Title

Differential regulation of protein- and polysaccharide-specific Ig isotype production in vivo in response to intact Streptococcus pneumoniae

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Abbreviations: Pn, intact *Streptococcus pneumoniae*; PS, polysaccharide; PPS, pneumococcal capsular polysaccharide; C-PS, C-polysaccharide; PC, phosphorylcholine; PspA, pneumococcal surface protein A; DC, dendritic cell; TLR, Toll-like receptor; TI, T cell-independent; TD, T cell-dependent;

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14. ABSTRACT
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Abstract. Adaptive humoral immunity to extracellular bacteria is largely mediated by antibody specific for both protein and polysaccharide antigens. Proteins and polysaccharides are biochemically distinct, and as a result are processed differently by the immune system, leading to different mechanistic pathways for eventual elicitation of specific Ig isotypes. Much of our current knowledge concerning the parameters underlying anti-protein and anti-polysaccharide Ig responses have come from studies using soluble, purified antigens. However, the lessons learned from these studies are not entirely applicable to the mechanisms underlying physiologic anti-protein and anti-polysaccharide Ig responses to intact bacteria. Specifically, unlike isolated, soluble antigens, intact bacteria are complex particulate immunogens in which multiple protein and polysaccharide antigens, and bacterial adjuvants (e.g. Toll-like receptor ligands) are co-expressed, indeed often physically linked. In this review, data from a series of recent studies are discussed in which heat-killed, intact *Streptococcus pneumoniae* was used as an immunogen to study the mechanisms underlying *in vivo* anti-protein and anti-polysaccharide Ig isotype induction. An unexpected role for $CD4^+$ T cells and dendritic cells for induction of IgG anti-polysaccharide responses by intact bacteria is discussed, and shown to have distinct mechanistic features from those that mediate anti-protein responses. The further role of cytokines, Toll-like receptors, and B cell receptor signaling in mediating these responses, and its implications for the effectiveness of anti-pneumococcal, polysaccharide-based vaccines, is also discussed.

HOST PROTECTION AGAINST INFECTIONS BY EXTRACELLULAR BACTERIA

Host protection against infections with extracellular bacteria, such as *Streptococcus pneumoniae* (Pn), is initially conferred by a rapid innate immune response leading to phagocytosis and killing of bacteria by phagocytes, such as neutrophils and macrophages [1]. Initiation of the innate response is largely mediated by Toll-like receptors (TLRs), expressed by innate immune cells, which recognize a limited number of conserved microbial structures [2, 3]. Early TLR-mediated signaling results in immune cell activation that drives the development of subsequent adaptive immunity, mediated by B and T cells. Although evidence has been recently cited suggesting a possible role for cell-mediated immunity in anti-Pn host protection from nasal colonization [4, 5], humoral immunity is currently considered the major adaptive mechanism for bacterial clearance from the blood and tissues [1]. IgM, IgG, or IgA specific for both bacterial protein and polysaccharide (PS) antigens can confer protection. Current, effective vaccines for protection from Pn infections are designed to elicit protective antibodies to the capsular PS [6]. Ig binding to the bacterial surface facilitates Fc- and complement-mediated opsonophagocytosis of bacteria by neutrophils and macrophages expressing Fc and complement receptors [1].

REGULATION OF ANTIBODY RESPONSES TO PURIFIED AND PS ANTIGENS

Much of our current thinking concerning the mechanism by which anti-protein and anti-PS Ig isotype responses are elicited has emerged from studies using purified antigens [7]. Purified protein antigens are typically ineffective at eliciting Ig responses in the absence of some form of adjuvant, whereas PS antigens induce Ig responses, albeit quantitatively modest, in the

absence of an added adjuvant. Proteins, when internalized by antigen-presenting cells (APCs) are enzymatically degraded into constituent peptides which then become associated with MHC-II molecules for transport to the cell surface. Such APCs present peptide/MHC-II complexes to $CD4^+$ T cells that lead to crosslinking of the specific T cell receptor (TCR). If the APC expresses sufficient levels of costimulatory molecules such as CD80 (B7-1) and CD86 (B7-2), as well as MHC-II molecules typically induced or upregulated by adjuvant, TCR crosslinking results in $CD4^+$ T cell effector function that promotes B cell proliferation, Ig class switching, and differentiation into Ig-secreting plasma cells, as well as B cell memory [8]. In addition to secreted cytokines, T cell expression of membrane CD40-ligand (L) [CD154] appears critical for induction of these T cell-dependent (TD) Ig responses [9].

Certain zwitterionic PSs such as polysaccharide A (PS-A) from *Bacteroides fragilis* and the capsular PS from type 1 Pn (Sp1) can be processed to low molecular weight carbohydrates, by a nitric oxide-mediated mechanism and presented to, and activate, $CD4^+$ T cells via the MHC-II endocytic pathway [10, 11]. C-polysaccharide (C-PS) [teichoic acid], present within the Pn cell wall, is also a zwitterionic PS [12], but its ability to activate T cells is currently unknown. Nevertheless, it is likely that most capsular PS, which are not zwitterionic, are not degraded within the APC for MHC-II association and presentation to T cells [13, 14]. For this reason, PS antigens typically do not elicit cognate $CD4^+$ T cell help, consistent with the observation that anti-PS responses in T cell-deficient athymic nude mice and TCR-knockout mice are comparable to those observed in wild-type mice [7]. Thus, capsular PS antigens are classified as T cell-independent (TI) type 2 (TI-2) antigens to distinguish them from the functionally distinct TI-1 antigens, such as LPS, which consist of a PS antigen linked to a TLR ligand (i.e. the TLR4

ligand, lipid-A) [15, 16]. Nevertheless, PS antigens may influence the ability of APCs to present associated protein antigens to $CD4^+$ T cells [17]. Thus, the ability of spleen cells from HLA-DR1 transgenic mice, pulsed with individual pneumococcal PS-CRM_{197} conjugates found within a heptavalent conjugate vaccine, to stimulate a CRM_{197}-specific, DR1-restricted $CD4^+$ T cell hybridoma, was dependent on the particular capsular PS serotype in the conjugate.

In contrast to proteins antigens, the inability of purified PS antigens to recruit classical cognate $CD4^+$ T cell help is associated with either absent or abortive germinal center (GC) formation [18-20] and a failure to induce a state of specific memory [7]. Unlike proteins, PS antigens which are composed of repeating, identical subunit sugars effect multivalent crosslinking of specific membrane (m)Ig on the surface of B cells. Multivalent mIg crosslinking has been shown to potently induce B cell proliferation [21], and in the presence of additional stimuli such as cytokines or TLRs, Ig class switching and differentiation into Ig-secreting cells [22]. The potential importance of mIg signaling in mediating anti-PS relative to anti-protein responses is perhaps underscored by the inability of purified PS antigens to induce Ig responses in the immature host [23], where B cells are known to have mIg signaling defects [24, 25]. In contrast, linkage of an immunogenic carrier protein to a PS antigen, which converts the subsequent anti-PS response from TI-2 to TD, leads to induction of a protective Ig response in the immature host [26]. The mechanism by which purified PS antigens, not known to be TLRs themselves, induce Ig responses *in vivo* has been a matter of interest, and will be discussed in detail below.

Antibody-antigen binding typically leads to fixation of complement, in particular the generation of distinct fragments of C3 [1]. Both B cells and follicular dendritic cells (FDCs) express complement receptor type 2 (CR2) [CD21], that binds the C3 fragment, C3d. Co-crosslinking of mIg and CD21 is markedly synergistic for B cell activation and proliferation, allowing for 100-1000-fold less antigen for comparable levels of immune induction [27]. In this regard, linkage of several copies of C3d to a purified PS antigen has been shown to significantly enhance anti-PS Ig responses [28]. Although endogenous C3d-mediated enhancement of anti-PS responses likely plays a physiologic role in augmenting humoral immunity to PS antigens [29], it is unlikely that mIg-CR2 co-crosslinking alone can induce Ig class switching and differentiation in the absence of additional signals.

The classification of capsular PS antigens as TI-2, however, appears to belie a more complicated mechanism for Ig induction. Thus, a number of early and recent studies from several laboratories have observed a role for both "amplifier" $CD4^+$ T cells (Ta) and "suppressor" $CD8^+$ T cells (Ts) in regulating anti-TI-2 responses in a non-classical manner (i.e. TCR recognition of B cell idiotypic determinants) [30]. In light of the observation that T cell-deficient mice have normal TI-2 responses, it has been postulated that the function of Ta and Ts cells are typically in balance. In this regard, although Ig responses to haptenated Ficoll (a widely used TI-2 antigen) have been shown to be CD40L-independent [31-33], more recently anti-PPS responses were shown to be inhibited by injection of the CD40L blocking mAb, MR1 [34, 35]. These apparent conflicts, utilizing different TI antigens, have not as yet been resolved into a more cohesive picture.

INDUCTION OF ANTI-PROTEIN AND ANTI-PS RESPONSES TO INTACT EXTRACELLULAR BACTERIA

Immunologic studies utilizing purified protein and PS antigens may render an incomplete view of the parameters that mediate *physiologic* humoral immune responses to intact pathogens. An intact bacterium contains protein and PS antigens that are intimately co-expressed within an organized particulate structure containing multiple adjuvanting TLR ligands [1]. In this regard, the immunologic behavior of these antigens may differ from their isolated, soluble counterparts. For example, mIg-mediated recognition and internalization, by PS-specific B cells, of specific PS antigen linked to protein within a bacterial fragment, could in theory confer TD properties to the PS antigen. This might be similar to what is observed for soluble conjugate vaccines [26]. Indeed, sequencing of variable regions of natural human PS-specific Ig has shown a striking degree of somatic hypermutation (SH) [36, 37]. This suggests that host contact with colonizing encapsulated bacteria may induce TD germinal center reactions where SH is known to occur. Similarly, repeated immunization of rabbits with formalinized capsular type III and type VIII pneumococci, followed by sequence analysis of the induced antibodies, has strongly suggested a high degree of somatic diversification [38]. Additionally, the particulate nature of the complex bacterial immunogen may in itself alter the immunologic characteristics of the constituent antigens [39]. Collectively, these considerations might further suggest a potential active role for DCs in mediating, perhaps differentially, both anti-protein and anti-PS Ig responses. The use of an intact bacterium to compare anti-protein and anti-PS responses has the further advantage of avoiding the potential artifacts of haptenation and in using comparable levels of adjuvanting.

If anti-PS responses to intact bacteria, like those to soluble protein-PS conjugates are in fact T cell-dependent, and if DCs play an active role, then *physiologically* are there in fact fundamental differences between the immunologic behavior of these two major classes of antigen? I will present data below that IgG anti-protein and-anti-PS responses to an intact bacterium do exhibit distinct characteristics, despite both being dependent on $CD4^+$ T cells and actively elicited by DCs. Perhaps the distinct manner in which PS antigens activate B cells, via multivalent mIg signaling, the engagement of functionally distinct subsets of B cells, and/or differential processing by DCs, will distinguish *physiologic* protein and PS-specific IgG responses, not whether they are TD or TI, respectively. Further, recent data suggests that $CD4^+$ T cell-dependent IgG anti-PS responses to intact bacteria and soluble conjugate vaccines may also exhibit distinct differences.

MARGINAL ZONE, B-1, AND FOLLICULAR B CELLS

As mentioned above, an understanding of the mechanisms underlying physiologic anti-protein and anti-PS responses must take into account the biochemical character of the antigens themselves, the structural context in which they are expressed within the bacterium, as well as whether functionally distinct B cell subsets are involved in the generation of the Ig responses. It is widely believed that TI responses to PS antigens are mediated by B-1 and marginal zone B cells (MZB), whereas follicular B cells (FB) mediate TD responses to proteins [40-42]. B-1 cells are divided into B-1a and B-1b and can be recognized by their characteristic cell surface phenotypes (e.g. B-1a=$B220^+CD11b^+CD5^+$, B-1b=$B220^+CD11b^+CD5^-$). In contrast, MZB and FB cells (collectively, B-2 cells) can be identified as $B220^+CD21^{high}CD23^{low}$ and

$B220^{+}CD21^{intermediate}CD23^{high}$, respectively. B-1 cells make up the majority of B cells in the pleura and peritoneum, but are also present as a minor population in the spleen (although at higher absolute numbers than B-1 cells found at the serosal surfaces). Attempts to identify the histologic location of B-1 cells in normal mouse spleen have proven difficult because of their paucity as well as their low level expression of CD5. However, a recent study using transgenic mice possessing elevated numbers of B-1 cells with an anti-phosphatidylcholine autoreactive specificity identify their main location as being within the center of the splenic B cell follicle. In contrast, FB cells are located somewhat more peripherally within the follicle [43]. B-1 cells appear to produce a significant percentage of natural Ig that is germline-encoded, low affinity, polyreactive, and specific for conserved microbial structures, including PS antigens. Indeed, the level of mIg signaling by endogenous antigen during B cell ontogeny appears to select which B cell subset emerges (B-1>MZB>FB) [44-47]. This may in turn lead to the hardwiring of different mIg triggering thresholds during immune responses to their corresponding foreign antigens. A recent study has further suggested that B-1a cells in particular, are responsible for production of natural serum Ig [48]. In contrast, B-1b, but not B-1a or MZB, cells were largely responsible for induction of anti-capsular pneumococcal PS, capsular type 3 (PPS3) antibodies following immunization with purified PPS3 or intact *Streptococcus pneumoniae*, capsular type 3. It was also suggested that induction of anti-phosphorylcholine responses to intact Pn3 are elicited by B-1a and MZB, but not B-1b cells.

With the above study noted, a likely role for MZB, in addition to B-1 cells, in TI responses to PS expressed by extracellular bacteria is still supported by previous studies [40-42]. This notion makes sense in light of the fact that extracellular bacteria are rapidly growing,

necessitating a relatively rapid immune response for host protection. Thus, the location of MZB in the marginal zone of the spleen, where blood-borne bacteria first enter, allow for immediate contact of B cell and pathogen [40, 41]. MZB also have a relatively high expression of CD21 for enhanced capture of, and activation by, complement-coated antigen, and of CD36 that facilitates TLR2 recognition of certain pathogen-derived adjuvanting ligands (49, 50]. Finally, MZB are somewhat larger in size than FB and respond more vigorously and rapidly to various stimuli [51-54] suggesting that they are constitutively in a state of partial activation.

Nevertheless, MZB express higher levels of MHC class II molecules, and B7.1 (CD80) and B7.2 (CD86) than FB. Thus, they are more effective at costimulating naïve $CD4^+$ T cells, suggesting a role in TD responses as well [55]. Indeed, adoptive transfer of MZB and FB into scid/scid mice followed by challenge with a TD protein antigen, demonstrated that MZB were the major source of Ig-secreting cells in the primary response, formed germinal centers (GCs) [with delayed kinetics] and underwent somatic hypermutation (SH) [56]. More recently, adoptive transfer studies were performed in which both HEL-specific MZB and FB from anti-HEL B cell knock-in mice were transferred into wild-type recipients, followed by immunization with HEL-SRBC. MZB, in contrast to FB, exhibited delayed expansion following Ag challenge, less efficient homing to the outer PALS (periarteriolar lymphoid sheath), delayed entry into germinal centers, and delayed onset of somatic hypermutation [57]. These results are consistent with the concept that MZB and FB are generally specialized for TI and TD responses, respectively, although MZB clearly can participate in a TD response. Thus, it remains unresolved which B cell subsets are primarily responsible for the $CD4^+$ TD IgG anti-protein and anti-PS responses to intact bacteria, as will now be discussed.

KINETICS, Ig CLASS SWITCHING, MEMORY GENERATION, AND T CELL-DEPENDENCE OF ANTI-PROTEIN AND ANTI-PS RESPONSES TO INTACT PN

We injected heat-killed, intact Pn i.p. into mice, followed by boosting on day 14. Sera were collected at weekly intervals (from day 0 to day 21) for measurement, by ELISA, of serum IgM and IgG isotype titers specific for various protein and PS antigens. In early studies we utilized the unencapsulated variant of Pn, capsular type 2 (R36A) for measurement of Ig specific for the cell wall protein, pneumococcal surface protein A (PspA) and the phosphorylcholine (PC) determinant of cell wall C-polysaccharide (C-PS) [teichoic acid]. In later studies we used the encapsulated Pn, capsular type 14 (Pn14) and extended our analyses to Ig responses specific for capsular PS type 14 (PPS14) and additional proteins, including pneumococcal surface adhesin A (PsaA) and pneumococcal surface protein C (PspC) [also called choline-binding protein, CbpA), as well as PspA and PC. These pneumococcal protein and PS antigen have been shown previously to be immunogenic (PspA [58], PsaA [59], PspC [60], PC [61], PPS [62]). The primary IgG anti-protein response to intact Pn developed over a more prolonged period with peak responses at day 10, whereas, IgM and IgG anti-PPS14 and anti-PC responses peaked on day 6 [63, 64]. Both anti-protein and anti-PS IgG consisted of all 4 IgG isotypes. Secondary immunization with Pn led to 10-20-fold enhancements in Ig titers for the various Pn proteins. The generation of memory was relatively long-lasting in that secondary immunization even 6 weeks after the primary led to similar boosted titers [65]. These observations were consistent with the ability of intact Pn to promote GC formation in the spleen. In contrast, no boosting was observed for IgM or IgG anti-PPS [64]. Although IgM and IgG anti-PC responses showed

modest (typically up to 3-fold) boosting upon secondary immunization on day 14, this memory was not long-lasting (unpublished). Of interest, both the anti-PPS14 and anti-PspA response to immunization with a soluble conjugate of PPS14-PspA showed the more prolonged kinetics and strong memory responses as that observed for the protein-specific IgG responses to intact Pn [66].

The more rapid kinetics and lack of memory generation for the anti-PS relative to the anti-protein response to intact Pn, mirrored what was generally observed using soluble purified antigens. However, the anti-PS response to intact Pn consisted of all 4 IgG isotypes, whereas IgG3 is generally the dominant IgG isotype elicited by purified PS [67]. We next wished to determine whether endogenous T cells regulated PS-specific Ig responses to intact Pn. Surprisingly, we observed that the IgG anti-PPPS14, anti-PC, and anti-PspA responses were all heavily dependent on TCR-α/β^{+}CD4^{+} T cells, with no apparent role for TCR-γ/δ^{+} T cells [63-65]. These studies were accomplished through the use of athymic nude mice with or without adoptive transfer of CD4^{+} T cells, TCR-$\beta^{-/-}$, TCR-$\delta^{-/-}$ or TCR-$\beta^{-/-}$ x $\delta^{-/-}$ mice (deficient in α/β, or γ/δ T cells or both, respectively) or of a depleting anti-CD4 mAb. In contrast, the IgM responses to PPS14 and PC were TI. Consistent with these data, earlier studies demonstrated that immunization of athymic nude mice or thymectomized-bone marrow reconstituted mice with intact, heat-killed group A Streptococcus resulted in a substantially reduced antibody response to group A streptococcal carbohydrate (GAC), relative to that seen in T cell-sufficient controls [68, 69]. Of interest, both the IgM and IgG3 anti-GAC response was reduced in the absence of T cells [69], whereas the IgM, though not IgG, anti-PC and anti-PPS14 response to intact Pn14 was largely TI.

Using CTLA4Ig, blocking anti-B7-1 and/or anti-B7-2 mAbs, and $CD28^{-/-}$ mice, we further showed that the primary IgG anti-protein and anti-PS responses were also heavily dependent on B7-2/CD28-dependent, but not B7-1-dependent, costimulation [70]. As discussed below, Pn induces a mixed type 1/type 2 cytokine and IgG isotype response. In this regard, although type 2 responses have been thought to be generally more dependent on B7-dependent T cell costimulation than type 1 responses [71], CTLA4Ig blocked both types of response to Pn to a comparable degree. Finally, studies using a blocking anti-CD40L mAb (MR1) or $CD40^{-/-}$ and $CD40L^{-/-}$ mice demonstrated a strong dependence on CD40/CD40L interactions for induction of the IgG anti-protein and anti-PS responses [63, 64]. *Collectively, these studies led to the surprising conclusion that despite a significant and apparently classic role for $CD4^+$ T cells in promoting IgG anti-PS responses to intact Pn, these IgG responses (encompassing all 4 IgG isotypes) still peaked more rapidly and failed to generate any long-lasting memory, in contrast to the IgG anti-protein responses.*

DISTINCT MODES OF $CD4^+$ T CELL HELP FOR INDUCTION OF IgG ANTI-PROTEIN AND ANTI-PS RESPONSES

As discussed above, distinct differences in the kinetics and the generation of memory between the IgG anti-protein and anti-PS responses to intact Pn are observed. Nevertheless, both responses show a strong dependency on both on TCR-α/β^+CD4^+ T cell help, B7-2/CD28-dependent costimulation, and CD40/CD40L interactions. These functional interactions are widely viewed as critical for the generation of memory in response to protein antigens. Thus, it

suggested the possibility that the nature of the T cell help for the anti-PS response might differ relative to that for the anti-protein response, resulting in a failure to elicit anti-PS memory. In light of the more rapid kinetics of the primary anti-PS response, we determined whether the duration of the required $CD4^+$ T cell was similarly shortened, relative to that necessary for an optimal anti-protein IgG response. $CD4^+$ T cells were depleted on different days following primary immunization with Pn, using anti-CD4 mAb (clone GK1.5) [64, 65]. Optimal IgG anti-PS responses required 1-2 days of $CD4^+$ T cell help, in contrast to the primary IgG anti-protein response which required >4 days. Consistent with these observations, injection of CTLA4Ig, to block both B7-1- and B7-2-dependent costimulation, likewise demonstrated a shortened requirement for T cell costimulation for the IgG anti-PS response [70]. As mentioned earlier, IgM anti-PS responses were unaffected by $CD4^+$ T cell depletion or B7 blockade, consistent with their TI nature. *Thus, the differences in kinetics between the anti-protein and anti-PS responses were correlated with a correspondingly different temporal involvement of $CD4^+$ T cells.*

We earlier suggested the possibility that uptake, by PS-specific B cells, of bacterial fragments containing both PS and protein, would mediate cognate interactions between PS-specific B cells and $CD4^+$ T cells, specific for peptide generated from the internalized bacterial protein. If so, $CD4^+$ T cells would require TCR specificity for Pn-derived peptides associated with MHC-II. To determine this, we utilized H-Y (male antigen)-specific TCR transgenic mice crossed with TCR-$\alpha^{-/-}$ mice (H-Y $\alpha^{-/-}$) [72]. Such mice were unable to generate endogenous TCR-α/β^+ T cells as evidenced by their inability to respond to a number of soluble proteins antigens, or elicit an anti-PspA response or GC reaction to challenge with intact Pn. Importantly, these mice, relative to wild-type mice, contained equivalent numbers of B cells, and both $CD8^+$ T

cells (specific for the H-Y antigen) and $CD4^+$ T cells, having TCR reactivity with MHC-II β-chain and some undefined peptide. Immunization of H-Y $\alpha^{-/-}$ mice with intact Pn14 resulted in an IgM anti-PS response equivalent to that elicited in the wild-type control [64, 65]. Of interest, both the IgG anti-PspA and IgG anti-PPS14 responses were markedly reduced in the H-Y $\alpha^{-/-}$ mice. Surprisingly, H-Y $\alpha^{-/-}$ and wild-type mice elicited equivalent IgG anti-PC responses, that in both cases could be strikingly reduced by prior injection of depleting anti-CD4 mAb [64, 65]. Consistent with these data, cathepsin $S^{-/-}$ mice, which are defective in formation of new MHC-II peptide complexes, and are defective in eliciting IgG responses to protein Ag in adjuvant [73, 74], exhibited a significantly reduced IgG anti-PspA response to intact Pn, but a normal IgG anti-PC response, relative to wild-type mice [65]. *These data thus demonstrate a requirement for TCR-specific $CD4^+$ T cell help for both the IgG anti-protein (prolonged kinetics) and IgG anti-PPS response (abbreviated kinetics), although a TCR-non-specific form of $CD4^+$ T cell (abbreviated kinetics) for the IgG anti-PC response.*

Our data on the requirement for TCR-specific $CD4^+$ T cell help for the anti-PPS14 response to intact Pn14 is consistent with a previous analysis using a soluble conjugate consisting of type III capsular polysaccharide from group B Streptococcus (GBSIII) linked to tetanus toxoid (TT). In this latter study the IgG anti-GBSIII response was shown to depend on MHC class II-TCR, B7-CD28, and CD40-CD40L interactions [26], similar to what we previously demonstrated using intact Pn [63]. This further draws parallels between intact bacteria and soluble conjugates in the regulation of anti-PS responses. However, recent unpublished data from our laboratory indicates that IgG anti-PPS14 responses to intact Pn versus a soluble PPS14-14-PspA conjugate differ significantly, despite both being dependent on $CD4^+$ T cell help. Thus,

serum titers of IgG-anti-PPS14 in response to intact Pn peak more rapidly and fail to develop memory, whereas IgG anti-PPS14 responses to the conjugate peak later (at the same time as the anti-PspA response) and elicit highly boosted responses following secondary immunization. Nevertheless, both responses appear to depend on cognate $CD4^+$ T cell help suggesting that the particulate nature of the intact Pn and/or its more complex biochemical composition alter the nature of the PS-specific response, relative to that observed using a simplified soluble conjugate.

The nature of the TCR-non-specific $CD4^+$ T cell help for the IgG anti-PC response to intact Pn is currently unknown. PC is an unusual antigenic determinant, in that anti-PC antibodies are highly restricted to the B-1 subset of B cells, although MZB have also been shown to play a role in this response [75]. Further, PC is present both as an autoantigen on the surface of apoptotic cells and oxidized low-density lipoprotein, as well as on commensal bacterial flora [76-78]. Not surprisingly PC-specific B cells, unlike PPS14-specific B cells, appear to be constitutively primed, as evidenced by the presence of significant titers of anti-PC, though not anti-PPS14, antibody in the sera of naïve mice. The B cell subset(s) responsible for secretion of anti-PPS14 antibody has not, as yet, been determined.

Thus, the activation state and/or the B cell subset derivation of the PC-specific B cell may confer unusual properties relative to its ability to interact with T cells. In this regard, peritoneal B-1 cells have been shown to express significantly higher levels of costimulatory molecules (CD80, CD86, CD40, and ICAM-1) than splenic B-2 cells [79], similar to that described for MZB, as mentioned earlier. This was associated with the ability of peritoneal B-1 cells, although not B-2 cells, to activate a small, but significant number of *syngeneic* thymocytes to undergo cell

division in the presence, but not absence of IL-2. Thus, B cell subsets (B-1 and MZB) that appear to preferentially mediate anti-PS responses may have the selective ability to rapidly recruit some form of T cell help to optimize early production of protective IgG. C-PS, of which PC is a major antigenic determinant, is also a zwitterionic PS as already discussed. Thus, C-PS has the potential to directly mediate APC-dependent, $CD4^+$ T cell activation. However, T cell activation induced by zwitterionic PSs appear to involve presentation of MHC-II/PS fragment complexes [10, 80, 81] suggesting a requirement for TCR specificity, not seen for the IgG anti-PC response to intact Pn.

In summary, the more accelerated kinetics of the IgG anti-PS response to intact Pn is correlated with a shorter duration of delivery of $CD4^+$ T cell help and B7-dependent costimulation, relative to that seen for the IgG anti-protein response. For IgG anti-PS responses both a TCR-specific (anti-PPS14) and TCR-non-specific (anti-PC) form of $CD4^+$ T cell help is observed. These abbreviated T cell kinetics may in part serve to limit sustained progression through the GC phase of the response, and thus curtail memory cell generation.

ENDOGENOUS $CD4^+CD25^+$ REGULATORY T CELLS (Treg) AND THE HUMORAL IMMUNE RESPONSE TO INTACT PN

Endogenous $CD4^+CD25^+$ T regulatory cells (Tregs) account for 5-10% of peripheral $CD4^+$ T cells, and due to their broad range of antigen specificities, can limit immune responses to many different self, as well as, foreign antigens [82, 83]. Although Tregs are well-known to down-regulate chronic cell-mediated immune responses such as those seen in autoimmunity,

tumor immunity, transplantation tolerance, and various infections that trigger cell-mediated immunity, very little is known regarding a potential role for Tregs in an acute humoral response to an extracellular bacteria. In this regard, a potential role for Treg in the induction of autoantibodies, including anti-double-stranded DNA antibodies [84], and a hyper IgE response in a B and T cell transgenic model system [85], has been reported.

CD25 (IL-2Rα) is constitutively expressed on Tregs. Injection of anti-IL-2Rα mAb (PC61) has been shown to selectively deplete Tregs *in vivo* and abrogate suppression [86]. Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) is also constitutively expressed on Tregs [87, 88]. An agonistic GITR-specific mAb, DTA-1, can abrogate the suppressor activity of Treg both *in vitro* and *in vivo* [87, 88]. GITR expression can also be induced on activated effector CD4+ T cells, where it can act as a costimulatory molecule [89]. Our observations that the IgG anti-protein and IgG anti-PS responses were stimulated by $CD4^+$ T cells, led us to ask whether these responses were under the negative control of Tregs. Injection of anti-IL-2Rα mAb to deplete Tregs, agonistic anti-GITR mAb to inhibit Treg function, or adoptive transfer of Treg-depleted $CD4^+$ T cells into athymic nude mice, each had no effect on either the primary or secondary protein- or PS-specific IgG response to intact Pn [90]. It was previously suggested that TLR-mediated innate immune activation, including the release of IL-6, inhibited Treg suppressor function, thus allowing for the initial immune response to a pathogen to progress [91, 92]. Surprisingly, we observed that anti-IL-2Rα mAb also had no effect on the IgG response to intact Pn in $MyD88^{-/-}$ mice or to a soluble protein-PS conjugate injected into wild-type mice in the absence of adjuvant [90]. *Collectively, these data suggest that, in contrast to their role in limiting chronic, cell-mediated immunity, Tregs may play no*

significant role in an acute humoral immune response to an intact extracellular bacterial pathogen, such as Pn.

A DIFFERENTIAL ROLE FOR DCs IN THE INDUCTION OF ANTI-PROTEIN AND ANTI-PS RESPONSES TO INTACT PN

In light of the $CD4^+$ T cell-dependence for both anti-protein and anti-PS induction in response to intact Pn, we wished to determine whether DCs played a role in either or both of these responses. We first demonstrated that immature bone marrow-derived myeloid dendritic cells (BmDC) undergo phenotypic maturation and secrete TNF-α, IL-6, IL-12 and IL-10 when pulsed *in vitro* with intact Pn [93] After transfer to naive mice, Pn-pulsed BmDC induce Ig isotype responses specific for both pneumococcal protein and PS antigens, having in common the requirement for viable BmDC (following the pulse and immediately prior to transfer into mice), as well as for T cells and B7-dependent costimulation in the recipient mice [93]. Whereas, primary Ig isotype responses to bacterial proteins uniformly required BmDC expression of MHC class II, CD40 and B7, and the secretion of IL-6, but not IL-12, similar requirements for anti-PS IgG responses were only observed for the IgG1 isotype. These data suggest that cognate interactions between DC and $CD4^+$ T cells are critical for anti-protein responses to intact Pn. In contrast, the cognate $CD4^+$ T cell help required for the IgG anti-PPS14 response [64] may be solely mediated at the level of $CD4^+$ T cell-B cell interactions. This further suggests that DCs may actively participate in IgG anti-PS responses through direct interactions with B cells, perhaps priming them for effective APC function with naïve $CD4^+$ T cells. Indeed, DCs have previously been shown to form synapses with B cells and to deliver B cell help [94].

Splenic DCs in naïve mice comprise at least 3 major subsets; “myeloid” ($CD11c^{high}CD11b^{high}CD8a^{-}$) and “lymphoid” ($CD11c^{high}DEC\text{-}205^{+}CD8a^{+}$) DCs located in the marginal zone and the outer T cell zone, respectively and plasmacytoid DCs ($CD11c^{low}Ly6G/C^{+}B220^{+}$) located within the T cell zone and the red pulp [95, 96]. These DC subsets show functional specialization including differences in cytokine production, adhesion properties, phagocytic capacity, and the induction of proliferation and cytokine production in T cells, and TLR expression. In particular murine lymphoid DCs have been reported to produce large amounts of IL-12 and direct Th1 development, whereas myeloid DCs produce lower amounts of IL-12 and preferentially induce Th2 responses. In this regard, as discussed below, we demonstrated that intact Pn induces a mixed type 1 and type 2 cytokine and IgG isotype response. In contrast, plasmacytoid DC produce large amounts of type I IFN especially important for anti-viral defense. This paradigm however appears oversimplified, because 1) a particular DC subset can be instructed by different pathogens or PAMPs to induce a type 1 versus type 2 cytokine response, 2) $CD8\alpha^{-}$ DCs may change to $CD8\alpha^{+}$ DCs and, 3) the two subsets may cross-inhibit each others function.

Although DC subsets have been the subject of intense research interest, their relative physiologic roles in mediating humoral immunity to intact extracellular bacteria remain largely unresolved. Recently a peripheral blood $CD11c^{low}CD11b^{high}$DC has been shown to play a major role in the uptake of systemically injected intact Pn, and the subsequent stimulation of *T cell-independent* IgM production upon entry into the spleen [97]. Upon systemic injection of intact Pn, these blood DC internalize bacteria, transiently disappear from the blood, and localize in the

spleen where they undergo phenotypic maturation. Blood DCs deliver survival signals to MZB and costimulate IgM secretion, at least in part through release of soluble TACI ligands [97]. Of interest, the splenic CD11c^{high} DC ("lymphoid" and "myeloid") shows no significant uptake of bacteria. Supporting this observation, we recently demonstrated that depletion of CD11c^{high} DC *in vivo* using CD11c-DTR transgenic mice treated with diphtheria toxin (DT) had no effect, and even led to enhancements in the anti-protein and anti-PS IgG response to intact Pn [unpublished]. Although it is quite possible that the CD11c^{low} DCs (e.g. the "blood DC") were not eliminated by DT treatment of CD11c-DTR mice, the presence of these DC were not evaluated in this experiment. *In summary, although much remains to be learned regarding the role of endogenous DC in regulating CD4^{+} T cell-dependent IgG anti-protein and anti-PS responses to intact Pn, it is likely that the mechanism by which DC promote these 2 types of response will differ significantly.*

PRO- AND ANTI-INFLAMMATORY CYTOKINES MEDIATE THE ANTI-PROTEIN AND ANTI-PS RESPONSES TO INTACT PN

The protective role of pro-inflammatory cytokines, such as IL-1, TNF-α, IL-6, and the "type 1" cytokine IFN-γ, a switch factor for the murine IgG3 [98] and IgG2a [99] isotypes, in early innate defense against extracellular bacteria is well-established [100], but their role in mediating the adaptive humoral response to these pathogens has been far less clear. Pro-inflammatory cytokines could, in theory, stimulate humoral immunity, especially through initial activation of peripheral DCs at sites of initial pathogen encounter, with subsequent migration to secondary lymphoid organs and functional maturation for enhanced APC function for naïve

$CD4^+$ T cells [101, 102]. Anti-inflammatory cytokines, such as IL-10, and the "type 2" cytokine IL-4, a switch factor for the murine IgG1 [103] and IgE [104] isotypes, are also induced in response to extracellular bacteria [105]. IL-10 could potentially down-regulate humoral responses during infections with extracellular bacteria, in part through direct and indirect inhibitory effects on APC recruitment and function [106]. In this regard we demonstrated that endogenous TNF-α, released within 2-6 hours by both non-B/non-T cells, as well as lymphoid cells, following primary immunization with intact Pn, was required during the first 48-72 hours for subsequent, optimal induction of both anti-PspA and anti-PC responses [107]. Further, mice genetically deficient in IL-6, IFN-γ, or IL-12, showed significant reductions in IgG anti-PspA responses. In contrast, IL-$4^{-/-}$ and IL-$10^{-/-}$ mice immunized with intact Pn showed a significant *elevation* in the IgG anti-PspA response, except for *decreased* IgG1 in IL-$4^{-/-}$ mice. A marked enhancement in the induction of pro-inflammatory cytokines was observed in the absence of IL-10, relative to controls. IgG anti-PC responses were similarly regulated, but to a more modest degree [107]. *These data suggest that endogenous pro-inflammatory and anti-inflammatory cytokines reciprocally regulate in vivo protein- and PS-specific Ig responses to intact Pn, analogous to what is observed at the innate level*.

THE ROLE OF TLRs IN REGULATING ANTI-PROTEIN AND ANTI-PS Ig ISOTYPE RESPONSES TO INTACT PN

A key role for TLR2 in mediating innate immunity to Pn has been demonstrated [108, 109]. This is likely due to the expression by Pn of various TLR ligands such as peptidoglycan, lipoteichoic acid, and lipoproteins [110]. TLRs other than TLR2 may also play a role in

responses to Pn. Thus, although TLR2$^{-/-}$ mice are more susceptible to experimental Pn meningitis, a substantial part of the inflammatory response is TLR2-independent [109]. Additionally, TLR2$^{-/-}$ mice inoculated intranasally with live Pn displayed only a modestly reduced inflammatory response in the lungs, and normal host immunity relative to wild-type mice, despite defective cytokine production from freshly-isolated TLR2$^{-/-}$ alveolar macrophages [111]. Pneumolysin, expressed in the cytoplasm by intact Pn, and released upon autolysin-mediated lysis, was recently shown to be a TLR4 ligand capable of stimulating IL-6 and TNF-α from macrophages [112]. In this regard, TLR4$^{-/-}$ mice were shown to be more susceptible to lethality following i.n. colonization with pneumolysin-positive Pn. Although Pn likely expresses stimulatory, unmethylated CpG-containing DNA, a role for TLR9 in immune responses to Pn has yet to be determined.

Less is known regarding the role of TLRs in shaping the adaptive humoral response to an intact pathogen. Injection of mice with purified antigens in adjuvant, demonstrated a role for the TLR adaptor protein, MyD88, in mediating an antigen-specific type 1 (i.e. IgG3, IgG2b, and/or IgG2a), but not type 2 (i.e. IgG1), *in vivo* IgG isotype response [3]. More recently, a normal pathogen-specific IgG isotype response to *Borrelia burgdorferi* was observed in TLR2$^{-/-}$ mice, although this was associated with a higher burden of pathogen [113]. However, MyD88$^{-/-}$ mice infected with *B. burgdorferi* demonstrated elevated *B. burgdorferi*-specific IgM and IgG1 responses, but diminished type 1 Ab [114]. Similarly, MyD88$^{-/-}$ mice exhibited reduced Leishmania-specific IgG2a, and elevated IgG1, in response to infection with *Leishmania major* [115]. Finally, a recent report demonstrated a role for endogenous TLR2 in stimulating an Ig response to a *Haemophilus influenzae* type b-outer membrane protein complex (OMPC)

glycoconjugate vaccine that was specific for both the *H. influenzae* capsular polysaccharide and the OMPC protein [116].

We recently demonstrated that MyD88$^{-/-}$, but not TLR2$^{-/-}$, mice were markedly defective in their induction of multiple splenic pro-inflammatory cytokine- and chemokine-specific mRNAs *in vivo*, and cytokine protein secretion *in vitro* in response to intact Pn14 [117]. Consistent with these data, naïve MyD88$^{-/-}$, but not TLR2$^{-/-}$ mice were more sensitive to killing following i.p. challenge with live Pn14, relative to wild-type mice. However, prior immunization of MyD88$^{-/-}$ mice with heat-killed Pn14 protected them against an otherwise lethal challenge with live Pn14 [117]. *These data have added further support to the notion that TLR ligands in addition to TLR2 are important in mediating innate immunity to Pn.* Surprisingly, both MyD88$^{-/-}$ and TLR2$^{-/-}$ mice exhibited striking defects in elicitation of type 1 IgG isotypes (IgG3, IgG2b, and IgG2a), but not IgG1, specific for several proteins (pneumococcal surface adhesin A [PsaA] and pneumococcal surface protein C [PspC]) and PSs (PPS14 and PC), in response to i.p. challenge with heat-killed Pn14 [117]. Of note, type 1 IgG isotype titers specific for PspA were also reduced in MyD88$^{-/-}$, but not TLR2$^{-/-}$ mice. Further, MyD88$^{-/-}$ mice had elevated IgG1 titers, whereas IgG1 titers in TLR2$^{-/-}$ and wild-type mice were comparable, suggesting a role for a TLR ligand(s) in addition to TLR2 in mediating the humoral response to intact Pn. *Thus, distinct TLRs may differentially regulate innate versus adaptive humoral immunity to intact Pn. These data are of further interest in light of earlier contrasting reports suggesting that immunization of soluble protein antigens with exogenous, isolated TLR2 ligands actually favor type 2 responses* [118-120].

A CRITICAL ROLE FOR CONTAMINATING TLR LIGANDS IN MEDIATING IN VIVO ANTI-PS RESPONSES TO PURIFIED, SOLUBLE PNEUMOCOCCAL PSs

As discussed above, intact Pn likely induces anti-PS responses *in vivo* through the combined action of TLR-mediated signaling and $CD4^+$ T cell- and DC-mediated B cell help. However, the mechanism by which *purified* pneumococcal capsular PS antigens (PPSs) induce Ig responses *in vivo* are poorly understood, especially since these PPSs are not known to be TLR ligands nor do they depend on T cell help for Ig induction. Indeed, previous studies from our laboratory, using an *in vitro* polyclonal model for multivalent membrane (m)Ig crosslinking in response to PS antigens (i.e. multiple anti-IgD antibodies conjugated to dextran [αδ-dex]) indicated that multivalent mIg crosslinking alone, or in concert with CD40-mediated activation, induced vigorous proliferation but no Ig secretion or isotype switching by highly purified B cells [22, 121]. However, addition of various Toll-like receptor (TLR) ligands, such as bacterial lipoprotein (TLR2), Neisserial porins (TLR2), unmethylated CpG-containing oligodeoxynucleotides (TLR9), or LPS (TLR4) to αδ-dex-activated B cell cultures induced substantial Ig secretion and isotype switching.

In this regard, we recently demonstrated that IgG anti-PPS responses to PPS3, PPS14, and C-polysaccharide (C-PS) were virtually undetectable in $TLR2^{-/-}$ mice, while specific IgM induction was variably reduced, relative to wild-type mice [122]. All PPS-containing preparations induced IL-6 and TNF-α from wild-type, but not $TLR2^{-/-}$, macrophages. The TLR2 activity was distinct from the PPS, in that it was phenol-extractable. Immunization of wild-type mice with phenol-extracted PPS14 also resulted in a marked reduction in the IgG, although not

IgM-anti-PPS14 response, relative to untreated PPS14. The commercial 23-valent PPS vaccine, Pneumovax-23 also contained TLR ligands (TLR2 and TLR4), which were absolutely critical for the IgG-inducing activity of the vaccine in mice. Finally, the commercial pneumococcal conjugate vaccine, Prevnar contained a TLR2 ligand(s) that substantially enhanced both the primary and secondary anti-PPS responses in mice, especially the type 1 IgG isotypes [122]. *These data strongly suggest the absolute need for a distinct, TLR-dependent second signal for inducing in vivo IgG TI humoral immune responses to isolated pneumococcal polysaccharide antigens and highlight the potential importance of previously unappreciated co-purified and/or contaminating TLR ligands in PPS vaccine preparations.*

INDUCTION OF IgG ANTI-PS RESPONSES TO INTACT PN14 OR TO A SOLUBLE PNEUMOCOCCAL PS-PROTEIN CONJUGATE IS MORE HEAVILY DEPENDENT ON BTK-MEDIATED BCR SIGNALING THAN ANTI-PROTEIN RESPONSES

The Bruton's tyrosine kinase (Btk) plays a major role in B cell receptor (BCR)-mediated signal transduction [123]. Btk is critical for the normal development of B-1, and to a lesser extent B-2 B cells. Thus, CBA/N (xid) mice [124], which have a loss-of-function point mutation in the Btk gene [125, 126] exhibit a marked reduction in peritoneal B-1a cells, although a more modest decrease in B-1b cells, and a 30-50% reduction in splenic B-2 cells, including both marginal zone and follicular subsets (127-129). Xid [130, 131] and $Btk^{-/-}$ [132] mice also exhibit marked defects in Ig induction in response to soluble T cell-independent type 2 (TI-2) antigens (e.g. polysaccharides). Btk appears to function as a BCR signal threshold modulator rather than as an essential component of the BCR signaling pathway [133]. Thus, xid B cells can respond to

particulate TI-2 antigens, such as TNP-sephadex or TNP-polyacrylamide [134]. Additionally, defective TI-2 responses in xid mice can be corrected by co-immunization with a TLR agonist such as 8-mercaptoguanosine [135, 136]. Finally, TI-2 responses in xid mice can be partially reconstituted through provision of T cell help [137, 138].

Defective humoral immune responses in xid or $Btk^{-/-}$ mice could result from a combination of defective B cell subset development as well as loss of Btk-mediated BCR signaling in the B cells that are present. In this regard, xid mice receiving one allele of a murine Btk transgene, driven by the Ig heavy chain promoter and enhancer, and expressing 25% of wild-type endogenous levels of Btk (xid^{1xtg} mice), restore splenic B-2 cell development to wild-type levels, and have a more modest decrease in peritoneal B-1a cells relative to xid mice [139]. Nevertheless, these mice still have defective BCR signaling and lower Ig responses to the soluble TI-2 antigen TNP-Ficoll, relative to wild-type mice. Essentially similar observations were made in xid mice containing a transgene encoding the anti-apoptotic protein Bcl-2 [140]. Since B-1 cells do not participate in the TNP-Ficoll response [141], these data collectively indicate a direct role for BCR signaling in Ig responses to soluble TI-2 antigens. These latter studies did not evaluate Ig responses to soluble TD antigens which are also reduced, albeit less dramatically, in xid mice.

These studies indicated that Ig responses to isolated PS antigens in xid or $Btk^{-/-}$ mice can vary dramatically depending upon the presence or absence of adjuvant, T cell help, and/or antigen particulation, and the level of restoration of B cell subset development. *Thus, the relative role of Btk-dependent BCR signaling in directly regulating anti-PS versus anti-protein Ig*

responses to an intact bacterium, such as Pn, in vivo has remained an open and important question. In this regard, we recently demonstrated that xid^{1xtg} mice immunized with intact Pn14 elicited a substantially reduced IgM and IgG anti-PS, but normal IgG anti-protein response, relative to wild-type mice [66]. Immunization of xid^{1xtg} mice with a soluble pneumococcal PS-protein conjugate in saline resulted in an even more profound defect in the anti-PS, but not anti-protein response, that nevertheless could be largely restored by inclusion of a Toll-like receptor (TLR) adjuvant (i.e. CpG-ODN, a TLR9 ligand [142]). As mentioned earlier, both the IgG anti-PS and anti-protein responses to intact Pn or to soluble PS-protein conjugate are dependent on CD4^{+} T cells. *These data demonstrated a greater dependence on Btk-mediated BCR signaling for physiologic anti-PS relative to anti-protein responses, as well as the existence of a compensatory TLR-mediated signaling pathway.* Consistent with these observations, were earlier findings, summarized by Press [143] that a subclass of TD antigens (designated "TD-2") elicited defective Ig responses in xid mice, in contrast to more normal responses induced by TD-1 antigens. Of note, TD-2 antigens comprised either certain PS antigens or proteins expressing repeating antigenic epitopes (similar to pneumococcal C-PS and PPS14 in the above studies), whereas TD-1 antigens included more classical soluble proteins such as KLH and hemocyanin (similar to PspA).

Neonatal, like xid or xid^{1xtg}, B cells also exhibit defective signaling following BCR crosslinking [24,, 25]. In this regard, similar to what is observed in xid mice, TI-2 responses are also markedly defective in the immature host, whereas T cell-dependent anti-protein responses are less affected [24, 25]. Thus, infants respond poorly to isolated PS vaccines, but elicit protective anti-PS responses to PS conjugated to an immunogenic carrier protein, which effects

recruitment of $CD4^+$ T cell help. Like xid B cells, the defective BCR-dependent function of neonatal B cells can be compensated for by TLR-dependent signaling [144]. Surprisingly however, only small minorities of infants less than 2 years of age elicit a detectable systemic or mucosal IgG anti-capsular PS response following natural infection with Pn, whereas a majority induce IgG specific for several Pn proteins [145-148]. In light of our current data, we propose that this may be partly due to an insufficient level of TLR signaling, provided during natural Pn infections, in order to overcome the defective BCR signaling that differentially impacts on the anti-PS response in the immature host.

FUTURE STUDIES

A number of unresolved questions are raised by the studies discussed above: 1) In contrast to anti-protein responses, why are anti-PS Ig isotype responses to intact Pn of much shorter duration, and why do they fail to generate memory despite the active participation of $CD4^+$ T cells? 2) Why are the kinetics for delivery of $CD4^+$ T cell help for anti-protein and anti-PS responses different, and what are the attendant functional consequences, especially on the generation of memory? 3) What is the mechanistic basis for the specific and non-specific T cell help for the anti-PPS14 and anti-PC responses, respectively? 4) How do DCs differentially process and present protein versus PS antigens derived from intact Pn to elicit Ig responses upon transfer into naïve hosts? 5) Which DC subset(s) plays the major role in IgM and IgG anti-Pn humoral immunity *in vivo* following immunization with free, intact Pn? 6) Which TLRs, and perhaps other innate receptors, are required for induction of innate and adaptive humoral immunity to intact Pn, and are the functional roles of distinct TLRs additive or synergistic? 7)

What are the implications of contaminating TLRs in pneumococcal PS-containing vaccines on vaccine effectiveness in humans, and thus perhaps on the manner in which PPSs are purified for commercial use? 8) Would a suitable adjuvant costimulate protective Ig responses to isolated PS antigens in the immature host, thus providing a cost-effective, alternative way to vaccinate infants in the developing world.

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