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BRIEF REPORT

The Immunoglobulin M Response to Pneumococcal Polysaccharide Vaccine Is Sufficient for Conferring Immunity

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In mice, pneumococcal polysaccharide (PPS) vaccines generate antigen-specific immunoglobulin M (IgM) and immunoglobulins G1, G2, and G3. Antibody and complement-dependent opsonophagocytosis correlates with the protection induced by PPS vaccines in vivo. Since IgM is a very efficient immunoglobulin isotype in activating the complement system, we evaluated whether anti-PPS IgM alone is sufficient to confer protective immunity to *Streptococcus pneumoniae*. We found that immunization of wild-type and activation-induced cytidine deaminase–deficient mice capable of producing only IgM with Pneumovax 23 generated comparable anti-PPS IgM and resistance to lethal systemic challenge with *S pneumoniae*. These data suggest that an IgM response to PPS vaccines is sufficient for conferring immunity.

Keywords. AID; B cells; IgM; pneumococcal polysaccharide vaccine; *Streptococcus pneumoniae*.

The capability of antibodies to recognize a broad range of antigens is attributed to diversity of the pre- and postimmune B-cell antigen receptor (BCR) repertoire. Following antigen exposure, postimmune BCR repertoire diversification is generated by somatic hypermutation (SHM) of the variable regions of immunoglobulin genes. This, combined with selection processes, result in affinity maturation of antibodies to a given antigen. In addition, DNA rearrangement can also occur in the heavy chain constant region of the immunoglobulin genes by class-switch recombination (CSR) resulting in the generation of isotypes other than immunoglobulin M (IgM), such as immunoglobulin G (IgG). In both humans and mice the processes of SHM and CSR require activation-induced cytidine deaminase (AID) [1]. Therefore, antigen-specific IgM is the only antibody that AID-deficient ($AID^{-/-}$) mice can produce upon immunization. AID induction in antigen-specific B cells is primarily mediated by CD40–CD40L interactions between antigen-specific B and T cells. Protein antigens such as tetanus and diphtheria toxins or protein-conjugated polysaccharide (PS) antigens (eg, pneumococcal polysaccharide conjugate vaccine [PCV]) are processed and presented to T cells for help by antigen-presenting cells including antigen-specific B cells. Vaccines comprised of protein antigens are therefore referred to as T-cell dependent and generate affinity-matured, isotype-switched antibodies such as IgG1 or IgG2.

Unlike protein antigens, native PS antigens (eg, Pneumovax 23, an unconjugated 23-valent pneumococcal PS vaccine) are typically resistant to degradation and do not bind major histocompatibility complex II, so T-cell help for PS-specific B cells is limited [2]. Thus, PS antigens are referred to as T-cell–independent (TI) antigens and activate PS-specific B cells primarily by cross-linking the BCR (ie, membrane IgM on B cells) to induce a rapid IgM responses [2].

Bacterial PS vaccines including Pneumovax 23 are often contaminated with Toll-like receptor (TLR) 2 and TLR4 ligands due to their isolation directly from bacteria [3]. TLR ligands can also induce AID expression, and BCR signaling can synergize in this process [4]. Thus, AID could also influence TI responses by promoting not only immunoglobulin isotype switching but also somatic mutations in V regions of antibody genes. We found that unmutated IgM responses generated in $AID^{-/-}$ mice protect from infection by *Borrelia hermsii*, an agent of relapsing fever and *Salmonella* Typhi, the causative agent of typhoid [5, 6]. Since B1b cells generate the majority of antigen-specific IgM to both Vi polysaccharide (ViPS) of *S* Typhi and serotype 3 pneumococcal polysaccharide (PPS3) [7], we evaluated whether unmutated IgM responses to Pneumovax 23 can also protect against serotype 3 *Streptococcus pneumoniae* challenge.

METHODS

Mice

The Institutional Animal Care and Use Committee approved these studies. Mice were housed in micro-isolator cages with free access to food and water and were maintained in a specific pathogen-free facility. Wild-type (C57BL6/J) mice (stock; 000664) and $AID^{-/-}$ (stock number 007770) mice on a C57BL/6J background were purchased from The Jackson Laboratories (Bar Harbor, Maine) [6]. B-cell–deficient mice (Mb1-Cre) on the C57BL6 background were obtained from Dr Ann Feeney (Scripps Research Institute, La Jolla, California) [8]. All mice were bred in our facility and 8- to 14-week-old mice of both sexes were used. To deplete C3,

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mice were treated intraperitoneally (i.p.) with 30 μg (14.0 units) cobra venom factor (CVF) (Quidel, San Diego, California) 1 day prior to infection [9].

Immunization

Ten micrograms of 23-valent PPS vaccine (Pneumovax 23; Merck & Co Inc, Whitehouse Station, New Jersey) were dissolved in 100 μL phosphate-buffered saline (PBS) and used to immunize mice i.p. [8]. Blood samples were obtained 0, 7, 14, 21, and 28 days following immunization and stored at −20°C.

Enzyme-Linked Immunosorbent Assay

Pneumovax 23 or PPS3-specific IgM and IgG were measured by coating 96-well plates (Nunc MultiSorp 467340; Nunc A/S, Roskilde, Denmark) with either 50 μL of Pneumovax 23 (5 μg/mL) or PPS3 (5 μg/mL; 169-X, American Type Culture Collection, Manassas, Virginia), in PBS overnight at room temperature [8]. All plates were washed and blocked with 2% bovine serum albumin in PBS pH 7.2 for 2 hours at room temperature. For measuring Pneumovax 23- and PPS3-specific antibody responses, serum samples were preincubated with C-PS (5 μg/mL; Statens Serum Institut diagnostica A/S, Denmark) to decrease background binding [8]. PPS-specific antibody levels in the present study were interpreted as ng/μL equivalents using normal mouse serum IgM or IgG standards (Bethyl Laboratories, Montgomery, Texas) [6, 8].

Infections

For pneumococcal infections, *S pneumoniae* WU2, a serotype 3 strain obtained from Dr David Briles (University of Alabama at Birmingham), was grown in Todd–Hewitt broth with 0.5% yeast extract to mid-log phase (optical density 0.5 at 600 nm) and resuspended in sterile PBS, and bacterial density was adjusted to approximately 5×10^2, 5×10^3, or 5×10^4 colony-forming units (CFU) in 100 μL PBS. Mice were injected i.p. and survival was monitored for 10 days [8].

Passive Immunization

A group of 7 wild-type (WT) or AID$^{-/-}$ mice was immunized with 10 μg of Pneumovax 23. Twenty-eight days postimmunization, mice were euthanized and serum was collected. Pooled serum was filter-sterilized (0.22 μm) and 250 μL of this serum was injected i.p. into B-cell–deficient mice [8]. Three hours later, mice were infected i.p. with 500 CFU of *S pneumoniae* (strain WU2) and survival of the infected mice was monitored for 10 days.

Statistical Analysis

Data presented throughout depict pooled data from at least 2 independent experiments. Statistics were performed using the Prism 5 software program (GraphPad Software, La Jolla, California).

RESULTS

PPS-Specific IgM Responses Occur in AID$^{-/-}$ Mice

In mice, B1b cells generate IgM and IgG3 to PPS3 and these antibodies confer protection against serotype 3 *S pneumoniae* [7]. Antibody responses to PPS3 are also induced in IgG3$^{-/-}$ mice, suggesting that IgG1 confers protection [10]. Antibody and complement-mediated opsonophagocytosis is considered the correlate for pneumococcal vaccine–induced protection [11]. IgM is highly efficient in activating the classical complement system, and Fc receptors for IgM also exist; we hypothesized that IgM should be sufficient to confer protective immunity to *S pneumoniae*. To test this, we immunized WT and AID$^{-/-}$ mice with Pneumovax 23. We found that WT and AID$^{-/-}$ mice produced comparable levels of anti-Pneumovax 23 IgM (Figure 1*A*). Because Pneumovax 23 is a polyvalent vaccine

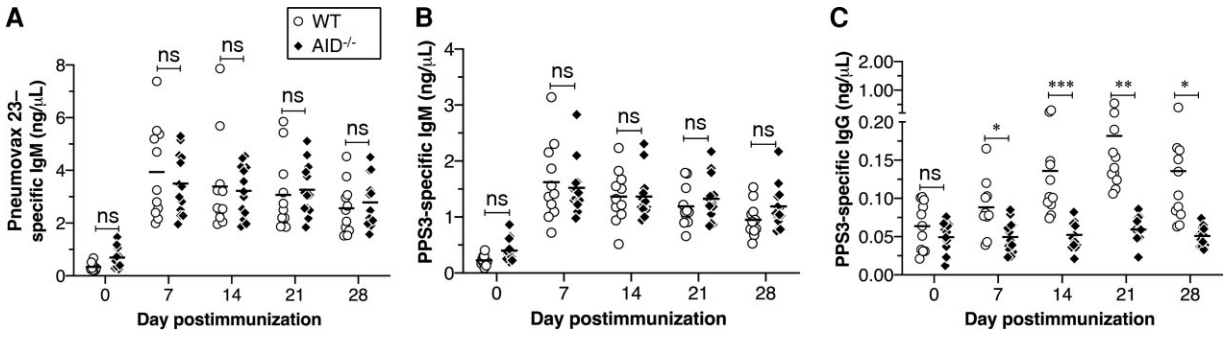

Figure 1. Immunization with Pneumovax 23 results in comparable anti– pneumococcal polysaccharide (PPS) immunoglobulin M (IgM) response in wild-type (WT) and activation-induced cytidine deaminase–deficient (AID$^{-/-}$) mice. WT or AID$^{-/-}$ mice were immunized intraperitoneally with 10 μg of Pneumovax 23 and levels of Pneumovax 23–specific IgM (*A*), serotype 3 PPS (PPS3)–specific IgM (*B*), and PPS3-specific immunoglobulin G (*C*) were measured by enzyme-linked immunosorbent assay. Each dot represents an individual mouse and the bar represents mean. The data represents pool of 2 independent experiments. Statistical significance was determined using the Bonferroni–Dunn method, with $\alpha = .05$. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. Abbreviations: AID, activation-induced cytidine deaminase; IgG, immunoglobulin G; IgM, immunoglobulin M; ns, not statistically significant; PPS3, serotype 3 pneumococcal polysaccharide; WT, wild-type.

containing 23 different polysaccharides, we also evaluated the antibody responses specific to 1 of the PSs expressed by the challenge strain WU2 of *S pneumoniae* PPS3 serotype [7, 8]. As was seen in the IgM responses to whole Pneumovax 23, IgM responses specific to PPS3 are also comparable in WT and AID$^{-/-}$ mice (Figure 1*B*). As expected, WT mice but not AID$^{-/-}$ mice generated PPS3-specific IgG responses, indicating the occurrence of isotype switching to antigen-specific IgG (Figure 1*C*).

Immunization of AID$^{-/-}$ Mice With Pneumovax 23 Can Confer Protection In Vivo

To evaluate whether anti-Pneumovax 23 IgM responses, in particular anti-PPS3 IgM responses, are sufficient in the absence of all other isotypes and confer similar levels of protection, we challenged immunized WT and AID$^{-/-}$ mice with serotype 3 *S pneumoniae* strain WU2. Compared to their unimmunized counterparts (Figure 2*A*), immunized WT and AID$^{-/-}$ mice survived when challenged with low (500) and high (50 000) CFU of *S pneumoniae* (Figure 2*B*).

To test whether the protection is mediated by antiserum, serum from Pneumovax 23–immunized WT or AID$^{-/-}$ mice was transferred to B-cell–deficient mice. These mice were challenged with serotype 3 *S pneumoniae*, strain WU2 [8]. B-cell–deficient mice that did not receive serum succumbed to infection, whereas mice that received immune serum from AID$^{-/-}$ mice or WT mice survived (Figure 2*C*). These data suggest that PPS3-specific serum IgM antibodies generated without somatic hypermutations can confer protection against serotype 3 *S pneumoniae* and that IgM class switching to IgG isotypes is not required.

To test whether the IgM-mediated protection is dependent on the complement system, we have depleted C3 in Pneumovax 23–immunized WT and AID$^{-/-}$ mice with CVF and a day after challenged the mice with 5000 CFU of *S pneumoniae* (Figure 2*D*). We found that this treatment had no statistically significant effect on the survival of mice (Figure 2*D*). Since we have not found a discernable role for C3, and several IgM Fc receptors have been identified, it is possible that IgM-mediated opsonophagocytosis could be a mechanism of control of *S pneumoniae*.

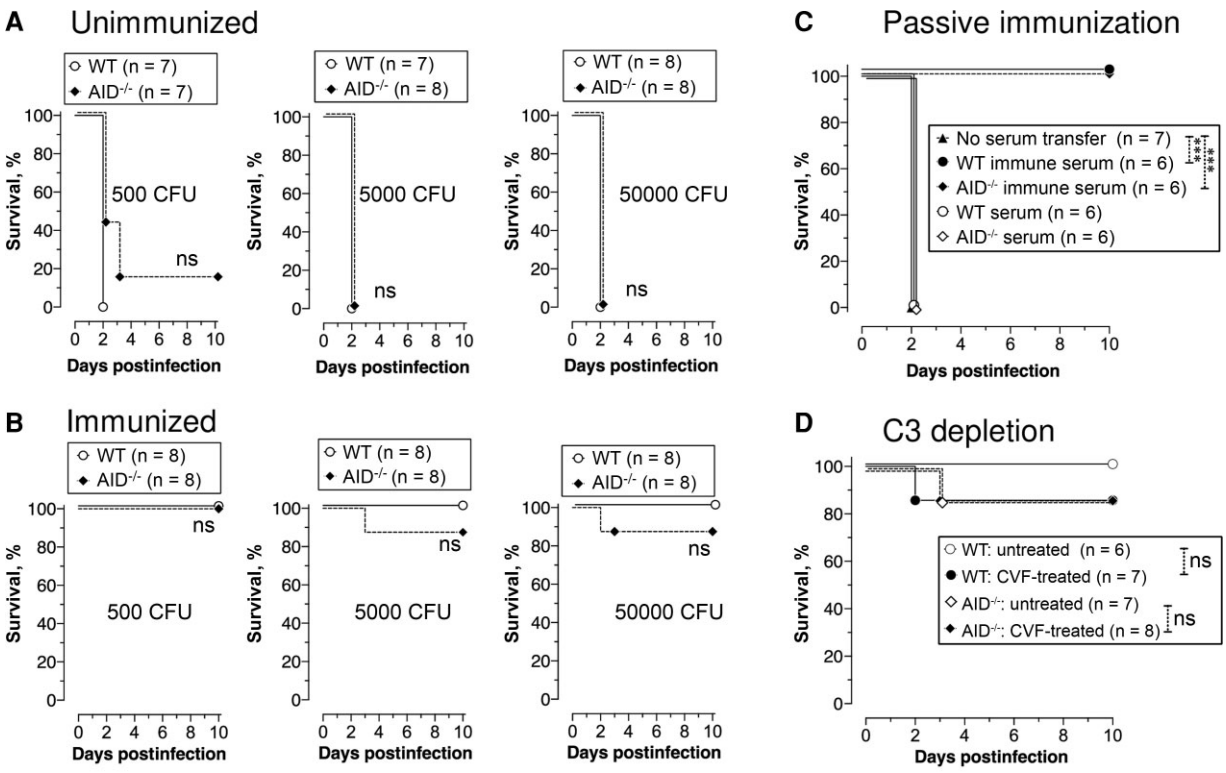

Figure 2. Pneumovax 23 immunization confers comparable protection against *Streptococcus pneumoniae* to wild-type (WT) and activation-induced cytidine deaminase–deficient (AID$^{-/-}$) mice. WT and AID$^{-/-}$ mice were immunized intraperitoneally (i.p.) with 10 μg of Pneumovax 23. Four weeks following immunization, unimmunized (*A*) and immunized (*B*) mice were infected i.p. with 500, 5000, or 50 000 colony-forming units (CFU) of *S pneumoniae* strain WU2 and survival was monitored. *C*, B-cell–deficient mice were injected with 250 μL of naive serum or immune serum from either WT or AID$^{-/-}$ mice immunized with Pneumovax 23. All mice were infected i.p. with 500 CFU of *S pneumoniae* strain WU2 and survival was monitored. *D*, WT and AID$^{-/-}$ mice were immunized i.p. with 10 μg of Pneumovax 23. Four weeks following immunization, some mice were treated with 14 units of cobra venom factor to deplete C3. A day after, mice were challenged with 5000 CFU of *S pneumoniae* strain WU2 and survival was monitored. Survival statistics were performed using log-rank (Mantel-Cox) test. ***$P < .001$. Abbreviations: AID, activation-induced cytidine deaminase; CFU, colony-forming units; CVF, cobra venom factor; ns, not statistically significant; WT, wild-type.

DISCUSSION

PPS3-immunized secretory $IgM^{-/-}$ or $IgG3^{-/-}$ mice survive serotype 3 *S pneumoniae* challenge, suggesting a redundancy in the immunoglobulin isotypes in conferring protective immunity [10, 12]. Previous research suggests that PPS3-immunized $IgG3^{-/-}$ mice are more susceptible compared to WT mice when challenged with high doses (10 000–100 000 CFU) of *S pneumoniae* serotype 3 strain A66.1 [10]. However, we have not seen an impact of challenge dose (ie, 5000–50 000 CFU) in the present study (Figure 2*B*). This difference could be due to the background of the mutant mouse strain (BALB/c vs C57BL6) and/or the serotype 3 *S pneumoniae* strain (WU2 vs A66.1) used in the 2 studies [10] (Figure 2*B*). It is important to note that, even with mouse and bacterial strain differences, PPS3-immunized $IgG3^{-/-}$ mice control *S pneumoniae* infection as efficiently as immunized WT mice when challenged with a low dose (500 CFU), suggesting that IgG3 is not essential for protective immunity [10]. In fact, it was shown that unmutated IgM monoclonal antibodies to PPS8, 1 of the 23 PPS serotypes in Pneumovax 23, when passively transferred confer protection against *S pneumoniae* serotype 8 [13]. Phosphorylcholine binding "natural" IgM produced by B1a cells in mice without apparent antigen stimulation also plays a significant role in protective immunity against *S pneumoniae* [7]. Our findings are consistent with the above studies [7, 10, 13] in showing that the unmutated IgM isotype is sufficient for conferring protection against *S pneumoniae*. Since immunoglobulin A is the dominant isotype in the mucosal secretions of mice, the ability of IgM to protect in the pulmonary challenge model remains to be determined.

Preimmune BCR repertoire diversity increases by several orders of magnitude due to the action of terminal deoxynucleotidyl transferase (TdT) during V(D)J recombination during B-cell development in the bone marrow. Interestingly, we have found previously that TdT-mediated junctional diversity of antibody V genes is not required for Pneumovax 23 and ViPS vaccine–mediated protection against *S pneumoniae* and *S* Typhi, respectively [8]. As shown with the anti-ViPS response in $AID^{-/-}$ mice [6], we now show that AID-mediated BCR diversity is also not required for Pneumovax 23–mediated protection to *S pneumoniae*. Our work therefore suggests that PS antigen–driven clonal selection of the preimmune BCR repertoire with appropriate V_H–D_H–J_H gene usage may be sufficient for protection.

We have previously demonstrated that B1b cells can generate a long-lasting TI IgM memory to *B hermsii* and that unmutated IgM specific to *B hermsii* is sufficient to control bacteremia in vivo [5]. Recently, it has been reported that IgM^+ memory B cells with reduced SHM were found to be longer-lived than hypermutated IgG^+ memory B cells, suggesting that antigen-specific unmutated IgM B cells can persist as memory B cells and confer long-lasting immunity [14]. Although PPS3 is recognized by B1b cells in WT mice [7], the antibody responses induced by TI antigens are short-lived, suggesting that hypermutated and isotype-switched antibodies influence unmutated IgM memory to TI antigens.

While PCVs as T-cell–dependent immunogens induce prolonged immunity, they require administration of multiple doses to boost optimal antibody responses in infants and young children. Moreover, such vaccines may be prohibitively expensive for developing countries where pneumococcal diseases are prevalent. Nevertheless, PCVs have been tested in a clinical trial in the Philippines and Pakistan [15]. For reasons that are unknown, despite the administration of multiple doses, PCV did not induce the expected IgG responses in these trials [15]. Identification of the mechanisms for persistence of long-lasting IgM responses in humans may lead to the development of novel TI PPS vaccines that serve as the vaccines of choice for individuals who do not respond to PCV due to inherent and acquired T-cell deficiencies.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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