

Role of toll like receptors in the antibody response to encapsulated bacteria

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1. ABSTRACT

Encapsulated bacteria are major pathogens in humans. The capsular polysaccharides (PS) of these bacteria are T-independent type 2 antigens, are not processed by antigen presenting cells and do not induce T cell help. PS antigens are poor immunogens in children less than two years, the peak age incidence of encapsulated bacterial infection. The TNF family receptors BAFFR and TACI interaction with the cytokines BAFF and APRIL are essential co-stimulatory factors for humoral responses to PS. Linkage of PS to a carrier protein to make glycoconjugate vaccines, enhances the immune response to PS similar to a T cell dependent antigen. Multiple doses of glycoconjugate vaccines are required to elicit protection, making their use in the developing world problematic. TLR engagement augments BAFF mediated PS antibody responses and TLR ligands serve as adjuvants for induction of anti-PS antibodies either for pure PS or for PS-protein conjugate vaccines. A variety of TLR ligands stimulate increased production of antibodies directed to both PS and protein components of encapsulated bacteria, and glycoconjugate vaccines, suggesting their future role in immunization strategies.

2. INTRODUCTION

Encapsulated bacteria continue to be serious human pathogens causing a variety of invasive infections. *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b cause sepsis, pneumonia and meningitis. Other pathogenic gram-positive and gram-negative organisms also contain polysaccharide (PS) capsules. These capsules interfere with host defense mechanisms such as complement deposition and opsonization of bacteria for uptake by phagocytes. Antibodies directed against the polysaccharide capsules of most of these organisms are protective against invasive infection. Thus, the major vaccine strategy over the last 50 years has been to utilize capsular PS of these pathogens as vaccines to elicit protective anti-PS antibodies.

T-dependent (Td) protein antigens are processed by B cells, and other antigen processing cells (APC), then antigen-derived peptides are presented to the T cell receptor (TCR) with MHC II. After presentation of the peptide-MHC II complex to the TCR, the B cell is activated by the T cell co-stimulatory molecule CD40L, which is upregulated when the TCR is stimulated releasing various cytokines. B cell interaction with T cells is critical for B

Table 1. Immune characteristics of bacterial polysaccharides

	Pure bacterial polysaccharide	Whole encapsulated bacteria	Polysaccharide-protein conjugate	Zwitterionic polysaccharide
T-independent	+	-	-	-
T-dependent	-	+	+	+

cell antibody responses to protein antigens. The outcomes of this physical interaction are clonal expansion, somatic hypermutation and affinity maturation of the antibody and class switching (1). Unfortunately, most bacterial capsular PS (with the exception of zwitterionic PS) are T independent type 2 antigens (TI), and are not processed and presented by antigen presenting cells, and thus do not engage T cells and do not elicit T cell help. PS antigens stimulate the B cell receptor (BCR) via cross linking in the absence of a second signal from T cells, yielding a relatively muted IgM-dominated, low affinity antibody response with little somatic hypermutation, and poor immunological memory (2,3,4). Thus vaccines derived from pure bacterial capsular PS may elicit protective antibodies in healthy adults, but the immunity is not long lasting and does not “boost” with a second immunization. Such vaccines are ineffective in small children whose “immature” B cells do not produce protective antibody titers to TI antigens such as bacterial PS (see below), and have reduced efficacy in the elderly (Table 1).

Recently, new data have shown that the humoral response to bacterial PS is more complex than simply cross linking the BCR followed by low affinity IgM production. In absence of the CD40-CD40L interaction between B cells and T cells that occurs after B cell activation with protein antigens, tumor necrosis family receptors (TNFR) and their ligands, as well as Toll like receptors (TLR), play crucial roles in the host response to PS and intact encapsulated bacteria, and in B cell differentiation and class switch recombination (5-7). These findings have laid the groundwork for new strategies to augment the antibody response to bacterial PS by using ligands that engage TLR.

3. TNFR AND THE ANTI-POLYSACCHARIDE ANTIBODY RESPONSE

3.1. TNFR BAFF and APRIL required for humoral response to polysaccharides

Despite the traditional concept that PS are TI type antigens and relatively “inert” to the mammalian immune system other than cross linking the BCR, the humoral response to PS involves several TNFR and their ligands. TACI (transmembrane activator and calcium modulator) receptor that engages with the cytokines BAFF (B cell activating factor) and APRIL (a proliferation inducing ligand) plays a key role in the production and regulation of antibodies to TI antigens. The ligands BAFF and APRIL bind to TACI, BAFFR and BCMA (B cell maturation antigen) on B cells and regulate B cell survival, activation and isotype switching particularly in the absence of CD40-CD40L signaling from T cells (5-7). BAFF is expressed by monocytes and dendritic cells and binds to BAFFR, BCMA, and TACI. APRIL binds to only TACI and BCMA. Deficiency in BAFF, or abnormality, blockade or absence of the receptor TACI decreases the number of B cells and disrupts humoral immunity to TI antigens including the PS of encapsulated bacteria (6, 9). TACI KO

mice, for example, respond with normal antibody production to T dependent antigens (Td) but are unable to make antibodies to TI antigens (4). Similarly, humans with common variable immune deficiency exhibit decreased humoral responses to carbohydrate antigens such as bacterial PS and have a mutation in TACI (5). The TNFR and their BAFF and APRIL ligands are thus a second set of important molecules after the BCR required for the antibody response to TI PS antigens. Finally, multiple doses of pure TI PS vaccines may induce hyporesponsiveness to PS antigens. The explanation for PS-induced hyporesponsiveness has been elusive. Recently, B cell hyporesponsiveness to PS was found in part due to suppressive effects of pure PS on the expression of TACI and BAFFR by PS-specific B cells (8).

3.2. Ontogeny of BAFF and APRIL receptors

Bacterial PS antigens are particularly poor immunogens in young children under the age of 24 months, the peak age incidence of invasive infections with the encapsulated bacterial pathogens. The explanation for the poor immunogenicity of polysaccharides in infants remains incompletely elucidated. Recent data show that under expression of TACI and BAFFR by B cells plays a key role in both humans and mice in the neonatal hyporesponsiveness to PS antigens (9,10). B cells obtained from cord blood of preterm human infants, for example, expressed significantly less TACI, BCMA and BAFFR compared to adult B cells, and exhibited a marked decrease in immunoglobulin secretion compared to both term baby and adult B cells. These B cells also could not proliferate as well as adult B cells when stimulated with human recombinant BAFF (10). Similarly, TACI expression was dramatically reduced on B cells from newborn mice compared to adult mice, and BAFF and APRIL were unable to induce immunoglobulin secretion in newborn B cells (9). In addition, although BAFF and APRIL were capable of stimulating plasma cell development in adult B cells, this did not occur with the neonatal B cells that expressed low quantities of TACI (9). Thus, the “immaturity” of infant B cells thought to cause hyporesponsiveness to bacterial PS is due at least in part to under expression of TNFR required for TI antibody responses. Bacterial PS antigens are also weak immunogens in the elderly, perhaps due to senescence of both B cell proliferative activity and T cell help. There are scant data on the expression and function of TNFR in the elderly.

4. TOLL LIKE RECEPTORS AND B CELL FUNCTION AND DIFFERENTIATION

4.1. TLR presence and effect on B cells

The TLR family of proteins is crucial to the innate immune response to pathogens. TLR are engaged by molecules present in microorganisms and mediate the activation of several nuclear transcription factors via

adaptor proteins such as MyD 88. The transcriptional activation of genes yields the production of a variety of pro inflammatory cytokines and up regulates receptors responsible for cell-cell immunological interactions. TLR are now also known to be instrumental in the regulation of antibody production but in a complex manner that depends on the maturational stage of the B-cell. TLR were initially thought to be absent or poorly expressed on B cells. However, we now know that human memory B cells and plasma cells express TLR. The ontogeny of TLR expression has been partially elucidated. Human hematopoietic stems cells express mRNA of TLR 1-9. Naïve B cells, however, do not express mRNA of TLR3, 4, 8, and have very low surface expression of the TLR, while plasma cells do express mRNA of TLR1-9 (11). Although naïve B cells have low or undetectable TLR expression, expression of TLR9, for example, is rapidly increased after the BCR is cross-linked (12). In order to obtain the most robust proliferation and differentiation of naïve B cells, BCR signaling, cognate T cell help and TLR stimulation all need to occur. These requirements prevent polyclonal activation of naïve B cells when TLR are engaged by intact bacteria, since only antigen specific B cells will be fully activated. Antigens such as PS that are not processed and presented to T cells, however, will also not elicit optimal activation of naïve B cells even in the presence of TLR engagement since cognate T cell help is not available. In contrast, human memory B cells constitutively express TLR2, (9,10,12) and TLR10 (11-13). Interestingly, human B cells do not express TLR4 rendering the cells unresponsive to LPS, although plasma cells have been found to express mRNA of TLR4 (11). Mouse B cells by contrast, have different patterns of TLR expression compared to human B cells, constitutively express TLR4, and LPS induces proliferation, class switch recombination and cytokine production (14). TLR stimulation of B cells may also be important during the initial exposure to PS antigens. Marginal zone (MZ) B cells and B1 B cells in the spleen generate a robust IgM response to TI PS antigens within hours after exposure. These cells give rise to plasmablasts and plasma cells with a more mature antibody response. Anti-IgM signals, however, are not sufficient to elicit optimal MZ and B1 B cell proliferation after initial PS antigenic exposure. TLR engagement also may be required particularly with intact bacteria (15,16). Neonatal MZ B cells in addition to under-expression of TACI, also have reduced expression of CD21 compared to adult MZ cells resulting in decreased responses to complement fragments and reduction in other “second” signals required for proliferation after BCR cross-linking (17).

4.2. TLR engagement augments BAFF and April mediated antibody secretion

Differentiation of naïve B cells involving class switch recombination and antigen-specific antibody production is now known to require integration of several receptors including the BCR, TNFR, and TLR. TLR engagement plays a crucial role to interact in a dynamic process with TNFR that is integral to the antibody response to bacterial PS, and is probably the molecular basis for TLR synergy with BCR activation (18,19). *In vitro* or *in vivo* exposure of newborn mouse B1 B-cells to the TLR9

engager CpG oligo-deoxynucleotide (CpG), for example, upregulates TACI expression on the neonatal B cells, renders them more responsive to BAFF and APRIL and increases their ability to produce IgA and IgG (9). Thus, TLR9 stimulation in mice corrects the under-expression of TACI on neonatal B cells, allowing increased immunoglobulin production. Similarly, CpG increases adult mouse B cell surface expression of BAFFR and TACI and augments the co-responsiveness to BAFF with increased proliferation and induction of IL-6 and IL-10 (18). CpG also can reverse the suppressive effects of pure PS on TACI and BAFFR expression, suggesting utility as an adjuvant for pure PS vaccines (8; see section 6.2 below). CpG and LPS increase the expression of TACI on follicular and marginal zone B cells, and increases their immunoglobulin secretion in response to BAFF and APRIL (20). MALP-2 a TLR2/6 agonist, also directly promotes activation and maturation of murine B cells and increases expression of molecules crucial to B cell T cell cross stimulation (21). Other TLR ligands such as Poly (I:C), a synthetic double stranded RNA that engages TLR3, enhances the TI antibody response by increasing the magnitude and speed of IgG antibody production by inducing type I interferon production (22). Finally, humans with common variable immune deficiency, some of whom have mutations in TACI, have B cells with markedly reduced activation in response to extracts of pneumococcus and *H. influenzae* type b and also show a defective response to TLR9 engagement. Thus, their significant hyporesponsiveness to TI antigens occurs due to either defective CpG upregulation of TACI and/or defective TACI production (2), showing the importance of TLR9 to the TI humoral immune response.

5. THE IMMUNE RESPONSE TO BACTERIAL POLYSACCHARIDE PROTEIN GLYCOCONJUGATE VACCINES

5.1. Glycoconjugate vaccine strategy

Covalent linkage of the PS to protein carrier molecules to make glycoconjugate vaccines improves the immunogenicity of PS in infants and children. Glycoconjugate vaccines perform more like Td antigens, and repeated immunizations induce a protective PS-specific memory response via induction of CD4⁺ T cell help and cytokine production (24). In addition, MHC class II-TCR, B7-CD28 and CD40-CD40L interactions between B cells and T cells are crucial to the enhanced PS specific antibody response (25). The mechanism of the enhanced PS-specific immunity of glycoconjugate vaccines includes antigen processing of the conjugate, presentation of carrier protein derived peptides to T cells, engagement of the TCR and cytokine production leading to clonal expansion of PS-specific B cells. However, the role of the PS attached to the protein carrier may be more complex than only being bound to the BCR of a PS-specific B cell. The PS in addition to the carrier protein, transits the APC and is expressed on the APC surface in association with MHC II (26). The efficiency of antigen processing of the conjugate vaccine may affect immunogenicity and the quality of T cell help, and improving antigen processing may be an adjuvant target (26). The precise mechanism by which the

PS is rendered more immunogenic by linkage to a protein, however, remains poorly defined. Glycoconjugate vaccines that use the capsules of *Haemophilus influenzae* type b (Hib), multiple serotypes of pneumococcus and of meningococcus covalently linked to carrier proteins, have proved remarkably safe and efficacious in the prevention of invasive disease from these pathogens (27-29). These PS-protein conjugate vaccines elicit high affinity anti-PS antibodies that are the result of clonal expansion of PS-specific B cells and somatic hypermutation, which does not occur with immunization of pure PS (4).

5.2. Limitations of glycoconjugate vaccines

Unfortunately, multiple doses of bacterial conjugate vaccines are required to induce protection, rendering the vaccines problematic in the developing world. The Hib conjugate vaccine was relatively simple since there was only one pathogenic serotype of the encapsulated *Haemophilus influenzae* type b, and one conjugate vaccine to be made to protect against infection. Since there are dozens of pathogenic serotypes of pneumococcus, current pneumococcal glycoconjugate vaccines rely on the separate purification of each serotype specific capsule, which is then separately conjugated to carrier protein. The first generation of the pneumococcal conjugate vaccine contained seven serotypes, each capsule separately conjugated to CRM₁₉₇. These serotypes were chosen due to their frequent prevalence in the United States (30). Although highly successful in the United States in reducing invasive pneumococcal infections in infants and children, infections with replacement serotypes not present in the vaccine became an increasing problem (31). In addition, dominant serotypes that caused invasive disease in the United States did not always match those serotypes prevalent in other parts of the world. Several vaccine companies increased the number of serotypes contained in the vaccine. The current formulation in the United States contains 13 different serotypes, each capsule separately purified and chemically linked to CRM197 carrier protein. Thus pneumococcal glycoconjugate vaccines are particularly expensive to manufacture. Finally, the elderly respond poorly to pure PS vaccines such as pneumovax, and the use of better vaccines to prevent pneumococcal infections would be of high clinical utility and may hold advantages over pure PS vaccines (32-34). The explanation for the mediocre response of the elderly to PS remains unclear. Antibody repertoire appears to remain intact, yet antibody titers to immunization with PS are lower than that achieved in healthy young adults (32). There are remarkably few data on B cell expression of TNFR or TLR in the elderly, although aged mice express significantly less TLR on splenocytes than young mice (35). Conjugate vaccines may offer a significant advantage to plain PS vaccines in the elderly but results obtained from immunization of elderly populations have been inconsistent (36, 37). Adjuvants that enhance immunogenicity with fewer doses in infants and the elderly would be of strong utility for the design of second-generation glycoconjugate and may include TLR agonists.

6. TLR STIMULATION ENHANCES THE ANTIBODY RESPONSE TO ENCAPSULATED BACTERIA

6.1. Role of TLR to immunity to intact encapsulated bacteria

TLR appear to be crucial to the host's initial defense against encapsulated bacteria, and to the subsequent immune response after challenge. The presence of MyD 88 but not TLR2 is required for normal cytokine responses after challenge with live *Streptococcus pneumoniae* (28). Both MyD 88 and TLR2 KO mice, however, have defective antibody responses to intact pneumococcus. MyD88 and TLR2 KO mice exhibited defects in production of IgG3, 2b, 2a but not IgG1 to immunization with intact organisms (38). The defect in antibody production also encompassed protein antigens present on the whole organism such as PspA in the TLR 2 KO mice. Although a second immunization with heat-killed organisms boosted the antibody responses to PC and PspA in TLR2 KO mice, no further increase in titer to anti-capsular PS occurred. Thus, TLR2 and its co-receptors TLR1 and 6 and MyD 88, perhaps mediating other TLR signals, are critical to the normal humoral response to intact pneumococcus.

A more detailed study found that the murine response to pneumococcal PC antigen which is TI but is optimized when CD4⁺ T cells are available, showed that absence of TLR2 greatly affected IgG3 (Td) but not IgM (TI) production. Thus, expression of TLR2 on both B-cells and CD4⁺ T cells is important for the antibody response to various components of intact pneumococcus (39).

6.2. TLR stimulation enhances the antibody response to pure bacterial PS

Since bacterial PS are known to be poor immunogens particularly in infants, utilization of TLR agonists as adjuvants to enhance the antibody responses poses a potential solution to the problem. Given the strong immunomodulatory effects of many TLR ligands, and their ability to influence B cell activation and BAFF and APRIL-mediated antibody secretion, TLR are logical targets for use of adjuvants with PS vaccines. Unmethylated CpG oligodinucleotides (CpG ODN), despite being directly mitogenic for B cells, were found to suppress the mouse anti-PS antibody response to the purified O-specific side chain of *Pseudomonas aeruginosa* when administered simultaneously with the PS (40). In contrast, CpG ODN when used 48 hours after PS immunization enhanced the anti-pneumococcal PS antibody response and increased the numbers of PS-specific antibody secreting cells (ASC). Enhancement of the humoral response to the PS was also seen when CpG ODN were administered 2 days after PS immunization of infant mice normally unable to mount a protective antibody response to pneumococcal PS (41). Protection against challenge with a lethal dose of pneumococcus was also significantly improved by using CpG ODN 2 days after PS immunization. Similar results were seen when different TLR agonists targeting TLR2/6, TLR3, TLR4 and TLR5 were administered 2 days after pneumococcal immunization. Interestingly, co-administration of any of the TLR agonists with PS vaccine

did not yield enhanced anti-PS antibody titers or protection as reported with pseudomonas O-specific side chain. The reason for the failure of co-administration of CpG and other TLR agonists and PS to yield enhanced PS-specific antibody production in these experimental animal models remains unclear. By contrast, contamination of pure pneumococcal PS vaccines with non-LPS TLR ligands may be important for immunogenicity. Commercially available 23-valent pneumococcal PS vaccine contained TLR2 activity that was crucial to immunogenicity (16). Phenol extraction removed the enhanced immunogenicity as did use in TLR2 KO mice. Taken as a whole, these data further support the concept that TLR co-stimulation is an important component of the immune response to TI antigens such as bacterial PS, and that utilization of TLR agonists may improve immunogenicity of PS. Clearly more work is required to determine timing of administration, TLR specificity and dose of TLR agonists as adjuvants to unconjugated PS vaccines.

6.3. TLR agonists are effective adjuvants for encapsulated bacterial glycoconjugate vaccines

The first commercially successful glycoconjugate vaccine utilized the purified capsular PS of Hib linked to CRM₁₉₇, a nontoxic mutant protein of diphtheria toxin (42). However, different pharmaceutical companies subsequently used a variety of different protein carriers in order to make Hib glycoconjugate vaccines. Unlike most glycoconjugate vaccines, the Hib vaccine in which the PS was covalently linked to the outer membrane protein complex of *Neisseria meningitidis* (OMPC) elicited protective anti-PS antibody titers after one dose in most human infants (43). Previous data showed that OMPC was mitogenic for B cells and contained porin proteins that were TLR2 agonists (44). Although the enhanced PS-specific immunogenicity of Hib-OMPC was initially assumed to be a characteristic of antigen processing and induced T cell help by the carrier proteins OMPC, subsequent studies showed that both unconjugated and conjugated OMPC were robust human and mouse TLR2 agonists (45). Hib-OMPC engaged TLR2 in a MyD88 dependent fashion and enhanced TNF release by dendritic cells. The Hib-OMPC was significantly less immunogenic in TLR2 KO mice showing that the enhanced immunogenicity was dependent on TLR2 engagement (45). No other Hib conjugate vaccines tested engaged any TLR, also suggesting that the unique immunogenicity in humans after one dose was a result of the TLR2 engagement. We do not yet know whether the use of a TLR2 engaging carrier protein such as OMPC provides longer-lasting immunity to Hib compared to other Hib conjugate vaccines. To date, the Hib-OMPC vaccine appears to elicit long-lived immunity to Hib, similar to that obtained with other Hib conjugate vaccines.

Since OMPC and TLR2 engagement enhanced the kinetics of the induction of protective anti-PS antibodies when conjugated to Hib PS, it seemed likely that OMPC and TLR2 engagement could enhance the anti-PS antibody response to other conjugated bacterial PS. The addition of OMPC to the seven-valent pneumococcal-CRM₁₉₇ glycoconjugate vaccine significantly enhanced anti-PS antibody titers in mice. In addition, OMPC- treated

mice made significantly more IgG3 anti-pneumococcal PS antibodies, the isotype associated with strong anti-encapsulated bacterial opsonic function, and the dominant isotype made in mice (analogous to IgG2 in humans) to TI antigens such as PS (46-49). Finally, the enhancement of anti-pneumococcal PS antibodies was associated with increases in splenocyte production of IL2, 4, 10 and TNF alpha (46). Since both Hib-OMPC and the pneumococcal-CRM₁₉₇ conjugate vaccine are licensed for use in human infants in the US, and since both Hib glycoconjugates and the pneumococcal glycoconjugate are given at the same time to infants, it may be possible to determine if the infants receiving Hib-OMPC have increased titers of antibody to pneumococcal capsular PS. In addition, since Hib-OMPC has been administered to many thousands of infants without significant problems, it seems clear that a TLR2 agonist is a safe and effective adjuvant for this bacterial polysaccharide in humans.

Engagement of other TLRs besides TLR2, also show promise as adjuvants for bacterial PS glycoconjugate vaccines. CpG oligodeoxynucleotides (TLR9) significantly increased the anti-pneumococcal PS antibody response to types 6B and 19F pneumococcal-CRM₁₉₇ glycoconjugate vaccines. Total anti-PS antibody titers were enhanced, as well as production of IgG2a and especially IgG3 (50).

Interestingly, the incidence of pneumococcal infection increases in the elderly, and the elderly have mediocre antibody responses to pure PS bacterial vaccines as noted previously (Section 5.2; 33,37). A variety of defects in aged mice parallel that seen in humans, and include decreased quantity and avidity of anti-PS antibodies, and decreased somatic hypermutation and plasma cell production. Indeed, conjugate vaccines, much as in elderly humans, show little advantage in aged mice compared to pure PS vaccines. The use of CpG in the aged mouse was found to significantly improve the production of anti-pneumococcal PS antibody responses to a type 14 pneumococcal-PspA glycoconjugate vaccine, and the IgG anti-type 14 PS antibody response was restored to young adult levels (51).

Taken together, the above findings suggest that future studies in humans will be important to conclusively determine the efficacy of TLR agonists as adjuvants for glycoconjugate vaccines and to reduce the number of doses required for protection against invasive infection by encapsulated bacteria in infants. Use of TLR agonists with glycoconjugate vaccines may also have benefits in the elderly human population, rendering improved immunity to pneumococcus after immunization.

6.4. TLR agonists improve humoral immunity to non-capsular protein antigens of encapsulated bacteria

Due to the challenges with immunogenicity of pure PS vaccines as well as the cost and frequency of administration of glycoconjugates described above, a variety of investigators have utilized non-capsular protein components of encapsulated bacteria as potential vaccines. *Neisseria meningitidis* serogroup B, for example, contains a

capsular PS that is poorly immunogenic even when conjugated to a protein, and which may cross react with human nervous tissue. Since meningococcal LPS is highly toxic, LPS-deficient, non-toxic outer membrane proteins have been utilized as experimental vaccines. Unfortunately, these antigens have mediocre immunogenicity and weak generation of bactericidal antibodies when the LPS is removed. Stimulation of TLR 3, 7 and 9 with TLR agonists was found to enhance the production of antigen specific IgG and significantly increase bactericidal titers compared to experimental vaccines that used outer membrane proteins alone (52). Several of the TLR agonists were much less toxic than LPS appeared to be safe potential adjuvants for this vaccine.

A more complex story emerges in terms of TLR 2 stimulation and the human antibody response to the pneumococcal protein antigen PspA. Bacterial lipoproteins known to engage TLR 2 (BLPs) augmented the production of anti-pneumococcal antibodies in naïve B cells via CD4⁺ T cell activation and augmented B cell proliferation. In contrast, TLR stimulation down regulated memory T cells and the subsequent antibody response in cells from children already colonized with pneumococcus (53). These data suggested that BLPs promoted naïve B cell responses in children, but reduced antibody responses where B cell memory responses existed. Since the antibody responses to these pneumococcal proteins are dependent on CD4⁺ T cells, BLPs also may downregulate the proliferation of memory CD4⁺ T cells resulting in a blunted memory antibody response due to decreased stimulation of T cells. Clearly further work is required to investigate these findings.

7. CONCLUSIONS

Encapsulated bacteria continue to be major pathogens in humans, particularly in infants and the elderly. Antibodies to the capsular PS are usually protective, but the TI nature of these PS, yield poor immunogenicity in infants and the elderly, the two human populations at highest risk of invasive infection with these organisms. Conjugation of the bacterial PS to protein carriers yields glycoconjugate vaccines with improved ability to generate protective levels of anti-PS antibodies in infants. These vaccines, however, require multiple doses and are expensive to manufacture, and may not have an advantage in the elderly, another target population for immunization. One mechanism to improve glycoconjugate vaccine protective efficacy is to utilize carrier proteins that also are antigens from pathogens. Pneumococcal capsular PS, for example, have been coupled with surface proteins as well as heat shock proteins as an alternative mechanism to induce T cell help and anti-pathogen antibodies against non-PS epitopes (54, 55). Others have linked pneumococcal PS to proteins from different pathogens such as protein D from non-typeable *Haemophilus influenzae* in an effort to prevent infections from related pathogens that colonize and infect the upper respiratory tract and cause otitis media (56). Since conjugation to the carrier protein is usually via random linkage, site directed conjugation also holds promise as a way to increase the density of peptides after the conjugate

is processed by an antigen processing cell in order to derive more robust T cell help.

Another mechanism to improve the immunogenicity and to reduce the number of doses of vaccine required to achieve protection will involve effective adjuvants. New information shows strong linkage between TNFR stimulation, TLR engagement, T cell help and activation and expansion of PS-antibody producing B cells. Thus, the use of TLR agonists, particularly TLR2 and 9 as adjuvants to increase anti-PS antibody production is logical based on these data showing direct regulation and or co-stimulation of the humoral response to PS via these TLR. Utilization of TLR agonists in experimental animal models shows promising efficacy in increasing both plain PS and glycoconjugate-induced antibody titers to bacterial PS. Finally, one glycoconjugate vaccine with enhanced immunogenicity already used in human infants, the Hib-OMPC vaccine, contains a carrier protein that is a robust TLR2 agonist, suggesting that further use of TLR agonists to enhance antibody titers to bacterial PS in humans may be highly effective and safe. Indeed, recent animal data suggest that using this TLR2 agonist with existing pneumococcal conjugate vaccines significantly increases the anti-PS antibody titer (46).

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