

Distinct types of T-cell help for the induction of a humoral immune response to *Streptococcus pneumoniae*

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Studies have indicated that purified soluble polysaccharide antigens can elicit T cell-independent Ig responses *in vivo*, although these responses can be modulated by T cells in a noncognate manner. Relatively little is known, however, concerning the parameters that regulate polysaccharide-specific, as well as protein-specific, Ig isotype responses to an intact extracellular bacterium. Using the murine *in vivo* humoral response to intact *Streptococcus pneumoniae* as a model it can be shown that CD4⁺ T-cell receptor $\alpha\beta$ ⁺ T cells deliver help for both polysaccharide- and protein-specific Ig responses. However, these responses differ fundamentally in their mechanism of action.

Relatively little is known concerning the parameters that mediate polysaccharide- and protein-specific Ig isotype responses to extracellular bacteria. In particular, much of our current understanding of Ig responses to polysaccharides has come from studies utilizing purified soluble polysaccharides, with or without haptentation^{1,2}. The inability of soluble polysaccharides to bind to MHC molecules³, in contrast to protein antigens, results in their lack of recruitment of cognate T-cell help, although noncognate forms of T-cell help can still modulate these responses. However, these studies might not be wholly applicable to our understanding of the mechanisms that regulate Ig responses to the polysaccharides and proteins expressed by an antigenically complex, intact bacterium.

The structural features of *Streptococcus pneumoniae* that are relevant to the studies described in this Opinion are illustrated in Fig. 1. The majority of our studies were conducted using a nonencapsulated mutant of *S. pneumoniae* capsular type 2 (strain R36A)^{12,13}, which was heat-killed before use. The induction of expression of serum Ig isotypes specific for the cell-wall protein pneumococcal surface protein A (PspA) and the phosphorylcholine (PC) determinant of

the cell-wall teichoic acid, was determined by ELISA (Ref. 14). Selected studies also determined Ig isotype responses specific for pneumococcal surface adhesin (PsaA) and pneumolysin and, through the use of *S. pneumoniae* capsular type 14, Ig responses to the Pn14 capsular polysaccharide.

Kinetics and memory

The anti-PC response to *S. pneumoniae* R36A shows more rapid kinetics than the anti-PspA response^{14,15}. Specifically, serum anti-PC titers are first detected on day four and peak at day six, whereas serum anti-PspA titers are first observed on day six and peak at day ten. PspA- but not PC-specific memory is generated¹⁴. Thus, boosting with R36A results in an increase in anti-PspA titers by 10–40 fold over that observed for the primary response, whereas little further increase in anti-PC titers is observed. In this regard, the anti-PC and anti-PspA responses are similar to classical antipolysaccharide and antiprotein Ig responses, respectively, to purified soluble antigens^{1,2}. Subsequent studies were designed to determine if different mechanisms regulated immune responses to the polysaccharide and protein determinants of the bacterium.

T-cell subsets

Earlier studies using mice that were immunodeficient as a result of a defective gene encoding Bruton's tyrosine kinase (Xid mice) demonstrated that Ig responses to polysaccharide antigens, including the PC determinant, were absent but responses to protein antigens were intact, suggesting differential regulation of these two responses^{16,17}. Both polysaccharide and protein responses were poor in mice with the *nu/nu* genotype (athymic mice with markedly reduced T-cell numbers), suggesting a role for T cells in these two responses. No detailed studies, however, have been published that describe the parameters that regulate polysaccharide- versus protein-specific Ig isotype responses to an intact extracellular bacterium. We found that both the IgG anti-PC and IgG anti-PspA, as well as the anti-PsaA and antipneumolysin, responses to R36A are dependent on TCR- $\alpha\beta$ ⁺, but not TCR- $\gamma\delta$ ⁺, T cells, as demonstrated using mice genetically deficient in either the TCR β chain or δ chain¹⁴ (Z-Q. Wu, unpublished). A role for TCR- $\gamma\delta$ ⁺ T cells might have been anticipated for the anti-PC response given the role of these cells in responses to nonprotein bacterial antigens¹⁸. Whereas the anti-PspA, anti-PsaA and antipneumolysin IgG responses were completely abrogated in TCR- β ^{-/-} mice, the anti-PC IgG response was typically reduced between five and seven times, but with little or no reduction in serum titers of anti-PC IgM. Both CD4⁺ and CD8⁺ T cells appear to be required for optimal T-cell help for the induction of PC-specific Ig, whereas CD4⁺ T cells, but not CD8⁺ T cells, are required for the antibody response to PspA (Ref. 14).

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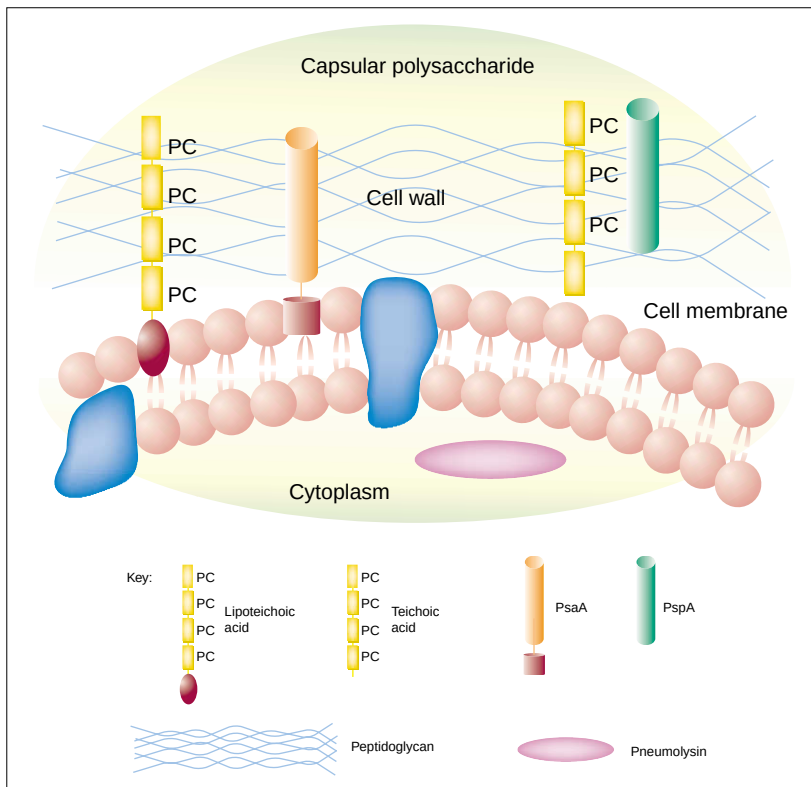


Fig. 1 The structural features of *Streptococcus pneumoniae*. The bacterium consists of a plasma membrane, cell wall and, typically, a polysaccharide capsule^{4,5}. The cell wall is composed of a peptidoglycan backbone, which anchors the capsular polysaccharide and also serves to bind the cell-wall C-polysaccharide (teichoic acid). Phosphorylcholine (PC) residues are covalently linked to the teichoic acid and bind several pneumococcal proteins through their PC-binding domains. The cell membrane also anchors a number of molecules including lipoteichoic acid (LTA). LTA contains a hydrophobic glycolipid anchor that mediates binding to the plasma membrane and, like teichoic acid, contains PC residues. The teichoic acid of LTA is chemically identical to the nonlipidated teichoic acid. In addition, pneumococcal surface adhesin A (PsaA), being a lipoprotein, is also anchored to the plasma membrane. Several pneumococcal molecules, including pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), pneumolysin, PC and capsular polysaccharide, could serve as useful targets for protective vaccines against *S. pneumoniae*^{6–11}.

Non-cognate T-cell help for the anti-PC response

Although both the anti-PC and anti-PspA antibody responses are dependent on CD4⁺ TCR- $\alpha\beta$ ⁺ T cells, the fact that PC is covalently linked to teichoic acid, a nonprotein cell wall constituent, raises an important question concerning the mechanism by which this T-cell help is delivered. Our initial hypothesis was that the bacterium might behave functionally like a protein–polysaccharide conjugate¹⁹. Specifically, proteins expressed by intact R36A or fragments of degraded R36A-containing teichoic acid might be internalized by PC-specific B cells and then presented as peptide–MHC class II complexes to specific T cells. To address this issue, we used a mouse model in which the H-Y (male antigen)-TCR transgenic insert was introduced into the TCR- $\alpha^{-/-}$ mouse²⁰ (mice were obtained from Osami Kanagawa, Washington University School of Medicine, St Louis, MO, USA). These mice express no endogenous TCR, but possess relatively normal numbers of CD4⁺ and CD8⁺ T cells and have normal numbers of B cells. When H-Y- $\alpha^{-/-}$ mice are immunized with R36A, no detectable anti-PspA response is observed (Z-Q. Wu *et al.*,

unpublished), consistent with the absence of MHC-restricted antigen-specific T-cell help predicted for this mouse model. The absence of an antibody response to PspA is associated with a complete absence of germinal center formation. By contrast, the anti-PC response to R36A in H-Y- $\alpha^{-/-}$ mice was similar to controls. When H-Y- $\alpha^{-/-}$ mice are acutely depleted of T cells before R36A immunization, a significant reduction in R36A-induced serum anti-PC titers is observed, comparable to that seen in acutely T-cell-depleted wild-type mice. Thus, T cells appear to augment the anti-PC response in a TCR-nonspecific manner and in the absence of germinal center formation, consistent with the lack of PC-specific memory.

‘...little is known regarding the role of DCs in inducing *in vivo* Ig isotype responses to intact extracellular bacteria....’

In collaboration with Harold Chapman (University of California at San Francisco, San Francisco, CA, USA) we also tested the ability of cathepsin S^{-/-} mice to induce anti-PC and anti-PspA responses to R36A. Antigen-presenting cells that lack the protease cathepsin S are unable to process the MHC class II-associated invariant chain beyond a 10 kDa fragment, resulting in a delay in peptide loading²¹. These mice were previously shown to contain normal numbers of B and T cells, but are significantly defective in specific IgG responses to protein antigen in adjuvant. We observed that cathepsin S^{-/-} mice have reductions in anti-PspA antibody titers of >4 times in response to R36A (Z-Q. Wu *et al.*, unpublished). By contrast, cathepsin S^{-/-} mice have a normal anti-PC response. These data suggest that, unlike the anti-PspA response, the anti-PC response might not require the generation of new MHC class II–peptide complexes, consistent with the role of TCR-nonspecific T cells in augmenting this response.

Dendritic-cell presentation of *S. pneumoniae in vivo*

The dendritic cell (DC) is considered to be the dominant antigen-presenting cell (APC) for the induction of primary T cell-dependent immune responses in naive mice^{22–24}. However, little is known regarding the role of DCs in inducing *in vivo* Ig isotype responses to intact extracellular bacteria and, in particular, the mechanism by which soluble or microbial cell-associated polysaccharide antigens are presented to the immune system. We generated a relatively pure population of immature CD11c⁺CD8⁻ (myeloid) DCs by culturing DC-enriched bone marrow (BM) cells in granulocyte–macrophage colony-stimulating factor (GM-CSF). DCs were incubated *in vitro* with type 14 *S. pneumoniae*, washed thoroughly to remove free bacteria and injected

intravenously into naive mice. *S. pneumoniae*-pulsed DCs elicit a primary anti-PspA response as well as PspA-specific memory (J. Colino *et al.*, unpublished). In addition, pulsed DCs elicit both an anti-PC and anti-Pn14 response. All three responses require viable DCs. Importantly, both the primary anti-PspA and anti-PC responses elicited by pulsed DCs are T cell-dependent.

'...the anti-PC response involves noncognate activation of TCR-nonspecific T cells.'

DCs from MHC class II^{-/-} mice²⁵ that were pulsed with *S. pneumoniae* and transferred into naive mice, are markedly defective at eliciting a primary anti-PspA response, as well as PspA-specific memory (J. Colino *et al.*, unpublished). By contrast, DCs lacking MHC class II elicit an anti-PC response comparable to that observed using wild-type DCs. In a complementary study, pulsed DCs transferred into an allogeneic recipient fail to elicit an anti-PspA response but induce an anti-PC response comparable to that observed when the same DCs are transferred into a syngeneic host. These data are thus consistent with the notion that the anti-PspA response requires cognate MHC class II-restricted interactions between DCs and T cells, whereas the anti-PC response involves noncognate activation of TCR-nonspecific T cells.

Costimulation requirements for the induction of anti-PC and anti-PspA responses

In light of the distinct forms of T-cell help that mediate the anti-PC and anti-PspA responses, we reasoned that costimulatory interactions between these T cells and APCs could also differ. In this regard, injection of a blocking anti-CD40 ligand (CD40L) antibody (MR1) completely abrogates the anti-PspA response to R36A, but has little, or only a modest, effect on the anti-PC response (P. Zelazowski *et al.*, unpublished). Similar effects of MR1 were also observed by Hwang *et al.*²⁶ in studies using *S. pneumoniae* type 6B. Crosslinking of CD40 on DCs by CD40L on activated T cells induces DC activation and maturation²⁷⁻²⁹. Whether CD40 is required for DC function *in vivo* will depend upon the nature of the immunogen. *S. pneumoniae*-pulsed CD40^{-/-} DCs are markedly defective in eliciting both a primary anti-PspA response and PspA-specific memory, when transferred into naive mice (J. Colino *et al.*, unpublished). By contrast, CD40^{-/-} DCs elicit an anti-PC response that is comparable to that observed for wild-type DCs. Thus, our CD40L blockade studies and the use of CD40^{-/-} DCs further support the notion of different mechanisms of T-cell help for the anti-PspA versus the anti-PC antibody responses.

The induction of T-cell effector functions generally requires two signals; one signal delivered through the

TCR by MHC-peptide complexes on the APC and the other through a costimulatory molecule, generally CD28, which binds to B7-1 and/or B7-2 on the APC (Refs 30,31). To determine a role for B7-dependent costimulation in the anti-PC and anti-PspA responses to R36A, we utilized cytotoxic T lymphocyte antigen 4 Ig (CTLA4Ig), a soluble fusion protein that binds to both B7-1 and B7-2 on the APC and prevents the interaction of these molecules with CD28 and CTLA4 on the T cell³². CTLA4Ig completely abrogates the primary anti-PspA response and the generation of PspA-specific memory, and reduces the anti-PC response by >5 times^{14,15}. This is associated with a complete inhibition of germinal center formation. A similar reduction in the anti-PC response is observed when CTLA4Ig is administered to H-Y- α ^{-/-} mice immunized with R36A. The primary anti-PC and anti-PspA responses are also reduced in mice treated with anti-B7-2, but not anti-B7-1, monoclonal antibodies (mAb), and in mice genetically deficient in CD28 (Ref. 15). Thus, despite the noncognate nature of the T-cell help for the anti-PC response to *S. pneumoniae*, an interaction between B7-2 and CD28 is still required, similar to that observed for the anti-PspA response.

Studies in which the timing of injection of CTLA4Ig (Ref. 15) or anti-CD4 and anti-CD8 mAbs was varied, indicate that the kinetics of delivery of these forms of T-cell help are different. Thus, the requirement for costimulation for an optimal anti-PC response is complete within one to two days after R36A immunization, whereas the costimulation requirement for anti-PspA continues until days five to six. Similarly, T-cell help for an optimal anti-PC response is required only during the first three to four days, whereas seven to eight days are required for the T cell-dependent induction of optimal PspA-specific Ig. Thus, the T-cell help for an optimal anti-PC response is delivered more rapidly than that required for an optimal anti-PspA response.

Concluding remarks

The mechanism(s) underlying the noncognate T-cell help for the anti-PC response are currently a matter of conjecture. However, the requirement for B7-2-dependent costimulation to mediate this help suggests that T cells expressing CD28 physically interact with B cells, macrophages and/or DCs expressing B7-2. This physical interaction is likely to occur optimally in the parafollicular T-cell zones of secondary lymphoid organs, where these cells are known to localize early in an immune response. Optimal B7-dependent costimulation typically requires upregulation of B7 on the APC. Bacterial DNA, peptidoglycan and lipoteichoic acid, which are all expressed by *S. pneumoniae*, can bind distinct Toll-like receptors (TLRs) on APCs (Ref. 33). TLRs mediate a signaling cascade that results in nuclear factor (NF)- κ B translocation to the nucleus, resulting in part in upregulation of B7. In addition, TLR

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activation, through APC contact with pathogen, will induce the release of multiple cytokines that can further costimulate T cells. Specific TCR recognition of foreign peptides does not appear to play a role in the anti-PC response. Nevertheless, T cells continually receive low-level TCR-mediated signals through recognition of the self-peptide-MHC complexes on which the T cells have been selected in the thymus^{34,35}. This sub-threshold signaling, in concert with B7-dependent and cytokine-mediated costimulation, might trigger sufficient T-cell effector function for PC-specific B-cell costimulation.

T cells, once activated, could either directly or indirectly costimulate PC-specific B cells, which ostensibly also receive potent signals through their B-cell receptors. Specifically, the PC moiety is expressed in a repetitive manner on the teichoic acid present within the bacterial cell wall, and thus might mediate multivalent membrane Ig (mIg) cross-linking on the

PC-specific B cell. We previously reported that such potent mIg signaling induces B-cell responsiveness to multiple cytokines, as well as microbial adjuvants, for the induction of B-cell maturation and Ig class-switching³⁶. Thus, the release of cytokines by activated T cells either in contact with, or in close proximity to, mIg-activated PC-specific B cells could augment the anti-PC response. Additionally, the induction of tumor necrosis factor (TNF) and/or TNF receptor family members on activated T cells, in contact with PC-specific B cells, could further costimulate B-cell activation³⁷. For example, CD27, CD30 and OX40 ligand, expressed on B cells, have all been shown to mediate positive signals for B-cell activation. Activated T cells could further stimulate APCs, and possibly other cytokine-producing cells, such as mast cells, neutrophils and endothelial cells, to release cytokines that might further amplify the B-cell Ig response to bacterial challenge.

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