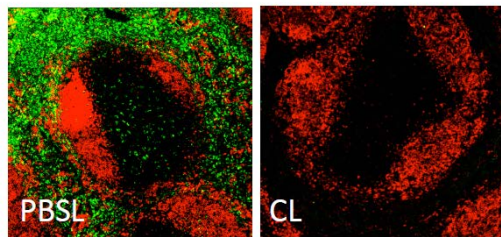
(A) Red pulp Macrophages, Monocytes and Neutrophils

**Fig 1**

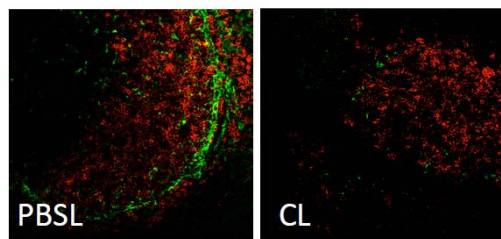


(B) Marginal Zone and Metallophilic Macrophages

ERTR9, B220 (MZM)



MOMA, B220 (MMM)



(C) Dendritic cells

Fig 1

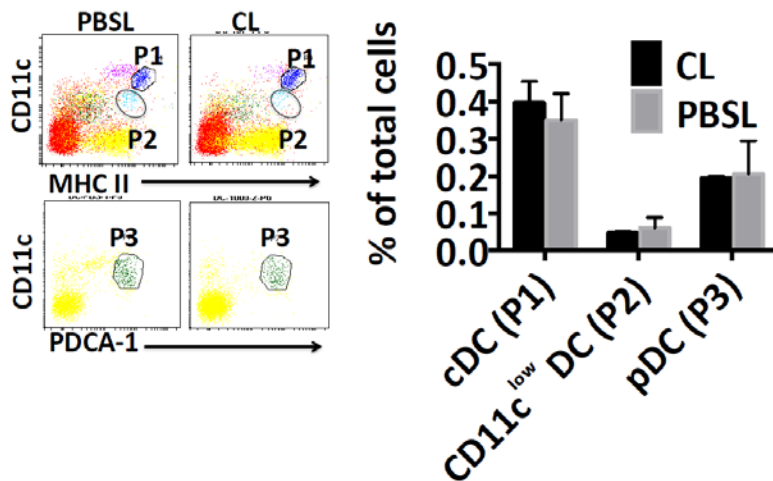
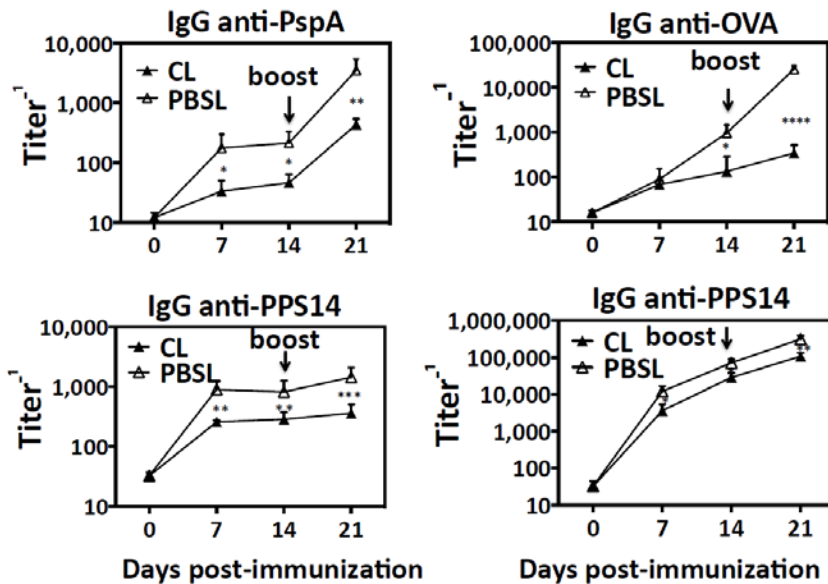


**Figure 1: Clodronate Liposomes (CL) depleted macrophages and monocytes/MoCs but not dendritic cells (DCs) or neutrophils.** BALB/c mice (3-5 per group) were injected intravenously (i.v.) with 200  $\mu$ l of CL or PBS. Spleens were harvested 18-24 h post-injection and processed for flow cytometry and/or confocal microscopy (see Tables I and II). Cells were gated to remove CD19<sup>+</sup> (B cells) and CD90<sup>+</sup> (T cells) cells from the analyses. (A) Flow cytometric analysis of red pulp macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>, P1), monocyte-derived cells [MoCs] (CD11b<sup>+</sup>CD11c<sup>low-int</sup>F4/80<sup>low</sup>Ly6C<sup>hi</sup>, P2), monocytes (CD11b<sup>+</sup>Ly6C<sup>+</sup>, P3), and neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>, P4). Color-coding defines the same cell populations in each of the 3 rows (B) Confocal microscopy demonstrating ERTR9<sup>+</sup> marginal zone macrophages (MZM) and MOMA<sup>+</sup> marginal metallophilic macrophages (MMM). (C) Flow cytometric analysis of conventional DC (cDC) [CD11c<sup>hi</sup>MHCII<sup>+</sup>, P1], CD11c<sup>low</sup> DC (CD11c<sup>low</sup>MHCII<sup>+</sup>, P2) and plasmacytoid DC (pDC) [B220<sup>+</sup>CD11c<sup>+</sup>PDCA-1<sup>+</sup>, P3]. The second row is further gated on B220<sup>+</sup> cells. \*  $p < 0.05$  (significance between the CL treated group relative to the PBS control treated group)

(A) Bacteria (R614-OVA<sup>323-339</sup>)(B) Conjugate (PPS14-OVA) **Fig 2**

(C) Protein (OVA)

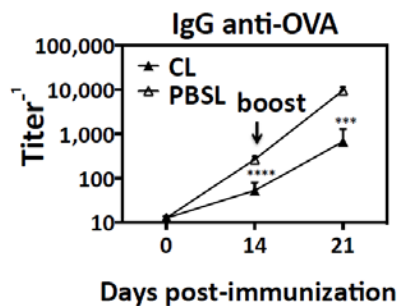
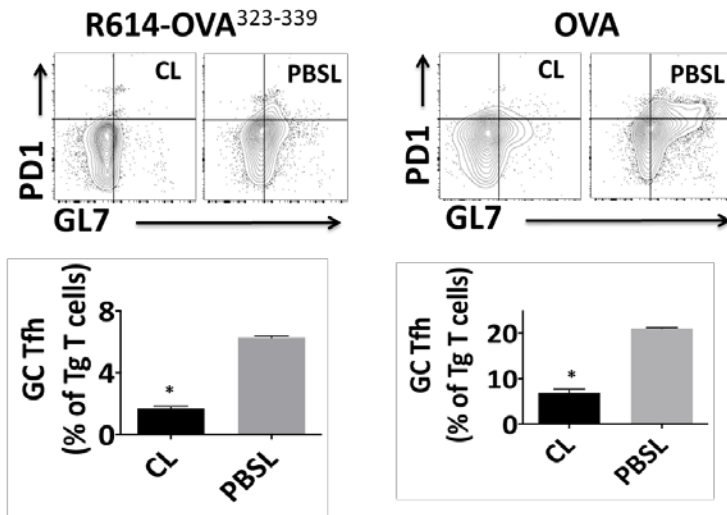


**Figure 2: CL inhibited the IgG anti-PspA response to R614-OVA<sup>323-339</sup> and the IgG anti-OVA responses to PPS14-OVA and OVA.** (A) BALB/c mice (7 per group) were injected i.v. with 200  $\mu$ l of CL or PBS liposomes at day -1 and immunized intraperitoneally (i.p.) at day 0, with  $2 \times 10^8$  CFU heat-inactivated intact R614-OVA<sup>323-339</sup>, (B)

5  $\mu$ g PPS14-OVA with 13  $\mu$ g of alum mixed with 25  $\mu$ g of CpG-ODN, or (C) 50  $\mu$ g OVA with 13  $\mu$ g of alum mixed with 25  $\mu$ g of CpG-ODN. Mice were boosted i.p. with the same dose of antigen on day 14. Serum titers of Ag-specific IgG were determined by ELISA (\*  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.0005$ , \*\*\*\*  $p < 0.00005$  (significance in antibody titers between CL treated group relative to PBS control treated group))

### A. GC T<sub>FH</sub> cells : Day 8 post Immunization

**Fig 3**



### B. GC formation : Day 10 post Immunization

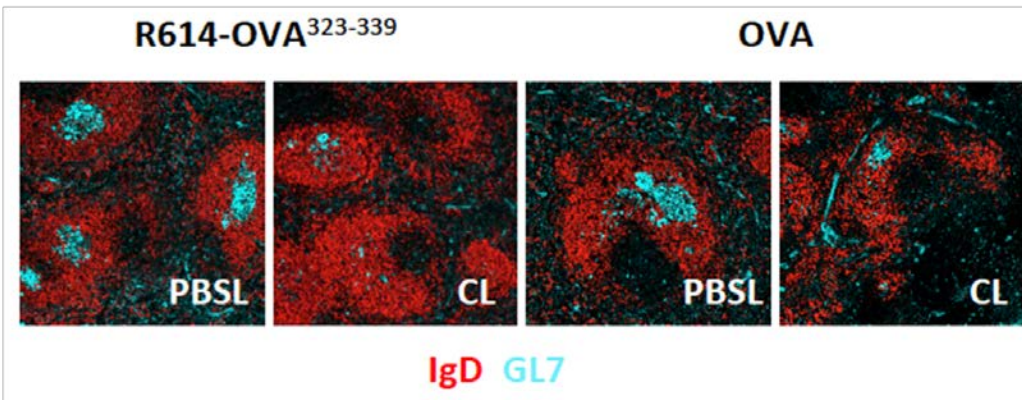


**Figure 3: CL markedly reduced germinal center (GC) T follicular helper (GC T<sub>FH</sub>) cells and GC formation in response to intact R614-OVA<sup>323-339</sup> and OVA.** BALB/c mice (3 per group) were injected i.v. with  $5 \times 10^5$  OVA-specific TCR Tg T cells (day -2). At day -1, mice were injected with CL or PBS liposomes. At day 0, mice were immunized i.p. with  $2 \times 10^8$  CFU R614-OVA<sup>323-339</sup> or 50  $\mu$ g OVA in alum and CpG. (A) At day 8, Tg T cells (B220-CD4<sup>+</sup>DO11.10 TCR<sup>+</sup>) from spleens of mice were analyzed by flow cytometry for GC T follicular helper (T<sub>FH</sub>) cells (GL7<sup>hi</sup>PD1<sup>hi</sup>CXCR5<sup>hi</sup>) [\* p<0.05] (B) Spleen sections were prepared on day 10 to determine GC formation (GL7+) by confocal microscopy.

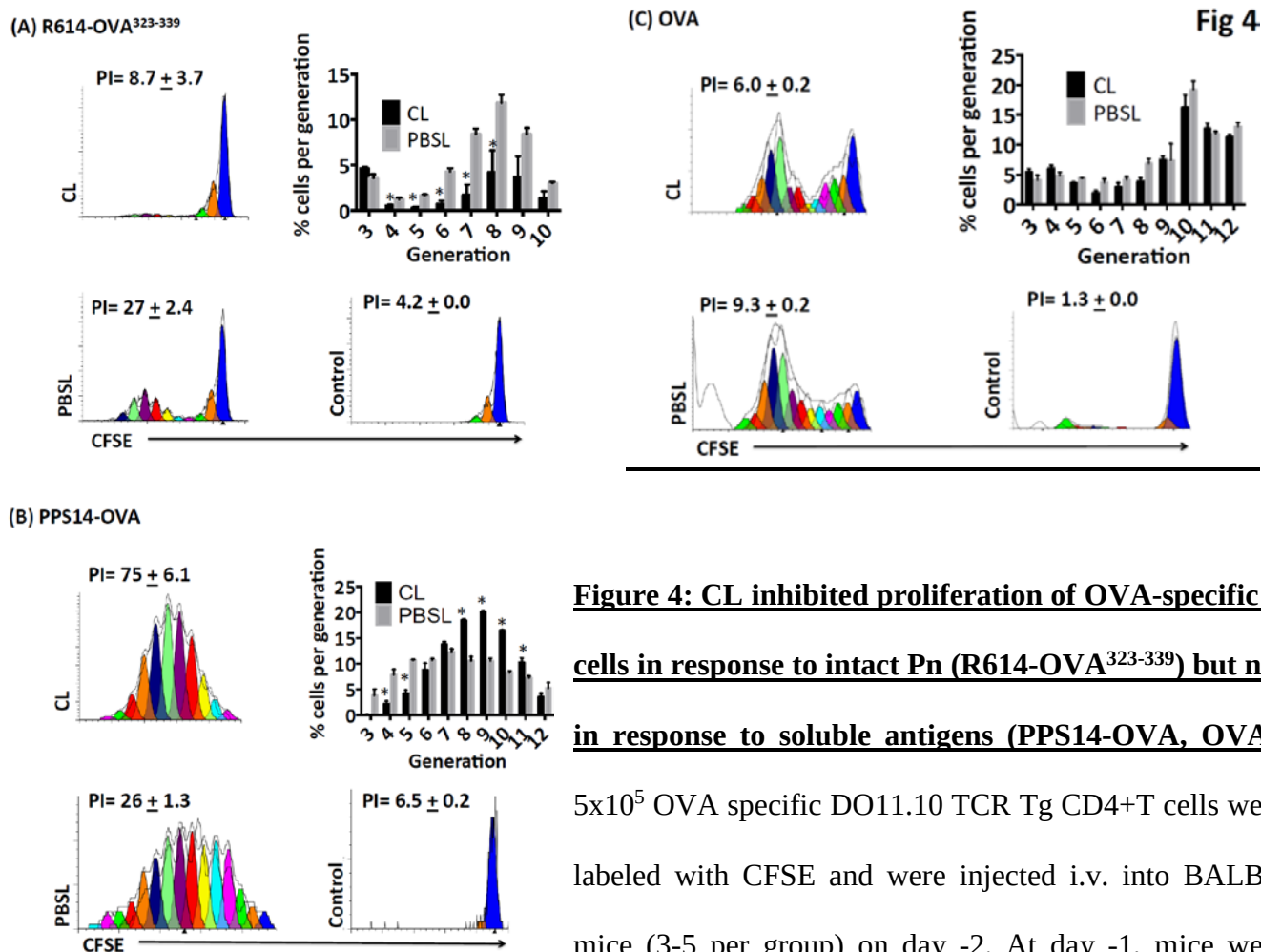
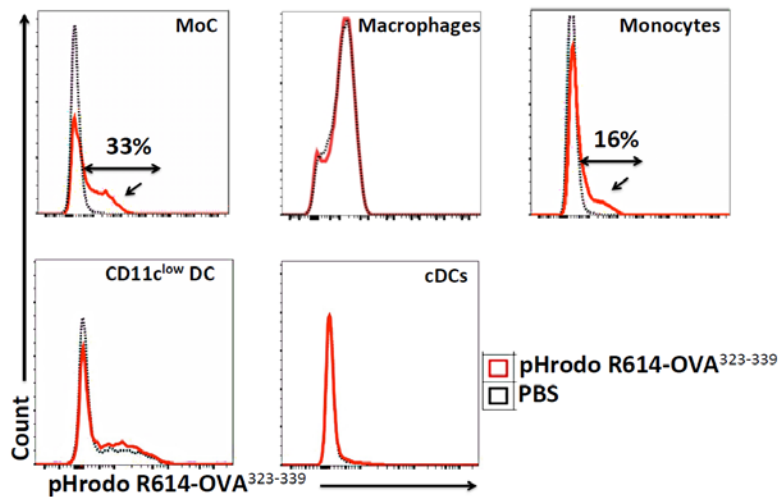


**Figure 4: CL inhibited proliferation of OVA-specific T cells in response to intact Pn (R614-OVA<sup>323-339</sup>) but not in response to soluble antigens (PPS14-OVA, OVA).**

5x10<sup>5</sup> OVA specific DO11.10 TCR Tg CD4<sup>+</sup>T cells were labeled with CFSE and were injected i.v. into BALB/c mice (3-5 per group) on day -2. At day -1, mice were injected i.v with 200 µl of CL or PBS liposomes. At day 0 mice were immunized with (A) R614-OVA<sup>323-339</sup>, (B) 5µg PPS14-OVA with 13 µg of alum mixed with 25 µg of CpG-ODN or (C) 50µg OVA with 13 µg of alum mixed with 25 µg of CpG-ODN. At day 2.5 or day 3 spleens cell suspensions were prepared and T cell proliferation by CFSE dilution was determined by flow cytometry. Tg T cells were identified as CD19<sup>-</sup>CD4<sup>+</sup>DO11.10 TCR<sup>+</sup>. PI=mean proliferative index for each group ± SEM (\* p<0.05).

(A) Uptake of pHrodo R614-OVA<sup>323-339</sup>

Fig 5



(B) Uptake of pHrodo OVA

Fig 5

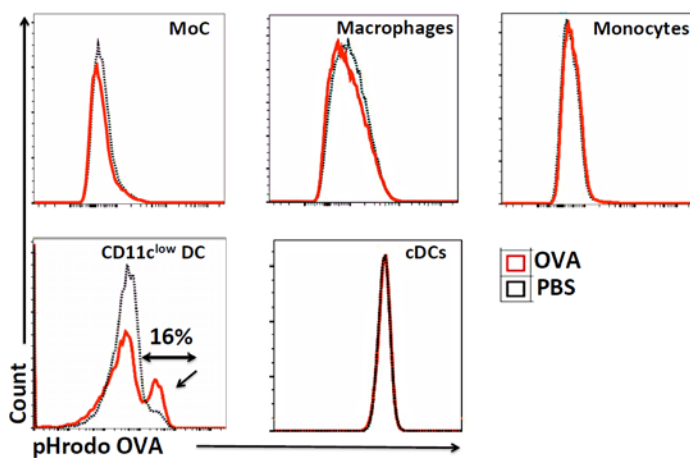


**Figure 5: pHrodo-labeled OVA was internalized by CD11c<sup>low</sup>MHC-II<sup>+</sup> cells whereas pHrodo-labeled intact R614-OVA<sup>323-339</sup> were internalized by moDCs.** BALB/c mice (3 per group) were immunized i.p. with pHrodo-labeled OVA or pHrodo-labeled R614-OVA<sup>323-339</sup>. 5h post-immunization, spleen cell suspensions were prepared for measurement of internalization, by flow cytometry of (A) pHrodo-labeled R614-OVA<sup>323-339</sup> or (B) pHrodo-labeled OVA.

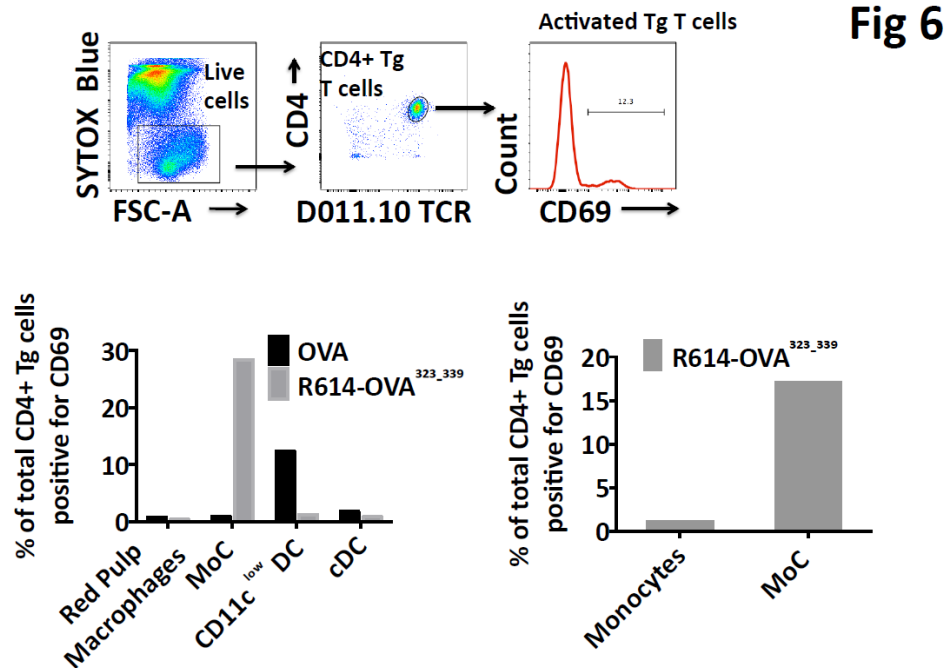


**Figure 6: MoC sorted from R614-OVA<sup>323-339</sup>-immunized mice and CD11c<sup>low</sup> DC sorted from OVA-immunized mice activated DO11.10 CD4+ Tg T cells in vitro.** BALB/c mice (3 per group) were immunized i.p. with R614-OVA<sup>323-339</sup> or OVA. 5h post-immunization, spleen cell suspensions were prepared for electronic cell sorting of cDC, CD11c<sup>low</sup> DCs, monocytes, MoCs and macrophages, and co-cultured with OVA-specific TCR Tg T cells. 16-24h later, surface CD69 expression on Tg T cells was determined by flow cytometry.

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## Chapter 3

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# MANUSCRIPT 2

## **Title**

Distinct mechanisms underlie boosted polysaccharide-specific IgG responses following secondary challenge with intact Gram-negative versus Gram-positive extracellular bacteria

## **Running title**

Elicitation of boosted secondary responses to bacteria

## **Authors**

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## **Abstract.**

Priming of mice with intact, heat-killed cells of Gram-negative *Neisseria meningitidis*, (MenC) or Gram-positive *group B Streptococcus* (GBS-III) bacteria resulted in augmented serum polysaccharide (PS)-specific IgG titers following booster immunization. Induction of memory required CD4<sup>+</sup> T cells during primary immunization. We determined whether PS-specific memory for IgG production was contained within the B cell and/or T cell populations, and whether augmented IgG responses following booster immunization were also dependent on CD4<sup>+</sup> T cells. Adoptive transfer of purified B cells from MenC- or GBS-III-primed, but not naïve mice resulted in augmented PS-specific IgG responses following booster immunization. Similar responses were observed when co-transferred CD4<sup>+</sup> T cells were from primed or naïve mice. Similarly, primary immunization with unencapsulated MenC or GBS-III, to potentially prime CD4<sup>+</sup> T cells, failed to enhance PS-specific IgG responses following booster immunization with their encapsulated isogenic partners. Further, in contrast to GBS-III, depletion of CD4<sup>+</sup> T cells during secondary immunization with MenC or another Gram-negative bacteria, *Acinetobacter baumannii*, did not inhibit augmented PS-specific IgG booster responses of mice primed with heat-killed cells. Also, in contrast with GBS-III, booster immunization of MenC-primed mice with isolated MenC-PS, a TI antigen, or a conjugate of MenC-PS and tetanus toxoid elicited an augmented PS-specific IgG response similar to booster immunization with intact MenC. These data demonstrate that memory for augmented PS-specific IgG booster responses to Gram-negative and Gram-positive bacteria are contained solely within the B cell compartment, with a differential requirement for CD4<sup>+</sup> T cells for augmented IgG responses following booster immunization.

## **Introduction.**

Infections with extracellular, polysaccharide (PS)-encapsulated bacteria are major sources of global morbidity and mortality among infants, the elderly and the immunosuppressed, causing sepsis, pneumonia and meningitis (1-4). Capsular PS serves as a major virulence factor by impeding phagocytosis and masking opsonins bound to underlying surface bacterial antigens (10). Isolated PS, with the exception of those that are zwitterionic (11), elicit antibodies in a T cell-independent (TI) fashion (17, 189) due to their inability to associate with MHC-II on antigen-presenting cells (APC) for presentation to cognate CD4<sup>+</sup> T cells (18, 19). However, immunization with a soluble conjugate vaccine formed by covalent attachment of PS with an immunogenic carrier protein facilitates recruitment of CD4<sup>+</sup> T cell help for PS-specific IgG responses (40, 41). Thus, unlike isolated PS, conjugate vaccines can elicit robust germinal center reactions, class switching, somatic hypermutation, and immunologic memory (42).

The presentation of PS expressed by intact bacteria differ from the isolated PS or conjugate vaccines as they are co-expressed, *non-covalently* with proteins within a particulate framework, along with multiple TLR, NLR, and scavenger receptor ligands (29). In this regard, we previously demonstrated that representative intact, heat-inactivated Gram-positive (GP) and Gram-negative (GN) extracellular bacteria elicit augmented and more rapid IgG responses following booster immunization of primed mice, similar to conjugate vaccines (29, 190). Induction of PS-specific B cell memory is dependent on CD4<sup>+</sup> T cell interactions with APC through costimulatory molecules, such as B7 and ICOS-L during the primary response. PS-specific IgG responses to intact bacteria are likely dependent on co-expressed bacterial proteins recognized by CD4<sup>+</sup> T cells (57). These data further suggested a general dichotomy between GP and GN bacteria, in which GP bacteria [i.e. *Streptococcus pneumoniae* (Pn) and Group B Streptococci (GBS)] elicited relatively rapid (peak day 7) CD4<sup>+</sup> T cell-dependent primary PS-specific IgG responses,

whereas GN bacteria [i.e. *Neisseria meningitidis* (Men) and *Acinetobacter baumannii* (AcB)] developed primary PS-specific IgG responses that developed more slowly (peak day 21) and were independent of T cell help (190). Immunity induced by intact bacteria also showed distinct response differences from soluble conjugate vaccines. For example, systemic immunization with intact Pn induced PS-specific IgG that derived from splenic marginal zone B cells and expressed a distinct idiotype, whereas a pneumococcal conjugate vaccine of the same PS serotype (type 14) induced PS-specific IgG largely lacking this idiotype and derived from splenic follicular B cells (61-63).

The general consensus regarding elicitation of memory IgG responses is that the memory B cells generated in the presence of CD4<sup>+</sup> T cell help during the primary response proliferate robustly upon re-exposure to antigen and generate plasma cells expressing high-affinity IgG that is dependent on help from cognate memory CD4<sup>+</sup> T cells (191). However, most of these conclusions have been derived from studies carried out with isolated proteins that left unresolved whether PS-specific memory for IgG in response to intact GP and GN bacteria was similarly contained within both the B cell and CD4<sup>+</sup> T cell compartments. Additionally, it was not determined whether the augmented PS-specific IgG responses following booster immunization with intact bacteria were also dependent on the presence of CD4<sup>+</sup> T cells.

To begin addressing these questions, we demonstrated that mice primed with intact GBS, capsular type III (GBS-III) elicited an augmented PS-specific IgG response only if CD4<sup>+</sup> T cells were present during both the primary and booster immunizations (192). This augmented response in GBS-III-primed mice did not occur following booster immunization with the corresponding isolated PS. In this report, we have extended these initial observations utilizing intact GBS-III, Men [capsular serogroup C (MenC)] and AcB

expressing the surface PS, poly-N-acetylglucosamine (PNAG), to determine the cellular nature of PS-specific memory for IgG and whether CD4<sup>+</sup> T cells are uniformly required for augmented IgG responses following booster immunization. In contrast to what has been previously observed for isolated proteins, which depend on both memory B cells and memory CD4<sup>+</sup> T cells for augmented anti-protein IgG responses, we now show that PS-specific memory for GP and GN bacteria are contained solely within the B cell compartment. Further, CD4<sup>+</sup> T cells are required during booster immunization for augmented PS-specific IgG responses for GP, but not for GN bacteria. For this reason, PS-specific IgG responses to GN-primed, but not GP-primed mice can be augmented using only isolated PS from the priming bacteria. These observations may have relevance for future vaccine design.

## **Methods.**

**Mice.** Athymic nude (BALB/c background), SCID/NCr (BALB/c background; strain code. 561) mice and BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Mice used were 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.

**Bacteria.** GBS-III (strain M781; ATCC BAA-22) and MenC (strain M1883; ATCC 53414) were obtained from American Type Culture Collection (Manassas, VA). COH1, a different strain of GBS-III expressing the same capsular type III PS and COH1-13, an isogenic COH1 mutant lacking a capsule were used in this study. The COH strains were a kind gift of Dr. Craig Rubens of Children's Orthopedic Hospital (Seattle, WA). The encapsulated and unencapsulated MenC strains, FAM18 C<sup>+</sup> and FAM18 C<sup>-</sup>

respectively, were used (193). Both M1883 and FAM18 C+ were similarly O-acetylated. Lyophilized or frozen stocks of bacteria were grown overnight on BBL blood agar plates (VWR International, Bridgeport, NJ). Isolated colonies of MenC or GBS-III on blood agar were grown to mid-log phase in Brain Heart Infusion media or Todd-Hewitt broth media (BD Biosciences, San Jose, CA), respectively. Harvested cells were heat-killed by incubation at 65°C for 2 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacteria were suspended in PBS and adjusted 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 for mouse immunizations. *Acinetobacter baumannii* (AcB) strain S1 $\Delta$ pga-c was constructed as described (194) by first deleting the chromosome pga locus ( $\Delta$ pga) encoding PNAG biosynthetic proteins then complementing the deletion in trans ( $\Delta$ pga-c) to achieve overexpression of PNAG on the cell surface. The frozen stocks of bacteria were grown overnight on lysogeny broth (LB) agar plates. Isolated colonies were grown in LB media to mid log phase, collected and heat-killed by incubation at 65°C for 2h. After thorough washings, bacteria were suspended in PBS and adjusted to give an absorbance reading of 0.4 at 600 nm, which corresponded to  $10^8$  CFU/ml. Bacteria were aliquoted at  $10^9$  CFU/ml and frozen at -20C.

**Reagents.** Purified MCPS was a kind gift of Dr. Andrew Lees (Fina BioSolutions LLC, Rockville, MD). A covalent conjugate of MCPS and tetanus toxoid (MCPS-TT) was prepared as previously described (144). Purified PPS14, which is structurally related to GBS-III PS but lacks the terminal sialic acid component (195) was purchased from American Type Culture Collection. Rat IgG2b anti-mouse CD4 monoclonal antibody (mAb) (clone GK1.5) was purchased from BioXcell (West Lebanon, NH). Purified polyclonal rat IgG was purchased from Sigma (St. Louis, MO). Alum (Allhydrogel 2%) was obtained from Brenntag Biosector (Frederikssund, Denmark). A stimulatory 30mer CpG-containing

oligodeoxynucleotide (CpG-ODN) was synthesized as previously described (145). B and T cell isolation kits were purchased from Miltenyi Biotec (Auburn, CA).

**Preparation of PNAG.** PNAG was prepared as previously described (194). Briefly, *Acinetobacter baumannii* strain S1 $\Delta$ *pga-c* was grown in LB media containing 1% glucose. Bacterial cells were treated with lysozyme followed by DNase I and RNase A and the cells were then removed by centrifugation, with the resulting supernatant precipitated with ethanol. The ethanol-insoluble material was collected by centrifugation, suspended in water, dialyzed against water, and freeze-dried until use. Before use, PNAG was dissolved at a concentration of 5 mg/ml in 5M HCl. An equal volume of NaOH was added to neutralize the solution by keeping the vial on ice.

**Immunizations.** Groups of seven mice each were immunized i.p. with  $2 \times 10^8$  CFU intact, heat-killed MenC in PBS,  $2 \times 10^9$  CFU intact, heat-killed GBS-III in PBS,  $1 \times 10^8$  CFU of intact, heat-killed AcB in PBS, 5  $\mu$ g purified MCPS or 1  $\mu$ g MCPS–TT, both adsorbed on 13  $\mu$ g alum mixed with 25  $\mu$ g CpG-ODN. Serum samples were prepared at different time points from blood obtained through the tail vein.

**Adoptive transfer of B and T lymphocytes into *scid* mice.** BALB/c mice were immunized i.p. with MenC or GBS-III as above. A separate control group was injected with PBS only. At day 21 post-immunization, spleen cells were obtained from immunized and naive mice. B cells and T cells were separately isolated by magnetic sorting using B and T cell isolation kits, respectively (Miltenyi Biotec). Isolated cells were analyzed by flow cytometry and determined to be 96–98% pure, with no detectable cross-contamination of B and T cells. The purified cells were adoptively transferred into *scid* mice at  $2 \times 10^7$  B cells/mouse and  $1 \times 10^7$  T cells /mouse in 4 different combinations: 1) naïve (n)B + nT, 2) nB + primed (p)T, 3) pB + nT and 4) pB + pT. The recipient *scid* mice were immunized with MenC or GBS-III, as above, a day later (d0) and sera were obtained on day 7.

**ELISA.** For measurement of serum titers of PPS14-specific IgG, Immulon 4 ELISA plates were coated overnight at 4°C with purified PPS14 (5 µg/ml, 100 µl/well) in PBS. For measurement of serum titers of MCPS-specific or PNAG-specific IgG, Immulon 4 ELISA plates were pre-coated with poly-L-lysine (Sigma) (5 µg/ml, 100 µl/well) in PBS for 1 h at 37°C. The plates were then washed three times with PBS plus 0.1% Tween 20 and then coated overnight at 4°C with purified MCPS (10 µg/ml, 100 µl/well) or PNAG (3 µg/ml, 100 µl/well). Plates were then washed three times with PBS plus 0.1% Tween 20 and were blocked with PBS plus 1.0% BSA for 1 h at 37°C. Three-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1.0% BSA were incubated overnight at 4°C, and plates were then washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG antibodies (200 ng/ml) in PBS + 1.0% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were washed three times with PBS plus 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in 1 M Tris plus 0.3 mM MgCl<sub>2</sub> (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 were determined as described previously (60).

**Statistics.** Serum titers of antigen-specific IgG were expressed as the geometric means ± SEM of the individual serum IgG titers. Significance was determined by performing comparative analysis of data using a one-way ANOVA test followed by Turkey's multiple comparisons (Fig 1), and repeated-measures Two-way ANOVA followed by Turkey's multiple comparisons (Fig 3, 5, 6) or Šídák multiple comparisons (Fig 2, 4). *p*-values of ≤ 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0 software. All experiments were performed at least two times.

## Results.

### **Adoptive transfer of B cells and CD4<sup>+</sup> T cells from naïve and/or MenC- or GBS-III primed mice into *scid* recipients for induction of PS-specific IgG responses.**

Intact, heat-killed MenC (196) and GBS-III (192) both elicit augmented PS-specific IgG responses following booster immunization of primed mice, which required CD4<sup>+</sup> T cells during the primary immunization. These data demonstrated that both MenC and GBS-III induce a state of CD4<sup>+</sup> T cell-dependent memory for PS-specific IgG responses, but left unresolved whether the specific memory was contained within the B cell and/or CD4<sup>+</sup> T cell populations. To determine this, we purified B cells or CD4<sup>+</sup> T cells 21 days after immunization of BALB/c mice with MenC or GBS-III. B cells or CD4<sup>+</sup> T cells were also purified from unprimed (naïve) mice for control purposes. Four separate combinations of B cells + T cells were injected into B cell/T cell-deficient BALB/c-*scid* mice as follows: 1) naïve (n)B + nT, 2) nB + primed(p)T, 3) pB + nT, and 4) pB + pT, followed by corresponding immunization with MenC or GBS-III. The number of B and CD4<sup>+</sup> T cells adoptively transferred per mouse was based on an earlier study on T cell-dependent IgG responses in reconstituted B cell/T cell-deficient mice (197). A separate group of *scid* mice received neither B or T cells as a negative control. Sera were obtained 7 days post-immunization to measure PS-specific IgG titers in order to determine whether a memory PS-specific IgG response occurred. The MenC PS is the capsular PS referred to as MCPS, whereas the GBS-III PS used is actually the core component of this antigen lacking a terminal sialic acid rendering the antigen structurally identical to the pneumococcal serotype 14 PS (PPS14) (198) (Figure 1). As expected, *scid* mice receiving neither B cells nor CD4<sup>+</sup> T cells failed to elicit a detectable MCPS-specific IgG response following immunization with MenC cells (Figure 1A) nor a PPS14-specific IgG response following immunization with GBS-III cells (Figure 1B). *Scid* mice receiving B cells from naïve BALB/c mice also failed to elicit a detectable MCPS- or PPS14-specific IgG response on day 7 following either MenC

(Figure 1A) or GBS-III priming (Figure 1B). In contrast, transfer of B cells, but not CD4<sup>+</sup> T cells, from primed mice was sufficient to elicit a substantial MCPS-specific IgG response or PPS14-specific IgG response on day 7 following immunization with MenC (Figure 1A) or to GBS-III (Figure 1B). Thus, these data strongly suggest that priming of B cells, but not CD4<sup>+</sup> T cells is critical for generating an augmented PS-specific IgG response following a booster immunization with intact bacteria.

**Primary immunization with unencapsulated isogenic mutants of MenC or GBS-III does not augment PS-specific IgG responses following booster immunization with the corresponding PS-encapsulated strains.**

In the next experiment we wished to further test the hypothesis that CD4<sup>+</sup> T cells from primed mice are no more effective than naïve CD4<sup>+</sup> T cells in promoting a boosted, PS-specific IgG response to secondary bacterial challenge (see Figure 1). Thus, we immunized mice with unencapsulated isogenic mutants of MenC (MCPS<sup>-</sup>) or GBS-III (GBS-III/PPS14<sup>-</sup>) in order to activate protein-specific CD4<sup>+</sup> T cells, without priming PS-specific B cells. Mice were then boosted 14 days later with encapsulated (MCPS<sup>+</sup>) MenC or (GBS-III/PPS14<sup>+</sup>) GBS-III, respectively. In this regard, we previously provided evidence that CD4<sup>+</sup> T cells specific for bacterial protein can provide help for PS-specific and protein-specific IgG responses to intact bacteria (57). Positive control mice received both a primary and booster immunization with MCPS<sup>+</sup> MenC or GBS-III/PPS14<sup>+</sup> GBS-III. As expected, mice primed with MCPS<sup>-</sup> MenC (Figure 2A) or GBS-III/PPS14<sup>-</sup> GBS-III (Figure 2B) made no detectable primary MCPS- or PPS14-specific IgG responses on either day 7 or day 14 post-immunization, whereas substantial primary MCPS- and PPS14-specific IgG responses were induced by the encapsulated bacteria. Upon booster immunization with MCPS<sup>+</sup> MenC (Figure 2A) or GBS-III/PPS14<sup>+</sup> GBS-III (Figure 2B), mice primed with MCPS<sup>-</sup> MenC or PPS14<sup>-</sup> GBS-III, respectively, elicited MCPS- and PPS14-specific IgG responses that were not significantly different

from the primary responses induced by the corresponding encapsulated bacteria. In contrast, mice primed and re-challenged with MCPS<sup>+</sup> MenC or PPS14<sup>+</sup> GBS-III elicited highly augmented MCPS- and PPS14-specific IgG responses following booster immunization. These data complement the observations shown in Figure 1, by demonstrating that CD4<sup>+</sup> T cells following primary immunization, by themselves do not enhance subsequent PS-specific IgG responses following an initial immunization with an intact bacterium.

**CD4<sup>+</sup> T cells are not required during booster immunization with MenC for augmented MCPS-specific IgG responses.**

We previously demonstrated that depletion of CD4<sup>+</sup> T cells during booster immunization with GBS-III completely inhibits the augmented PPS14-specific IgG response in GBS-III-primed mice (192). In this regard, we wished to determine whether there was a similar requirement for CD4<sup>+</sup> T cells during secondary immunization with MenC for boosted MCPS-specific IgG responses in MenC-primed mice. To determine this, we injected a depleting anti-CD4 mAb or negative control rat IgG, 1 day prior to secondary immunization with MenC, into BALB/c mice that received MenC 21 days earlier, in the absence of any additional antibody treatments. As a control separate groups of mice received anti-CD4 mAb or rat IgG, 1 day prior to primary immunization with MenC followed by boosting with MenC, 21 days later in the absence of antibody. These latter two groups were included to confirm, functionally that anti-CD4 mAb was effective in depleting CD4<sup>+</sup> T cells by blocking the development of MCPS-specific memory for IgG as previously described (196). Injection of anti-CD4 mAb depleted CD4<sup>+</sup> T cells by >95%, as determined 24 h later by flow cytometry, whereas rat IgG had no effect (data not shown). As illustrated in Figure 3, injection of anti-CD4 mAb prior to secondary immunization with MenC had no effect on the boosted MCPS-specific IgG response, whereas as expected, injection of anti-CD4 mAb prior to primary immunization with MenC completely inhibited the boosted MCPS-specific IgG response following

secondary challenge. Thus, MenC differs from GBS-III (192) in exhibiting no apparent requirement for CD4<sup>+</sup> T cells during secondary challenge, to elicit boosted PS-specific IgG responses.

**The requirement for CD4<sup>+</sup> T cells during primary and booster immunization for elicitation of PS-specific IgG responses to *Acinetobacter baumannii* (AcB), another GN bacteria, is similar to that for MenC.**

We next wished to determine whether the dichotomy between GBS-III (GP bacterium) and MenC (GN bacterium) for CD4<sup>+</sup> T cell help during primary and secondary PS-specific IgG responses might reflect a more general distinction between GP and GN bacteria. Thus we utilized a strain of AcB, a GN bacterium that was engineered to overexpress the polysaccharide PNAG on its surface. In a first set of experiments, BALB/c mice were immunized with intact, heat-killed AcB in the presence of either depleting anti-CD4 mAb or negative control rat IgG and then boosted 21 days later with AcB in the absence of additional antibody treatments. Complementary studies were performed in which BALB/c or athymic nude (BALB/c background) mice, which lack T cells, were immunized with AcB and boosted 21 days later. As shown in Figure 4A, depletion of CD4<sup>+</sup> T cells prior to primary immunization with AcB, had only a minimal effect on primary PNAG-specific IgG response, similar to MenC (Figure 3), and in contrast to GBS-III (192). However, similar to both MenC (Figure 3) and GBS-III (192), CD4<sup>+</sup> T cells were required during primary immunization for induction of an augmented PNAG-specific IgG response following booster immunization (day 28>day 21,  $p \leq 0.05$ ). Immunization of athymic nude mice with AcB confirmed the essentially TI nature of the primary PNAG-specific IgG response, but the requirement for T cells to elicit a boosted PNAG-specific IgG response following secondary challenge (Figure 4A). In a second set of experiments, BALB/c mice were immunized with AcB alone, and then boosted 21 days later with AcB 1 day following injection of depleting anti-CD4 mAb or negative control rat IgG. As indicated in Figure 4B,

anti-CD4 mAb injected prior to a booster immunization had no effect on the augmented PNAG-specific IgG response, similar to MenC and in contrast to GBS-III. These data thus lend support to the notion of a potential, general dichotomy in the role of CD4<sup>+</sup> T cells during primary and secondary PS-specific IgG responses to GP versus GN bacteria.

### **Mice primed with MenC elicit augmented MCPS-specific IgG responses following booster immunization with isolated MCPS.**

The lack of a requirement for CD4<sup>+</sup> T cells following booster immunization with MenC for induction of boosted PS-specific IgG suggested the possibility that a boosted PS-specific IgG response could be elicited in MenC-primed mice, by isolated MCPS, a TI antigen. To determine this, mice were primed with MenC and boosted with either MCPS, or MenC as a positive control. Since MCPS is a TI antigen that does not by itself elicit memory MCPS-specific IgG responses (196), as further controls, separate groups of mice were also primed with MCPS and then boosted with either MCPS or MenC. MCPS was injected with alum + CpG-ODN. As depicted in Figure 5, in MenC-primed, but not MCPS-primed mice, booster immunization with either MCPS or MenC elicited augmented MCPS-specific IgG responses. We previously reported that contaminating TLR ligands in preparations of isolated pneumococcal PS were critical for induction of PS-specific IgG in vivo (199). We also previously demonstrated that the preparation of MCPS used in this study elicited PS-specific IgG in vivo, when injected in saline only, thus suggesting the possibility of contaminating TLR ligands also playing a stimulatory role (196). To determine whether contaminating TLR ligands might be critical for the augmented PS-specific IgG response following booster immunization with isolated MCPS in MenC-primed mice, we utilized a second MCPS preparation that was unable to elicit MCPS-specific IgG responses in vivo (200) and was confirmed to lack innate stimulating activity on macrophages in vitro (data not shown). Using this MCPS preparation,

which was injected only in saline for booster immunization, a similar level of augmentation in the MCPS-specific IgG response was observed in MenC-primed mice (data not shown), strongly arguing against a critical role for contaminating TLR ligands in eliciting an augmented booster IgG response from primed MCPS-specific B cells.

**Mice primed with either MenC or a soluble conjugate of MCPS and tetanus toxoid (MCPS-TT) elicit augmented MCPS-specific IgG responses following booster immunization with either MenC or MCPS-TT.**

Previous studies using intact heat-killed *S. pneumoniae* and a soluble pneumococcal conjugate vaccine strongly suggested that PS-specific IgG responses were derived from marginal zone and follicular B cells, respectively (61, 63), with elicitation of distinct idiotypes on the PS-specific (serotype 14) IgG (62). Indeed, mice primed with intact serotype 14 *S. pneumoniae* do not elicit an augmented PS-specific IgG response following booster immunization with a soluble type 14 pneumococcal conjugate vaccine, in which the carrier protein is pneumococcal surface protein A [PspA] (201), but do elicit an augmented response following booster immunization with intact GBS-III expressing type 14 PS (192). These findings suggested that mice primed with an MCPS conjugate vaccine might not elicit an augmented MCPS-specific IgG response following booster immunization with MenC, and vice versa, since these responses might be derived from distinct B cell subsets. However, as illustrated in Figure 6, augmented MCPS-specific IgG responses were observed regardless of whether MenC or MCPS-TT was used for priming and regardless of whether MenC or MCPS-TT was used for booster immunization. Of interest, of the 4 groups, mice primed with MenC and subsequently challenged with MCPS-TT or primed with MCPS-TT and subsequently challenged with MenC gave significantly higher secondary serum titers of MCPS-specific IgG compared to the other two groups. In addition, mice primed with MenC and subsequently

challenged with MCPS-TT demonstrated a significantly higher MCPS-specific IgG titer compared to mice primed with MCPS-TT and challenged with MenC. Since boosted MCPS-specific IgG responses in primed mice do not require CD4<sup>+</sup> T cells following booster immunization (see Figure 3) the presence of TT in the MCPS-TT conjugate, but not in intact MenC, should not preclude augmented MCPS-specific IgG responses when using MenC and MCPS-TT for immunization in sequence. These data demonstrate further potential differences between GP and GN bacteria that may have important implications for future vaccine design.

## **Discussion.**

We previously demonstrated that capsular PS-specific IgG responses to intact extracellular bacteria in vivo are markedly influenced by the architecture and/or the composition of the underlying sub-capsular domain (144, 190, 192, 196). Thus, the primary PS-specific IgG response to intact *S. pneumoniae* peaks relatively early (by day 7), is dependent on CD4<sup>+</sup> T cells, and demonstrates no augmentation following booster immunization (144). In contrast, the primary PS-specific IgG response to intact MenC, peaks relatively late (day 14-21), is independent of CD4<sup>+</sup> T cells, and demonstrates substantial augmentation following booster challenge (196). Moreover, the primary PS-specific IgG response to GBS-III which expresses PS biochemically similar to PS expressed by *S. pneumoniae* (capsular type 14) in which there is a shared core structure, also peaks by day 7 and is dependent on CD4<sup>+</sup> T cells, like *S. pneumoniae*, but in contrast, demonstrates a highly augmented PPS14-specific IgG booster response (192). This demonstrates that structurally similar capsular PS expressed by two distinct bacteria may be associated with different PS-specific IgG responses to their shared epitopes, in vivo. The induction of memory for PS-specific IgG in response to GBS-III and MenC requires CD4<sup>+</sup> T cells during the primary immunization. These data left unresolved whether memory for PS-specific IgG was contained within the B cell and/or memory T

cell population, whether CD4<sup>+</sup> T cells were required for boosted IgG responses during secondary challenge, and whether a more general dichotomy might exist in how induction of PS-specific IgG is regulated in response to GP versus GN bacteria.

In this report, we demonstrate that induction of memory for PS-specific IgG in response to either MenC or GBS-III is contained within the B cell, but not CD4<sup>+</sup> T cell population. We demonstrated earlier that the augmented PPS14-specific IgG response to the GP bacterium, GBS-III expressing PPS14 as its core capsular component also requires CD4<sup>+</sup> T cells during the booster immunization (192). This is consistent with the lack of an augmented PS-specific IgG response in GBS-III-primed mice following booster immunization with isolated PPS14, a TI antigen. By contrast, in this study we show that the augmented MCPS-specific IgG response to MenC is independent of CD4<sup>+</sup> T cells following the booster immunization. This is consistent with the augmented MCPS-specific IgG response observed in MenC-primed mice following booster immunization with isolated MCPS, also a TI antigen, or a soluble covalent conjugate of MCPS and TT. Results similar to that obtained with MenC are also observed using another GN bacteria, AcB expressing PNAG, i.e. a primary PNAG-specific IgG response that peaks relatively late (by day 14-21) and that is independent of CD4<sup>+</sup> T cells, and an augmented PNAG-specific IgG response following booster immunization with AcB that requires CD4<sup>+</sup> T cells during the primary, but not during the booster immunization. These data further support the notion that the regulation of capsular PS-specific IgG responses to intact bacteria are dependent on the nature of the sub-capsular domain, and suggest a potential, general dichotomy between GP and GN bacteria.

The distinct structural and compositional differences between GP and GN bacteria may play an important role in distinguishing anti-PS antibody responses among these two sub-groups of pathogens. Thus, 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 (50, 72). PS expressed by GN bacteria, which express a thin peptidoglycan cell wall, is covalently attached to the acyl glycerol moiety of the outer membrane, which contains highly immunogenic proteins, including porins, and lipopolysaccharide (LPS), a potentially potent stimulator of the innate immune system depending on its biochemical composition (73, 74). Shedding of vesicles containing the outer membrane/PS complex is a unique property of GN bacteria that may have distinct immunologic consequences for the anti-PS response (76, 77). How these structural and biochemical differences between GP and GN bacteria lead to differences in the cellular regulation of PS-specific IgG responses remains to be determined. One possible difference may lie in the different TLR ligands expressed by these two classes of bacteria. In this regard, previous studies from our laboratory demonstrated a key role for endogenous TLR2 and TLR4 in augmenting IgG responses to *S. pneumoniae* (202) and *N. meningitidis* (196), respectively.

Early studies using isolated haptenated polysaccharides observed that booster immunization fails to augment specific serum antibody titers unless primed B cells are first adoptively transferred into a naïve host (203, 204). The requirement for adoptive transfer to observe an augmented antibody response from TI-specific memory B cells was subsequently shown to be due to an inhibitory effect of circulating antigen-specific IgG induced during primary immunization (205). TI, in contrast to TD, memory B cells are derived from the B-1b subset, and memory is manifested by rapid induction of specific IgM, but not IgG following secondary immunization (118, 119). In the current study, the PS-specific B cells isolated following immunization with intact bacteria, in contrast to TI memory B cells, mediate augmented PS-

specific IgG responses following booster immunization without the need for adoptive transfer to circumvent inhibitory antigen-specific IgG, and require CD4<sup>+</sup> T cell help for their generation, thus strongly suggesting their derivation from a different mechanistic pathway.

Our current data in mice may have relevance for vaccination of humans with polysaccharide-based vaccines. IgG<sup>+</sup>CD27<sup>+</sup> human memory B cells specific for MCPS have been demonstrated following vaccination with a MenC-TT vaccine. These memory B cells can be subsequently triggered by intact MenC to differentiate into plasma cells, similar to our data, although in this case requiring contact-dependent (in part through CD40) but non-cognate help from bystander CD4<sup>+</sup> T cells specific for MenC proteins (206). Meningococcal, *Haemophilus influenzae* type b, and pneumococcal conjugate vaccines also induce memory B cells that can be subsequently triggered for PS-specific IgG responses by the respective unconjugated PS, implying that only memory B cells, and not T cells play a key role in these IgG responses (207-213). It has been proposed that natural priming of humans by exposure to PS-encapsulated bacteria may induce memory B cells that can be subsequently triggered in a TI manner, since vaccination of adults with capsular PS vaccines show features of a secondary antibody (i.e. hypermutated IgG) (52, 214-217).

Our data using intact MenC are similar to a previous study on the CD4<sup>+</sup> T cell requirements for eliciting a secondary, memory response to human cytomegalovirus (HCMV) or tick-borne encephalitis virus (TBEV). Thus, transfer of B cells from virus-primed mice into B cell/T cell-deficient RAG-1<sup>-/-</sup> recipients resulted in an antigen-specific memory response, including rapid IgG induction with long-term persistence, that was independent of CD4<sup>+</sup> T cells during the secondary challenge (218). Further, transfer

of memory B cells into immunocompetent mice, demonstrated no evidence of helper T cell-mediated enhancement of the memory IgG response. Finally, similar to our study with MenC, secondary immunization with HCMV, of mice primed with a soluble HCMV-derived protein (i.e. glycoprotein B), elicited a gB-specific memory response. A more recent study also demonstrated that secondary IgG anti-Env responses to virus-like particles of simian immunodeficiency virus or adenoviral vectors expressing Env were TI (219). These data are in contrast to experimental models using soluble proteins and hapten-carrier conjugates, in which CD4<sup>+</sup> T cells are required during secondary immunization in order to activate specific memory B cells for IgG production (220). In contrast, an earlier study using vesicular stomatitis virus or lymphocytic choriomeningitis virus (221), demonstrated a requirement for T cells for elicitation of the secondary response, although this may have reflected the brief time (20 min) after which virus was injected following adoptive transfer of memory B cells (218).

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## **Footnotes.**

1. Mandatory disclaimer: The opinions expressed herein are those of the authors and are not necessarily representative of those of the Uniformed Services University of the Health Sciences (USUHS) the Department of Defense (DOD), or the United States Army, Navy, or Air Force.

2. Supported by: USUHS Research and Education Endowment Fund (CMS).

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4. Abbreviations: AcB, *Acinetobacter baumannii*; GBS-III, *Streptococcus agalactiae* (group B Streptococcus), capsular type III; GN, Gram-negative; GP, Gram-positive; MCPS, serogroup C capsular polysaccharide of *Neisseria meningitidis*; MenC, *Neisseria meningitidis*, capsular serogroup C; PNAG, poly-N-acetylglucosamine; PS, polysaccharide; PPS14, type 14 capsular polysaccharide of *Streptococcus pneumoniae*; TLR, Toll-like receptor; TT, tetanus toxoid.

5. Keywords: *Acinetobacter baumannii*, antibody, B cell, bacteria, conjugate vaccine Gram-negative, Gram-positive, Group B Streptococcus, memory, *Neisseria meningitidis*, polysaccharide, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, T cell, T cell-independent.

*pneumoniae*, T cell, T cell-independent.

### 3.6. Figures and Figure legends:

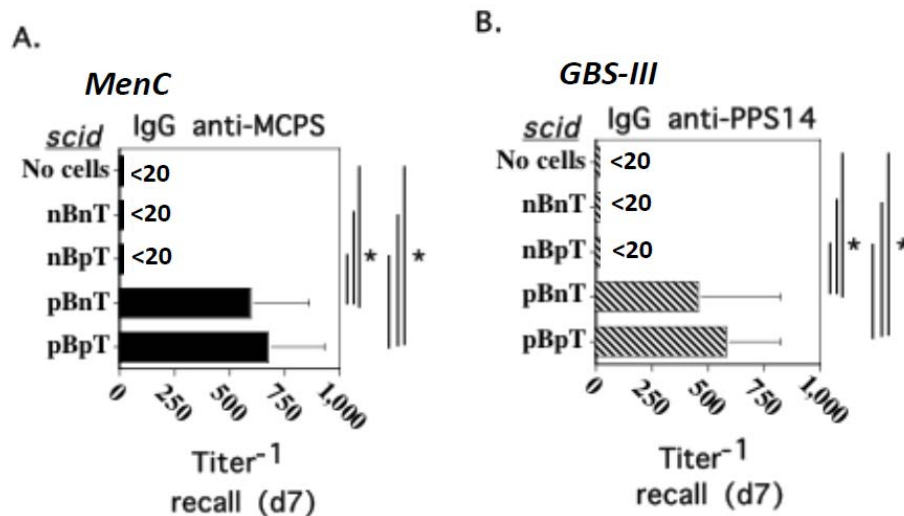


**Figure 1. Adoptive transfer of B cells and CD4<sup>+</sup> T cells from naïve and/or MenC- or GBS-III primed mice into *scid* recipients for induction of PS-specific IgG responses.** BALB/c mice were immunized with A)  $2 \times 10^8$  CFU/mouse of intact heat-inactivated MenC or B)  $2 \times 10^9$  CFU/mouse of intact heat-inactivated GBS-III. At day 21 post-immunization, spleen cells were obtained from immunized and unimmunized (naïve) mice, B and CD4<sup>+</sup> T cells were isolated by magnetic sorting and then adoptively transferred into *scid* mice (7 per group), as follows: (a) naïve (n) B and naïve (n) T cells, (b) nB and primed (p) T cells, (c) pB and nT, and (d) pB and pT. One day following adoptive transfer, *scid* recipients were boosted with A) MenC ( $2 \times 10^8$  CFU/mouse) or B) GBS-III ( $2 \times 10^9$  CFU/mouse), respectively. Serum titers of A) MCPS-specific or B) PPS14-specific IgG were determined by ELISA. \* $p \leq 0.05$ .

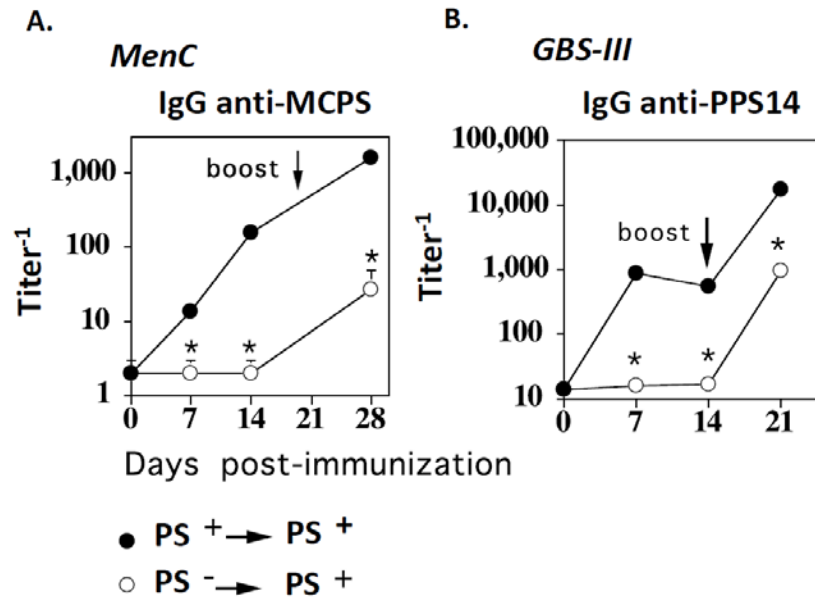


**Figure 2. Primary immunization with unencapsulated isogenic mutants of MenC or GBS-III does not augment PS-specific IgG responses following booster immunization with the corresponding PS-encapsulated strains.** BALB/c mice (7 per group) were immunized i.p. with A)  $2 \times 10^8$  CFU/mouse intact, heat-inactivated PS+ (strain FAM18C+) MenC or PS- (strain FAM18C-) and boosted on day 14 with the same dose of PS+ MenC, or B) with  $2 \times 10^9$  CFU/mouse PS+ (strain COH1) GBS-III or PS- (strain COH1-13) GBS-III and boosted on day 14 with the same dose of COH1. Serum titers of A) MCPS-specific or B) PPS14-specific IgG were determined by ELISA. \* $p \leq 0.05$  (significance between primary and secondary titers of PS+ versus PS- primary groups).

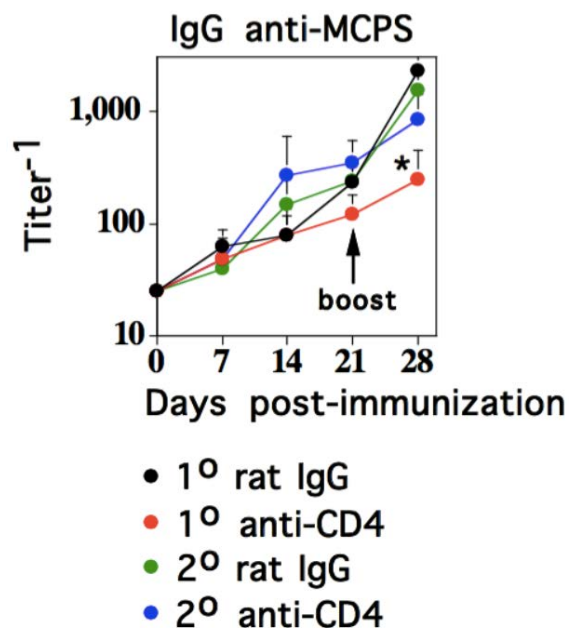


**Figure 3. CD4<sup>+</sup> T cells are not required during booster immunization with MenC for augmented MCPS-specific IgG responses.** BALB/c mice (7 per group) were immunized i.p. with  $2 \times 10^8$  CFU/mouse of intact heat-inactivated MenC (day 0) and boosted i.p. with the same dose of MenC on day 21. Depleting anti-CD4 mAb (clone GK1.5, 0.5 mg/mouse) or control rat IgG (0.5 mg/mouse) were injected on either day -1 or day 20. Serum titers of MCPS-specific IgG were determined by ELISA (\*  $p \leq 0.05$ ) (significance between secondary titers of rat IgG-injected versus anti-CD4 mAb-injected groups).

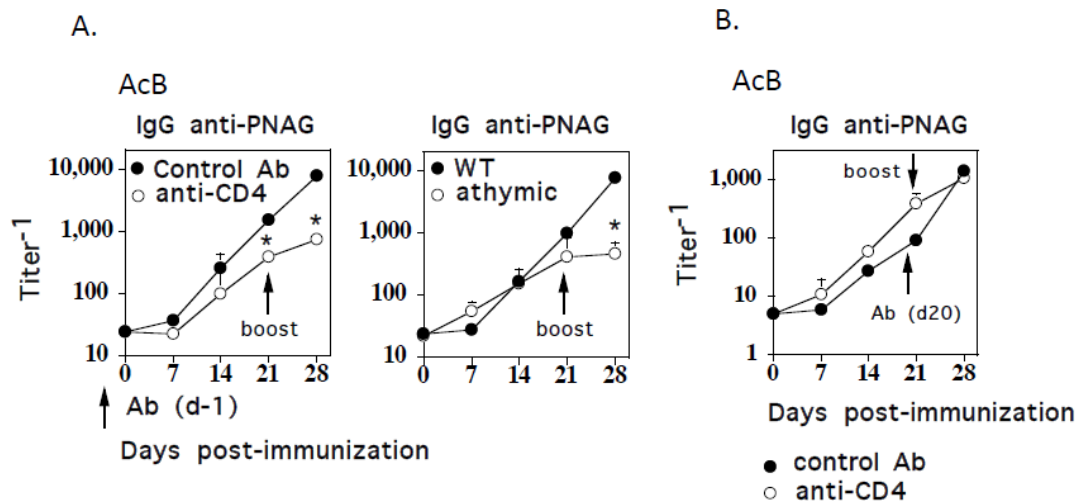


**Figure 4. The requirement for CD4<sup>+</sup> T cells during primary and booster immunization for elicitation of PS-specific IgG responses to *Acinetobacter baumannii* (AcB), another GN bacteria, is similar to that for *MenC*.** A) BALB/c (left) or athymic mice (right) (7 per group) were immunized i.p. (day 0) with  $1 \times 10^8$  CFU/mouse of intact, heat-inactivated AcB and boosted i.p. with the same dose of AcB on day 21. For BALB/c mice (left), anti-CD4 mAb (clone GK1.5, 0.5 mg/mouse) or control rat IgG (0.5 mg/mouse) were injected on day -1. B) BALB/c mice (7 per group) were immunized as in “A” except that anti-CD4 mAb and rat IgG were injected on day 20. Serum titers of PNAG-specific IgG were determined by ELISA (\*  $p \leq 0.05$ ) (significance between rat IgG-injected versus anti-CD4 mAb-injected groups or between wild type (WT) versus athymic groups).

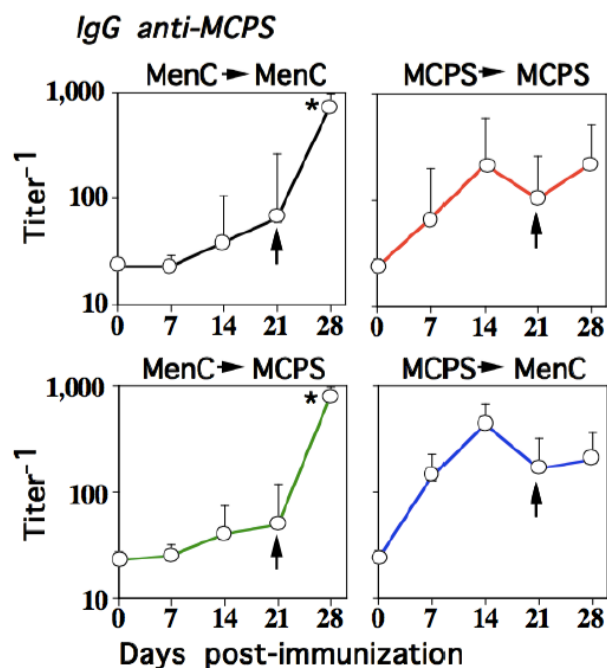


**Figure 5. Mice primed with MenC elicit augmented MCPS-specific IgG responses following booster immunization with isolated MCPS.** BALB/c mice (7 per group) were immunized i.p. with  $2 \times 10^8$  CFU/mouse of intact heat-inactivated MenC or 5  $\mu$ g of MCPS adsorbed on 13  $\mu$ g alum mixed with 25  $\mu$ g CpG-ODN and were boosted i.p. with the same dose of MenC or MCPS, on day 21. Serum titers of MCPS-specific IgG were determined by ELISA. (\*  $p \leq 0.05$ ) (Significance between serum titer on day 21 [pre-boost] versus day 28 [post-boost]).

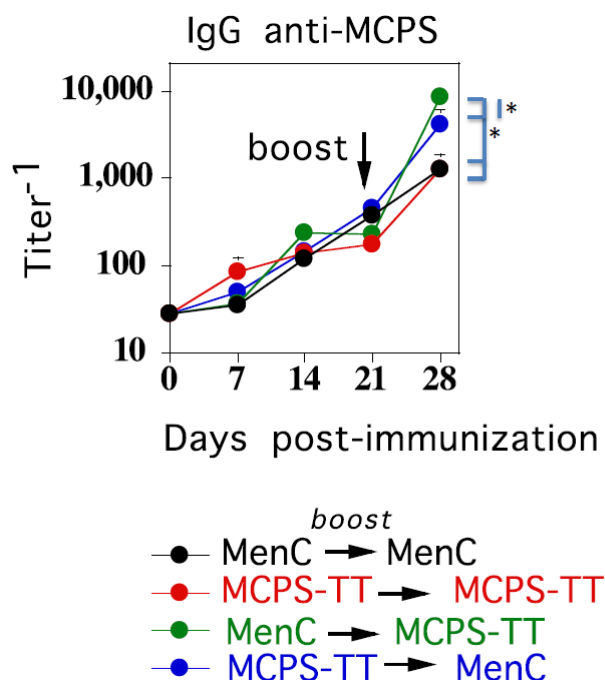


**Figure 6. Mice primed with either MenC or a soluble conjugate of MCPS and tetanus toxoid (MCPS-TT) elicit augmented MCPS-specific IgG responses following booster immunization with either MenC or MCPS-TT.** BALB/c mice (7 per group) were immunized i.p. with  $2 \times 10^8$  CFU/mouse of intact, heat-inactivated MenC or 1  $\mu$ g of MCPS–TT adsorbed on 13  $\mu$ g alum mixed with 25  $\mu$ g CpG-ODN, and then boosted i.p. with the same dose of MenC or MCPS–TT on day 21. Serum titers of MCPS-specific IgG were determined by ELISA. (\*  $p \leq 0.05$ ). (Significant differences in secondary serum titers of MCPS-specific IgG between the indicated groups).

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## **Chapter 4**

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# **CONCLUSION**

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# Conclusion

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## ***Project 1: Distinct cellular pathways for induction of CD4<sup>+</sup> T cell-dependent antibody responses to antigen expressed by intact bacteria versus isolated soluble antigen.***

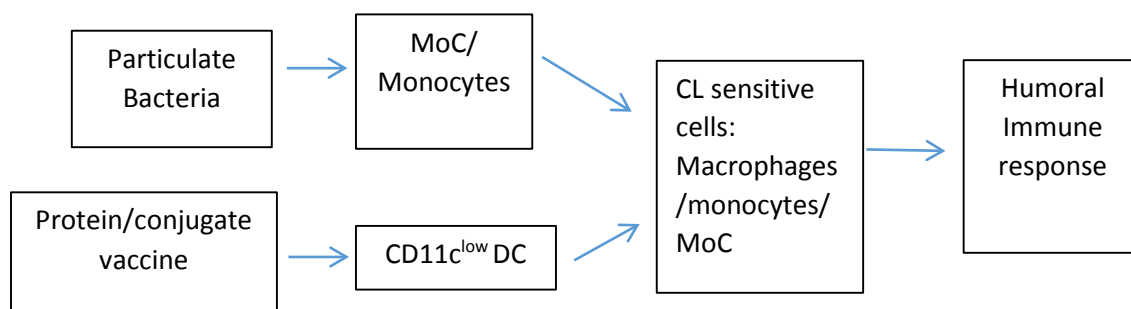
Previous studies from our laboratory suggested that though the regulation of IgG responses to intact bacteria and conjugate vaccines share some properties, striking differences exist between the two in the characteristics of the PS-specific IgG responses to the PS expressed on the surface of the intact bacteria versus the same PS in a conjugate vaccine (63, 222). Moreover we also demonstrated that when PPS14 conjugate vaccine was attached to 1  $\mu$ m latex beads, it exhibited similar immunologic properties to that displayed by intact bacteria (222), which indicated that the differences exhibited between the intact bacteria and conjugate vaccine might be the particulate nature of the former. Internalization of protein antigens by APC followed by priming of CD4<sup>+</sup> T cells is an early event in the initiation of an Ag-specific antibody response. Based on the differences in the mechanism of PS-specific IgG responses to intact extracellular bacteria versus its soluble counterpart, we hypothesized that distinct APCs mediate activation of CD4<sup>+</sup> T cells in response to immunization with a soluble form of Ag versus an intact extracellular bacterium. Specifically, we wished to determine the potential role of macrophages and monocytes/MoC, cells efficient in phagocytosis of particulate Ags, in mediating antibody production in response to systemic immunization with Ag expressed by an intact bacterium versus an isolated, soluble antigen. To test this hypothesis, we utilized CL to selectively deplete phagocytic cells in order to determine its effect on antibody responses to these two distinct forms of Ag.

We demonstrated here that CL differentially depleted macrophages and monocytes (and MoC) but not

dendritic cells (DCs) or neutrophils. Depletion of MMM, MZ and red pulp macrophages, and monocytes, including MoC, by CL injection in BALB/c mice resulted in significant inhibition of the primary and secondary PspA- and PPS14-specific IgG responses to both soluble Ag and intact bacteria, compared to control mice treated with PBSL. The PspA/OVA-specific IgG responses were consistently reduced to a significantly greater degree than that observed for PPS14, in response only to intact bacteria but not in response to soluble antigens. To better define the basis for the reduced PspA/OVA- and PPS14-specific IgG responses following treatment with CL, we investigated the effect of CL on T<sub>FH</sub> and GC formation. We then determined that the percentages of GC T<sub>FH</sub> were significantly reduced in CL relative to PBSL-treated mice following immunization with either R614-OVA<sup>323-339</sup> or OVA. We also demonstrated that CL relative to PBSL injection led to a marked reduction in the GC response following immunization with either R614-OVA<sup>323-339</sup> or OVA. We next wanted to determine if the inhibition of T<sub>FH</sub> formation and reduction in the GC reaction was a consequence of an earlier inhibitory effect on OVA-specific T cell proliferation. We demonstrated that CL-treated mice immunized with R614-OVA<sup>323-339</sup> exhibited a significant reduction in the proliferation of the Tg<sup>+</sup> T cells relative to PBSL-treated mice with a significantly diminished percentage of cells per generation. In contrast, CL-treated mice immunized with PPS14-OVA exhibited a significantly higher proliferative response of Tg<sup>+</sup> T cells relative to PBSL-treated mice. CL- and PBSL-treated mice immunized with OVA alone exhibited a similar degree of proliferation. These data indicated that the inhibition of GC T<sub>FH</sub>, GC, and Ag-specific IgG secretion in response to PPS14-OVA or OVA, resulting from treatment with CL, could not be explained by the early inhibition of specific CD4<sup>+</sup> T cell proliferation. Consistent with these data, we demonstrated that MoC and monocytes were involved in internalization of R614-OVA<sup>323-339</sup>, with little or no detectable uptake by macrophages, CD11c<sup>low</sup> DC or cDC. In contrast, uptake of soluble OVA was observed in CD11c<sup>low</sup> DC but not in cDC, macrophages, monocytes or MoC. Similarly, we further demonstrated that MoC, isolated from mice

immunized with R614-OVA<sup>323-339</sup>, were able to activate Tg<sup>+</sup> cells. In contrast, only CD11c<sup>low</sup> DC activated Tg<sup>+</sup> following immunization with OVA.

Collectively, these data suggest that the CL-sensitive phagocytic cells are critical for uptake and early antigen presentation CD4<sup>+</sup> T cells in response to an intact bacterium (R614-OVA<sup>323-339</sup>), but not in response to soluble antigens (PPS-OVA or OVA). CD11c<sup>low</sup> DCs, not depleted by CL, appear to play a larger role than cDCs as APCs for activating CD4<sup>+</sup> T cells in response to soluble protein. Further, CL-sensitive cells are critical for induction of GC T<sub>FH</sub>, GC, and antibody production in response to soluble antigens, as evident from the inhibition of T<sub>FH</sub>, GC reaction and IgG responses to soluble antigen following CL treatment. A similar, independent role for CL-sensitive cells in these latter responses to intact bacteria cannot be evaluated in this study since CL also inhibited early T cell proliferation. These data suggest the existence of two different pathways associated with humoral responses to two different forms of the same antigen.



***Project 2: Distinct mechanisms underlie boosted polysaccharide-specific IgG responses following secondary challenge with intact Gram-negative versus Gram-positive extracellular bacteria.***

Previously, we established that the nature of the in vivo PS-specific IgG response to capsular PS antigens expressed by intact extracellular bacteria is markedly influenced by the the distinct architecture and/or the composition and/or the differential attachment of the PS to the underlying sub-capsular domains of intact GP and GN bacteria. Overall, these studies suggested a potential dichotomy in the regulation of in vivo PS-specific Ig responses to intact GN MenC and GP GBS-III and Pn14 (29).

The general consensus regarding elicitation of memory IgG responses is that memory B cells generated during the primary response in the presence of CD4<sup>+</sup> T cell help, will proliferate robustly in response to antigen re-exposure and generate plasma cells expressing high-affinity IgG that is dependent on help from cognate memory CD4<sup>+</sup> T cells that were also generated during the primary immunization (191). However, the majority of these studies utilized isolated soluble proteins, which may be induce immunologic events that are distinct from proteins that are expressed by intact extracellular bacteria. In this study, we wished to determine if the PS-specific memory for IgG in response to intact GP and GN bacteria was similarly contained within both the B cell and CD4<sup>+</sup> T cell compartments, and whether the boosted PS-specific IgG responses following secondary immunization with intact bacteria also depended on the presence of CD4<sup>+</sup> T cells. To determine this, we used GP GBS-III and GN MenC and AcB.

We demonstrated that primed B cells were critical for generating an augmented PS-specific IgG response following boosting with intact bacteria. Naïve CD4<sup>+</sup> T cells were as effective as CD4<sup>+</sup> T cells from primed mice for eliciting the augmented secondary response. We further supported this hypothesis by demonstrating that priming of BALB/c with unencapsulated isogenic mutants of MenC or GBS-III, to

potentially activate CD4<sup>+</sup> T cells without activating PS-specific B cells, did not induce augmented PS-specific IgG responses following boosting with the corresponding encapsulated bacteria. In contrast to GBS-III, which required T cells during the booster immunization for augmented PS-specific IgG responses, elicitation of augmented PS-specific IgG booster responses to MenC was independent of CD4<sup>+</sup> T cells during the boost. This was consistent with the observation that boosting with either MCPS or MenC elicited augmented MCPS-specific IgG responses in MenC-primed, but not MCPS-primed mice. Further, we also demonstrated that unlike GBS-III, mice primed with either MenC or a soluble conjugate of MCPS and tetanus toxoid (MCPS-TT) elicited boosted MCPS-specific IgG responses following secondary challenge with either MenC or MCPS-TT. Overall results similar to that observed using MenC were obtained with a second GN bacteria, AcB, which demonstrated a CD4<sup>+</sup> T cell-independent elicitation of an augmented PS-specific IgG response following booster immunization, similar to MenC.

These data suggest that induction of memory for PS-specific IgG responses to either MenC or GBS-III is contained within the B cell, but not CD4<sup>+</sup> T cell population. At least, in the absence of B cell priming, CD4<sup>+</sup> T cells following primary immunization, by themselves do not enhance subsequent PS-specific IgG responses to an intact bacterium, relative to naïve CD4<sup>+</sup> T cells. MenC, and as demonstrated by a second GN bacteria, AcB differs from GBS-III in the lack of a requirement for CD4<sup>+</sup> T cells during booster immunization of primed mice, to elicit an augmented PS-specific IgG response. Unlike GBS-III, the boosted MCPS-specific IgG responses observed regardless of whether MenC or MCPS-TT was used for priming and regardless of whether MenC or MCPS-TT was used for booster challenge suggests that the responses were derived from the same B cell subset. These data further highlight the immunologic differences between GP and GN bacteria that may have important implications for future vaccine design.

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## Clinical relevance

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*Streptococcus pneumoniae* a gram-positive, alpha-hemolytic diplococcus was isolated as the etiologic agent of pneumonia. There are around 90 known serotypes of *S. pneumoniae*. Pneumonia is the 6th leading cause of death in the US and *S. pneumoniae* is the main bacterial pathogen responsible for this disease, and causes over 40,000 deaths a year in our country. The current vaccines available for pneumonia are A) The pneumococcal conjugate vaccine (PCV13 or Prevnar 13<sup>®</sup>) and B) The pneumococcal polysaccharide vaccine (PPSV23 or Pneumovax 23<sup>®</sup>) that contains 23 different pneumococcal capsular serotypes, and confers protection against ~90% of invasive pneumococcal isolates found in the developed world (23). Prevnar 13<sup>®</sup> prevents pneumococcal pneumonia and invasive disease caused by 13 *Streptococcus pneumoniae* strains (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) and is approved for adults 50 years of age and older. It is conjugated to CRM<sub>197</sub>, a protein toxoid derived from the bacterium, *Corynebacterium diphtheriae* (46). It has been shown to confer protection against Pn infections in young children. But Prevnar 13<sup>®</sup> is not 100% effective and will only help protect against the 13 strains included in the vaccine. The complexity is also associated with its proper formulation. Moreover, conjugate vaccines with more serotypes, are relatively expensive and hence, there are limitations for its use in developing countries. Therefore, there is a need for improvements in design and better understanding of the mechanisms underlying PS-specific Ig responses that would assist in the design of improved vaccines.

This first project elucidates a very innovative concept that the mechanism associated with the induction of PS specific IgG responses to intact bacteria is different from that of soluble/conjugate vaccine. We have also demonstrated that distinct APCs are involved in activating CD4<sup>+</sup> T cells in response to the same protein expressed by intact heat-killed bacteria versus soluble antigen. Therefore a novel idea would be

to target the antigens, using monoclonal antibodies directed against receptors on the surface of the APCs, (e.g: Phase I clinical trials targeting DEC205 (CDX-1401, Celldex) and mannose receptor/CD206 (CDX-1307, Celldex). This would then initiate a targeted humoral immune system against a specific form of antigen. Another novel concept to mimic natural infection would be non-covalent association of protein and capsular polysaccharide on bacteria-sized latex beads, as a vaccine candidate. This is based on the fact that we have demonstrated earlier that the noncovalent association in a particle, of polysaccharide and protein, recapitulates essential immunologic characteristics of intact bacteria that are distinct from soluble covalent conjugates of these respective Ags (57).

In the second study we demonstrate that not only induction of memory for PS-specific IgG response to either MenC or GBS-III is contained within the B cell, but also that primed B cells, independent of T cells, can elicit boosted secondary antibody response and hence, mice primed with MenC could elicit augmented antibody response to a purified MenC polysaccharide, a T independent Ag. A common outcome of meningococcal (MenC) infection is meningitis, which is more commonly diagnosed among infants, adolescents, and young adults. Most disease worldwide are caused by the five serogroups (“strains”) of *MenC*: A, B, C, W, and Y. Three of these serogroups (B, C, and Y) cause most of the illness seen in the United States. Current licensed meningococcal conjugate vaccines, polysaccharide vaccines and Serogroup B meningococcal vaccines help provide protection against all four serogroups of the MenC bacteria that are commonly seen in the United States. However, these vaccines are expensive and therefore not for use in developing countries. But the fact that MCPS, which is relatively inexpensive to produce, could elicit an augmented antibody responses in MenC primed mice suggested that this could be a potential vaccine candidate for secondary immunization, specially in cases where patients were already exposed to the bacteri or who received a prior dose of MenC conjugate vaccine. Hence, this would be an inexpensive way of providing protection especially, during a normal course of exposure to MenC.

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# *Follow-up studies*

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## **Project 1:**

Our present study provide evidence that distinct APCs are responsible for the induction of CD4<sup>+</sup> T cell activation and proliferation in response to protein antigens expressed by an intact extracellular bacterium versus an isolated, soluble, protein. These data raise a number of unresolved questions that can form the basis for future studies:

1) Can we identify the specific APC that mediates CD4<sup>+</sup> T cell activation to intact bacteria in vivo? Since administration of CL to mice eliminates a number of phagocytic cell types, experiments effecting more selective depletion of CL-sensitive cell types are required either using constitutive or conditional knockout mice or mice expressing diphtheria toxin receptor (DTR) linked to genes expressed in a cell-type specific manner. In the latter instance, administration of diphtheria toxin would selectively eliminate the DTR<sup>+</sup> cell. For example CD169-DTR mice deplete splenic MMM and MZM following DT, but not cDC (223).

2) What is the nature of the CD11c<sup>low</sup> DC in relation to the more conventional CD11c<sup>+</sup> DC? Flow cytometric analysis utilizing a wider range of phenotypic markers, and gene expression arrays may help to better define the functional differences between these two cells types.

3) What is the identity of the CL-resistant APC in vivo required for CD4<sup>+</sup> T cell activation in response to soluble proteins. A similar approach as that described in “1)” is required. For example, CD11c-DTR mice treated with DT, which selectively eliminates CD11c<sup>+</sup> cells (224) or Batf3<sup>-/-</sup> mice (225) which are selectively depleted in CD8α<sup>+</sup> DC might be of value.

4) Are the same OVA-specific Tg<sup>+</sup> T cells activated by the CL-sensitive and CL-resistance APC

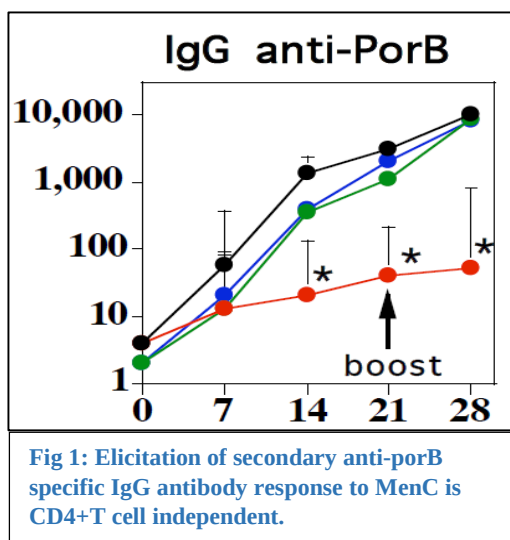
functionally similar? Isolation of these Tg<sup>+</sup> T cells after adoptive transfer and immunization with intact OVA-expressing bacteria versus soluble OVA with phenotypic analysis of Tg<sup>+</sup> T cells by flow cytometry and gene expression arrays, followed by a systems biology analysis would elucidate potential functional differences in these T cells.

5) What is the identity and function of the CL-sensitive cells that co-stimulate Ig production in response to soluble proteins, and are these same cells also important for downstream events in response to intact bacteria? One approach is to delay the addition of CL to determine potential effects on Ig secretion in response to intact bacteria, i.e. depleting CL-sensitive cells after initial antigen presentation to T cells has occurred. Another approach is to immunize mice after varying time periods following CL injection, since specific cell types reconstitute at early and later times following CL treatment. Again, the use of DTR and KO mice to selectively deplete particular CL-sensitive cells may help to distinguish between effects on early antigen presentation and more downstream events critical for induction of Ig secretion, e.g. preservation of early T cell activation but inhibition of Ig secretion in response to intact bacteria.

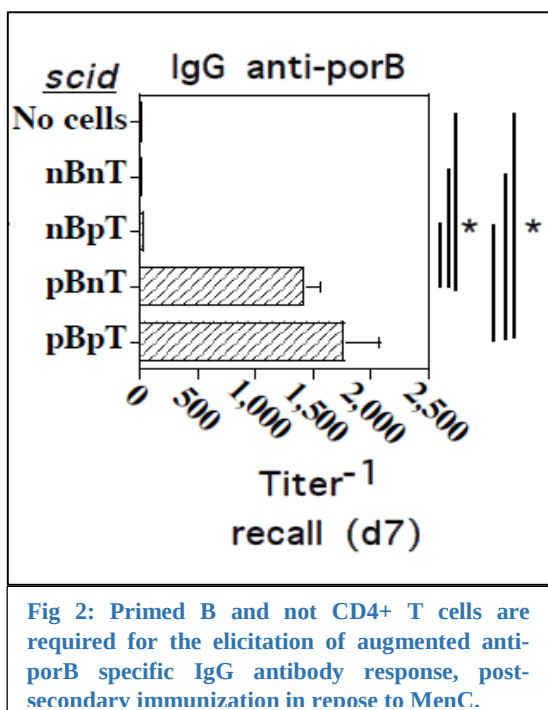
## Project 2:

In the current study, we demonstrated that elicitation of augmented PS-specific booster IgG responses to MenC was CD4<sup>+</sup> T cell-independent. These data raise a number of unresolved questions that can form the basis for future studies:

1. What is the role of CD<sup>+</sup> T cells in elicitation of protein (PorB) specific boosted IgG response to MenC?
2. Can we determine the mechanism that resulted in the distinction in the antibody response between gram negative and gram positive bacteria?
3. Is there a real dichotomy between Gram positive and negative bacteria?
4. If yes, is it based on the different physical/chemical composition of the bacteria.



primary and memory Ab response, but elicitation of augmented IgG anti-PorB booster response was independent of CD4<sup>+</sup> T cells during the booster immunization (Fig 1). Moreover, the elicitation of the augmented secondary anti-PorB IgG response was dependent on primed B cells (Fig 2). These data demonstrate that the elicitation of both an augmented PS- and protein-specific IgG booster response to



the effect of this on anti-PorB specific IgG response. Depleting CD4<sup>+</sup> T cells before booster

To follow up further, we started with investigating the role of CD4<sup>+</sup> T cells in elicitation of protein (PorB) specific secondary IgG response to MenC.

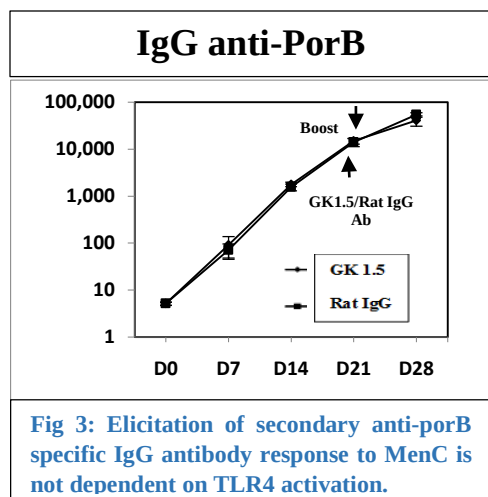
Preliminary Data:

We showed that, similar to MCPS-specific IgG response, mice primed and boosted with MenC resulted in the induction of a CD4<sup>+</sup> T cell dependent PorB-specific (a MenC OM protein)

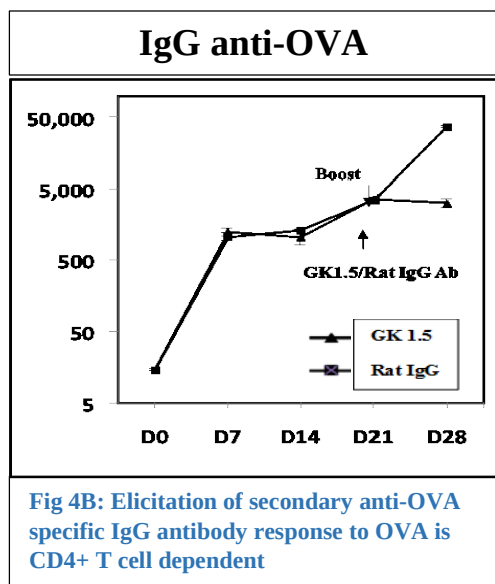
MenC, is-independent of CD4<sup>+</sup> T cells.

Lipopolysaccharide (LPS), a component of the outer membranes of GN bacteria has been shown to be a ligand for TLR4, and a potent activator of the innate immune system (226). Hence, to determine if the TI- independent property of MenC is by virtue of the activation signal provided by TLR4, we primed and boosted C3H/HeJ(Tlr4<sup>Lps-d</sup>) mice with MenC in presence/absence of

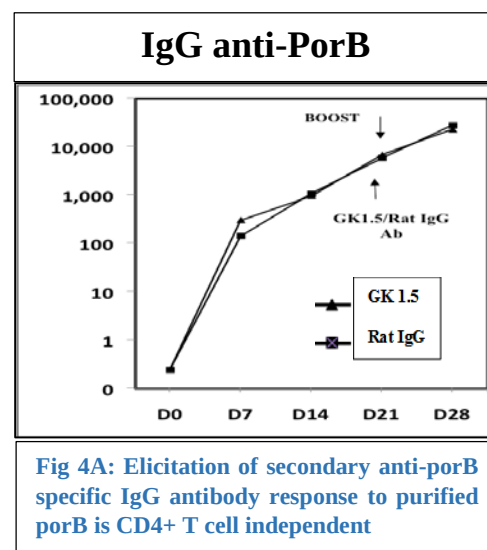
CD4<sup>+</sup> T cells, before booster immunization, and determined



immunization in the TLR4 defective mouse (C3H/HeJ(Tlr4<sup>Lps-d</sup>)) model had no effect on the elicitation of the IgG anti-PorB response (Fig 3). This confirmed that the TI property is independent of TLR4 activation. To determine if the TI elicitation of the augmented PorB-specific IgG booster response to MenC is an intrinsic property of the PorB protein only, we primed and boosted BALB/c mice with PorB purified from MenC, instead of MenC, as an antigen (Fig 4A). Soluble OVA was used as a control protein for this study (Fig 4B). Of interest, we demonstrated that elicitation of augmented PorB-specific IgG response to purified PorB, is CD4<sup>+</sup> T cell-independent. In contrast, the augmented OVA-specific IgG response, following booster immunization, in OVA-primed BALB/C mice, was CD4<sup>+</sup> T-cell dependent. Previous studies



currently in progress.



have shown that porB is a ligand for TLR2 (226). Hence, we wanted to determine if the activation of TLR2 by PorB is providing the necessary stimulation, in the absence of CD4<sup>+</sup> T cells, for elicitation of the augmented Ab response, post secondary immunization. To determine this, we primed and boosted TLR2<sup>-/-</sup> mice with PorB, in presence/absence of CD4<sup>+</sup> T cells, before booster immunization.. This experiment is

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