Intact Bacteria Inhibit the Induction of Humoral Immune Responses to Bacterial-Derived and Heterologous Soluble T Cell-Dependent Antigens[1]

Gouri Chattopadhyay,* Quanyi Chen,* Jesus Colino,* Andrew Lees,† and Clifford M. Snapper[2]*

During infections with extracellular bacteria, such as *Streptococcus pneumoniae* (Pn), the immune system likely encounters bacterial components in soluble form, as well as those associated with the intact bacterium. The potential cross-regulatory effects on humoral immunity in response to these two forms of Ag are unknown. We thus investigated the immunologic consequences of coimmunization with intact Pn and soluble conjugates of Pn-derived proteins and polysaccharides (PS) as a model. Coimmunization of mice with Pn and conjugate resulted in marked inhibition of conjugate-induced PS-specific memory, as well as primary and memory anti-protein Ig responses. Inhibition occurred with unencapsulated Pn, encapsulated Pn expressing different capsular types of PS than that present in the conjugate, and with conjugate containing protein not expressed by Pn, but not with 1-μm latex beads in adjuvant. Inhibition was long-lasting and occurred only during the early phase of the immune response, but it was not associated with tolerance. Pn inhibited the trafficking of conjugate from the splenic marginal zone to the B cell follicle and T cell area, strongly suggesting a potential mechanism for inhibition. These data suggest that during infection, bacterial-associated Ags are the preferential immunogen for antibacterial Ig responses. ***The Journal of Immunology,*** **2009, 182: 2011–2019.**

During infections with *Streptococcus pneumoniae* (Pn),[3] as well as with other extracellular bacteria, the immune system likely encounters a variety of microbial components in soluble form, as well as those associated with the intact bacterium (1, 2). Thus, secreted hydrolases such as hyaluronidases, neuraminidases, and endoglycosidases can mediate bacterial spread and destruction of host tissue through degradation of hyaluronan, mucins, and glycolipids. Additionally, during the stationary growth phase, Pn expresses a major autolysin (LytA amidase) that degrades its own peptidoglycan cell wall, resulting in release of cytoplasmic proteins (3, 4). One such protein is pneumolysin, which can induce host cell injury through formation of cell membrane pores (5) and at lower concentrations can stimulate release of proinflammatory mediators (6) and directly accelerate cell death of neutrophils (7), the major phagocytic cell that mediates innate immunity to extracellular bacteria. Additionally, since both capsular polysaccharide (PS) and a number of proteins are covalently attached to the bacterial cell wall peptidoglycan (8, 9), the release of soluble PS-protein conjugates upon bacterial lysis is also likely.

Adaptive immunity to extracellular bacteria is largely mediated by Ab. Although soluble and particulate Ags may exhibit distinct immunologic properties (10–12), their potential cross-regulatory effects on the humoral immune response following concomitant immunization, as might occur during bacterial infections, are unknown. In particular, the context in which the Ag is expressed may affect the manner in which it is transported and/or processed within the secondary lymphoid organ. This, in turn, may significantly impact the quality and quantity of the subsequent immune response. The size of the immunogen (13–15), its soluble or particulate nature (16, 17), the valency (18–20) and biochemical nature of the antigenic epitope (21), and the presence of associated innate immune cell activators, such as TLRs (22, 23), and mediators of cellular uptake, such as scavenger receptor ligands (24–26), in turn can influence the outcome of these processes.

The binding of Ag by B cells via their BCR is a particularly critical event for the initiation of a specific humoral immune response. Depending on the nature of the immunogen and the site of immunization, delivery of intact Ag to B cells within the secondary lymphoid organ can occur in a number of distinct ways, including diffusion through the conduit system (13–15), or transport by immune cells, including marginal zone B (MZB) cells (27, 28), macrophages (29–32), or dendritic cells (33–37). Additionally, the specific B cell (38, 39), macrophage (40, 41), or DC subset (42–44) that initially encounters a particular immunogen may further

*Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; and †Fina BioSolutions, Rockville, MD 20850

Received for publication August 8, 2008. Accepted for publication December 5, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by National Institutes of Health Grant 1R01 AI49192 (to C.M.S.) and the Uniformed Services University of the Health Sciences Dean's Research and Education Endowment Fund (to C.M.S.), and by funding from the intramural program of the National Human Genome Research Institute (to P.L.S.).

Opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

[2] Address correspondence and reprint requests to Dr. Clifford M. Snapper, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. E-mail address: csnapper@usuhs.mil

[3] Abbreviations used in this paper: Pn, intact *Streptococcus pneumoniae*; PS, polysaccharide; MZB, marginal zone B; FDC, follicular dendritic cell; FB, follicular B; TI, T cell independent; TD, T cell dependent; GC, germinal center; PPS14, pneumococcal capsular polysaccharide, serotype 14; PspA, pneumococcal surface protein A; cOVA, chicken OVA; Pn14, intact *S. pneumoniae*, capsular type 14; R36A, unencapsulated variant of *S. pneumoniae*, capsular polysaccharide, serotype 2 (strain D39); CBP, choline-binding protein; CpG-ODN, CpG-containing oligodeoxynucleotide.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802615

influence the nature of subsequent B cell, as well as T cell, signaling and the functional outcome. B cell contact with Ag might occur via direct transfer by Ag-transporting cells or by initial transfer of Ag from these latter cells to follicular dendritic cells (FDCs) in the B cell follicle, followed by B cell binding to the FDC-bound Ag (45, 46).

Studies directly comparing the parameters that mediate a PS- and protein-specific Ig response to intact Pn relative to those that regulate a humoral response to a soluble pneumococcal PS-protein conjugate have indeed revealed two distinct pathways of immune activation. Specifically, although the IgG anti-PS responses to Pn and conjugate are both dependent on $CD4^+$ T cells, B7/CD28 costimulation, and CD40/CD40L interactions, the IgG anti-PS response to Pn is essentially extrafollicular, with more rapid kinetics of primary induction and failure to generate PS-specific memory, whereas the same response to conjugate is follicular in nature, with more prolonged kinetics and the generation of PS-specific memory (47–52). Recent data strongly suggest that secretion of PS-specific IgG in response to Pn and conjugate is largely effected by MZB and follicular B (FB) cells, respectively (53). Additionally, the IgM anti-PS responses to Pn and conjugate are T cell independent (TI) and T cell dependent (TD), respectively (47, 51, 52). In contrast, the protein-specific IgG responses to both Pn and conjugate appear to be mediated by FB cells that give rise to a germinal center (GC) reaction followed by the generation of protein-specific memory (53). Collectively, these studies form a strong basis for testing the immunologic consequences of coimmunization with intact Pn and soluble Pn-derived conjugate as a model for understanding potential cross-regulatory immune pathways mediated by intact pathogens and the soluble products that they secrete. In this study we demonstrate the ability of the intact bacterium to suppress systemic Ig responses to soluble Ags, likely through an inhibitory effect on soluble TD Ag trafficking from the marginal zone to the B cell follicles and T cell areas of the spleen.

Materials and Methods

Mice

Female BALB/c mice were purchased from The National Cancer Institute (Frederick, MD) and were used between 7 and 10 wk of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and they were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Reagents

Purified *S. pneumoniae* capsular polysaccharide type 14 (PPS14) was purchased from the American Type Culture Collection. Recombinant pneumococcal surface protein A (PspA) was expressed in *Sacharomyces cerevisiae* BJ3505 and purified as previously described (54). A His-tagged, truncated EBV gp350 protein consisting of the first 470 aa was expressed in SF9 insect cells transfected with a baculovirus vector containing the gp350 cDNA. The gp350 protein was purified by Ni-NTA affinity chromatography. Soluble conjugates comprising PPS14 (2×10^6 m.w.) covalently linked to PspA or gp350 were synthesized using CDAP (1-cyano-4-dimethyaminopyridinium tetrafluoroborate) chemistry as previously described (55). The molar ratios of protein to PPS14 were ~15–20 for each conjugate. Chicken OVA (cOVA; "Inject OVA") was purchased from Pierce.

Preparation of Pn strains

Frozen stocks of Pn14 (*S. pneumoniae*, capsular type 14), D39, R36A (unencapsulated variant of *S. pneumoniae*, capsular polysaccharide, serotype 2 (strain D39)), WU-2, and JD11 were thawed and subcultured on BBL pre-made blood agar plates (VWR International) as previously described. Briefly, isolated colonies on blood agar were grown in Todd-Hewitt broth (BD Biosciences) to mid-log phase, collected, and heat-killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates.

Preparation of R36A depleted of choline-binding proteins (CBPs)

Cultures of R36A were collected by centrifugation, washed twice with PBS, and the bacterial pellet was treated for 20 min at room temperature with 2% choline chloride (Sigma-Aldrich) to release CBPs from the bacterial cell wall (56). The resulting CBP-depleted bacteria were washed by centrifugation, heat-killed, and stored as indicated above. PspA content of the CBP-depleted R36A preparations was ≤60 ng per 10^9 CFU.

Immunizations

Mice ($n = 7$/group) were immunized i.p. with 2×10^8 CFU heat-killed bacteria in saline or 1 μg (weight of PS) of conjugate (PPS14-PspA, PPS14-gp350, or PPS14-tetanus toxoid) adsorbed on 13 μg of alum (Alhydrogel, 2%; Brenntag Biosector) mixed with 25 μg of a stimulatory phosphorothioated 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN) (57, 58), and similarly boosted. Coimmunization studies were performed by injecting Pn14 and conjugate plus alum/CpG-ODN separately at two different i.p. sites. Biodegradable polystyrene latex beads (1.1 μm mean particle size) were purchased from Sigma-Aldrich (catalog no. LB11) and injected i.p. at 2×10^8 particles per mouse. Serum samples for measurement of Ag-specific IgM and IgG titers were prepared from blood obtained through the tail vein.

Measurement of serum Ag-specific Ig isotype titers

ELISA was performed as previously described. Briefly, Immulon 4 ELISA plates (Dynex Technologies) were coated with PPS14, PspA, or gp350 (5 μg/ml) in PBS, and plates were washed with PBS plus 0.1% Tween 20 and then blocked with PBS plus 1% BSA. Five-fold dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1% BSA were then added and plates were washed three times with PBS plus 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgM or IgG Abs (200 ng/ml final concentration) in PBS plus 0.05% Tween 20 were then added, and plates were washed with PBS plus 0.1% Tween 20 and substrate (*p*-nitrophenyl phosphate, disodium; Sigma-Aldrich) was added at 1 mg/ml in TM buffer (1 M Tris plus 0.3 mM $MgCl_2$ (pH 9.8)) for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems).

Fluorescence microscopy

R36A was labeled with SYTO 83 fluorescent nucleic acid stain (Molecular Probes), and conjugate was labeled with Alexa Fluor 405 (Solulink). Following immunization, spleens were removed and incubated at least 6 h in 15 ml of PLP buffer (0.05 M PBS containing 0.1 M L-lysine (pH 7.4), 2 mg/ml $NaIO_4$, and 10 mg/ml paraformaldehyde). The fixed samples were washed in PBS and dehydrated in 30% sucrose in PBS. Tissues were snap-frozen in Tissue-Tek (VWR International). Twenty- to 30-μm-thick frozen sections were cut and stained with rat anti-mouse B220-PE (clone RA3-6B2; BD Bioscience) and rat anti-mouse CD169-FITC (clone MOMA-1; AbD Serotec) for 45 min followed by washing three times in Tris buffer. Sections were mounted with ProLong antifade kit (Molecular Probes). Immunofluorescence imaging was performed with a Zeiss Pascal laser scanning confocal microscope. Separate images were collected for each fluorochrome and overlaid to obtain a multicolor image. Final image processing was performed with ImageJ software (National Institutes of Health, Betheda, MD) and Adobe Photoshop.

Statistical analysis

Serum Ig isotype titers were expressed as geometric means ± SEM of the individual serum Ig isotype titers. Significance was determined by Student's t test. Values of $p < 0.05$ were considered statistically significant. Each experiment was performed at least twice to ascertain reproducibility.

Results

Pn14 fails to induce memory and inhibits memory generation by pneumococcal conjugate

We previously reported that mice immunized i.p. with intact, heat-inactivated Pn14 fail to elicit an enhanced PPS14-specific IgG secondary response upon boosting with additional Pn14, in distinct contrast to priming and boosting with a pneumococcal conjugate

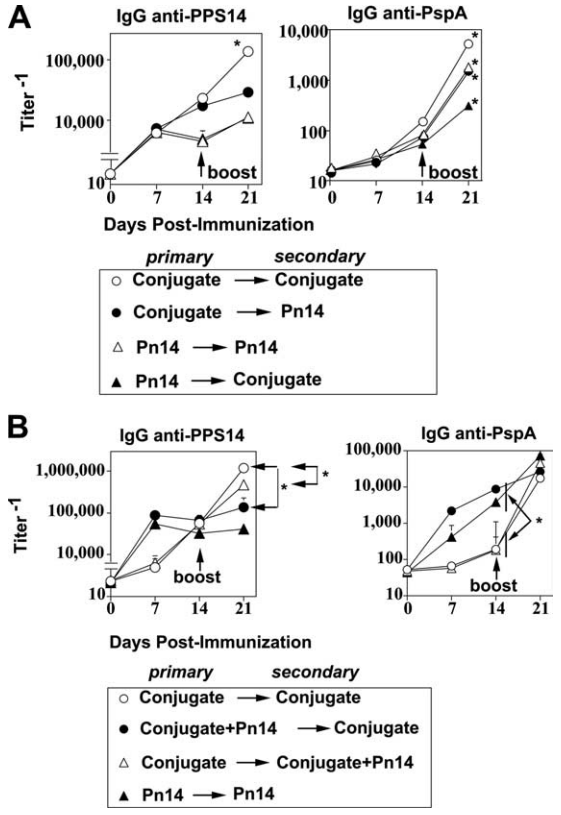

FIGURE 1. Pn14 fails to induce memory and inhibits memory generation by pneumococcal conjugate. *A*, Mice were initially immunized i.p. with either PPS14-PspA (conjugate) in alum + CpG-ODN or Pn14, and boosted on day 14 with either conjugate or Pn14. Serum titers of IgG anti-PPS14 and IgG anti-PspA were determined by ELISA. *, $p < 0.05$ between primary (day 14) and secondary (day 21) titers. *B*, Mice were initially immunized i.p. and boosted on day 14 as indicated. Conjugate refers to PPS14-PspA in alum + CpG-ODN. Serum titers of IgG anti-PPS14 and IgG anti-PspA were determined by ELISA. *, $p < 0.05$ between groups indicated by arrows.

consisting of PPS14 covalently linked to PspA (PPS14-PspA) adsorbed to alum and the TLR9 agonist (57) CpG-ODN (49, 54). To better define the nature of this dichotomy, we wanted to determine whether Pn14-primed mice could be effectively boosted by secondary immunization with conjugate or, conversely, whether conjugate-primed mice could be boosted with Pn14. In prior dose-response studies, we determined the amount of Pn14 or PPS14-PspA that generated maximal Ig-inducing responses in vivo (data not shown), and we have used these doses in both previous and in the present experiments. As illustrated below (see Fig. 3*A*), the primary IgG anti-PPS14 response to conjugate peaks on day 14, and thus unless otherwise indicated, mice were boosted on day 14 to elicit a secondary response. As illustrated in Fig. 1*A*, an enhanced secondary PPS14-specific IgG response was elicited only when mice were both primed and boosted with conjugate. In contrast, enhanced secondary PspA-specific IgG responses were observed both when Pn14-primed mice were boosted with either conjugate or Pn14, or when primary conjugate immunization was followed by secondary challenge with Pn14 or conjugate. These data were thus consistent with our recent observation that the Pn14- and conjugate-induced PPS14-specific IgG responses were derived from different B cell subsets (i.e., MZB and FB cells, respectively), whereas the PspA-specific IgG responses to either Pn14 or conjugate appeared to arise from FB cells (53).

In light of the failure of Pn14 to elicit memory for the PPS14-specific IgG response, we next asked whether Pn14 could prevent induction of memory when coimmunized with conjugate. Mice were thus coimmunized with Pn14 and conjugate adsorbed to alum/CpG-ODN at two different i.p. sites and boosted with conjugate alone, or first primed with conjugate alone and boosted with Pn14 and conjugate, again at two different i.p. sites (Fig. 1*B*). As controls, mice were both primed and boosted with conjugate alone or Pn14 alone. In contrast to mice primed with conjugate alone, primary coimmunization with Pn14 and conjugate resulted in the complete abrogation of the enhanced PPS14-specific IgG response following secondary immunization with conjugate. A partial, although significant, reduction in the secondary response was also observed in conjugate-primed mice boosted with Pn14 and conjugate. In contrast, secondary PspA-specific IgG responses were essentially equivalent in all four immunization groups (Fig. 1*B*). Thus, Pn14 appeared to prevent the generation of PPS14-specific IgG memory responses to conjugate.

Inhibition of conjugate-induced PS-specific IgG memory responses is not dependent on Pn capsular PS or particulation per se

Pn typically expresses >90 different non-cross-reacting capsular serotypes (59). Previous studies indicated that free PPS could inhibit the IgG anti-PPS response to conjugate in a PPS serotype-specific manner (60, 61). In this regard, we wanted to determine whether the inhibitory effect of Pn14 on the induction of conjugate-induced PPS14-specific IgG memory was dependent on coimmunization of the same PPS serotype, and/or whether Pn was even required to express any PPS capsule to mediate its inhibitory effect. We thus utilized PPS2 (strain D39) and PPS3 (strain WU-2) encapsulated Pn strains and their respective nonencapsulated isogenic mutants (strains R36A and JD11) in coimmunization studies with the PPS14-PspA conjugate. As illustrated in Fig. 2*A*, coimmunization of conjugate with either of the two encapsulated Pn or their unencapsulated mutants significantly inhibited the enhanced conjugate-induced PPS14-specific IgG response following secondary immunization with conjugate alone. D39 and R36A were more effective inhibitors than WU-2 or JD11, although in each case the same dose of Pn was used. In light of these data, we utilized R36A to effect inhibition in all subsequent studies.

The IgM, like the IgG, anti-PS response to conjugate is also dependent on T cell help (51, 52), in contrast to the IgM anti-PS response to Pn14 that is TI (47, 50). In Fig. 2*B* we further demonstrate that coimmunization of conjugate with R36A also inhibits the enhanced IgM anti-PPS14 response to secondary immunization with conjugate alone. Collectively, these data demonstrate that the mechanism of bacterial inhibition of the PPS14-specific Ig response to conjugate is independent of the strain or PPS serotype of the bacteria, and indeed occurs in the complete absence of a PPS capsule. Since particulate and soluble Ags exhibit distinct immunologic features (16, 17), we next wanted to determine whether the particulate nature of Pn itself was mediating the inhibitory effect on the soluble conjugate. Thus, we coimmunized mice with 1.1-mm biodegradable latex beads in alum plus CpG-ODN with PPS14-PspA conjugate, also in alum plus CpG-ODN. Blood-borne inert microspheres, like intact particulate pathogens, upon entering the spleen are concentrated largely within macrophages present within the splenic marginal zone (62, 63). As illustrated in Fig. 2*C*, we observed no significant inhibitory effects of the beads on the IgG anti-PPS14 response to conjugate.

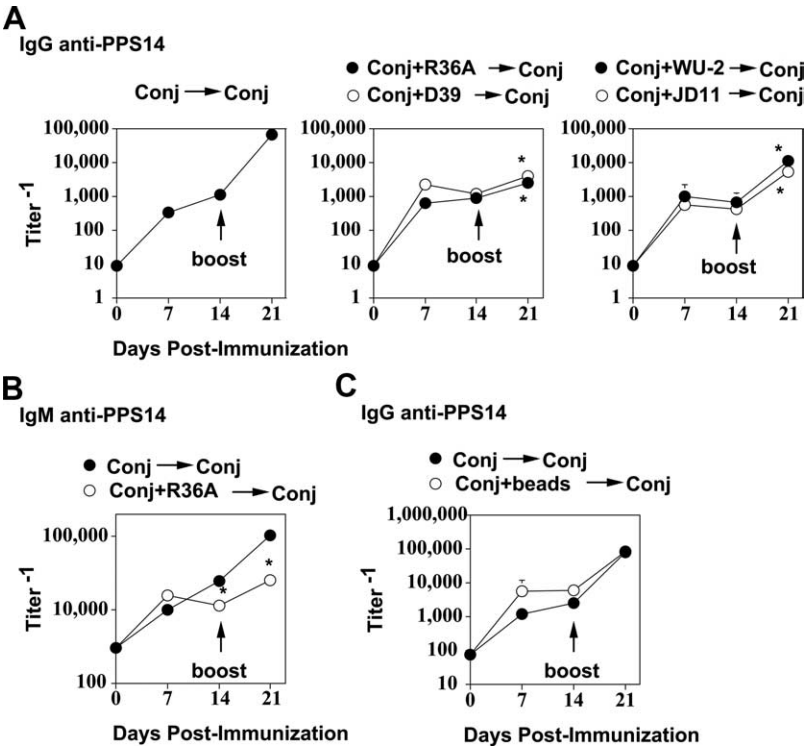

FIGURE 2. Inhibition of conjugate-induced PS-specific memory is not dependent on Pn capsular PS or particulation per se. *A*, Mice were initially immunized i.p. with PPS14-PspA in alum + CpG-ODN (conjugate) without or with coimmunization with one of the following Pn strains: D39, R36A, WU-2, or JD11. All groups of mice were boosted with conjugate alone on day 14. Serum titers of IgG anti-PPS14 were measured by ELISA. $*$, $p < 0.05$ between mice initially injected with conjugate alone (*left panel*) vs mice coinjected with conjugate and a Pn strain (*center* and *right panels*). *B*, Serum samples from *A* (*left* and *center panels*) for measurement of IgM anti-PPS14 titers by ELISA. $*$, $p < 0.05$) between two groups of mice. *C*, Mice were initially immunized i.p. with PPS14-PspA in alum + CpG-ODN (conjugate) without or with coimmunization with 1.1-μm polystyrene latex beads in alum + CpG-ODN. Both groups of mice were boosted on day 14 with conjugate alone. Serum titers of IgG anti-PPS14 were measured by ELISA. $*$, $p < 0.05$ between two groups of mice.

Pn inhibits maintenance of primary IgG anti-PPS14 response to conjugate and induces long-lasting abrogation of memory, but not tolerance

A primary TD Ig response to systemic immunization with a protein Ag is typically characterized by an early and transient extrafollicular plasma cell response in the spleen followed by a GC reaction that produces long-lived BM plasma cells and memory B cells (64, 65). In light of our observation that Pn inhibits the conjugate-induced PPS14-specific IgG memory response, we wanted to determine whether it also inhibited the maintenance of primary serum Ig titers, with this latter process also being dependent on a GC reaction. Additionally, we wanted to determine whether the Pn-mediated inhibition of conjugate-induced memory was relatively long-lasting. Thus, we coimmunized mice with R36A and conjugate and, instead of boosting with conjugate on day 14 (see Figs. 1 and 2), we delayed secondary immunization until day 42. As illustrated in Fig. 3*A*, primary immunization with conjugate alone resulted in peak IgG anti-PPS14 serum titers at day 14 that were maintained at an equivalent level until day 42. In contrast, coimmunization with R36A, while having no significant effect on conjugate-induced PPS14-specific IgG serum titers at day 14, as we demonstrated in Figs. 1 and 2, resulted in a steady decline in serum titers until day 42, resulting in >10-fold lower titers relative to mice immunized with conjugate alone. Additionally, whereas mice primed and boosted with conjugate alone elicited >30-fold greater secondary vs primary IgG anti-PPS14 titers, mice coimmunized with R36A and conjugate initially elicited secondary titers no greater than those observed for mice initially injected with either PBS or R36A alone followed by secondary immunization with conjugate (Fig. 3*A*). However, over the ensuing 3 wk, IgG anti-PPS14 titers gradually and significantly increased over those observed for the peak primary response, but they were still significantly below serum titers observed in mice immunized and boosted with conjugate alone (Fig. 3*A*).

In light of the ability of R36A to inhibit the generation of PPS14-specific memory in response to conjugate, we wanted to determine whether this also induced a state of tolerance for generating a memory response. Mice were immunized with conjugate in the absence or presence of R36A and boosted with conjugate alone on day 14 with or without an additional boost on day 28 (Fig.

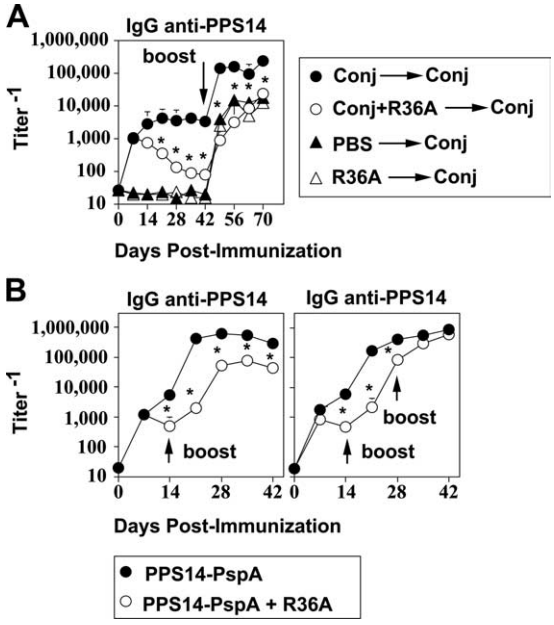

FIGURE 3. Pn inhibits maintenance of primary IgG anti-PPS14 response to conjugate and induces long-lasting abrogation of memory. *A*, Mice were initially immunized as indicated and then boosted on day 42 with conjugate alone. Conjugate refers to PPS14-PspA in alum + CpG-ODN. *B*, Mice were initially immunized with PPS14-PspA in alum + CpG-ODN with or without R36A and then boosted with PPS14-PspA in alum + CpG-ODN alone either once on day 14 (left box) or twice (day 14 and day 28, right box). Serum titers of IgG anti-PPS14 were measured by ELISA. $*$, $p < 0.05$ between mice initially immunized with conjugate vs conjugate + R36A.

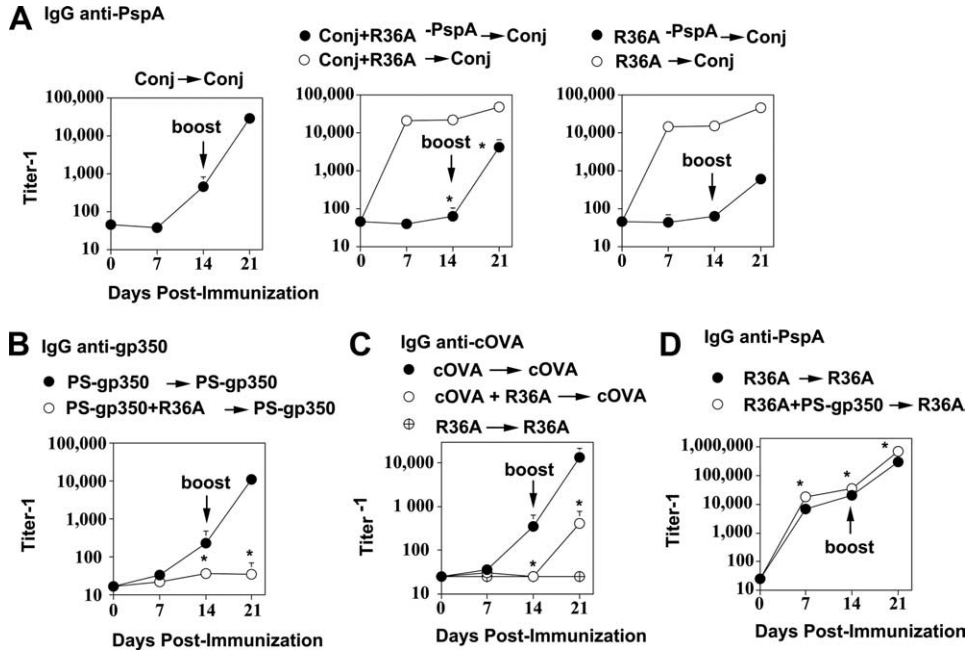

FIGURE 4. Pn also inhibits induction of protein-specific IgG memory in response to conjugate. *A*, Mice were initially immunized i.p. as indicated and boosted on day 14 with conjugate alone. Conjugate refers to PPS14-PspA in alum + CpG-ODN; R36A−PspA, choline chloride-treated R36A to remove PspA. Serum titers of IgG anti-PspA were measured by ELISA. ∗, $p < 0.05$ between mice initially immunized with conjugate alone (*left panel*) vs mice initially immunized with conjugate + $R36A^{-PspA}$ (*center panel*, ●). *B*, Mice were initially immunized i.p. with PPS14 (PS)-gp350 in alum + CpG-ODN without or with coimmunization with R36A. Both groups were boosted on day 14 with PS-gp350 alone. Serum titers of IgG anti-gp350 were measured by ELISA. ∗, $p < 0.05$. *C*, Mice were initially immunized with R36A in the presence or absence of PS-gp350 in alum + CpG-ODN. Both groups were boosted on day 14 with R36A alone. Serum titers of IgG anti-PspA were measured by ELISA. ∗, $p < 0.05$. *D*, Mice were immunized i.p. with cOVA (5 μg/mouse) or cOVA + R36A in saline and boosted with cOVA alone (5 μg/mouse) in saline on day 14. Serum titers of IgG anti-cOVA were measured by ELISA. ∗, $p < 0.05$ serum IgG anti-cOVA titers between mice primed with cOVA vs cOVA + R36A.

3*B*). Similar to that observed in Fig. 3*A*, mice immunized with conjugate plus R36A showed both delayed and absolutely inhibited secondary IgG anti-PPS14 titers following a single boost with conjugate relative to mice primed with conjugate alone (Fig. 3*B*). However, a second boost of conjugate in mice initially primed with conjugate plus R36A induced serum titers of IgG anti-PPS14 to levels similar to those observed in mice primed and boosted once with conjugate alone (Fig. 3*B*). Collectively, the data illustrated in Fig. 3 demonstrate that R36A inhibits the maintenance, although not early induction, of primary serum titers of IgG anti-PPS14 in response to conjugate, and it both delays and absolutely inhibits the generation of memory in a relatively sustained manner, without producing a state of tolerance for induction of a memory response, upon reimmunization.

Pn also inhibits induction of protein-specific IgG memory in response to conjugate

Conjugate induces both an anti-PS and anti-protein response that is TD and that leads to the generation of PS- and protein-specific memory, respectively. We thus sought to determine whether R36A also inhibits the induction of protein-specific memory in response to conjugate. To accomplish this, we took two separate approaches. In the first approach, since PspA is a CBP, we depleted R36A of PspA ($R36A^{-PspA}$) by using choline chloride as a competitor (56), and coimmunized $R36A^{-PspA}$ with PPS14-PspA conjugate, followed by boosting with conjugate alone. As illustrated in Fig. 4*A* (*right panel*), primary immunization with R36A alone induced a robust IgG anti-PspA response, whereas essentially no induction was observed using $R36A^{-PspA}$, confirming that PspA was largely depleted from the bacteria. Boosting of $R36A^{-PspA}$-primed mice with conjugate resulted in only a modest enhancement in the secondary IgG anti-PspA response, 7 days later, compared with what was observed 7 days following primary immunization with conjugate alone (Fig. 4*A*, compare *right* and *left panels*). Of interest, coimmunization of mice with $R36A^{-PspA}$ and conjugate resulted in a nearly complete inhibition in the conjugate-induced primary anti-IgG anti-PspA response and in a partial, although significant, inhibition (10-fold reduction) of the secondary IgG anti-PspA response following boosting with conjugate alone (Fig. 4*A*, compare *left* and *center panels*).

In a second set of studies we coimmunized mice with R36A and a conjugate consisting of PPS14 and gp350 (PS-gp350) followed by boosting with PPS14-gp350 and measurement of serum IgG anti-gp350 titers. Gp350 is an EBV-derived protein (66) not expressed by any Pn strain. Coimmunization of PS-gp350 with R36A resulted in a marked inhibition of both the primary IgG anti-gp350 response and in the enhanced secondary response following boosting with PS-gp350 alone (Fig. 4*B*). These two sets of experiments demonstrate that coimmunization of mice with R36A and conjugate results in inhibition of not only conjugate-induced PS-specific memory and maintenance of the primary response, but also in the elicitation of both the primary and secondary conjugate-induced IgG anti-protein response.

We next wanted to determine whether R36A could inhibit a specific IgG response to a soluble, unconjugated protein, not derived from a pathogen, immunized in the absence of adjuvant and mixed together for i.p. injection at one site. Thus, mice were injected i.p. with cOVA in saline either alone or mixed with R36A and then boosted i.p. on day 14 with cOVA alone in saline. As illustrated in Fig. 4*C*, the presence of R36A during priming with cOVA inhibited both the primary and memory IgG anti-cOVA

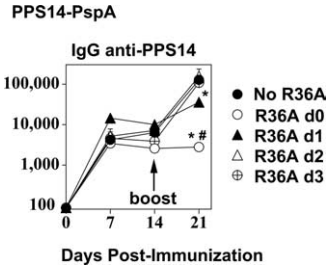

FIGURE 5. Pn acts only within the first 24 h following conjugate immunization to inhibit memory. Separate groups of mice were immunized i.p. with PPS14-PspA in alum + CpG-ODN. On different days following immunization, mice were coimmunized (R36A d0) or subsequently immunized (R36A d1, d2, or d3) with R36A or not given R36A at all (No R36A). Serum titers of IgG anti-PPS14 were measured by ELISA. *, $p < 0.05$ between mice not coimmunized with R36A (No R36A) vs other groups (R36A d0, d1, d2, or d3); #, $p < 0.05$ between R36A d0 and R36A d1.

response relative to mice primed with cOVA alone. In a final experiment, we sought to determine the converse ability of the soluble conjugate to inhibit an anti-protein (i.e., anti-PspA) IgG response to R36A. We used a conjugate containing a carrier protein not present within R36A (i.e., the EBV protein gp350), which was conjugated to PPS14. As illustrated in Fig. 4*D*, the conjugate failed to significantly alter either the primary or secondary IgG anti-PspA response to R36A.

Pn acts largely within the first 24 h following conjugate immunization to inhibit memory

To better understand the mechanism underlying the R36A-mediated inhibition of conjugate-induced Ig production, we determined during what time period following coimmunization that R36A exerts its inhibitory effect. Separate sets of mice were immunized with PPS14-PspA, and R36A was injected on either day 0 (coimmunization), 1, 2, or 3. All mice were boosted with PPS14-PspA alone on day 14. R36A had no significant effect on the conjugate-induced primary IgG anti-PPS14 response in any of the groups (Fig. 5). R36A coinjected with PPS14-PspA (R36A d0) resulted in a complete inhibition of the conjugate-induced secondary response. Delay of addition of R36A by 1 day resulted in only a partial, although significant, inhibitory effect, whereas no effect was observed if R36A was injected either 2 or 3 days following immunization with conjugate. Thus, R36A acts relatively early (largely within the first 24 h) during the immune response to conjugate to inhibit the subsequent generation of conjugate-induced PPS14-specific IgG memory.

Pn inhibits the accumulation of conjugate within the splenic white pulp

Our observation that R36A exerts its inhibitory effect on the conjugate-induced Ig response largely within the first 24 h following immunization with conjugate suggests the possibility that R36A is interfering with transport and/or processing of conjugate within the spleen, the primary site where Ig responses are elicited in response to systemic immunization. Mice were thus immunized with Alexa Fluor 405-labeled PPS14-PspA in the absence or presence of unlabelled R36A, and spleens were removed 4 h later for fluorescence confocal microscopic analysis. Sections were stained with PE-labeled anti-B220 (B cells) and FITC-anti-CD169 (MOMA-1, marginal metallophilic macrophages). As illustrated in Fig. 6*A*, mice immunized with PPS14-PspA alone exhibited large amounts of conjugate within both B cell follicles and the T cell compartment of the splenic white pulp, as well as conjugate within the marginal zone and red pulp. In contrast, conjugate was localized almost entirely within the marginal zone and red pulp in mice coimmunized with labeled PPS14-PspA and unlabelled R36A, with only small amounts present within the white pulp. Further analysis of spleen sections removed 1, 4, 8, and 24 h following immunization with labeled conjugate alone demonstrated peak entry of conjugate into the B cell follicles and T cell regions at 1 h, with maintenance at 4 and 8 h, and disappearance of fluorescent signal within the white pulp, but not marginal zone, by 24 h (data not shown). However, at no time point during this 24-h period was significant entry of labeled conjugate into the white pulp observed when mice were coimmunized with unlabelled R36A, relative to those immunized with conjugate alone (data not shown). In contrast, coinjection of latex beads with labeled conjugate (see Fig. 2*C*) had no apparent effect on conjugate accumulation within the

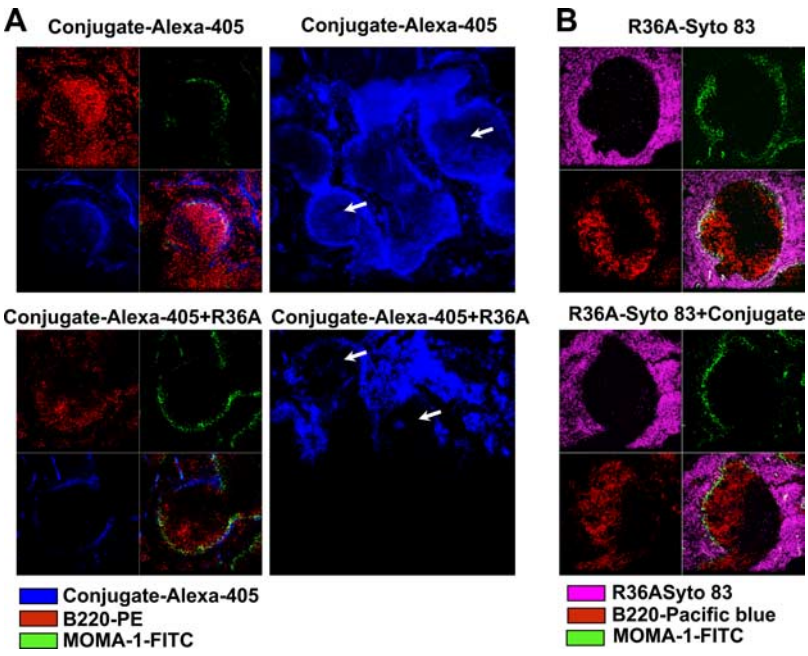

FIGURE 6. Pn inhibits trafficking of conjugate from splenic marginal zone into white pulp. *A*, Mice were immunized with Alexa Fluor 405-labeled PPS14-PspA in alum + CpG-ODN in the absence or presence of unlabelled R36A, and spleens were removed 4 h later for confocal fluorescence microscopic analysis. Sections were stained with PE-labeled anti-B220 (B cells) and FITC-anti-CD169 (MOMA-1, marginal metallophilic macrophages). *B*, Mice were immunized with SYTO 83-labeled R36A with or without unlabelled PPS14-PspA. Spleens were removed 8 h later and sections were stained with PE-anti-B220 and FITC-anti-CD169.

white pulp relative to that observed upon injection of conjugate alone (data not shown).

In contrast to conjugate, immunization with SYTO 83-labeled R36A alone resulted in only small amounts of R36A entering into the B cell follicles and T cell regions from the marginal zone, detectable only by 4 h (but not 1 h), being maintained at 8 h, and with fluorescent signal disappearing from the white pulp, but not marginal zone, by 24 h (Fig. 6*B* (8 h time point) and data not shown). Coimmunization of unlabelled conjugate and labeled R36A did not alter the distribution pattern of R36A at any time point (Fig. 6*B* and data not shown) relative to mice immunized with R36A alone. These data strongly suggest that the ability of R36A to inhibit the conjugate-induced Ig response is secondary to its blocking of conjugate transport from the marginal zone into the splenic B cell follicle and T cell region, steps otherwise required to generate a TD follicular response and induction of memory.

Discussion

In this study we demonstrate that the maintenance of the TD primary PS-specific IgG response and the generation of memory, but not the initial primary PS-specific response to soluble pneumococcal conjugate, is inhibited upon coimmunization with intact Pn. Additionally, Pn inhibits both the primary and secondary IgG antiprotein (i.e., PspA or gp350) responses to either conjugate (PPS14-PspA or PPS14-gp35, respectively) or to a soluble, unconjugated nonbacterial protein (i.e., cOVA). Although Ab responses to purified, soluble PS Ags are TI (21), capsular PS expressed by bacteria is covalently linked to the cell wall peptidoglycan, to which a number of bacterial proteins are also covalently attached (8, 9), potentially conferring TD properties to the PS Ag shed during bacterial autolysis.

Pn-mediated inhibition of the Ig response to the soluble TD Ags occurs whether the two immunogens are injected i.p. at separate sites or mixed together and injected at one i.p. site. The mechanism of this inhibition is Ag-nonspecific, independent of Pn expression of PS capsule, mediated by several Pn strains, and is not related to the particulation of the Ag per se. Instead, inhibition is directly correlated with the ability of intact Pn to prevent conjugate transport from the marginal zone to the interior of the white pulp, where it can initiate a GC reaction, important for long-term Ig production and the generation of memory. This notion is supported by the loss of most of the inhibitory effect when injection of Pn is delayed by 24 h following conjugate immunization. That the effect of Pn is dominant to that of soluble Ag is evidenced by the use of optimal Ig-inducing dosages of each immunogen, as well as the inability of the soluble conjugate or isolated protein to inhibit protein-specific Ig induction in response to intact Pn.

Several studies have also demonstrated different types of cross-regulation between Ags delivered concomitantly. The conjugate vaccine itself is a notable example, whereupon covalent linkage of an immunogenic protein to a PS Ag converts the PS from a weak TI to a strong TD Ag, including the capacity of the latter to generate PS-specific immunologic memory and stimulate anti-PS responses in infants (67, 68). Of interest, the presence of free PS in the conjugate has been shown to inhibit the anti-PS response to the conjugate itself (60, 61). Additionally, distinct PS Ags comprising conjugate vaccines may also influence the ability of APCs to present the associated protein Ags to $CD4^+$ T cells (69).

During bacterial infections, both soluble products secreted or released by the pathogen as well as the intact pathogen itself concurrently enter the secondary lymphoid organ where they likely get transported and processed in distinct ways and activate different, although perhaps overlapping, immune cellular pathways. However, little is known about whether these pathways proceed independently or are cross-regulatory, and thus how this might affect the individual immune responses elicited to the pathogen's component Ags. Both soluble conjugate and intact Pn have relatively large molecular masses that preclude their passive diffusion through the conduit system present within the spleen (13–15). Our unpublished observations that both immunogens elicit an ICOS-dependent, protein-specific IgG memory response dependent upon FB cells indicate that at least a fraction of the total conjugate and Pn entering the spleen is transported from the marginal zone to the B cell follicle (see Fig. 6) via one or more immune cell types. One cellular candidate for transport of conjugate and/or Pn into the follicle is the MZB cell. MZB cells can bind Ag-associated complement components of C3 via CD21 and CD35 and therefore capture Ag in a BCR-independent manner (70–72). Upon migration to the follicle, MZB cells are able to deposit Ag onto FDC that can then serve as a platform for FB cell capture of Ag in a BCR-dependent manner and subsequent initiation of a GC reaction (28, 29, 73).

Conjugate and Pn likely activate complement in vivo via the classical pathway, as evidenced in part by the ability of natural IgM to enhance immune responses to both soluble and particulate Ags in a CD21/CD35-dependent manner (74). The ability of repetitive surfaces, as manifested by intact pathogens, to efficiently activate complement (75) suggests that Pn might bind MZB cells via CD21 more efficiently than does conjugate and thus directly block the latter's binding to and transport by MZB cells. This could in turn result in enhanced trapping of conjugate by macrophages within the marginal zone (28, 76). Arguing against this scenario, however, is a report that although the pneumococcal conjugate vaccine Prevnar is indeed transported from the marginal zone to the follicle, it does not bind to MZB cells (77). Both macrophages (29–32) and dendritic cells (33–37) are also known to bind and transport intact Ag for delivery to FDC and/or FB cells. Thus, direct Pn-mediated inhibition of conjugate binding and transport via these latter cells are other potential mechanisms for Pn-mediated inhibition of the conjugate-induced Ig response. The possibility also exists that Pn induces rapid migration of Ag transport cells to the follicle before conjugate has sufficient time to bind, again potentially resulting in conjugate trapping by macrophages in the marginal zone.

Infections with extracellular bacteria develop in an acute, life-threatening manner, and thus they require not only a robust innate immune response, but also the rapid elicitation of specific Ab to enhance opsonophagocytosis (59). Upon entry into the blood, these bacteria quickly travel to the spleen where they are initially delivered into the marginal sinus and red pulp. In this location they are exposed to several distinct macrophage subsets that mediate phagocytosis and pathogen killing, as well as to MZB cells (78) that are programmed to differentiate rapidly into short-lived PS-specific Ig-secreting plasma cells in an extrafollicular immune response that can be both TI (IgM) and TD (IgG) (39, 79). Upon further transport into the splenic white pulp, Ag over a more prolonged period can trigger a GC reaction by FB cells critical for the generation of immunologic memory and sustained Ig secretion from long-lived bone marrow plasma cells (80). We speculate that the ability of Pn to block soluble Ag entry into the white pulp, without its own entry being inhibited, might allow for both a rapid primary MZB cell-mediated, PS-specific, and more prolonged FB-mediated, protein-specific memory Ig response that is specifically focused on pathogen-associated, but not pathogen-released, Ag, thus optimizing Ab-mediated pathogen recognition and subsequent opsonization for effective phagocyte killing. However, the possibility that secreted or released soluble bacterial virulence factors may also effectively escape adaptive immune recognition in the

presence of intact pathogen could alternatively provide a mechanism that promotes bacterial pathogenesis.

Disclosures

The authors have no financial conflicts of interest.

References

1. Jedrzejas, M. J. 2004. Extracellular virulence factors of *Streptococcus pneumoniae*. *Front. Biosci.* 9: 891–914.
2. Rigden, D. J., M. Y. Galperin, and M. J. Jedrzejas. 2003. Analysis of structure and function of putative surface-exposed proteins encoded in the *Streptococcus pneumoniae* genome: a bioinformatics-based approach to vaccine and drug design. *Crit. Rev. Biochem. Mol. Biol.* 38: 143–168.
3. Lopez, R., J. L. Garcia, E. Garcia, C. Ronda, and P. Garcia. 1992. Structural analysis and biological significance of the cell wall lytic enzymes of *Streptococcus pneumoniae* and its bacteriophage. *FEMS Microb. Lett.* 79: 439–447.
4. Ronda, C., J. L. Garcia, E. Garcia, J. M. Sanchez-Puelles, and R. Lopez. 1987. Biological role of the pneumococcal amidase: cloning of the lytA gene in *Streptococcus pneumoniae*. *Eur. J. Biochem.* 164: 621–624.
5. Boulnois, G. J., J. C. Paton, T. J. Mitchell, and P. W. Andrew. 1991. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. *Mol. Microbiol.* 5: 2611–2616.
6. Houldsworth, S., P. W. Andrew, and T. J. Mitchell. 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect. Immun.* 62: 1501–1503.
7. Martner, A., C. Dahlgren, J. C. Paton, and A. E. Wold. 2008. Pneumolysin released during *Streptococcus pneumoniae* autolysis is a potent activator of intracellular oxygen radical production in neutrophils. *Infect. Immun.* 76: 4079–4087.
8. Navarre, W. W., and O. Schneewind. 1999. Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol. Mol. Biol. Rev.* 63: 174–229.
9. Sorensen, U. B., J. Henrichsen, H. C. Chen, and S. C. Szu. 1990. Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of *Streptococcus pneumoniae* revealed by immunochemical methods. *Microb. Pathog.* 8: 325–334.
10. Kovacsovics-Bankowski, M., K. Clark, B. Benacerraf, and K. L. Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. USA* 90: 4942–4946.
11. Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu. Rev. Immunol.* 20: 621–667.
12. Ziegler, H. K., C. A. Orlin, and C. W. Cluff. 1987. Differential requirements for the processing and presentation of soluble and particulate bacterial antigens by macrophages. *Eur. J. Immunol.* 17: 1287–1296.
13. Gretz, J. E., C. C. Norbury, A. O. Anderson, A. E. Proudfoot, and S. Shaw. 2000. Lymph-borne chemokines and other low molecular weight molecules reach high endothelial venules via specialized conduits while a functional barrier limits access to the lymphocyte microenvironments in lymph node cortex. *J. Exp. Med.* 192: 1425–1440.
14. Sixt, M., N. Kanazawa, M. Selg, T. Samson, G. Roos, D. P. Reinhardt, R. Pabst, M. B. Lutz, and L. Sorokin. 2005. The conduit system transports soluble antigens from the afferent lymph to resident dendritic cells in the T cell area of the lymph node. *Immunity* 22: 19–29.
15. Nolte, M. A., J. A. Belien, I. Schadee-Eestermans, W. Jansen, W. W. Unger, N. van Rooijen, G. Kraal, and R. E. Mebius. 2003. A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *J. Exp. Med.* 198: 505–512.
16. Vidard, L., M. Kovacsovics-Bankowski, S. K. Kraeft, L. B. Chen, B. Benacerraf, and K. L. Rock. 1996. Analysis of MHC class II presentation of particulate antigens of B lymphocytes. *J. Immunol.* 156: 2809–2818.
17. Nayak, J. V., D. A. Hokey, A. Larregina, Y. He, R. D. Salter, S. C. Watkins, and L. D. Falo, Jr. 2006. Phagocytosis induces lysosome remodeling and regulated presentation of particulate antigens by activated dendritic cells. *J. Immunol.* 177: 8493–8503.
18. Thyagarajan, R., N. Arunkumar, and W. Song. 2003. Polyvalent antigens stabilize B cell antigen receptor surface signaling microdomains. *J. Immunol.* 170: 6099–6106.
19. Snapper, C. M., M. R. Kehry, B. E. Castle, and J. J. Mond. 1995. Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. *J. Immunol.* 154: 1177–1187.
20. Snapper, C. M., and J. J. Mond. 1996. A model for induction of T cell-independent humoral immunity in response to polysaccharide antigens. *J. Immunol.* 157: 2229–2233.
21. Mond, J. J., A. Lees, and C. M. Snapper. 1995. T cell-independent antigens type 2. *Annu. Rev. Immunol.* 13: 655–692.
22. Blander, J. M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440: 808–812.
23. Barton, G. M., and R. Medzhitov. 2002. Control of adaptive immune responses by Toll-like receptors. *Curr. Opin. Immunol.* 14: 380–383.
24. Kang, Y. S., J. Y. Kim, S. A. Bruening, M. Pack, A. Charalambous, A. Pritsker, T. M. Moran, J. M. Loeffler, R. M. Steinman, and C. G. Park. 2004. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen. *Proc. Natl. Acad. Sci. USA* 101: 215–220.
25. Lanoue, A., M. R. Clatworthy, P. Smith, S. Green, M. J. Townsend, H. E. Jolin, K. G. Smith, P. G. Fallon, and A. N. McKenzie. 2004. SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. *J. Exp. Med.* 200: 1383–1393.
26. Arredouani, M., Z. Yang, Y. Ning, G. Qin, R. Soininen, K. Tryggvason, and L. Kobzik. 2004. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J. Exp. Med.* 200: 267–272.
27. Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, Jr., and J. G. Cyster. 2008. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat. Immunol.* 9: 54–62.
28. Ferguson, A. R., M. E. Youd, and R. B. Corley. 2004. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int. Immunol.* 16: 1411–1422.
29. Groeneveld, P. H., T. Erich, and G. Kraal. 1986. The differential effects of bacterial lipopolysaccharide (LPS) on splenic non-lymphoid cells demonstrated by monoclonal antibodies. *Immunology* 58: 285–290.
30. Mueller, C. G., I. Cremer, P. E. Paulet, S. Niida, N. Maeda, S. Lebeque, W. H. Fridman, and C. Sautes-Fridman. 2001. Mannose receptor ligand-positive cells express the metalloprotease decysin in the B cell follicle. *J. Immunol.* 167: 5052–5060.
31. Karlsson, M. C., R. Guinamard, S. Bolland, M. Sankala, R. M. Steinman, and J. V. Ravetch. 2003. Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. *J. Exp. Med.* 198: 333–340.
32. Martinez-Pomares, L., M. Kosco-Vilbois, E. Darley, P. Tree, S. Herren, J. Y. Bonnefoy, and S. Gordon. 1996. Fc chimeric protein containing the cysteine-rich domain of the murine mannose receptor binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers. *J. Exp. Med.* 184: 1927–1937.
33. Huang, N. N., S. B. Han, I. Y. Hwang, and J. H. Kehrl. 2005. B cells productively engage soluble antigen-pulsed dendritic cells: visualization of live-cell dynamics of B cell-dendritic cell interactions. *J. Immunol.* 175: 7125–7134.
34. Wykes, M., A. Pombo, C. Jenkins, and G. G. Macpherson. 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol.* 161: 1313–1319.
35. Bjorck, P., L. Flores-Romo, and Y.-J. Liu. 1997. Human interdigitating dendritic cells directly stimulate CD40-activated naive B cells. *Eur. J. Immunol.* 27: 1266–1274.
36. Kushnir, N., L. Liu, and G. G. MacPherson. 1998. Dendritic cells and resting B cells form clusters in vitro and in vivo: T cell independence, partial LFA-1 dependence, and regulation by cross-linking surface molecules. *J. Immunol.* 160: 1774–1781.
37. Bergtold, A., D. D. Desai, A. Gavhane, and R. Clynes. 2005. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity* 23: 503–514.
38. Attanavanich, K., and J. F. Kearney. 2004. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J. Immunol.* 172: 803–811.
39. Song, H., and J. Cerny. 2003. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J. Exp. Med.* 198: 1923–1935.
40. Kang, Y. S., S. Yamazaki, T. Iyoda, M. Pack, S. A. Bruening, J. Y. Kim, K. Takahara, K. Inaba, R. M. Steinman, and C. G. Park. 2003. SIGN-R1, a novel C-type lectin expressed by marginal zone macrophages in spleen, mediates uptake of the polysaccharide dextran. *Int. Immunol.* 15: 177–186.
41. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5: 953–964.
42. Itano, A. A., S. J. McSorley, R. L. Reinhardt, B. D. Ehst, E. Ingulli, A. Y. Rudensky, and M. K. Jenkins. 2003. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* 19: 47–57.
43. Grdic, D., L. Ekman, K. Schon, K. Lindgren, J. Mattsson, K. E. Magnusson, P. Ricciardi-Castagnoli, and N. Lycke. 2005. Splenic marginal zone dendritic cells mediate the cholera toxin adjuvant effect: dependence on the ADP-ribosyltransferase activity of the holotoxin. *J. Immunol.* 175: 5192–5202.
44. Liu, Y. J., H. Kanzler, V. Soumelis, and M. Gilliet. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol.* 2: 585–589.
45. Tew, J. G., R. P. Phipps, and T. E. Mandel. 1980. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* 53: 175–201.
46. Szakal, A. K., M. H. Kosco, and J. G. Tew. 1989. Microanatomy of lymphoid tissue during humoral immune responses: structure function relationships. *Annu. Rev. Immunol.* 7: 91–109.
47. Wu, Z. Q., Q. Vos, Y. Shen, A. Lees, S. R. Wilson, D. E. Briles, W. C. Gause, J. J. Mond, and C. M. Snapper. 1999. In vivo polysaccharide-specific IgG isotype responses to intact *Streptococcus pneumoniae* are T cell dependent and require CD40- and B7-ligand interactions. *J. Immunol.* 163: 659–667.
48. Wu, Z. Q., A. Q. Khan, Y. Shen, J. Schartman, R. Peach, A. Lees, J. J. Mond, W. C. Gause, and C. M. Snapper. 2000. B7 requirements for primary and secondary protein- and polysaccharide-specific Ig isotype responses to *Streptococcus pneumoniae*. *J. Immunol.* 165: 6840–6848.
49. Khan, A. Q., G. Sen, S. Guo, O. N. Witte, and C. M. Snapper. 2006. Induction of in vivo antipolysaccharide immunoglobulin responses to intact *Streptococcus pneumoniae* is more heavily dependent on Btk-mediated B-cell receptor signaling than antiprotein responses. *Infect. Immun.* 74: 1419–1424.

50. Khan, A. Q., A. Lees, and C. M. Snapper. 2004. Differential regulation of IgG anti-capsular polysaccharide and antiprotein responses to intact *Streptococcus pneumoniae* in the presence of cognate $CD4^+$ T cell help. *J. Immunol.* 172: 532–539.
51. Guttormsen, H. K., A. H. Sharpe, A. K. Chandraker, A. K. Brigtsen, M. H. Sayegh, and D. L. Kasper. 1999. Cognate stimulatory B-cell-T-cell interactions are critical for T-cell help recruited by glycoconjugate vaccines. *Infect. Immun.* 67: 6375–6384.
52. Guttormsen, H. K., L. M. Wetzler, R. W. Finberg, and D. L. Kasper. 1998. Immunologic memory induced by a glycoconjugate vaccine in a murine adoptive lymphocyte transfer model. *Infect. Immun.* 66: 2026–2032.
53. Chattopadhyay, G., A. Q. Khan, G. Sen, J. Colino, W. Dubois, A. Rubtsov, R. M. Torres, M. Potter, and C. M. Snapper. 2007. Transgenic expression of Bcl-x_L or Bcl-2 by murine B cells enhances the in vivo antipolysaccharide, but not antiprotein, response to intact *Streptococcus pneumoniae*. *J. Immunol.* 179: 7523–7534.
54. Chen, Q., G. Sen, and C. M. Snapper. 2006. Endogenous IL-1R1 signaling is critical for cognate $CD4^+$ T cell help for induction of in vivo type 1 and type 2 antipolysaccharide and antiprotein Ig isotype responses to intact *Streptococcus pneumoniae*, but not to a soluble pneumococcal conjugate vaccine. *J. Immunol.* 177: 6044–6051.
55. Lees, A., B. L. Nelson, and J. J. Mond. 1996. Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate for use in protein-polysaccharide conjugate vaccines and immunological reagents. *Vaccine* 14: 190–198.
56. Briles, D. E., J. D. King, M. A. Gray, L. S. McDaniel, E. Swiatlo, and K. A. Benton. 1996. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 14: 858–867.
57. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745.
58. Sen, G., Q. Chen, and C. M. Snapper. 2006. Immunization of aged mice with a pneumococcal conjugate vaccine combined with an unmethylated CpG-containing oligodeoxynucleotide restores defective immunoglobulin G antipolysaccharide responses and specific $CD4^+$-T-cell priming to young adult levels. *Infect. Immun.* 74: 2177–2186.
59. AlonsoDeVelasco, E., A. F. Verheul, J. Verhoef, and H. Snippe. 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. *Microbiol. Rev.* 59: 591–603.
60. Peeters, C. C., A. M. Tenbergen-Meekes, J. T. Poolman, B. J. Zegers, and G. T. Rijkers. 1992. Immunogenicity of a *Streptococcus pneumoniae* type 4 polysaccharide-protein conjugate vaccine is decreased by admixture of high doses of free saccharide. *Vaccine* 10: 833–840.
61. Rodriguez, M. E., G. P. van den Dobbelsteen, L. A. Oomen, O. de Weers, L. van Buren, M. Beurret, J. T. Poolman, and P. Hoogerhout. 1998. Immunogenicity of *Streptococcus pneumoniae* type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled polysaccharide on the antigen-specific immune response. *Vaccine* 16: 1941–1949.
62. Aichele, P., J. Zinke, L. Grode, R. A. Schwendener, S. H. Kaufmann, and P. Seiler. 2003. Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. *J. Immunol.* 171: 1148–1155.
63. Kraal, G., H. Ter Hart, C. Meelhuizen, G. Venneker, and E. Claassen. 1989. Marginal zone macrophages and their role in the immune response against T-independent type 2 antigens: modulation of the cells with specific antibody. *Eur. J. Immunol.* 19: 675–680.
64. Shapiro-Shelef, M., and K. Calame. 2005. Regulation of plasma-cell development. *Nat. Rev. Immunol.* 5: 230–242.
65. MacLennan, I. C., K. M. Toellner, A. F. Cunningham, K. Serre, D. M. Sze, E. Zuniga, M. C. Cook, and C. G. Vinuesa. 2003. Extrafollicular antibody responses. *Immunol. Rev.* 194: 8–18.
66. Nemerow, G. R., C. Mold, V. K. Schwend, V. Tollefson, and N. R. Cooper. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J. Virol.* 61: 1416–1420.
67. Robbins, J. B., and R. Schneerson. 1990. Polysaccharide-protein conjugates: a new generation of vaccines. *J. Infect. Dis.* 161: 821–832.
68. Avery, O. T., and W. F. Goebel. 1931. Chemo-immunological studies on conjugated carbohydrate-proteins: V. The immunological specificity of an antigen prepared by combining the capsular polysaccharide of type III pneumococcus with foreign protein. *J. Exp. Med.* 54: 437–447.
69. Leonard, E. G., D. H. Canaday, C. V. Harding, and J. R. Schreiber. 2003. Antigen processing of the heptavalent pneumococcal conjugate vaccine carrier protein CRM_{197} differs depending on the serotype of the attached polysaccharide. *Infect. Immun.* 71: 4186–4189.
70. Nossal, G. J., C. M. Austin, J. Pye, and J. Mitchell. 1966. Antigens in immunity: XII. Antigen trapping in the spleen. *Int. Arch. Allergy Appl. Immunol.* 29: 368–383.
71. Mitchell, J., and A. Abbot. 1971. Antigens in immunity: XVI. A light and electron microscope study of antigen localization in the rat spleen. *Immunology* 21: 207–224.
72. Brown, J. C., G. Harris, M. Papamichail, V. S. Sljivic, and E. J. Holborow. 1973. The localization of aggregated human -globulin in the spleens of normal mice. *Immunology* 24: 955–968.
73. Gray, D., D. S. Kumararatne, J. Lortan, M. Khan, and I. C. MacLennan. 1984. Relation of intra-splenic migration of marginal zone B cells to antigen localization on follicular dendritic cells. *Immunology* 52: 659–669.
74. Ochsenbein, A. F., and R. M. Zinkernagel. 2000. Natural antibodies and complement link innate and acquired immunity. *Immunol. Today* 21: 624–630.
75. Matsushita, M., and T. Fujita. 2001. Ficolins and the lectin complement pathway. *Immunol. Rev.* 180: 78–85.
76. Youd, M. E., A. R. Ferguson, and R. B. Corley. 2002. Synergistic roles of IgM and complement in antigen trapping and follicular localization. *Eur. J. Immunol.* 32: 2328–2337.
77. Breukels, M. A., A. Zandvoort, G. T. Rijkers, M. E. Lodewijk, P. A. Klok, G. Harms, and W. Timens. 2005. Complement dependency of splenic localization of pneumococcal polysaccharide and conjugate vaccines. *Scand. J. Immunol.* 61: 322–328.
78. Lopes-Carvalho, T., J. Foote, and J. F. Kearney. 2005. Marginal zone B cells in lymphocyte activation and regulation. *Curr. Opin. Immunol.* 17: 244–250.
79. Phan, T. G., S. Gardam, A. Basten, and R. Brink. 2005. Altered migration, recruitment, and somatic hypermutation in the early response of marginal zone B cells to T cell-dependent antigen. *J. Immunol.* 174: 4567–4578.
80. Allen, C. D., T. Okada, and J. G. Cyster. 2007. Germinal-center organization and cellular dynamics. *Immunity* 27: 190–202.