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Neisseria meningitidis PorB, a TLR2 Ligand, Induces an Antigen-Specific Eosinophil Recall Response: Potential Adjuvant for Helminth Vaccines? 1

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Efficacious adjuvants are important components of new vaccines. The neisserial outer membrane protein, PorB, is a TLR2 ligand with unique adjuvant activity. We demonstrate that PorB promotes Th2-skewed cellular immune response to the model Ag, OVA, in mice, including Ag-specific recall eosinophil recruitment to the peritoneum. PorB induces chemokine secretion by myeloid cells using both TLR2-dependent and -independent mechanisms, suggesting that anatomical distribution of TLR2+ cells may not be a limiting factor for potential vaccine strategies. The results from this study suggest that PorB, and other TLR2 ligands, may be ideal for use against pathogens where eosinophilia may be protective, such as parasitic helminths. The Journal of Immunology, 2007, 179: 3222–3230.

Vaccines have been the most efficacious immunological advance of the 20th century (1). The prevention of a wide variety of diseases has been made possible through progress in vaccinology, which includes the coupling of protein Ags to poorly immunogenic polysaccharide capsules, as well as recent advances in genomics that may identify previously unknown vaccine targets (2–4). Yet obstacles remain in the development of immunotherapies for pathogens such as HIV, Plasmodium sp., parasitic helminths, mycobacterium, serotype B of Neisseria meningitidis, and for emerging pathogens, such as Ebola virus (1).

Because infection does not always induce a sufficient protective immune response to a pathogen, a goal of vaccination is to induce immunity greater than that induced to the pathogen during natural infection (5). In many cases, a vaccine Ag would have to be immunologically enhanced, such as by the addition of an adjuvant. TLR ligands provide a new and propitious group of molecules that have been shown to be promising candidates for vaccine adjuvants (6). Signaling through TLRs has been shown to be immunostimulatory, inducing host cells to proliferate, secrete cytokines and chemokines, and up-regulate costimulatory molecules (6–8). The latter characteristic renders TLR ligands a potent addition to the vaccine adjuvant repertoire by possessing the capability to link the innate and adaptive immune systems, thus inducing not only an inflammatory response but also activation of the adaptive arm of the immune system. Several TLR ligands are being investigated in clinical trials as adjuvants for human vaccines presently, including CpG oligodeoxynucleotides (ODN)3 (TLR9), monophosphoryl lipid A (TLR4), and Resiquimod (TLR7 and -8) (9–11).

Our laboratory has focused on characterizing the immunostimulatory TLR2/TLR1 ligand PorB (12), an outer membrane protein (OMP) of the bacteria Neisseria meningitidis (13, 14). Neisserial porins are trimeric β-barrel structures that serve as pores for ion exchange between the bacteria and its surroundings (15). PorB has been shown to induce the costimulatory capacity of murine dendritic cells (DCs) and stimulate B cell proliferation in vitro as well as enhancing the humoral response to meningococcal capsule in vivo (13, 16, 17). PorB is a component of the OMP preparation originally used as a carrier protein for the Haemophilus influenzae type B (HIB) vaccine. Upon further characterization, the OMP was shown to have adjuvant activity as well, thus serving two purposes in the vaccine. The adjuvant activity of the OMP has been shown to be dependent on stimulation through TLR2 in vitro (18).

The potent immunostimulatory capacity of PorB and its characterization as a TLR2 ligand suggested that PorB could be effectively used as a vaccine adjuvant. We sought to determine the best approach for examining the adjuvanticity of PorB in vivo using model Ags. In this study, we demonstrate a unique feature of PorB-mediated adjuvanticity, the ability to stimulate an eosinophilic recall response. This recall response is similar to the recall of eosinophils seen in a secondary helminth infection (19). Because eosinophils have been shown to be cytotoxic to pathogens, especially to helminths, and are often associated with a Th2 phenotype, these results indicate promise for a PorB-parasite Ag vaccine, which may stimulate protective immunity against helminth parasites. We then examined the possible mechanisms of PorB-mediated recall eosinophilia, findings that could have implications for PorB’s future adjuvant use.

1 Abbreviations used in this paper: ODN, oligodeoxynucleotide; DC, dendritic cell; OMP, outer membrane protein; HIB, Haemophilus influenzae type B; LOS, lipooligosaccharide; WT, wild type; KO, knockout; PEC, peritoneal exudate cell; PMN, polymorphonuclear neutrophil; FSC/SSC, forward/side scatter; PGN, peptidoglycan; Myo, myoglobin; BMMC, bone marrow-derived mast cell; MALP-2, macrophage activating lipopeptide-2; im, intermediate.

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Materials and Methods

**Mice**

Male C57BL/6 × +/− (wild-type (WT)) and WBB6F1/J-KitW/KitW × (KitW/−; mast cell-deficient (20)) mice and their heterozygous littersmates between the ages of 6 and 8 wk were purchased from The Jackson Laboratory. Mice deficient (knockout (KO)) in TLR2 and TLR4 (on C57BL/6 background, backcrossed five times) were a gift from Dr. S. Akira (Research Institute of Medical Science, University of Tokyo, Japan). All mice were maintained in accordance with institutional standards and Institutional Animal Care and Use Committee protocols at the Laboratory Animal Science Facility at Boston University School of Medicine.

**Isolation of N. meningitidis PorB**

PorB was isolated from N. meningitidis strain H44/76 lacking both PorA and RmpM, as previously described (21). This mutant strain allowed for the purification of PorB without contamination from other OMPS. The porins were purified by detergent extraction and column chromatography (21). PAGE and silver staining demonstrated negligible contamination by other proteins and lipopolysaccharides (LOS) (data not shown).

**ELISAs for the detection of total serum IgE and chemokine concentrations**

Total IgE levels were measured by coating an ELISA plate (Immulon II; Fisher Scientific) with unlabelled goat-anti-mouse IgE overnight at 4°C in coating buffer (0.1 M carbonate buffer (pH 9.8)). Results were based on a standard curves with a known concentrations of purified murine IgE (BD Biosciences), Chemokines RANTES, MIP-1α, MIP-2, eotaxin (R&D Systems), and MCP-1 (BD Biosciences) were analyzed by sandwich ELISA according to the manufacturer’s protocol. Results were based on a standard curves with a known concentrations of chemokine. ELISA plates were read with a SpectraMax 340 ELISA plate reader from Molecular Devices.

**In vivo cellular recruitment experiments**

WT, KitW/−, TLR2 KO, and TLR4 KO mice were used for in vivo studies. Four to five mice were injected i.p. with 10 μg of PorB in PBS or PBS alone and were sacrificed at 4, 15, 24, 48, 64, and 72 h postinjection. The peritoneal cavity was lavaged with ice-cold RPMI 1640 (BioWhittaker), and the resulting peritoneal exudate cells (PECs) were washed and labeled with fluorochrome-conjugated Abs to the following cell surface molecules: Mac-1-FITC, CD19-biotin, CD23-PE, CD8-Chrome, CD3-FITC, Gr-1-PE, CD117-biotin, CD54-FITC, CD69-FITC, CCR3-UNLB, and CD81-PE purchased from BD Biosciences. Anti-TLR2-PE, -TLR4-PE, and -Fc ε purchased from BD Biosciences. Anti-TLR2-PE, -TLR4-PE, and CD117-PE were purchased from eBioscience. Goat anti-rabbit-FITC was purchased from Sigma-Aldrich. Cells were assessed for surface marker expression via flow cytometry on a FACScan (BD Biosciences) and analyzed with WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA). Cells were identified as follows using forward/side-scatter (FSC/SSC) plots as previously described (22, 23) and with the following combinations of specific markers: B1 cells: Mac-1−, CD19−, CD23−; B2 cells: Mac-1+, CD19+, CD23−; neutrophils (polymorphonuclear neutrophils (PMNs)): Mac-1−, Gr-1−; macrophages: Mac-1+, Gr-1−; eosinophils: Mac-1−, CCR3+; Gr-1−, mast cells: Mac-1−, CD117−. Eosinophil levels were also measured electronically by gating on FSC/SSC, as previously described (22). In preliminary experiments, PECs from naive mice were quantified by flow cytometry to determine whether any differences in basal cell populations existed between genetically deficient and WT mice. No significant differences were found, excluding the absence of mast cells in KitW/− mice (data not shown).

**Determination of cellular recall response**

Four mice per group were primed by i.p. or s.c. injection on day 0 with either 10 μg of OVA or equine myoglobin (Mog; Sigma-Aldrich)/10 μg of PorB, or 10 μg of OVA or Myo alone, each in 100 μl of PBS, or PBS alone. On day 14, primed mice were challenged with either OVA or Myo alone and were sacrificed at 4, 15, 24, 40, 48, 64, and 72 h after challenge. Mice were euthanized by carbon dioxide asphyxiation and murine peritoneal cavities were lavaged as above. This time point assures maximum PMN influx and compared with PBS injection. No other cell type was significantly increased between the PorB-injected and the PBS-injected mice (data not shown). To differentiate between an overall increase in eosinophils, peritoneal lavage fluid (100 μl) from PorB-injected mice was centrifuged in a microfuge for 30 s. Cells were resuspended in 100 μl of PBS containing 5% BSA and spun in a Shandon cytoflex at 500 rpm for 5 min and stained with Fisher Hema 3 Manual Staining System (Fisher Scientific). Cytospin slides were then examined with a Nikon Eclipse 6400 Phase Contrast Microscope to determine the cell populations present.

**Isolation of murine PMNs**

Naïve WT mice were injected with 1 ml of sterile thioglycolate medium (Remel) to generate a neutrophil (PMN) influx. Fifteen hours postinjection, mice were sacrificed via CO2 asphyxiation and murine peritoneal cavities were lavaged as above. This time point assures maximum PMN influx and minimum macrophage recruitment. PMNs were washed and resuspended in RPMI 1640 supplemented with 10% FBS (Gemini Bioproducts) and 100 μM penicillin and 100 μM streptomycin and 2 mM l-glutamine (all from BioWhittaker), referred to as R10. Cells were examined by flow cytometry for expression of CD19 (B cells), CD4 (T cells), Mac-1− (macrophages), Mac-1− and Gr-1− double positive (PMNs). Purity was assessed to be ~95%. PMNs were rested for 4 h, followed by stimulation with 10 μg of PorB for 18 h. Supernatants were collected and frozen at −20°C for subsequent chemokine analysis.

**Peritoneal macrophage isolation**

Naive WT and TLR2 KO mice were sacrificed by CO2 asphyxiation and the peritoneal cavity was lavaged with 5 ml of ice-cold RPMI 1640. Cells were washed, counted, and plated and allowed to adhere overnight in R10 medium. Nonadherent cells (lymphocytes) were removed by washing twice with PBS. Adherent cells were removed, counted, and replated. Cells were allowed to rest for 4 h before stimulation. Both adherent macrophages and nonadherent cells were maintained in R10. Cells were stimulated with 10 μg/ml PorB, and 18 h later supernatants were collected and frozen and cells were analyzed by flow cytometry to determine purity of macrophage isolation (found to be 85–92% pure).

**Generation of bone marrow-derived mast cells (BMMCs)**

After sacrifice, naïve WT, TLR2 KO, and TLR4 KO bone marrow was flushed from the femur and hip joints of mice with a 30-gauge needle using ice-cold sterile RPMI 1640. Cells were washed, plated, and allowed to rest overnight. Nonadherent cells were removed, counted, and plated at a density of 2 × 10^5 cells/ml. Recombinant murine IL-3 and stem cell factor (Sigma-Aldrich) were added at 10 ng/ml, two times per week, for 4–5 wk to expand and enrich mast cell cultures (modified from Ref. 24).

**Statistical analysis**

Student’s t tests were calculated using Microsoft Excel. Values of p < 0.05 with 95% confidence level were considered statistically significant. GraphPad Instat using Tukey-Kramer multiple-comparisons test was used to generate p values and 95% confidence intervals.

**Results**

PorB induces the recruitment of eosinophils in vivo

The main purpose of this study was to characterize the inflammatory response induced by PorB. An in vivo model of inflammation and cellular recruitment was used to track the influx of cells into the murine peritoneum. PorB or sterile PBS was injected i.p., and cell populations were determined by flow cytometric analysis at 4, 15, 24, 40, 48, 64, and 72 h. Eosinophils were measured by electronically gating on FCS/SSC as described previously (Fig. 1A) (22). Results are shown from 15, 40, and 64 h (Fig. 1B). PorB injection resulted in the recruitment of a significant number of eosinophils to the peritoneum for each time point (p < 0.05) when compared with PBS injection. No other cell type was significantly increased between the PorB-injected and the PBS-injected mice (data not shown). To differentiate between an overall increase in...
inflammation induced with PorB priming and a specific recruitment of eosinophils, neutrophil (PMN) numbers were compared between the PorB- and PBS-injected mice. There was no significant difference in PMN numbers between the two groups of mice, indicating that there was an increase specifically in the eosinophil population in PorB-injected mice (Fig. 1C). Eosinophils constituted between 8 and 31% of total cells recruited in response to PorB, with 0.5–1 × 10⁶ cells typically recruited at 24 h. Cytospin analysis of lavage fluid confirmed the presence of eosinophilic cells with donut-shaped nuclei (data not shown). To assess the activation status of the eosinophils recruited in response to PorB, cells were labeled with fluorescent Abs against a panel of activation markers and assessed by flow cytometry. Eosinophils expressed Mac-1 (CD11b/CD18; Fig. 1D) and CCR3, but low levels of CD81 and CD54 (data not shown).

PorB as an adjuvant induces an Ag-specific eosinophilia recall

To determine whether PorB could induce an Ag-specific eosinophilic recall response, two model Ags were used, OVA and Myo. Data are shown from OVA immunizations, but similar results were found using Myo. Mice were primed i.p. or s.c. on day 0 with one of the following: PorB/OVA, OVA alone, or PBS. Subsequent challenge of the PorB/OVA group with OVA alone at day 14 induced a significant increase in the number of eosinophils recruited by the PorB/OVA-primed group after challenge with OVA alone compared with mice primed and challenged with OVA alone (p = 0.02; Fig. 2A). PMN numbers between PorB/Ag-primed mice and mice primed with Ag alone were similar, suggesting that PorB specifically enhanced eosinophil recruitment (Fig. 2B). At the level of practical applicability, s.c. primed mice demonstrated recall peritoneal eosinophils to the same level as mice primed and challenged i.p., suggesting that this vaccine regimen induces a systemic response, and therefore, anatomical location of the initial vaccination would not be a limiting factor in the field. Interestingly, both the specific eosinophilic recall and nonspecific PMN accumulation mirror responses seen in secondary infections with the filarial parasite *Brugia malayi* (Fig. 2C) (experiments performed at University of Connecticut Health Center (Farmington, CT) by L. M. Ganley-Leal (Ph.D. dissertation) under the direction of Dr. T. V. Rajan; data used with permission from Dr. T. V. Rajan).

Because PorB is a known TLR2 ligand, we sought to determine whether the induction of eosinophilia recall was a global characteristic of TLR2 ligands. Mice were primed with Pam3CSK4/OVA or PGN/OVA to determine whether eosinophilia recall in response to OVA would be generated. Interestingly, the TLR2/TLR1 ligand Pam3CSK4 induced recall eosinophilia, whereas the TLR2/TLR6/ NOD2 (25) ligand PGN did not, even at a relatively high dose of 50 μg/ml (shown are results from 10 μg/ml PGN; Fig. 2A).

Ag specificity is essential for a vaccine to be efficacious against a pathogen and nonspecific inflammation can be potentially dangerous; thus, we sought to confirm that the PorB/OVA-associated recall eosinophilia was truly Ag specific. Mice were immunized with PorB/OVA and challenged with either OVA (Ag specific) or Myo (Ag nonspecific). Eosinophilia recall was observed only in the mice challenged with OVA, indicating that recall is Ag specific (Fig. 2E). The reverse experiment was also performed, priming with PorB/Myo and challenging with OVA on day 14. Again, eosinophilia was only observed in an Ag-specific manner, in this case, to Myo (Fig. 2E).

Eosinophil recall response is reduced in TLR2 KO mice, but is intact in TLR4 KO mice

To further evaluate the role of TLR2 in the eosinophilia recall response to OVA, mice genetically deficient in TLR2 or TLR4 were primed with PorB/OVA, and challenged with OVA alone 2 wk following priming. Priming with PorB induced a significantly
lower influx of eosinophils in TLR2 KO mice in response to OVA compared with WT mice, whereas eosinophil recall was similar in TLR4 KO and WT mice (Fig. 3). These results suggest that TLR2 plays a role in the “priming” aspect of the PorB-mediated augmentation of the recall eosinophilia.

Peritoneal macrophages up-regulate TLR2 after stimulation with PorB and secrete chemokines in a TLR2-dependent manner

We sought to examine the potential mechanism(s) of PorB-mediated eosinophil recruitment. Because PorB is a TLR2 ligand, we assessed TLR2 expression on cells in the peritoneal cavity. The lymphocytic population (B, T, NK cells) demonstrated low levels of TLR2 (data not shown). In contrast, 25% of peritoneal macrophages were TLR2+ ex vivo (Fig. 4A). After injection with PorB, macrophages increased surface levels of TLR2 compared with injection with PBS alone (Fig. 4A).

We then assessed the production of chemokines from peritoneal macrophages to determine their contribution to PorB-mediated eosinophilia. Although the eotaxin is a major chemoattractant for optimal eosinophil recruitment (26), we found that PorB induced very low levels of eotaxin from cultured macrophages. This was probably due to the fact that eotaxin is produced mainly by epithelial cells rather than by myeloid cells (27). In contrast, incubation of naive peritoneal macrophages with PorB induced high levels of the MIP-1α (CCL3) and RANTES (CCL5), other chemokines implicated in eosinophil mobilization (Fig. 4B) (28, 29).

To further dissect the mechanism of PorB-mediated chemokine secretion, macrophages were isolated from TLR2 KO mice and cultured in the presence of PorB. Macrophages lacking TLR2 secreted lower levels of both MIP-1α and RANTES when compared with WT mice, demonstrating that PorB likely induces production of chemokines largely in a TLR2-dependent manner (Fig. 4B). These data indicate that TLR2-mediated stimulation of macrophages by PorB could be an important source of eosinophil-specific chemokines in this murine model of inflammation.

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**FIGURE 2.** Intraperitoneal priming with PorB/OVA induces an Ag-specific recall eosinophilia response upon challenge with OVA alone. A, C57BL/6 (n = 4 mice per group) were injected i.p. or s.c. with PorB/OVA, PGN/OVA, Pam3CSK4/OVA, or OVA alone on day 0 as described in Materials and Methods. On day 14, mice were challenged with OVA alone or PBS and sacrificed. Cellular populations in the peritoneum were characterized via flow cytometric analysis. Mice primed with PorB/OVA had significantly more eosinophils than mice primed with OVA alone (p < 0.05). B, PMN recruitment in C57BL/6 mice primed with PorB/OVA or OVA alone and challenged with OVA on day 14 are not statistically different. n = 5 mice per group. C, Experimental primary and secondary B. malayi infections in mice result in cell recruitment similar to that seen in response to priming with PorB. Two groups of mice (n = 5 mice per group) were injected with 50 L3 (TRS Laboratory). B. malayi on day 0 (primary infection). One of the groups of mice was challenged with 50 L3 B. malayi on day 28 (secondary infection), and all mice were necropsied on day 29. Cell recruitment to the peritoneum was assessed as above and as previously described (79). PMN numbers in secondary infections remain similar between the primary and secondary infections, whereas there is a significant and specific increase in eosinophil numbers. n = 5 mice/group. Experiments were performed at University of Connecticut Health Center (Farmington, CT).

**FIGURE 3.** Recall eosinophilia is reduced in TLR2 KO mice. C57BL/6 and TLR2 KO mice were concurrently injected with 10 μg of OVA or 10 μg of OVA plus 10 μg of PorB on day 0. On day 14, all mice were challenged with 10 μg of OVA. Cell recruitment was determined as previously described; n = 4 mice per group. Each figure is pooled data from four experiments. Values of p are noted where significant.
PMNs stimulated with PorB express TLR2 and secrete macrophage and eosinophil chemotactants

In general, injection of PBS into the peritoneal cavity induces an acute neutrophil (PMN) influx, which peaks at ~15 h postinjection (Fig. 1B). PMN recruitment and expression of TLR2 in response to PorB was not greater than in response to PBS (Fig. 5A) or thioglycolate (data not shown), demonstrating that basal levels of TLR2 are high on murine PMNs. We sought to determine whether recruited PMNs might also secrete chemokines implicated in eosinophil recruitment in response to PorB. To assess chemokine production from a highly pure population of PMNs (~95% purity), thioglycollate-elicted PMNs were isolated from murine peritoneal cavities and cultured in the presence of PorB. PMNs secreted high levels of the chemokine MCP-1 (CCL2, a macrophage chemotactant), MIP-1α (CCL3), and RANTES (CCL5) in response to PorB when compared with unstimulated cells (Fig. 5B).

Mast cells do not express TLR2 after stimulation with PorB, but secrete chemokines in response to PorB

Although TLR2 appeared to play a central role in eosinophil recruitment, residual eosinophil chemotaxis to the peritoneum was still evident in TLR2 KO mice (see Fig. 3). We sought to determine possible TLR2-independent mechanisms of eosinophil recruitment induced by PorB. Mast cells play a major role in cellular recruitment, because they can rapidly secrete chemokines and cytokines as well as degranulate in response to stimulation (30–32). In contrast to macrophages, CD117+ mast cells recovered from the peritoneum of WT mice were TLR2neg (Fig. 6A). Similarly, cultured WT BMMCs (~98% pure; see Fig. 6A) were also TLR2neg, and stimulation with PorB did not induce expression of TLR2 (Fig. 6B). Despite the apparent lack of surface TLR2, BMMCs stimulated with PorB secreted both MIP-1α and RANTES (Fig. 6C). BMMCs derived from TLR2 KO mice produced equal amounts of MIP-1α and slightly decreased levels of RANTES in response to PorB (Fig. 6C). These results suggest that mast cells likely respond to PorB in a TLR2-independent manner and could be involved in the recruitment of eosinophils when PorB is used in these vaccine regimens.

Mast cells can become activated by cross-linking IgE bound to surface FceRI receptors. There are several mechanisms whereby IgE can be cross-linked. IgE can be specifically cross-linked by Ag that binds to the Fab region of the bound Ab. Additionally, IgE can be nonspecifically cross-linked by Ag that binds to either the Fc or the Fab portion of the IgE Ab, such as in the case of HIV gp120 (33) and Schistosoma mansoni IL-4-inducing principle of S. mansoni eggs (34). Cross-linking in either manner usually has similar functional consequences and induces the mast cells to degranulate, releasing various protease and other mediators, including histamine, PGs, and leukotrienes (35). To assess whether mast cell-bound IgE may be nonspecifically cross-linked by PorB, exogenous trinitrophenyl-specific IgE (non-PorB-specific) was added to BMMCs to occupy high-affinity IgE receptors, because there was no measurable endogenous PorB-specific IgE in the mice used in

FIGURE 4. Macrophages stimulated with PorB up-regulate TLR2 and secrete high levels of TLR2 ex vivo after stimulation with PorB for 24 h. Mice were injected with 10 μg of PorB or PBS and sacrificed 24 h later. Peritoneal lavage was performed to isolate PECs. TLR2 expression was assessed on Mac-1+Gr-1− cells (macrophages). Gray fill, Isotype control; thick black line, PBS injected; thin black line, PorB injected. Representative of five mice/group.

FIGURE 5. Murine PMNs express high basal levels of TLR2 and secrete chemokines in response to PorB. A, PMNs express TLR2 at high basal levels ex vivo after injection with PBS or PorB. Gray fill, Isotype; thin black line, PBS injected; thick black line, PorB injected. Representative of five mice per group. B, PMNs secrete chemokines implicated in macrophage and eosinophil recruitment in vitro in response to stimulation with PorB. PMNs were obtained from the peritoneum 18 h after injection with thioglycollate medium. PMN purity was assessed to be ~95%. Cells were plated in R10 at 5 × 105 stimulated and with 10 μg of PorB for 18 h. ND, Undetectable levels. Cell-free supernatants were analyzed by standard ELISA.
this study (data not shown). Addition of PorB to IgE+ mast cells did not increase chemokine production, suggesting that non-Ag-specific mechanisms of IgE cross-linking by PorB was inducing mast cell activation. In contrast, the addition of anti-IgE, which cross-links surface-bound IgE induced very high levels of MIP-1α release (Fig. 6D).

*Mast cell-deficient mice have reduced eosinophil response upon injection with PorB*

Because PorB induced potent chemokine secretion from BMMCs, we sought to determine whether mast cells played a role in the recruitment of eosinophils in vivo. Mast cell-deficient KitW-v mice and their heterozygous littermates (controls) were injected with PorB, and cellular influx was evaluated at 24 postinjection. KitW-v mice injected with PorB showed a specific, albeit moderate, decrease in eosinophil numbers when compared with their WT littermates. Influx of other cell types, including neutrophils, was comparable between the KitW-v and their heterozygous littermates (Fig. 7 and data not shown). These results suggest that mast cells play a role, although likely minor, in PorB-mediated eosinophil recruitment and that both TLR2-dependent and -independent mechanisms are involved.

**Discussion**

An adjuvant is described as a substance that, when mixed with an Ag, enhances the immunogenicity of that Ag (36). Mechanisms of adjuvant augmentation of the immune response are not entirely understood, but they appear to have one or more effects: prolonging Ag persistence, enhancing costimulatory signals, inducing granuloma formation, and/or stimulating lymphocyte proliferation in a nonspecific fashion (37). The activity of aluminum potassium sulfate, or alum, is associated with its ability to prolong the persistence of Ag (38). Alum-adjuvanted vaccines are the only FDA-approved vaccines; therefore, there is an increased call for novel adjuvants that can enhance both the innate and the acquired immune system to maximize an immune response (4, 10). Additionally, adjuvants are needed to augment immunity in situations where natural infection is not protective, such as in the case of *Plasmodium*, HIV, and helminth infections (39). TLR ligands have recently become the main targets of vaccine researchers for their unique ability to couple these traits (4, 6).
vaccine. The adjuvant chosen for a vaccine preparation should enhance specific protective immunity unique to the pathogen targeted. Even distinct forms of the same TLR ligand can activate cells differentially. In the case of CpG DNA acting through TLR9, D-type ODN has been shown to stimulate plasmacytoid DCs to secrete IFN-α, promote monocyte maturation to DCs, as well as prime NK cells for IFN-γ production. In contrast, K-type ODN has been shown to stimulate B cells and monocytes to secrete IgM, IL-10, and IL-6 (40). Therefore, if CpG is to be used as an adjuvant, the type of immune response that would be elicited would be vastly different depending on which type of ODN was used; thus, the nature of protective immunity required for a specific pathogen should be considered when choosing an appropriate adjuvant.

Similar to TLR9 ligands, TLR2 ligands can stimulate a diverse array of effects. Signaling via TLR2 has been implicated in the skewing of immune response to a Th2 phenotype (41, 42), which favors eosinophil activation (43). TLR2 recognizes a myriad of molecules, including bacterial lipopeptides, Gram-positive PGN, and yeast zymosan (44). The diversity in TLR2 ligands is mirrored on which of the TLRs is expressed by host cells. Whether TLR2 is heterodimerized with TLR1 or TLR6, or depending perhaps TLR2 ligands may activate cells differently based on the nature of protective immunity required for a specific pathogen invasion (10) by rapidly degranulating and/or releasing eosinophil adhesion to the endothelium by blocking CD18, part of the Mac-1 complex (60, 61). Additionally, Mac-1 expression on myeloid cells has also been shown to promote aggregation, phagocytosis, particle-induced oxidative burst, and Ab-mediated cellular cytotoxicity (65).

The mechanisms for primary PorB-mediated recruitment of eosinophils to the peritoneum were analyzed in this study. PorB stimulated macrophages to secrete chemokines implicated in eosinophil recruitment, namely RANTES (28), and MIP-1α (66) in a TLR2-dependent manner. The small amount of chemokines secreted from macrophages lacking TLR2 may be due to PorB’s ability to insert into biological membranes and create a pore, thereby allowing mediators to leach from the cell (67). Furthermore, PMNs expressed high basal levels of TLR2 and may also contribute to eosinophil recruitment directly or indirectly by recruiting macrophages, which may, in turn, secrete additional chemokines. We hypothesized that the residual PorB-mediated eosinophilia noted in TLR2 KO mice may be in part due to TLR2-independent activation of TLR2⁺⁺ mast cells. Mast cells are tissue resident and are often associated with blood vessels or mucosal surfaces, making them uniquely positioned to monitor pathogen invasion (10) by rapidly degranulating and/or releasing chemokines and cytokines in response to stimulation (35). Despite the apparent lack of TLR2, WT BMMCs stimulated with PorB secreted relatively equivalent amounts of chemokines as did mast cells from TLR2 KO. Mast cell-deficient mice had reduced eosinophilia following injection with PorB. These results are similar to a recent study whereby PGN induced lymph node hypertrophy in a mast cell-dependent- but TLR2- and MyD88-independent manner, indicating that other TLR2 agonists can stimulate mast cells in a TLR2-independent manner (68). Recently, it has also been reported that the TLR2 ligands PGN and zymosan can stimulate cell
recruitment and activate cells independently of TLR2 via the complement components C3 and C5a (69, 70); therefore, we cannot discount that other receptors, such as complement receptors, may play a role in PorB-mediated activation in vivo. TLR2-independent cellular activation may be a boon to an adjuvant candidate, because a more broad distribution of cells may be activated upon stimulation with PorB. Indeed, as mentioned above, porins may interact with cells by inserting into or binding to the membrane during infection (67). Furthermore, porins can interact with different surfaces, including artificial membranes, planar lipid bilayers (71), and eukaryotic cell membranes (72, 73). The porins form aqueous transmembrane channels leading to a decrease in membrane potential (74) and interruption of normal cell signaling (75) demonstrating that PorB can interact with cells in a TLR2-independent manner.

It should be noted that no reports of eosinophilia have been observed after immunization with the Hib-OMPC vaccine. Experiments by our group have shown that primary PorB-mediated eosinophilia is ablated upon coinjection with meningococcal LOS (our unpublished observations); thus, we speculate that the Hib-OMPC vaccine does not induce eosinophilia due to the presence of small amount of LOS that are present in the outer membrane (76) and that only purified TLR2 agonists induce eosinophilia. In fact, it has recently been reported that TLRs may not be required for the augmentation of Ab responses by adjuvants containing multiple TLR agonists, suggesting that mixed preparations stimulate immunity differently (77).

At the level of practical applicability, one of the major classes of pathogens that have been shown to be vulnerable to eosinophils are the parasitic helminths (49, 50, 78). Thus, results indicate that PorB may be able to be used as an effective adjuvant for vaccines directed against parasitic helminths, for example, and may not be dependent on the anatomical distribution of TLR2. Because multiple vaccination protocols are complicated in the field where parasitic diseases are endemic, another advantage of these findings is that Ag-specific eosinophilia was observed after one s.c. immunization with PorB, suggesting that PorB would be a viable candidate for an adjuvant in a tropical clinical setting. Importantly, PorB was well tolerated by mice, because none of the animals exhibited any outward signs of discomfort or irritation after injection, which would be advantageous for a candidate vaccine adjuvant as well. These findings demonstrate a new application for the use of TLR ligands as vaccine adjuvants.

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Disclosures

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