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Differential Idiotype Utilization for the In Vivo Type 14 Capsular Polysaccharide-Specific Ig Responses to Intact *Streptococcus pneumoniae* versus a Pneumococcal Conjugate Vaccine

Jesus Colino,* Leah Duke,* Swadhinya Arjunaraja,† Quanyi Chen,* Leyu Liu,‡ and Clifford M. Snapper*

Murine IgG responses specific for the capsular polysaccharide (pneumococcal capsular polysaccharide serotype 14; PPS14) of *Streptococcus pneumoniae* type 14 (Pn14), induced in response to intact Pn14 or a PPS14–protein conjugate, are both dependent on CD4+ T cell help but appear to use marginal zone versus follicular B cells, respectively. In this study, we identify an idiotype (44.1-Id) that dominates the PPS14-specific IgG, but not IgM, responses to intact Pn14, isolated PPS14, and Group B *Streptococcus* (strain COH1-11) expressing capsular polysaccharide structurally identical to PPS14. The 44.1-Id, however, is not expressed in the repertoire of natural PPS14-specific Abs. In distinct contrast, PPS14-specific IgG responses to a soluble PPS14–protein conjugate exhibit minimal usage of the 44.1-Id, although significant 44.1-Id expression is elicited in response to conjugate attached to particles. The 44.1-Id elicited in response to intact Pn14 was expressed in similar proportions among all four IgG subclasses during both the primary and secondary responses. The 44.1-Id usage was linked to the *Igh*<sup>a</sup>, but not *Igh*<sup>b</sup>, allotype and was associated with induction of relatively high total PPS14-specific IgG responses. In contrast to PPS14–protein conjugate, avidity maturation of the 44.1-Id–dominant PPS14-specific IgG responses was limited, even during the highly boosted T cell-dependent PPS14-specific secondary responses to COH1-11. These results indicate that different antigenic forms of the same capsular polysaccharide can recruit distinct B cell clones expressing characteristic idiotypes under genetic control and suggest that the 44.1-Id is derived from marginal zone B cells. *The Journal of Immunology*, 2012, 189: 000–000.

Humoral immune responses to non-zwitterionic bacterial capsular polysaccharides (PS) have traditionally been considered T cell-independent (TI) (1). This conclusion was based almost exclusively on studies using isolated PS. Although these PS are degraded into smaller fragments within endosomes through an oxidative pathway (2), they fail to associate with MHC class II molecules necessary for recruitment of CD4+ T cell help (3, 4). Covalent linkage of immunogenic proteins and PS to create a soluble conjugate vaccine results in a T cell-dependent (TD) PS-specific IgG response and the induction of PS-specific memory (5–8). Although CD4+ T cells generated in response to a conjugate vaccine include individual specificities for both the protein and PS components (9), a recent study strongly suggests that PS-specific CD4+ T cells may be the dominant source of help for induction of PS-specific IgG (2). Conjugate vaccines have had a major impact on the prevention of infections by PS-encapsulated extracellular bacteria, especially in the infant population, although their use in adults remains controversial (10).

Although humoral immune responses to isolated PS and conjugate vaccines have been studied in some detail, the mechanism underlying PS-specific IgG responses to intact extracellular bacteria has been largely neglected. These latter studies are important in that the intact bacterium is the natural antigenic form of PS during infections with these pathogens that contributes to host protection (11). Intact bacteria are complex particulate Ags in which PS is associated with proteins in a noncovalent manner and with ligands that engage the innate immune system. Particulate Ags such as intact bacteria, in contrast to their soluble counterparts, exhibit unique immunologic properties. For example, particulates concentrate within the marginal zone of the spleen (12), are more efficiently internalized by APC (13, 14), although requiring more Ag processing time (15), and are presented poorly by B cells. Further, internalization of particulate Ag by APC results in quantitative and qualitative differences in the epitopes generated relative to soluble Ag (16). Finally, distinct differences between individual bacteria in the composition of their subcapsular domains may also impact the associated PS-specific response (17). Thus, PS associated with intact bacteria are likely to behave in a unique manner relative to isolated PS or conjugate vaccines.

Ab responses to isolated PS are oligoclonal (18, 19), reflecting the involvement of a few dominant B cell clones and probably the limited epitope diversity of PS. Dominant B cell clones can be...
identified by sequencing the V region of the BCR or serologically using anti-idiotypic Abs. Although the phenomenon of B cell dominance is poorly understood, dominant idiotype (Id) could be generated by random somatic mutation and selection of Ag-driven B cell clones or reflect the use of germline-encoded Ig genes, conserved during evolution. The frequent linkage observed between the allotype of the H chain of Igks (Igh-C) and the individual expression of the dominant Id (20–23) suggest that they may be under the control of regulatory genes linked to the Igh-C allotype (21). Individual lack of expression of a particular dominant Id does not typically affect the quantity of the Ag-specific Ig response, with the exception of the murine response to o1-3 dextran (22).

Human primary Ig responses after immunization with isolated PS, despite being TI, exhibit somatic mutations (19, 24, 25) at a rate that is similar to that observed at least early in the response to conjugate vaccines (19, 25). To explain this surprising result, it has been proposed that vaccination of adults with isolated or protein-conjugated PS reactivates PS-specific memory B cells induced in response to PS conjugated to protein cell wall components of extracellular bacteria to which the host had been previously exposed (25, 26). This PS is perhaps in a TD form via association with bacterial protein. In this regard, studies from our laboratory utilizing inactivated, intact Streptococcus pneumoniae capsular type 14 (Pn14) demonstrated that the murine pneumococcal PS (pneumococcal capsular polysaccharide serotype 14; PPS14)-specific IgG, but not IgM, response was indeed dependent on CD4+ T cells and B7–CD28 and CD40–CD40L interactions, similar to a pneumococcal conjugate vaccine (27). Nevertheless, the PPS14-specific IgG response to intact Pn14, in contrast to the conjugate, exhibited accelerated primary kinetics and was mediated by an ICOS-independent, apoptosis-prone, extrafollicular pathway that failed to produce a boosted response after secondary immunization. Of note, the TD PPS14-specific IgG responses to Pn14 versus pneumococcal conjugate vaccine were dependent on marginal zone (MZ) versus follicular (FO) B cells, respectively (13, 28). Thus, the PPS14-specific Ig response to intact Pn14, in contrast to conjugate, appears to combine both TI and TD features. Surprisingly, however, a more recent study from our laboratory demonstrated a highly boosted, ICOS-dependent PS-specific IgG secondary response to intact Streptococcus agalactiae (Group B Streptococcus capsular type III; GBS-III) expressing a capsular PS identical to PPS14 (29). These studies emphasize the importance of the subcapsular domain in influencing the associated PS-specific Ig response.

In this report, we have extended our studies on the distinct humoral immune responses to different antigenic forms of PPS14 and identified an Id (44.1-Id) that dominates the PPS14-specific IgG response to intact Pn14 and to isolated PPS14, but not to a soluble PPS14–protein conjugate. However, the usage of the 44.1-Id is partly restored in the responses to PPS14–protein conjugate coupled to particles. In addition, the 44.1-Id is elicited in mice expressing the Igch, but not Igkch, allotype, and this is associated with relatively high serum titers of PPS14-specific IgG in Igch-expressing mouse strains. Collectively, these and our previous data demonstrate that distinct forms of the same PS Ag target distinct B cell clones expressing unique Id under genetic control. Additionally, although not directly demonstrated in this study, these data further suggest that 44.1-Id usage is relatively specific for the MZ B cell subset. These results provide a mechanism to explain our previous observation that immunization with intact bacteria does not result in a more robust PS-specific IgG response upon subsequent vaccination with soluble conjugates of the same PS that is covalently linked to protein, and vice versa, a phenomenon that may have clinical relevance to the future design of PS-based vaccines.
Production of syngeneic anti-Id 2B6.2 mAb specific for 44.1 mAb

The 2B6.2 B cell hybridoma secreting IgG1k mAb (clone 2B6.2) specific for an Id of 44.1 was made as follows: female BALB/c mice were immunized s.c. with 50 μg 44.1–KLH in CFA and boosted 6 wk later with 50 μg 44.1–KLH in IFA. Three weeks after the second immunization, mice received an i.p. injection of 44.1–KLH (10 μg) in saline. Three days after the i.p. booster, spleens were taken for hybridoma production using previously published methods (33). Hybridomas were screened using ELISA for the ability to inhibit the binding of 44.1 to PS14. An IgG1k anti-Id mAb (clone SC11.1) specific for the anti-PepA mAb, DC10-IA5, was similarly produced and used as a negative control.

Purification and biotinylation of mAb

All mAbs were purified from hybridoma culture supernatant. IgG was purified by protein G Sepharose affinity chromatography and IgM by ammonium sulfate precipitation. IgG mAbs were oxidized with 10 mM metaperiodate (Sigma) for conjugation to biotin-LC-hydrazide through the IgG carbohydrate moieties (34).

Determination of the fine specificity of anti-PPS14 mAb by radioantigen binding assay inhibition

The ability of different monosaccharide and disaccharide motifs of PPS14 (lactose, aminophenyl-lactoside, N-acetylamino-saccharose, and melibiose) to inhibit the binding of a panel of anti-PPS14 mAbs to PS14 was determined by radioantigen binding assay (RABA) (18). All the inhibitors were tested at a concentration of 40 mM.

Capture-sandwich ELISA to determine Id specificity of 2B6.2 anti-Id mAb

Immuno-4 HB-X microtiter plates (Dynex Technologies, Milford, MA) were coated with 1 μg/ml of one of the indicated PPS14-specific (including 44.1) or control mAbs. Wells were blocked with 2% BSA in PBS (PBS–BSA), washed with PBS, and then incubated overnight at 4°C with serial dilutions of purified 2B6.2 anti-Id mAb. Captured 2B6.2 was detected by incubation at 4°C with 1 μg/ml of biotinylated 44.1 mAb. Negative controls included wells not coated with mAb and wells coated with each mAb but not incubated with 2B6.2. Wells were then incubated for 1 h at 37°C with a conjugate of streptavidin and alkaline phosphatase (streptavidin–AP; Southern Biotechnology Associates, Birmingham, AL) and the enzymatic reaction developed as above.

Indirect ELISA to measure the capacity of 2B6.2 to inhibit binding of 44.1 and PS14 in solution

Wells were coated with 44.1, blocked with PBS–BSA, then incubated with decreasing amounts of 2B6.2 mAb or purified PS14 for 18 h at 4°C. SC11.1 was used as a negative control. Wells incubated with PS14 were used as positive controls of inhibition and as standards to compare the potency of 2B6.2 and PS14 to inhibit the PS14 binding to 44.1 mAb. Wells were subsequently incubated for 18 h at 4°C with biotinylated PS14 (65 ng/ml), followed by incubation with streptavidin–AP.

Indirect ELISA to measure the capacity of 2B6.2 to inhibit the binding between 44.1 and PS14 in solid phase

Inhibitory mixtures containing decreasing amounts of 2B6.2 and a constant amount (0.5 μg/ml) of biotinylated 44.1 were incubated for 18 h at 4°C. This concentration of 44.1 was the highest in the linear part of the titration curve of the binding to PS14 in this assay. To compare the inhibitory capacities of 2B6.2 and PS14, mixtures with decreasing amounts of PS14 and 44.1 were also included in the assay and used as positive controls of inhibition. After incubation, the inhibitory mixtures were transferred to wells previously coated with 1 μg/ml PS14. Other controls included inhibition mixtures with the inhibitor but without 44.1 (background) and without inhibitor but containing 44.1 (positive control, no inhibition). Mixtures of 44.1 and SC11.1 were also used as negative controls. After 18 h at 4°C, wells were incubated for 1 h at 37°C with streptavidin–AP and the enzymatic reaction developed as above.

Mouse immunizations and sera collection

Unless indicated, mice were immunized i.p. at day 0 and boosted on day 14. Heat-killed bacteria were suspended in saline and the PS14–PspA conjugate adsorbed on 13 μg alun (Brentnag Biosector, Frederikssund, Denmark) mixed with 25 μg of a stimulatory 30-mer CpG-containing oligodeoxynucleotide (CpG-ODN). Sera were prepared from blood obtained through the tail vein.

Inhibition ELISA to quantify serum 44.1-Id+ PPS14-specific Ig

The inhibition ELISA described earlier was adapted to quantify 44.1-Id+ PPS14-specific IgG1 and IgG and semiquantitatively estimate 44.1-Id+ IgM. Serum titers of PPS14-specific Ig were previously measured in the absence of inhibitor. The dilution of each individual serum sample at the inflection point of the titration curve and dilutions 3-fold lower were selected for analysis. Preimmune sera were tested to a 1/25 final dilution due to the low content of PPS14-specific Ig. Sera producing OD405nm <0.5 were discarded. Inhibition mixtures were prepared by mixing sera at the specified dilutions with 10 μg/ml 2B6.2 and incubated for 24 h at 4°C before being transferred to wells previously coated with 1 μg/ml PS14 and blocked with PBS–BSA. MOPC-21 or SC11.1, instead of 2B6.2, were included as negative controls (i.e., no inhibition). This concentration of 2B6.2 completely inhibits the binding of 44.1 to PS14 in the range of 0.06–500 ng of 44.1. All ELISA determinations were conducted using PPS14-specific Ig concentrations within this range (<100 ng/ml), resulting in OD405nm within the linear portion of the titration curve. Three replicate plates were included in each analysis. In each plate, serial dilutions of 44.1 (150–0.03 ng/ml) were included in the absence (standard curve) or presence (control for inhibition) of 2B6.2 inhibitor. In the final step, plates were incubated with alkaline phosphatase (AP)-conjugated polyclonal goat anti-mouse IgG, IgE, or IgM to develop the binding of 44.1-Id+ IgG or IgM. Serum concentrations of PPS14-specific IgM content were determined in similar manner but expressed semi-quantitatively as titer. Titers were the dilution of sera giving an OD405nm equal to 1.0 in reference to a titration curve of pooled PPS14-specific IgM-containing sera. The low and variable functional avidity of IgM precludes use of a PPS14-specific IgM mAb for quantitation. Nevertheless, when the PPS14-specific IgG titers were used to obtain the percentage of 44.1-Id+ IgG1 nearly identical results were obtained using the quantitative method. In some experiments, this semi-quantitative approach was used to estimate 44.1-Id expression in the other IgG subclasses (IgG2a, IgG2b, and IgG3).

Determination of the avidity of serum PPS14-specific IgG

The average avidity of serum PPS14-specific IgG was determined essentially as described previously (35, 36). Briefly, sera dilutions producing absorbance readings at the top of the titration curve were incubated in 16 wells coated with 5 μg/ml PPS14. Duplicate wells were then incubated for 15 min at room temperature with increasing concentrations (0 to 4 M) of the chaotrope, sodium thiocyanate (NaSCN; Fluka Analytical/Sigma). Bound PPS14 is not released by treatment with NaSCN. After incubation, NaSCN and eluted Ig were removed by extensive washings, and the wells were then incubated with AP-conjugated polyclonal goat anti-mouse IgG for 1 h at 37°C. The enzymatic reaction was developed until the OD405nm in the controls without NaSCN reached predetermined values. All plates included dilutions of 44.1 (75–0.2 ng/ml) as a standard. The amount of IgG that remained bound to PS14 after treatment with NaSCN was determined by extrapolation from this standard curve. Avidities were expressed as avidity index (AI) (i.e., the molar concentration of NaSCN eluting 50% of PPS14-specific IgG in the serum sample). The avidity of the interaction is proportional to the resistance to elution by the chaotrope (35).

Determination of the avidity of 44.1-Id+ PPS14-specific IgG

The AI of 44.1-Id+ PPS14-specific IgG was determined as for the total PPS14-specific IgG, except that the serum was previously incubated overnight with 10 μg/ml 2B6.2 to inhibit the binding of 44.1-Id+ IgG to PS14. Avidity of total and 44.1-Id+ IgG was always determined in parallel.
Statistics

Data were expressed as geometric mean ± SEM of the individual serum samples. Significance between groups was determined by the Student t test, and p values <0.05 were considered statistically significant.

Results

**The PPS14-specific 44.1 mAb belongs to the VHV/Vk21 family**

The IgG1 44.1 mAb is highly specific for PPS14 and shows no cross-reactivity with cell wall and other S. pneumoniae PS or host tissues, with the exception of a cross-reactive PPS14 glycoconjugate constitutively expressed by murine dendritic cell exosomes (37). Analysis of the rearranged sequences of the variable regions of the H and L chain genes, using the IgBLAST database (http://www.ncbi.nlm.nih.gov/igblast/), indicates that 44.1 mAb belongs to the VHV/Vk21 protein family. The H chain of 44.1 mAb is derived from the J558 VH germline family rearranged to a linear backbone of Table I. Because the repeating unit of PPS14 is formed by 9.2 mAbs but minimally or not at all with 17.1 and 23.1 mAbs aminophenyl-lactoside interacted significantly only with 44.1 and cross-reactivity with cell wall and other The IgG1 and JH2 gene. There is no evidence of DH gene usage. The L chain of 44.1 mAb uses the 21-12 Vκ germline subfamily and Jκ2 genes.

**The 2B6.2 anti-Id mAb is specific for an Id expressed by the PPS14-specific 44.1 mAb**

Ab responses to purified PPS14 are known to be oligoclonal in humans (18). To determine the relevance of the B cell clone represented by the 44.1 mAb in the murine response to PPS14, we developed a syngeneic anti-Id mAb (clone B6.2) from splenocytes of BALB/c mice immunized with a covalent conjugate of 44.1 and KLH. The specificity of the 2B6.2 mAb was initially tested against a panel of PPS14-specific mAb of close but different fine specificity (Table I). All anti-PPS14 mAbs interacted with different avidities with N-acetyl-lactosamine but poorly or not at all with melibiose (Galα1→6Glc). However, lactose and aminophenyl-lactoside interacted significantly only with 44.1 and 9.2 mAbs but minimally or not at all with 17.1 and 23.1 mAbs (Table I). Because the repeating unit of PPS14 is formed by a linear backbone of −(4Glc) (44.1-Id) expressed by 44.1, which may not be shared by Abs with specificities similar to 44.1.

2B6.2 recognizes an Id in or near the paratope of 44.1

To determine the topological relation between the Id recognized by 2B6.2 and the paratope of 44.1, different formats of an Ag-binding inhibition ELISA were used (Fig. 2B, 2C). The paratope represents a 15–22 aa region of the Ab Fv region that is the Ag binding site. As shown in Fig. 2B, 2B6.2, but not an anti-Id mAb (clone 5C11.1) specific for the anti-PspA mAb, DC10-IA5, completely inhibited the interaction between 44.1 (coated to the well, capture ELISA) and biotinylated PPS14 in solution. Similar results were obtained when both 44.1 and 2B6.2 were allowed to interact in solution before transfer to PPS14-coated plates (Fig. 2C, indirect inhibition ELISA). 2B6.2 has similar inhibitory potency as purified PPS14 (Fig. 2B, 2C). These results demonstrate that the Id recognized by 2B6.2 is the paratope of 44.1, or is at least located close enough to prevent the binding of PPS14 by steric hindrance.

Using the IC50 from the indirect inhibition ELISA (Fig. 2C), the relative avidity of 2B6.2 for 44.1 is estimated to be 2.2 × 1010 M−1. However, although 2B6.2 has similar inhibitory potency as purified PPS14 (Fig. 2B, 2C), its avidity of binding to 44.1 is likely to be at least one order of magnitude more than the interaction of 44.1 and PPS14. Thus, the repetitive unit of PPS14 (tetrascarbohydrate, m.w. ≈ 761) is the minimal structure containing a single PPS14-specific epitope (40, 41), as evidenced by the ability of aminophenyl-lactoside to provide most of the energy of binding for 44.1 (Table I). Due to steric hindrance, only 1 in 3.4 repeating units of PPS14 are occupied by specific Ab at saturation (42), indicating that 39 ± 19 more PPS14 epitopes than 2B6.2 paratopes are needed to provide the same inhibition. Correcting the PPS14 molar concentration by the number of functional epitopes at saturation, the relative avidity of 44.1 for an epitope on PPS14 is 9.6 × 104 M−1 (from Fig. 2C). The higher avidity interaction of 44.1 and 2B6.2 is supported by the slightly greater slope of the inhibitory curves obtained for 2B6.1 than for PPS14 (43), which is more evident when the 44.1 is multimerized on the surface of the plastic well (Fig. 2B) than in solution (Fig. 2C). In summary, the paratope of 44.1 is part of the Id recognized by 2B6.2, and the interactions of 44.1 with PPS14 (through the paratope) and 2B6.2 (through the Id) are of moderate to high avidity. BALB/c mice elicit a PPS14-specific IgG response to intact Pn14 that is dominant for the 44.1-Id

The 44.1 mAb was derived from BALB/c mice immunized with intact Pn14. Thus, we wished to determine the relative contribution of the 44.1-Id to the Pn14-induced PPS14-specific Ig response of the parental BALB/c mouse. An inhibition ELISA was used based on the ability of 2B6.2 completely to inhibit the binding of 44.1-Id+ Ig to PPS14 (Fig. 2). Thus, the contribution of the 44.1-Id can be

![Image](https://www.jimmunol.org/)
estimated as the percentage of the total amount of PPS14-specific IgM or IgG that is inhibited by 2B6.2. The IgG anti-PPS14 response elicited 7 d after immunization with Pn14 (strain R6-14) was dominated by 44.1-Id+ IgG (60.6 ± 9.5%). This Id dominance remained unchanged (p = 0.71) after secondary immunization (Fig. 3). In a further, retrospective analysis of serum samples from several past experiments, some individual mouse variation in the usage of the 44.1-Id was noted, with half of the mice using the 44.1-Id in a dominant fashion (>65% IgG anti-PPS14) and half in sizable amounts (>15% to <65% IgG anti-PPS14) (Supplemental Table I). The average contribution of the 44.1-Id to the IgG anti-PPS14 response remained relatively constant during at least the first 2 wk after a primary or a secondary immunization with Pn14 (Supplemental Table I). Sera collected as early as day 5 post-immunization had similar 44.1-Id content as those collected at day 7 (p > 0.2, data not shown). Thus, overall, the kinetics of the 44.1-Id and 44.1-Id+ PPS14-specific IgG responses were similar. Pn14 elicits a mixed type 1 and type 2 PPS14-specific IgG isotype response (44, 45). However, no preferential usage of the 44.1-Id was found among the four IgG isotypes elicited. Thus, although the 44.1-Id usage for IgG1 was the lowest (45.0 ± 6.5%), it was not significantly different (p > 0.1) from the percentage of 44.1-Id+ IgG2a (50.7 ± 5.5%), IgG2b (57.1 ± 5.3%), or IgG3 (63.2 ± 7.4%).

The prevalent usage of the 44.1-Id in the PPS14-specific IgG response to Pn14 contrasts with the lack of detectable 44.1-Id in the preimmunization titers of natural PPS14-specific IgM and IgG (Fig. 3, Supplemental Table I). Percentages <10% and lower than two times the SEM were considered, in practical terms, negative. Natural 44.1-Id+ IgM was also undetectable in a capture ELISA in which Ab captured with 2B6.2 was detected with AP-labeled goat anti-mouse IgM. This lack of detection is unlikely due to the low amounts of PPS14-specific Ig present in the sera of naive mice. On the contrary, because we used an inhibition assay for the detection of 44.1-Id, lower amounts of PPS14-specific Ig could result in an overestimation of the percentage of 44.1-Id+ IgG. Moreover, there was no correlation (r² = 0.00012) between the concentration of serum PPS14-specific IgG, over a range of 0.32–16.5 μg/ml, and the percentage of 44.1-Id+ IgG in Pn14-immunized BALB/c mice. This is consistent with the deviation from the germline Vλ sequence of the 44.1 hybridoma. This conclusion is in agreement with the minor usage of the 44.1-Id in the PPS14-specific IgM response to Pn14 (18.4 ± 2.5%), which is significantly lower than that of the IgG response (p = 2.9 × 10⁻¹², paired t test), but still significantly higher than in preimmune sera (2.7 ± 3.0%, p = 0.013) (Fig. 3, Supplemental Table I). Secondary immunization decreased the percentage of 44.1-Id+ IgM (Fig. 3) to preimmune levels (p = 0.93) although this reduction was not significant (p > 0.13) when a greater number of sera were sampled (Supplemental Table I). No correlation was observed (r² = 0.029) between the percentage of 44.1-Id+ IgM and serum titers of PPS14-specific IgM. In summary, these data strongly suggest that the expression of the 44.1-Id, which is absent from the natural repertoire, is generated or selected, in an IgG isotype-independent manner, after Pn14-mediated activation of PPS14-specific B cell clones that rapidly undergo class switching to IgG.

The 44.1-Id is dominant in PPS14-specific IgG responses to Pn14 in Igκb, but not Igλb, mouse strains

The influence of mouse genetic background on elicitation of the 44.1-Id was next determined. BALB/c mice (H-2b) were compared with allogeneic C57BL/6 (H-2b) and FVB (H2q) mouse strains. FVB is an inbred strain derived from a colony of outbred Swiss mice (46) and expresses an Igk allotype closer to BALB/c (Ighκ) than to C57BL/6 (Ighκ). The expression of the 44.1-Id was even more prevalent in the primary and secondary PPS14-specific IgG responses of FVB mice (80 ± 5%) than in BALB/c mice (Fig. 3). The usage of the 44.1-Id in the elicited PPS14-specific IgG1 and IgG2a, but not in IgG3, was higher in these mice, but as in BALB/c, 44.1-Id was not detected in the natural PPS14-specific Ig from naive mice. In distinct contrast, the 44.1-Id made only a minor contribution (14–18%) to the overall PPS14-specific IgG response in C57BL/6 mice and a negligible contribution to the PPS14-specific IgM response (<8%), which are not significantly (p > 0.4) different from preimmune sera (Fig. 3). To investigate further whether the expression of the 44.1-Id was linked to the Igh-Cκb allotype, as described for other antigenic systems (20–23), PPS14-specific responses to R6-14 in C.BKa mice were determined (Fig. 3). This congenic strain expresses the Igh-Cκb allotype from C57BL/Ka mice, in an essentially BALB/c background. Of note, C.BKa mice were similar to C57BL/6 mice in their minor expression of the 44.1-Id by PPS14-specific IgG and IgM in response to Pn14 on day 7 (Fig. 3) and day 14 (data not shown). Overall serum titers of PPS14-specific IgG in response to R6-14 in both C57BL/6 and C.BKa mice were similar (p > 0.26) and markedly lower than in BALB/c or FVB mice (Fig. 3) for all IgG subclasses (i.e., Fig. 3 for IgG1; data not shown). PPS14-specific Ig responses in C57BL/6 and C.BKa mice only differed in the amount of IgG1 elicited, which was detectable in C.BKa (94 ± 16 ng/ml) and barely detectable in 20% of C57BL/6 (24 ± 19 ng/ml). Thus, these results establish a linkage between the expression of the 44.1-Id and the Igh-Cκb, but not Igh-Cλb, allotype,
and strongly suggests that the magnitude of the PPS14-specific IgG responses to Pn14 are linked to both the H chain allotype, but not H-2 haplotype, and the ability of the individuals to generate an efficient combinatorial site represented by the 44.1-Id.

The 44.1-Id is expressed in BALB/c mice in response to isolated PPS14 and intact Pn14 but not to PPS14–PspA conjugate

Capsular PS expressed by Gram-positive bacteria, such as Pn14, is covalently linked to a thick, underlying cell wall peptidoglycan to which a number of proteins are also covalently attached (47, 48). Thus, the immune response to intact Pn14 might resemble that elicited in response to a PPS14–protein conjugate. In this regard, the PPS14-specific IgG response to Pn14, in contrast to isolated PPS14, is dependent on CD4+ T cell help, although in contrast to a PPS14–protein conjugate, secondary immunization fails to elicit a boosted response (17) (Fig. 4). Of note, the PPS14-specific IgG responses to intact Pn14 and pneumococcal conjugate are derived from MZ versus FO B cells, respectively (13, 28). Thus, we wished to compare the expression of the 44.1-Id in BALB/c mice immunized with these different PPS14-containing compositions.

Doses of each immunogen were selected to induce similar amounts of PPS14-specific IgG during the primary response. The contribution of the 44.1-Id to the PPS14-specific IgG response to isolated PPS14 was similar to that observed in response to intact R6-14, as well as minimal if any contribution of 44.1-Id to the IgM response (Fig. 4). In the experiment shown, PPS14 was admixed with alum and CpG-ODN, although neither altered the nature of the PPS14-specific IgM or IgG response (data not shown). In distinct contrast, the primary PPS14-specific IgG and IgG1 responses to PPS14–PspA conjugate exhibited no significant 44.1-Id expression (<7%). IgG1 is the major IgG subclass in response to PPS14–PspA. However, secondary immunization with PPS14–PspA conjugate elicited significant expression of 44.1-Id in the PPS14-specific IgG and IgG1 responses, although in contrast to isolated PPS14 and R6-14, this still represented only a minor contribution (~20%). In C57BL/6 mice, no significant expression of the 44.1-Id was observed in the PPS14-specific IgG or IgM responses to PPS14–PspA (data not shown). Thus, the PPS14-specific IgG response to PPS14–PspA conjugate utilizes a different Id profile relative to that elicited by isolated PPS14 or intact Pn14.
The dominant expression of 44.1-Id is preserved during the boosted, secondary PPS14-specific IgG responses to GBS-III and its isogenic desialylated variant

The maintenance of the dominant 44.1-Id in the PPS14-specific IgG response to Pn14 may reflect, in contrast to PPS14–PspA conjugate, the inability of Pn14 to elicit a boosted, secondary PPS14-specific IgG response (Figs. 3–5), precluding the further expansion and potential somatic hypermutation of PPS14-specific B cell clones. To test this hypothesis, we used intact, inactivated GBS-III (strain COH1), a bacterium that expresses a PS structurally similar to PPS14, except for the presence of a terminal N-acetyl neuraminic acid linked (2→3) to the side chain of the β-d-Galp-(1→4) residue of the PPS14 repetitive unit (49). This modification is absent in the isogenic variant COH1-11, which expresses the desialylated PS structurally identical to PPS14 (31). In this regard, PS-specific Ig responses to both COH1 and COH1-11 are mostly cross-reactive with PPS14 (50). However, in contrast to Pn14, COH1-11 induce highly boosted, secondary PPS14-specific IgG as well as IgM responses (Fig. 5). In addition, secondary immunization with COH1-11 also boosts the PPS14-specific IgG response in mice primed with Pn14 (strain R6-14) (Fig. 5). The data illustrated in Fig. 5 indicate that the 44.1-Id is dominant in both the primary and boosted secondary PPS14-specific IgG responses to COH1-11 and in mice primed with R6-14 and boosted with COH1-11. Of note, mice primed with either R6-14 or COH1-11 followed by secondary immunization with COH1-11 elicited a significant increase in 44.1-Id usage in the boosted PPS14-specific IgM response (Fig. 5). Collectively, these data suggest that 44.1-Id+ B cell clones dominate the PPS14-specific Ig response to intact bacteria, in contrast to PPS14–protein conjugate vaccine, due to the physicochemical differences between the immunogens themselves. 44.1-Id+, in contrast to 44.1-Id−, PPS14-specific IgG undergoes minimal avidity maturation

Avidity maturation as a result of somatic hypermutation could result in the loss of the expression of the 44.1-Id. Thus, the persistent dominance of the 44.1-Id+ IgG responses to R6-14 and COH1-11 may be due to the lack of avidity maturation in these B cell clones or because changes in avidity do not affect the expression of the 44.1-Id. Therefore, we analyzed the average avidity of PPS14-specific IgG during the response to different immunogenic forms of PPS14 by measuring the eluted amount of PPS14-specific IgG at increasing concentrations of NaSCN (Fig. 6A). Although PPS14-specific IgG induced by immunization with R6-14 or COH1-11 underwent a significant (p = 0.004) increase in avidity between days 7 and 14 after priming with bacteria, the average avidity 7 d after secondary immunization remained unaffected or was reduced relative to the primary (Fig. 6A). This was unexpected for the PPS14-specific response to COH1-11, in light of the boosted response after secondary immunization. Of note, the average avidity of PPS14-specific IgG induced by bacteria is only slightly lower than the avidity of the 44.1 mAb, which was generated in response to intact Pn14 (Fig. 6A, dashed line). In contrast, the PPS14-specific IgG response to soluble PPS14–PspA conjugate underwent avidity maturation throughout both the primary and secondary response (Fig. 6A). Although the 44.1-Id minimally contributed to the primary IgG response to PPS14–PspA (Fig. 4), the average avidity was similar to that seen for bacteria (Fig. 6A). These results further support the hypothesis that distinctive B cell clones are involved in the PPS14-specific IgG responses to bacteria versus conjugate and that the lack of usage of the 44.1-Id in the response to conjugate is unlikely to be only the result of affinity maturation.

As expected, the PPS14-specific 44.1-Id− IgG induced in response to soluble PPS14–PspA conjugate exhibited similar avidity as total PPS14-specific IgG (Fig. 6B). However, although the avidity of the minority 44.1-Id− IgG induced during the primary response to R6-14 or COH1-11 has essentially identical average avidity as the total PPS14-specific IgG, the avidity of 44.1-Id− IgG induced during the secondary response to R6-14 (p = 0.0004) or COH1-11 (p = 0.025) increased significantly with respect to the avidity of the total PPS14-specific IgG, which is dominated by 44.1-Id+ IgG (Fig. 6B). Similar results were obtained in mice primed with R6-14 and boosted with COH1-11 (data not shown). These results suggest that during the response to bacteria, distinct B cell clones may contribute to the minor, 44.1-Id− PPS14-specific IgG response.

Particulation of PPS14–PspA conjugate increases the recruitment of 44.1-Id− Ig into the PPS14-specific response

We previously demonstrated that the PPS14-specific IgG response to soluble PPS14–PspA conjugate versus intact Pn14 derives from

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** 44.1-Id expression in the PPS14-specific IgG responses to GBS-III variants expressing a PS structurally identical to PPS14. Female BALB/c mice (n = 7) were immunized with 1 × 10^9 CFU/mouse S. pneumoniae strain R6-14 or GBS-III strain COH1-11 (expressing a PS structurally identical to PPS14). Fourteen days later, every group was challenged with the same strain, except for a group of mice immunized at day 0 with R6-14 and challenged at day 14 with COH1-11 (“R6-14; COH1-11”). The content of PPS14-specific IgG, IgG1, and IgM expressing the 44.1-Id (top panels) in the sera collected prior to the immunizations (preimmune), 14 d after the first (primary response), and after the second immunization (secondary response) was determined by inhibition ELISA using plates coated with PPS14. *p < 0.05 (PPS14-specific Ig responses of each group relative to mice receiving two immunizations with R6-14), †p < 0.05 (primary relative to preimmune response). Levels of 44.1-Id+ Ig of each isotype are shown as overlay with black bars. NQ, Not quantifiable.
FO versus MZ B cells, respectively (13, 28). However, when the PPS14–PspA conjugate was stably adsorbed to the surface of intact unencapsulated *S. pneumoniae*, in which the bacterial cell wall PspA was removed by choline treatment (R36A*ch*), the resulting IgG anti-PPS14 response was almost entirely derived from MZ instead of FO B cells (13). A major but incomplete switch in B cell subset usage was also observed using PPS14–PspA covalently attached to latex particles of 30 mm in diameter. This switch from FO to MZ B cells in the response to conjugate attached to latex particles did not affect the ability to generate avidity maturation (Fig. 6A). In this regard, we wished to determine whether conjugate particulation affects the usage of the 44.1-Id in the PPS14-specific Ig response. PPS14-specific IgG and IgG1 responses after primary immunization with PPS14–PspA adsorbed to R36A*ch* or covalently coupled to latex beads induced a significant (p < 0.001) increase in the relative usage of the 44.1-Id, relative to that observed using free conjugate (Fig. 7). Secondary immunization significantly increased the relative percentage of 44.1-Id* + B cell clone generated in response to particulate forms of PPS14 may be contained within the population of PPS14-specific MZ B cells.

**FIGURE 6.** Average avidity of PPS14-specific IgG responses after immunization with different antigenic forms of the PPS14. (A) The average avidity of PPS14-specific IgG in the serum of mice immunized at days 0 and 14 with the same antigenic form of PPS14 was estimated by elution with NaSCN. The Ags compared were 1 µg free PPS14–PspA conjugate ("soluble") or 0.1 µg PPS14–PspA covalently attached to 2 × 10⁹ aldehyde sulfate latex beads 0.96 µm in diameter ("beads"), both admixed with alum and CpG-ODN, and 1 × 10⁹ CFU/mouse R6-14 or 1 × 10⁹ CFU/mouse COH1-11 in saline. Sera from mice immunized at day 0 with R6-14 and at day 14 with COH1-11 were also included in the analysis. *p < 0.05 (AI for day 14 relative to day 7, and day 21 relative to day 14). The AI of the 44.1 mAb is indicated by a dashed line. (B) Comparison of the avidity of whole (solid line) and 44.1-Id* (dashed line) PPS14-specific IgG present in the sera of mice immunized with free PPS14–PspA conjugate, R6-14, or COH1-11. Data show the AI as the molar concentration of NaSCN eluting 50% of the total content of PPS14-specific IgG in the serum sample. *p < 0.05 (AI of 44.1-Id* relative to whole IgG).

**FIGURE 7.** Expression of the 44.1-Id in the PPS14-specific Ig responses to soluble and particulate forms of the PPS14–PspA conjugate. Female BALB/c mice (n = 7) were immunized on days 0 and 14 with free PPS14–PspA conjugate ("soluble"; 1 µg), covalently attached to 2 × 10⁹ aldehyde sulfate latex beads/mouse ("latex beads"; 0.1 µg), or adsorbed to 2 × 10⁹ CFU/mouse unencapsulated *S. pneumoniae* (strain R36A) previously depleted of native PspA by treatment with choline chloride ("R36A*ch*"; 0.020 µg). Alum and CpG-ODN were used as adjuvant for all immunizations. The content of PPS14-specific IgG, IgG1, and IgM expressing the 44.1-Id (top panel) was determined, as in previous figures, 14 d after the first and second immunization. *p < 0.05 (between the particulate forms of PPS14–PspA relative to free PPS14–PspA), *p < 0.05 (second primary relative to primary responses). Levels of 44.1-Id* Ig of each isotype are shown as overlay of black bars.
Discussion

These data demonstrate that the physicochemical context in which PS is presented to the immune system can determine the dominant 44.1-Id expression of the elicited PS-specific IgG. Specifically, the dominance of 44.1-Id expression in response to intact Pn14 or GBS-III was not observed in the PPS14-specific IgG response to PPS14–PspA conjugate. However, immunization with PPS14–PspA on the surface of a particle [i.e., intact unencapsulated *S. pneumoniae* or latex bead (both ~1 μm in diameter)] led to significant 44.1-Id expression. PPS14-specific IgG responses to intact Pn14 or particulate PPS14–PspA versus soluble PPS14–PspA were previously shown to derive from MZ and FO B cells, respectively (13, 28). Thus, the expression of the 44.1-Id is correlated with the activity of MZ B cells and can be induced in response to conjugate vaccine that is in particulate, but not soluble, form. This is consistent with the observed sequestration of particulate Ag within the MZ that promotes prolonged interactions with resident immune cells (51). Of note, as will be discussed later, the TI IgG response to isolated, soluble PPS14 was also dominated by the 44.1-Id. In contrast to IgG, only minor 44.1-Id expression was observed for PPS14-specific IgM elicited in response to either particulate or isolated PPS14, indicating that the 44.1-Id was generated at the time of IgG class switching. Collectively, these results have implications for understanding PS-specific Ig responses to vaccination, in which a host that is vaccinated with either isolated PS or PS–protein conjugate may also have been naturally exposed to the PS-expressing bacterium. In addition, this study suggests that particulation of select Ags may redirect the adaptive immune response from predominantly FO to MZ B cell involvement, with attendant differences in the fine specificity and/or kinetics of induction of the elicited Ig.

The expression of the 44.1-Id in response to the particulate forms of PPS14–PspA conjugate did not limit the development of affinity maturation during the secondary response, which was similar to that induced by the soluble form of the conjugate. Progressive affinity maturation is typically associated with immune responses within germinal centers in which cognate B cell–T cell interactions occur over a sustained period of time (52–54), although B cells participating in an extracellular, short-lived plasma cell response can also exhibit somatic hypermutation, albeit to a much more limited degree (55, 56). Extracellular responses can be either TI or TD (57). A more recent study further demonstrated that immunization of mice with the CD1d-restricted glycolipid, α-GalCer, conjugated to the hapten NP, induced an NKT cell-dependent germinal center reaction and NP-specific IgG memory B cells, but very limited affinity maturation relative to immunization with NP-OVA that recruits classical MHC class II-dependent CD4+ T cell help (58). Thus, lack of further increases in avidity in serum PPS14-specific IgG after secondary immunization with COH1-11, despite a substantial boost in serum titers, whereas significant affinity maturation was observed after secondary immunization with PPS14–PspA conjugate, suggests the possibility that Pn14 either failed to induce memory B cells, but perhaps only memory CD4+ T cells, or induced memory B cells primarily as part of an extracellular response and/or germinal center reaction in which the degree of affinity maturation is at best modest (59). The potential role of NKT cells in this response remains to be determined.

In humans, Ig elicited in response to isolated PS, a TI Ag, exhibits somatic mutation (19, 24, 25). Although it was earlier suggested that vaccination with isolated PS activates PS-specific memory B cells previously generated in a TD response to the same PS naturally expressed by intact bacteria to which the host had been exposed (25, 26), our data and that demonstrating the possibility of TI induction of somatic hypermutation (55, 56) suggest that naive B cells responding to isolated PS may exhibit somatically mutated BCR, independent of prior encounter with bacteria. Although the nature of the CD4+ T cell help for the PPS14-specific IgG response to Pn14 remains unclear, our data support the notion that direct covalent attachment of PS with protein, as exists with a conjugate vaccine, but not intact extracellular bacteria, may be critical for allowing PS-specific B cells to receive the prolonged, MHC class II-dependent cognate help from specific CD4+ T cells that may be critical for induction of both memory B cells and substantial avidity maturation of the secondary IgG response (13). Whether, in general, these CD4+ T cells are specific for peptides derived from the conjugate or for the PS itself in which attached peptide is required for association with MHC class II will require further study to determine (2, 9). Thus, the major factor determining the usage of the 44.1-Id in a TD PPS14-specific IgG response appears to be the particular nature of the PPS14-containing immunogen and not the type of CD4+ T cell help recruited or the ability to generate progressive affinity maturation. Thus, particulation and direct covalent linkage between PS and proteins could lead to improved vaccines for eliciting PS-specific humoral immunity.

Dominant expression of the 44.1-Id in response to intact Pn14, but not after immunization with PPS14–PspA conjugate, likely reflects differential VH gene usage of these clones. This is consistent with the suggestion that the 44.1-Id clone is selectively contained within the MZ B cell subset. MZ B cells have a distinctive repertoire of BCR specificities (60–62). During B cell development, differentiation of B cell precursors into MZ, relative to FO, B cells appears to be favored by exposure to Ags that induce weaker BCR signaling (63–65), which then has a major impact on the selection of Ag specificities. Thus, it is likely that 44.1-Id precursor B cell clones are selectively retained and differentiate in the MZ, where they have preferential access over FO B cells to particulate, blood-borne Pn14 that becomes sequestered within the marginal sinus. The likelihood, on the basis of previous selection, that 44.1-Id+ B cell precursor cells will be recruited into the immune response upon even weak BCR signaling, and the intrinsically rapid response of MZ B cells after activation (66, 67), may confer a survival advantage during acute infections with live Pn14. Alternatively, 44.1-Id precursor cells could theoretically be equally represented within the FO B cell compartment that preferentially encounters soluble PPS14–protein conjugate relative to intact Pn14. Dominant expression of 44.1-Id+ B cells might then reflect their ability to outcompete 44.1-Id+ cells as a result of higher rates of somatic mutation and affinity maturation in response to PPS14–PspA conjugate. However, this seems less likely in light of our observation that 44.1-Id expression in response to Pn14 is relatively stable over time, despite at least moderate increases in overall avidity of the PPS14-specific IgG response. Further, 44.1-Id+ and 44.1-Id+ PPS14-specific IgG expression is predominant early in the primary responses to Pn14 and PPS14–PspA conjugate, respectively, when overall avidity of PPS14-specific IgG is comparable. Thus, we suggest that the 44.1-Id is a marker of MZ B cell involvement in PPS14-specific IgG responses to particulate PPS14-containing immunogens, although confirmation of this notion will require further studies.

In this regard, the IgG response to isolated, soluble PPS14 was also dominated by the 44.1-Id. We previously demonstrated that murine IgG responses to isolated pneumococcal PS are critically dependent on a contaminating TLR2 ligand(s) (68). Of note, TLR-mediated stimulation can promote rapid plasma cell differentiation in B1 and MZ B cells but not in naive FO B cells (69) with synergistic signals provided via multivalent BCR engagement (70).
Thus, this may explain the 44.1-Id dominance of the response to isolated PPS14, where CD4+ T cells are not recruited and thus cannot provide the necessary help for PPS14-specific FO B cell differentiation. These data do not necessarily distinguish between the relative involvement of B-1b versus MZ B cells, expressing the 44.1-Id, in response to isolated PPS14, especially in light of the functional similarities of B-1b and MZ B cells (60, 71, 72).

The expression of the 44.1-Id shows a clear linkage to the murine IgH allele. Thus, in addition to its dominant expression in BALB/c but not C57BL/6 mice, minimal 44.1-Id usage is also observed in C.BKa mice, which express the IgH allele in an essentially BALB/c background. Although allotype-specific linkages have been described in other experimental systems using various PS Ags (20–23, 73), the 44.1-Id differs from these systems in several aspects. First, 44.1-Id is prevalent in elicited PPS14-specific IgG, but not IgM, and it was undetectable in natural PPS14-specific Ig. In contrast, in BALB/c mice, the dominant T15-Id is expressed by both PC-specific IgM and IgG induced in response to intact S. pneumoniae and is constitutively expressed by PC-specific natural Ig. PC is a haptenic component of the S. pneumoniae cell wall teichoic acid and membrane lipoteichoic acid. Further, T15-Id+ Ig is produced predominantly by B-1 cells (74), utilizing germline V genes. Thus, T15-Id relative to 44.1-Id is generated in a mechanistically distinct manner.

Second, the lack of expression of the 44.1-Id is associated with reduced PPS14-specific IgG, but not IgM, responses. Thus, in contrast to BALB/c mice, PPS14-specific IgG responses in C.BKa mice were low, as in C57BL/6 mice, despite their essentially BALB/c background. To our knowledge, a similar association between murine allotype, Id, and magnitude of the IgG response has only been reported for α1-3 dextran, in which the dominant J588-Id is also linked to the IgH allele and is derived from the same VIK gene family as 44.1-Id (22). TΔT, which plays a major role in generating diversity during V(D)J recombination, is essential for the expression of the J588-Id (75). TΔT is absent during fetal development and in fetal-derived B cells, such as B-1 cells. The similarities between the J588-Id and 44.1-Id strongly suggest a similar dependence on TΔT activity for 44.1-Id expression, although we did not find evidence of D gene usage in the 44.1 mAb. Reduced TΔT activity could be related to the poor PPS14-specific IgG responses in C57BL/6 mice. Thus, the adult MZ of C57BL/6 mice is enriched in B cells possessing a shorter CDR3, similar to fetal-derived B1 cells (61). In humans, the magnitude, although not avidity, of pneumococcal PS-specific and other bacterial PS-specific IgG responses has also been associated with individual allotype (76).

Our earlier observation that priming with Pn14 followed by secondary immunization with GBS-III (29), but not PPS14–PspA (77), results in a boosted PPS14-specific IgG response supports the notion that intact bacteria and conjugate target different PS-specific B cell clones. This is also consistent with a study in which SCID mice reconstituted with PBL from the same unvaccinated human donor showed significant differences in human V gene usage in response to immunization of the recipient mice with purified PS versus conjugate vaccine (78). In addition, distinct Id usage of human Ig specific for Haemophilus influenzae b polysaccharide (Hib PS), with associated differences in affinity and protective capacity, was observed in response to Hib PS conjugate vaccines comprising varying m.w. Hib PS and different carrier proteins (33, 79, 80), further emphasizing the role of biochemical context of a given PS Ag in the selection of specific Id. However, the dichotomy in murine Id usage in response to isolated PPS14 versus PPS14 conjugate vaccine, as described in this report, may not be universally applicable to other PS and/or in the human host.

Thus, 85% of humans immunized with either free Hib PS or a conjugate of Hib PS and diphtheria toxoid expressed anti-Hib PS Ig bearing the same Id (i.e., Hibl-1 expressed by anti-Hib PS having the germline κ1-A2 variable L chain) (33). In each case, this Id composed ~60% of the Hib PS-specific Ig. Further, recurring usage of similar V genes and rates of mutation were found in humans in response to isolated PS and PS–protein conjugate using B cell hybridomas (26) and combinatorial cloning (25) of PBMC from vaccinated individuals.

Disclosures
The authors have no financial conflicts of interest.

References