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# Meningococcal Porin PorB Binds to TLR2 and Requires TLR1 for Signaling<sup>1</sup>

Paola Massari,<sup>2,3\*</sup> Alberto Visintin,<sup>2†</sup> Jay Gunawardana,\* Kristen A. Halmen,<sup>†</sup> Carol A. King,\* Douglas T. Golenbock,<sup>†</sup> and Lee M. Wetzler\*

TLR2 plays a key role in the initiation of the cellular innate immune responses by a wide range of bacterial products. TLRs signaling, including TLR2 and its coreceptors TLR1 and TLR6, is mediated by a number of specific ligands. Although many of the TLR-mediated cell signaling pathways have been elucidated in the past few years, the molecular mechanisms that lead to cell activation are still poorly understood. In this study, we investigate the interaction of PorB from *Neisseria meningitidis* with TLR2 and describe the direct binding of a bacterial protein to TLR2 for the first time. Using labeled PorB, we demonstrate its binding to TLR2 both in its soluble form in vitro, and when it is over-expressed on the surface of human embryonic kidney 293 cells. We also show that TLR2-mediated binding of PorB is directly related to cellular activation. In addition, using 293 cells expressing the chimeric TLR2/TLR1 and TLR2/TLR6 complexes, we report the selectivity of PorB binding to the TLR2/TLR1 heterodimer, which is required for initiating signaling in transfected 293 cells and in murine B cells. Together, these data provide new evidence that TLR2 recognizes PorB through direct binding, and that PorB-induced cell activation is mediated by a TLR2/TLR1 complex. *The Journal of Immunology*, 2006, 176: 2373–2380.

**N***eisseria meningitidis* is a Gram-negative pathogenic bacterium responsible for human diseases such as bacterial meningitis and septicemia (1, 2). Meningococcal porins such as PorA and PorB, are the most represented neisserial outer membrane proteins (3). Neisserial PorB elicits a variety of different responses in several cell types, including human and murine lymphoid cells and epithelial cells. One of its best described activities is its effect as adjuvant of the immune response, eliciting B lymphocyte proliferation in vitro (4), enhancement of Ab secretion, and amplification of the immune response (5) in a TLR2 and MyD88-dependent fashion (6), which makes it the first nonlipid containing bacterial protein capable of acting through TLR2. The mechanism of PorB adjuvanticity correlates with its ability to up-regulate the expression of the costimulatory molecule CD86 on the surface of B cells (4, 7), dendritic cells (8), and macrophages (H. Macleod and L. Wetzler, unpublished data). Among the variety of intracellular signal transduction events elicited by PorB, NF- $\kappa$ B nuclear translocation has been shown to occur (6) as well as Erk, Jnk, and p38 phosphorylation (H. Macleod and L. M. Wetzler, unpublished observation). All these functions are completely abrogated in TLR2 and MyD88 knockout mice (6).

Thus, TLR2 is essential for PorB response, among other meningococcal products such as lipoprotein (9), soluble factors (10),

outer membrane protein complex (11), and live bacteria (12). It has been shown that the selectivity of the TLR-mediated cellular response to such different bacterial components is due to TLR2 heterodimerization with accessory coreceptors, namely TLR1 and TLR6 (13). The initial events that lead to TLR activation by their agonists (i.e., if recognition of specific bacterial products is mediated through selected binding of these products to the TLR they activate) are still poorly understood. To address this question, we investigated the correlation between the nature of the interaction of PorB with the target cells and its ability to activate them upon TLR2 and TLR6 or TLR1 engagement, observing the direct physical interaction of PorB with TLR2 in a cell-free system and on the surface of TLR2-transfected cells. Furthermore, the hypothesis that involvement of coreceptors, such as TLR1 or TLR6, might be required for PorB-mediated cell activation is explored.

## Materials and Methods

### Bacterial strain and PorB isolation

PorB was purified from *N. meningitidis* strain H44/76  $\Delta$ -1/4 (14) with the method described by Massari et al. (15), which allows for complete removal of endotoxin and lipopeptide. Purified PorB was labeled with biotin according to Visintin et al. (16). Briefly, PorB was incubated with a 10-fold molar excess of NHS-biotin in 10 mM borate buffer (pH 9) for 1 h at 25°C according to the manufacturer's instructions (Pierce). The coupling reaction was stopped with 0.1 M Tris-HCl for 10 min at 25°C. Biotinylated PorB was washed three times in PBS, and the efficiency of the biotinylation was detected by Western blotting with a HRP-conjugated anti-biotin serum (New England Biolabs). Alternatively, PorB was labeled with the fluorescent dye AlexaFluor-594 (Molecular Probes) per manufacturer specifications, with the exception that removal of unbound dye was obtained by five consecutive washes with 1.5 ml of PBS until the UV absorbance of the wash buffer returned to baseline. Labeled PorB retained its ability to induce cell activation as measured by NF- $\kappa$ B nuclear translocation and/or CD86 up-regulation (data not shown).

### Cell cultures and lymphocytes isolation

Human embryonic kidney 293 cells that were stably transfected with different constructs labeled with either cyan-fluorescent protein (CFP)<sup>4</sup> or

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<sup>4</sup> Abbreviations used in this paper: CFP, cyan-fluorescent protein; YFP, yellow-fluorescent protein; MFI, mean fluorescent intensity; LOS, lipooligosaccharide; BAP<sup>Flag</sup>,

yellow-fluorescent protein (YFP) were used. Human-TNFR-CFP (293-TNFR), TLR2-YFP (293-TLR2), TLR4-CFP/MD2 (17), TLR2-CFP/TLR1-YFP (293-TLR2/TLR1), TLR2-CFP/TLR6-YFP (293-TLR2/TLR6), and TLR2-CFP/TLR2-YFP (293-TLR2/TLR2) (18) were grown in DMEM (Cellgro; Mediatech) supplemented with 10% FBS, 2 mM L-glutamine, 10  $\mu\text{g}/\text{ml}$  ciprofloxacin, and 500  $\mu\text{g}/\text{ml}$  G418. HeLa cells were grown in DMEM (BioWhittaker) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Murine splenic B cells were isolated from wild-type C57BL/6J mice and TLR2 (19), TLR6 (20), or TLR1 (21) knockout mice as previously described (4). Cells were grown in RPMI 1640 (Mediatech) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 10 mM HEPES, and 10 mM 2-ME.

#### NF- $\kappa$ B luciferase reporter assay

Cellular activation of 293 cells via TLR2 or TLR4/MD-2 was measured by luciferase production driven by an NF- $\kappa$ B luciferase reporter vector, as previously described (22). Fifteen microliters of GeneJuice (Novagen) were equilibrated at 25°C for 5 min in 250  $\mu\text{l}$  of DMEM, 2  $\mu\text{g}$  of the reporter plasmid were added and incubated for 15 min further, and the mixture was added to  $\sim 5 \times 10^6$  cells. The cells were immediately seeded in 96-well plates and allowed to adhere for up to 24 h. Different concentrations of PorB or *E. coli* LPS (0111:B4) were added to the cells in fresh medium. After a period of 4–16 h, luciferase activity was measured using commercial reagents (Promega) per the manufacturer's instructions, and the luminescence was assessed from duplicate wells using a Wallac Victor2 luminometer.

#### TLR2/PorB ELISA-like binding assay

The first 587 aa of the human TLR2 protein were fused in frame with the C-terminal 233 aa of the Fc portion of the mouse IgG2a and modified by the addition of the linker sequence AAAGG. The cDNA encoding for the resulting chimeric protein was cloned in the retroviral vector pCLCX4 (23), and 293 cells stably secreting TLR2:Fc were generated (24). TLR2:Fc was purified from the cell culture supernatant by standard protein A affinity chromatography and eluted with 0.1 M glycine (pH 2.2). As a control, a similar immunoadhesin encoding for the extracellular domain of TLR4 was created. High protein ELISA binding plates were coated with increasing concentrations of PorB, Pam2CSK4 (EMC Microcollections), or *E. coli* LPS for 3 h at 25°C, blocked for 10 min with 200  $\mu\text{l}$  per well of superblock solution (Pierce) and incubated with 2  $\mu\text{g}/\text{ml}$  of the TLR2:Fc immunoadhesin. After three washes with PBS-T, an HRP-labeled anti-mouse polyclonal Ab (Bio-Rad) was used for detection of captured TLR2:Fc, followed by the appropriated chromogenic substrates (DakoCytomation). Alternatively, 20  $\mu\text{g}/\text{ml}$  of the TLR2:Fc or TLR4:Fc immunoadhesins were plated in carbonate buffer (pH 9.0) on protein A-coated plates. The plates were blocked with 1% BSA, 5% sucrose, 0.1% Tween in PBS for 1 h at 25°C, and incubated at 37°C for 1 h with increasing concentrations of biotinylated PorB or Flag-tagged bacterial alkaline phosphatase (BAP<sup>Flag</sup>) (Sigma-Aldrich) as a control. Biotinylated PorB bound to TLR2:Fc was detected using an anti-biotin-HRP Ab and detected as above. BAP<sup>Flag</sup> was detected using a HRP-tagged anti-Flag mAb (M2, Sigma-Aldrich). The absorbance at 450 nm was measured with a Wallac Victor2 plate reader. Each experiment was performed in triplicate, and the results represent the average readings  $\pm$  SD. The binding affinity constant,  $K_d$ , was calculated according to the law of mass action, which predicts the fractional receptor occupancy at equilibrium as a function of ligand concentration in moles per liter.

#### Immunoprecipitation

The interaction between biotinylated PorB and TLR2 was determined according to Visintin et al. (16). Briefly, 293-TLR2 cells ( $\sim 7\text{--}8 \times 10^6$  cells) were incubated with 1  $\mu\text{g}/\text{ml}$  of biotinylated PorB in 5 ml of medium for up to 3 h. The cells were then washed extensively in Hanks' buffer and lysed with 1 ml of ice-cold lysis buffer (20 mM Tris (pH 8), 137 mM NaCl, 1% Triton X-100, 2 mM EDTA, 10% glycerol) containing protease inhibitors PMSF (1 nM), leupeptin, and aprotinin (10  $\mu\text{g}/\text{ml}$ ). Lysates were centrifuged at 10,000  $\times g$  for 5 min, and the supernatants were collected and incubated with 20  $\mu\text{l}$  of packed streptavidin beads (Sigma-Aldrich) for 1 h at 4°C. The pellets were washed extensively in lysis buffer lacking the protease inhibitors, and analyzed by SDS-PAGE on 10% polyacrylamide gels and Western blotting. A cross-reacting anti-GFP anti-rabbit polyclonal Ab (Molecular Probes) was used to detect the YFP fluorochrome for 30

min at 25°C, followed by HRP-conjugated anti-rabbit polyclonal Ab (Bio-Rad) for 15 min and ECL.

#### Binding assay

Fluorescently labeled PorB (Alexa<sup>594</sup>-PorB) was used to measure binding to the cell surface by flow cytometry. Fifty microliters of transfected 293 cells, HeLa cells, or murine B cells were seeded at  $2 \times 10^6$  per milliliter in 96 U-bottom microplates in PBS/2% FBS and incubated with increasing concentrations of fluorescent PorB for 1 h at 0°C or 37°C. Unbound PorB was removed by two washes with 200  $\mu\text{l}$  of PBS/2% FBS for 5 min at 4°C. The cells were resuspended in 300  $\mu\text{l}$  of PBS/2% FBS and immediately analyzed by flow cytometry. For the competition assay, cells were incubated with fluorescent PorB and 100  $\mu\text{g}/\text{ml}$  of unlabeled PorB, Pam3CSK4, or diacylated macrophage-activating lipopeptide-2 (MALP-2) (EMC Microcollections). Cell-bound fluorescence was analyzed with a FACScan flow cytometer using CellQuest acquisition and analysis software (BD Biosciences). Gating was used to exclude cellular debris associated with necrosis. The results represent the average mean fluorescence intensity (MFI)  $\pm$  SD of multiple experiments. Statistics were calculated using the Student's *t* test.

#### Detection of IL-8 production

Transfected 293 cells were incubated with increasing concentrations of PorB (0.15–10  $\mu\text{g}/\text{ml}$ ) or Pam3CSK4 (0.15–100 ng/ml) and MALP-2 (0.15–100 ng/ml) for 18 h, and IL-8 secretion in the supernatant of the stimulated cells was measured by ELISA in triplicate wells with the BD OptEIA h-IL-8 ELISA set (BD Pharmingen) as specified by the manufacturer. Each experiment was performed in triplicate and the results represent the average readings  $\pm$  SD.

#### B cell activation and flow cytometric analysis

B lymphocytes ( $5 \times 10^6/\text{ml}$ ) were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 18 h with 10  $\mu\text{g}/\text{ml}$  PorB, 500 ng/ml Pam3CSK4, and 200 ng/ml neisserial lipooligosaccharide (LOS; a gift from M. Apicella, University of Iowa, Iowa City, IA). The cells were washed, incubated for 30 min on ice with a FITC-labeled murine anti-CD86 Ab, and washed again, and the expression of surface Ags was examined by flow cytometric analysis.

## Results

### Binding of PorB to TLR2 is measurable

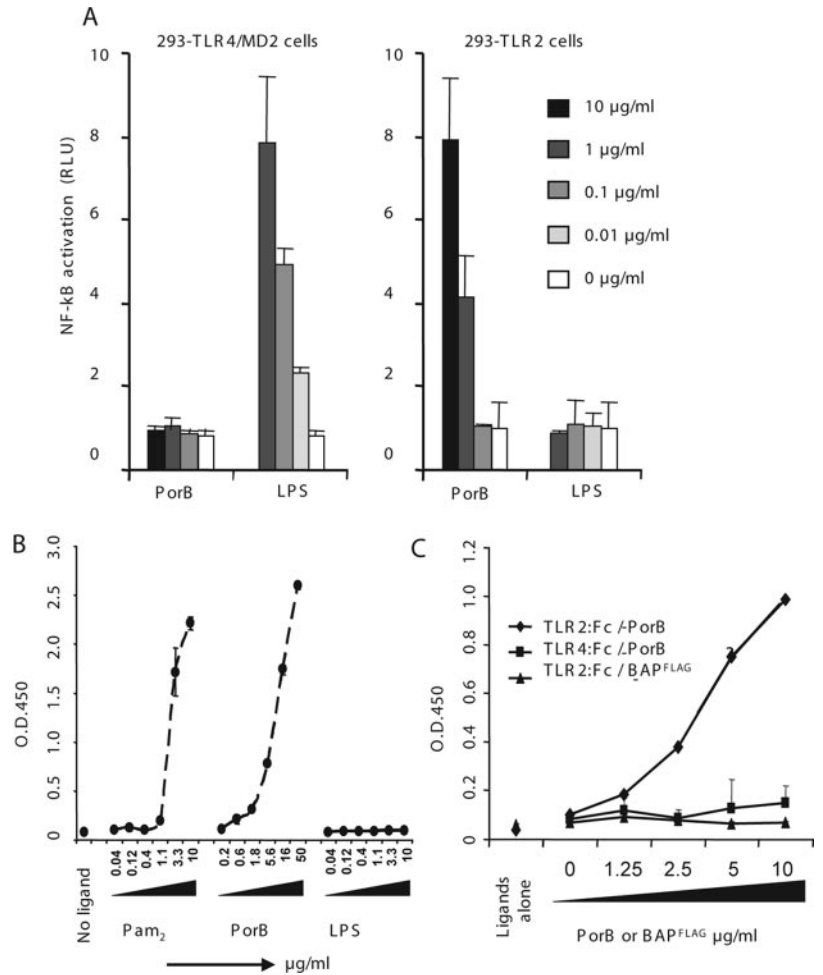
Compelling evidence exist suggesting that PorB interacts with TLR2 expressing cells. It has been shown that PorB is capable of activating the expression of a reporter gene encoding for NF- $\kappa$ B in cells expressing TLR2 (6) but not TLR4 (Fig. 1A). This strongly establishes a link between gene activation and the triggering of TLR2 via PorB in living cells. To assess whether direct binding of PorB to TLR2 occurs, we first examined this question in a cell-free system. A chimeric TLR2:Fc fusion protein was developed, consisting of the extracellular domain of TLR2 fused in frame with the Fc portion of the mouse IgG2a. This was used as a source of TLR2 extracellular domain in two ELISA-like assays. In Fig. 1B, high-protein binding ELISA plates were coated with increasing concentrations of Pam2CSK4, purified PorB, or *E. coli* LPS, and the chimeric protein TLR2:Fc was used to detect the plated ligand. The TLR2:Fc fusion protein readily bound to Pam2CSK4 and PorB with apparent  $K_d$  of  $\sim 1.5$  and 10 nM, respectively, calculated as described in *Materials and Methods*. As expected, TLR2 did not interact with LPS in this system. In the second ELISA-like assay, plates were coated with the TLR2 immunoadhesin, and biotinylated PorB was used to detect the capturing protein (Fig. 1C,  $\blacklozenge$ ). As a negative control, either a Flag-tagged protein similar to PorB in size, BAP<sup>Flag</sup> (Fig. 1C,  $\blacktriangle$ ) was used to detect TLR2:Fc, or a related immunoadhesin (TLR4:Fc) was used to capture the biotinylated PorB (Fig. 1C,  $\blacksquare$ ). Using this approach, an apparent  $K_d$  of  $\sim 5$  nM was observed for the interaction between biotinylated PorB and TLR2.

### Direct evidence of physical interaction between PorB and TLR2

As proof of the direct binding of PorB to TLR2 in living cells, an immunoprecipitation assay was performed. PorB was labeled with

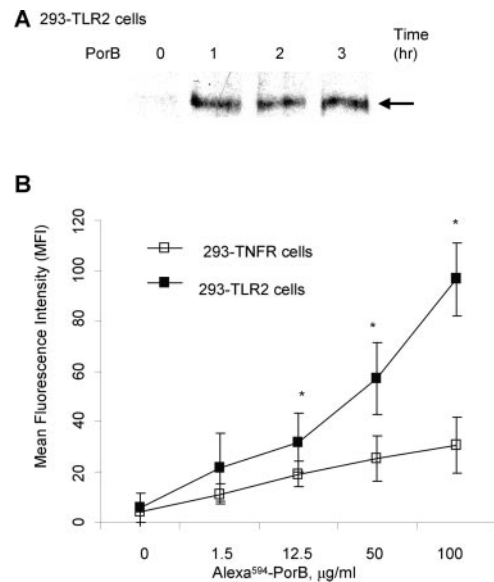
Flag-tagged bacterial alkaline phosphatase; MALP-2, diacylated macrophage-activating lipopeptide-2.

**FIGURE 1.** *A*, 293 cells stably transfected with human TLR2 or TLR4/MD2 and an NF- $\kappa$ B luciferase reporter vector were incubated with increasing concentrations of PorB (0.1, 1, 10 mg/ml) or *E. coli* LPS (0.01, 0.1, 1 mg/ml) for 16 h, and the activity of the NF- $\kappa$ B-dependent luciferase reporter gene was assessed by luminometry. Results are expressed as average luciferase units (relative light units) of triplicate readings  $\pm$  SD, normalized to the untreated cells. *B*, Increasing concentrations of Pam2CSK4, PorB, and LPS were adsorbed on ELISA plates, and 2  $\mu$ g/ml of the soluble TLR2 extracellular domain (TLR2:Fc) was used as soluble phase. Specific TLR2 binding was detected through the Fc domain of the fusion protein using an HRP-conjugated mouse serum. Average readings  $\pm$  SD of absorbance readings at 450 nm from duplicate wells are shown. *C*, ELISA plates were coated with TLR2:Fc or TLR4:Fc (20  $\mu$ g/ml) and increasing concentrations of biotinylated PorB or Flag-tagged BAP were used as soluble phase. TLR-bound proteins were detected using an HRP-labeled anti-biotin polyclonal Ab or an HRP-labeled anti-Flag mAb, respectively. Detection was performed as in *B*. Each experiment is representative of two similar ones.



biotin, and the efficiency of the biotinylation procedure was assessed by Western blot with an anti-biotin Ab (data not shown). Stable cell lines of 293-TLR2 cells were incubated with 1  $\mu$ g/ml of biotinylated PorB for up to 3 h, lysed, and the cell lysates were subjected to immunoprecipitation with streptavidin. The pellets containing the beads and the proteins associated with them were then separated by SDS-PAGE followed by Western blotting with an anti-GFP polyclonal Ab. By cross-reacting with the YFP fluorochrome, this Ab allowed the detection of YFP-labeled TLR2 that may have been precipitated by PorB. As shown in Fig. 2*A*, PorB could efficiently precipitate TLR2 as early as 1 h after incubation with the cells, suggesting that the two molecules form a detergent-stable complex in living cells.

To further investigate the interaction of PorB with cells, 293-TLR2 cells and 293 cells expressing a truncated, inactive form of TNFR (in which the p65 extracellular domain was fused with a CFP fluorochrome in the cytoplasmic portion (17)) were used. PorB was labeled with a red fluorescent fluorochrome, AlexaFluor 594 (Alexa<sup>594</sup>-PorB). The immune-stimulating activity of PorB was tested as a control for the labeling procedure, and was not affected by the addition of the fluorochrome (data not shown). To investigate the binding of PorB to TLR2, both transfected cell types were incubated with increasing concentrations of Alexa<sup>594</sup>-PorB at 0°C for 1 h, to allow surface receptors engagement but not internalization of the receptor-ligand complex. The cells were washed to remove unbound porin and resuspended in PBS/2% FBS, and cell-bound fluorescence was analyzed by flow cytometry. Histograms of each cell sample were generated, and the MFI of the positive cell population was calculated, which directly



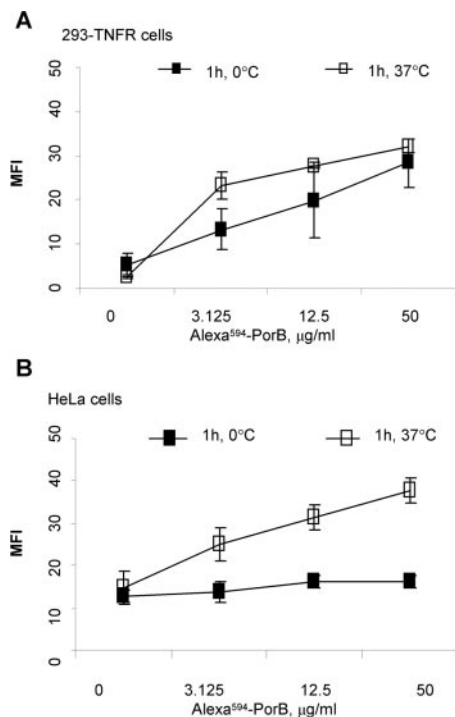
**FIGURE 2.** *A*, 293-TLR2 cells were incubated with 1  $\mu$ g/ml of biotinylated PorB for 1, 2, and 3 h and lysed. The cell lysates were subjected to streptavidin pull-down and analyzed by Western blot with an anti-GFP Ab, which cross-reacts with the TLR2-YFP fluorochrome tag. The band corresponding to TLR2 pulled down by the biotinylated PorB-streptavidin complex is indicated by the arrow. *B*, Increasing concentrations of fluorescent PorB (Alexa<sup>594</sup>-PorB) were incubated at 0°C for 1 h with 293-TLR2 cells (■) or with 293-TNFR cells (□). The fluorescence associated with the cells was measured by flow cytometry and expressed as MFI  $\pm$  SD. \*,  $p < 0.05$  by Student's  $t$  test.

relates to the surface density of Alexa<sup>594</sup>-PorB bound to the cell membrane. After incubation of TLR2-transfected cells with Alexa<sup>594</sup>-PorB, higher MFI values were detected (Fig. 2B, ■), as compared with 293-TNFR cells (Fig. 2B, □). The titration of the fluorescence associated to 293-TLR2 cells suggested that binding to the receptor was dose-dependent.

It has been previously shown that neisserial porins have the ability of inserting in the cell membrane (25, 26), and the possibility of this nonspecific interaction was addressed. Transfected 293 cells were incubated with Alexa<sup>594</sup>-PorB at 37°C to confer membrane mobility and thus allow for the insertion process. In Fig. 3A, a comparison of 293-TNFR cells incubated with Alexa<sup>594</sup>-PorB for 1 h at 0°C or at 37°C shows that the fluorescence associated with the cells was not dependent on the incubation temperature. A similar behavior was noted when 293-TLR2 cells were used and higher MFI values were detected, consistent with the binding experiments (data not shown). This data suggests that in transfected 293, the ratio of PorB association with the cells due to insertion at 37°C vs receptor-mediated binding at 0°C was negligible. HeLa cells, which are known to interact with PorB (26, 27) and do not express endogenous TLR2 (28), were then tested in the binding assay. After incubation of HeLa cells with Alexa<sup>594</sup>-PorB for 1 h at 0°C, minimal cell-associated fluorescence was detected (Fig. 3B, ■). When the incubation was performed at 37°C PorB was found to associate with the cells (Fig. 3B, □). This data demonstrates a temperature-dependent interaction of PorB with HeLa cells, likely due to its insertion in the membrane. Moreover, HeLa cells do not appear to express hypothetical surface-exposed receptors for PorB, due to the lack of cell membrane association at 0°C.

#### Other TLR2 ligands compete for PorB binding site

Having established an effective binding assay, we then determined whether PorB binding could be prevented in competition assays.

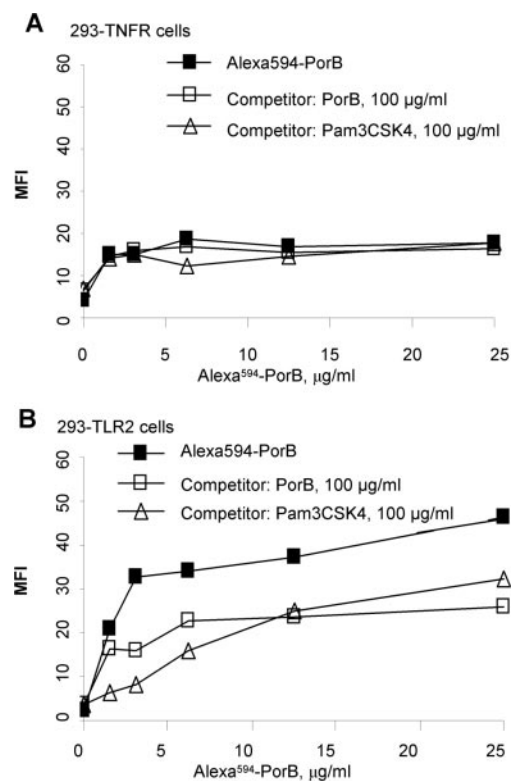


**FIGURE 3.** A, 293-TNFR cells were incubated with increasing concentrations of fluorescent PorB (Alexa<sup>594</sup>-PorB) for 1 h at 0°C (■) or at 37°C (□). B, HeLa cells were incubated with (Alexa<sup>594</sup>-PorB) as above. The cell-associated fluorescence was measured by flow cytometry and expressed as MFI ± SD of triplicate experiments.

293-TNFR and 293-TLR2 cells were incubated with Alexa<sup>594</sup>-PorB alone (Fig. 4, A and B, ■) or coincubated with an excess amount (100 µg/ml) of nonfluorescent PorB as competitor, and the binding was measured by flow cytometry as above. In the absence of TLR2 over-expression, fluorescent PorB interaction with 293-TNFR cell membrane was not inhibited by unlabeled PorB (Fig. 4A, □). When 293-TLR2 cells were used, a decrease in Alexa<sup>594</sup>-PorB fluorescence was detected as a result of competition for the binding site between the two species of porin (Fig. 4B, □). Pam3CSK4 was also used as competitor for PorB binding to 293-TNFR cells and 293-TLR2 cells. When 293-TNFR cells were incubated with 100 µg/ml Pam3CSK4 and Alexa<sup>594</sup>-PorB, no effect on PorB interaction with the cells was detected (Fig. 4A, △). However, when 293-TLR2 cells were used, Pam3CSK4 successfully competed with Alexa<sup>594</sup>-PorB for binding (Fig. 4B, △).

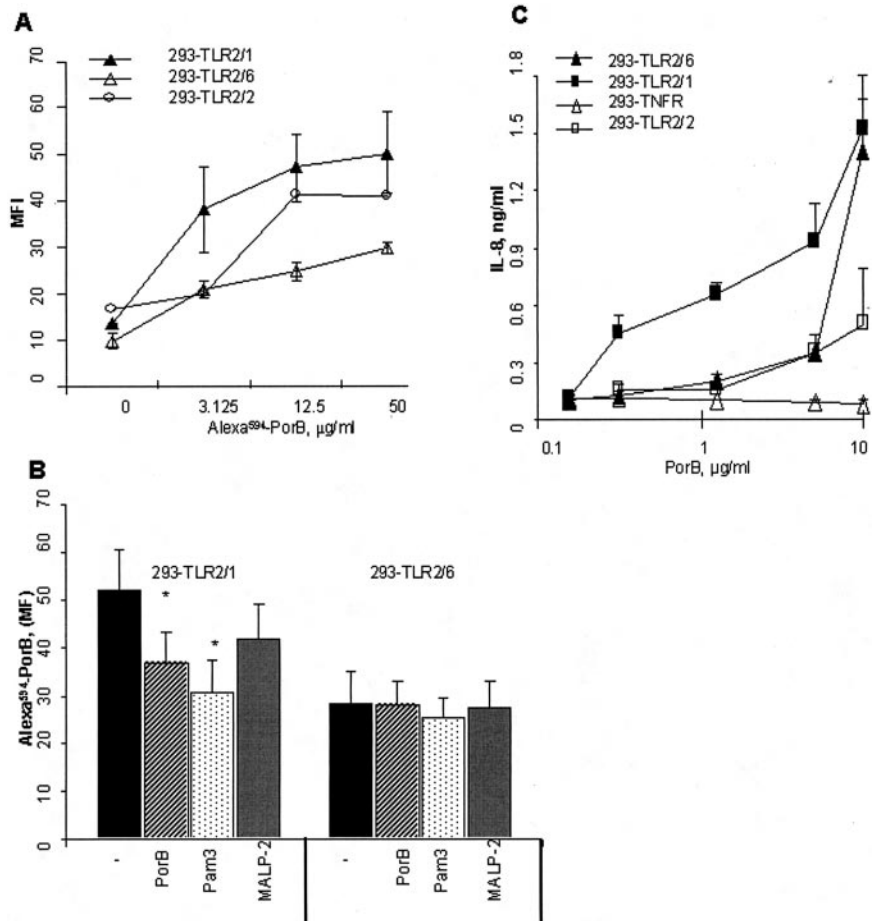
#### TLR1 enhances PorB binding to TLR2

TLR2 heterodimerization with TLR1 or TLR6 is required for specific recognition of different ligands (10, 20, 29). Because 293 cells only express low endogenous levels of TLR1 and TLR6 (30), cells expressing these TLRs in conjunction with TLR2 were used for the binding assay. Alexa<sup>594</sup>-PorB binding was measured in 293-TLR2/TLR6 cells and 293-TLR2/TLR1 cells, and compared with 293-TLR2/TLR2 cells as a control, which express both CFP- and YFP-labeled TLR2 constructs. Fig. 5A shows that more PorB bound to 293-TLR2/TLR1 cells (Fig. 5A, ▲) than to cells coexpressing TLR2 and TLR6 (Fig. 5A, △), TLR2 alone (Fig. 5A, ○), or TNFR, shown in Fig. 2B. Competition for binding to TLR2/TLR1-expressing cells was demonstrated by using an excess amount of unlabeled PorB (Fig. 5B, left panel, dashed bar) or Pam3CSK4 (Fig. 5B, left panel, dotted bar) as competitors, whereas MALP-2



**FIGURE 4.** Competition binding assay was performed using nonlabeled PorB (100 µg/ml, □) or Pam3CSK4 (100 µg/ml, △) and increasing concentrations of Alexa<sup>594</sup>-PorB (■) as in Fig. 3. A, 293-TNFR cells; B, 293-TLR2 cells, one representative experiment.

**FIGURE 5.** A, Increasing concentrations of Alexa<sup>594</sup>-PorB were incubated with 293-TLR2/TLR1 cells (▲), 293-TLR2/TLR6 cells (△), and 293-TLR2/TLR2 cells (○) at 0°C for 1 h. The cell-associated fluorescence was measured by flow cytometry and expressed as MFI ± SD of triplicate experiments. B, *Left panel*, A fixed amount of Alexa<sup>594</sup>-PorB (25 μg/ml) was incubated for 1 h at 0°C with 293-TLR2/TLR1 cells alone (black bar) and with 100 μg/ml of nonlabeled PorB (dashed bar), Pam3CSK4 (dotted bar), or MALP-2 (gray bar). *Right panel*, 293-TLR2/TLR6 cells incubated as above. The fluorescence was measured and expressed as MFI ± SD. \*,  $p < 0.05$  by Student's *t* test. C, IL-8 production was measured by ELISA after incubation with increasing concentrations of PorB for 18 h with 293-TLR2/TLR1 cells (■), 293-TLR2/TLR6 cells (▲), 293-TLR2/TLR2 cells (□), and 293-TNFR cells (△). Each point is the average of absorbance readings at 450 nm from triplicate wells ± SD.



(Fig. 5B, *left panel*, gray bar) failed to displace Alexa<sup>594</sup>-PorB binding. The interaction of PorB with TLR2/TLR6-expressing cells was unaffected, as shown in Fig. 5B, *right panel*.

Furthermore, B cells from spleens of TLR6 and TLR1 knockout mice in a C57BL/6J background, and from their hemizygous counterparts were used. The cells were incubated with Alexa<sup>594</sup>-PorB, and the binding was measured. When Alexa<sup>594</sup>-PorB was incubated with cells lacking TLR1, less fluorescence was detected compared with wild-type B cells and B cells lacking TLR6 (data not shown). This data suggested that the presence of TLR2 alone on the surface of murine B cells was sufficient for PorB binding, but that this binding was enhanced if TLR1 was also expressed.

#### Importance of TLR1 in PorB mediated-cell activation

Induction of IL-8 upon PorB stimulation of transfected 293 cells was measured by ELISA. Fig. 5C shows dose-dependent IL-8 production from 293-TLR2/TLR1 cells in response to PorB (Fig. 5C, ■). IL-8 secretion was decreased in cells expressing TLR2/TLR6 (Fig. 5C, ▲), and it was absent in 293-TNFR cells (Fig. 5C, △). Cells cotransfected with TLR2 alone secreted IL-8 in a similar manner to 293-TLR2/TLR6 cells (Fig. 5C, □). Interestingly, when 10 μg/ml PorB were used, TLR2/TLR6-transfected cells produced a similar amount of IL-8 as TLR2/TLR1-transfected cells, likely due to the heterodimerization of TLR2 with the endogenous TLR1 and increased activation due to the higher amount of PorB used. As positive controls, Pam3CSK4 and MALP-2 were also used, and a dose-response IL-8 production was detected (data not shown). Below-saturation concentrations of Pam3CSK4 and MALP-2 were chosen, and the amount of IL-8 secreted at these concentrations is shown in Table I. MALP-2 specifically induced higher IL-8 se-

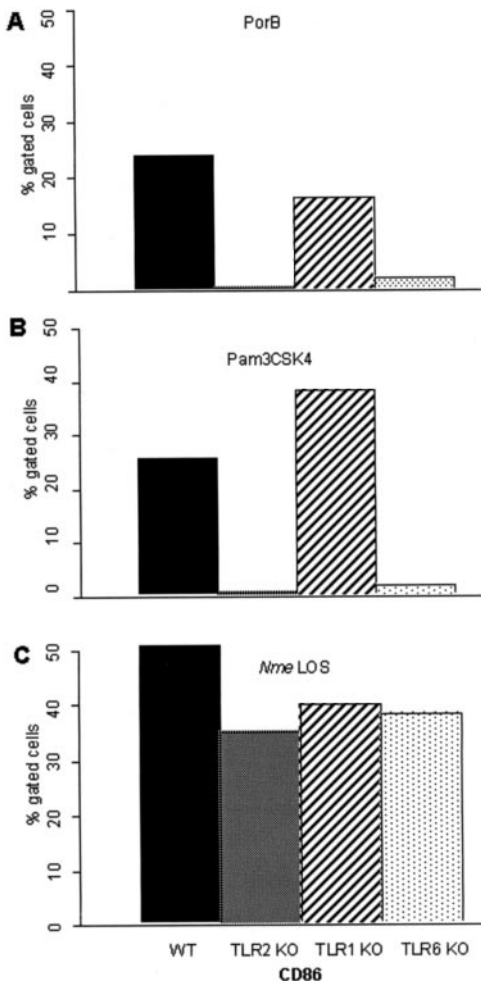
cretion in 293-TLR2/TLR6 cells, whereas Pam3CSK4 induced slightly higher IL-8 production in 293-TLR2/TLR1 cells. 293-TNFR cells failed to respond to either ligand.

To investigate whether coexpression of TLR2 and TLR1, or TLR6 was also required for PorB immune stimulatory activity, B cells isolated from spleens of TLR6 and TLR1 knockout mice and from the wild-type controls were incubated for 18 h with medium alone, 10 μg/ml PorB, 500 ng/ml Pam3CSK4, or 200 ng/ml neisserial LOS as a non-TLR2 control. The cells were labeled with anti-CD86 fluorochrome-conjugate Ab, and the level of surface expression was determined by flow cytometric analysis. The percentage of gated cells expressing higher surface expression of CD86 in response to PorB was calculated and it is shown in Fig. 6A, as a representative experiment. B cells from wild-type mice (Fig. 6A, black bar), TLR2 knockout mice (Fig. 6A, gray bar), TLR6 knockout mice (Fig. 6A, dashed bar), or TLR1 knockout mice (Fig. 6A, dotted bar) are compared. PorB did not up-regulate

**Table I.** IL-8 production measured by ELISA of supernatants from 293-TLR2/TLR1 cells, 293-TLR2/TLR6 cells, and 293-TNFR cells incubated with Pam3CSK4 and MALP-2 for 18 h<sup>a</sup>

	293-TLR2/1	293-TLR2/6	293-TNFR
Pam3CSK4 (6.25 ng/ml)	1.64 ± 0.3	1.23 ± 0.05	0.1 ± 0.02
MALP-2 (25 ng/ml)	0.29 ± 0.06	1.11 ± 0.05	0.07 ± 0.03

<sup>a</sup> The values represent the average of absorbance readings at 450 nm from triplicate wells ± SD.



**FIGURE 6.** B cells were isolated from wild-type mice (black bar), TLR2 (gray bar), TLR6 (dashed bar), and TLR1 (dotted bar) knockout mice and incubated with PorB (10 mg/ml; A), Pam3CSK4 (500 ng/ml; B), or neisserial LOS (200 ng/ml; C) for 18 h. Up-regulation of CD86 was measured by flow cytometry with anti-CD86 FITC-labeled Ab. The percentage of gated cells is shown.

CD86 surface expression in B cells from TLR2 knockout mice (6) or TLR1 knockout mice. CD86 expression on B cells from TLR6 knockout mice was similar to expression induced on B cells from wild-type mice. Similar results were obtained for MHC class II up-regulation (data not shown), suggesting that B cell activation was dependent on the normal expression of TLR1 in addition to TLR2 on the cell surface. As a control for TLR2-mediated activation of B cells, Pam3CSK4 was used, and as a non-TLR2-dependent control, neisserial LOS was used (Fig. 6, B and C, respectively).

## Discussion

The interaction of meningococcal PorB with the cell surface appears to have two components. Nonspecific interaction has been shown to be due to direct insertion of the porin in its native trimeric form in the cell membrane (24, 25, 31), artificial membranes, and planar lipid bilayers (32), with a mechanism appearing to involve the formation of a functional channel (33). The second, specific, mechanism is mediated by binding to TLR2 on the cell surface (this report). The binding of PorB to TLR2 has been demonstrated in both an indirect manner, by ELISA and by flow cytometry using fluorescently labeled porin, and in a direct manner, by coimmunoprecipitation. The indirect evidence of PorB

binding to TLR2 has been obtained by a modified ELISA, using a TLR2-Fc fusion protein. The  $K_d$  of PorB binding, or concentration of ligand that occupies half of the receptors at equilibrium, was calculated empirically. When PorB was coated on the plate, it bound the chimera protein with high affinity, with a  $K_d$  of  $\sim 10$  nM. An alternate TLR2 ligand tested, Pam2CSK4, showed a  $K_d$  of  $\sim 1.5$  nM in this assay. When PorB was used in solution, its  $K_d$  appears to be decreased to  $\sim 5$  nM. Assuming that one trimer of PorB ( $\sim 100$  kDa) binds to one single site on TLR2, this result is consistent with a model in which TLR2:Fc binds monovalently to PorB, when the latter is blocked on the plate. Alternatively, half the amount of biotinylated PorB might be needed, when in solution. This discrepancy could be explained by the incomplete availability of binding sites due to structural distortion of the TLR2:Fc protein bound on the plate, if each chimera protein binds to one trimer at the time. One of the most interesting implications of these studies is that the direct interaction between TLR2 and at least two of its putative ligands (the synthetic lipoprotein Pam2CSK4 and the lipid-free protein PorB) in vitro does not appear to be dependent on additional TLRs or other proteins of cellular origin, suggesting that TLR2 bears at least one binding site for these two ligands. Although PorB and Pam2CSK4 can bind to TLR2 alone, cell activation mediated only by TLR2 has not been demonstrated. Previous published data have demonstrated that TLR2 signaling depends on its "cooperation" with accessory TLRs (13), namely TLR1 (21) and TLR6 (20). Human embryonic kidney 293 fibroblasts express low levels of several endogenous TLRs including TLR1 and TLR6, but lack the expression of endogenous TLR2, TLR4, and the associated molecules CD14 and MD2. These cells have been shown to respond to TLR2 ligands, including PorB (6), when transfected with human TLR2. Interaction of fluorescent PorB to 293-TLR2 cells measured by flow cytometry indicated that PorB bound to cells expressing TLR2 in a dose-dependent manner, suggesting the existence of a unique ligand binding domain. A similar model has been previously reported for another TLR2 ligand, peptidoglycan (34). Most importantly, direct interaction of PorB with TLR2 has been demonstrated in an immunoprecipitation experiment in 293-TLR2 cells. A complex between biotinylated PorB and TLR2 was pulled down as early as 1 h after coincubation of living cells with as low as 1  $\mu$ g/ml PorB, demonstrating a specific ligand-receptor interaction.

Because neisserial porins can insert into the cell membrane, the possible contribution of this nonspecific interaction was addressed. A TLR-independent insertion could, in fact, account for the interaction with cells lacking TLR2 (see Fig. 2B), and which is not followed by cellular activation. According to the membrane fluid mosaic model postulated by Singer and Nicholson in 1972 (35), a cell membrane holds a fluid state at physiological temperature ( $37^\circ\text{C}$ ) while it becomes more tightly packed in a "gel" state at lower temperatures. However, low temperature must be used in binding experiments to prevent endocytosis of the cell surface receptors ligand complexes. If, hypothetically speaking, PorB inserts into the cell membrane in addition to binding to a receptor, more PorB would be detected after incubation at  $37^\circ\text{C}$  than at  $0^\circ\text{C}$ , due to the cumulative effect of both insertion and binding. Comparison of 293-TNRF cells and 293-TLR2 cells incubated with fluorescent PorB did not show a temperature-dependent increase of fluorescence, suggesting that in 293 cells the contribution of the insertion process was negligible. Previous work from our group and others has shown that PorB interacts with HeLa cells (26, 27). Although HeLa cells do not express endogenous TLR2 (28) or TLR1 (10) on their surface, a recent report describes weak levels of most TLRs mRNA (34). In agreement with these reports, TLR1 mRNA was detected by RT-PCR (P. Massari, unpublished observation), but

anti-TLR2 or anti-TLR1 Abs failed to detect surface expression of these receptors in the HeLa cells used in this work. After incubation of HeLa cells with fluorescent PorB at 0°C, minimal membrane association was detected. On the contrary, incubation at 37°C demonstrated a dose-dependent association. If PorB can only insert in the membrane of HeLa cells, this process would be inhibited when the cell membrane is less fluid at 0°C. Moreover, it appears that HeLa cells lacked any additional surface-exposed receptor for PorB, because minimal binding was detected at 0°C. The involvement of another receptor on 293 cells (such as endogenous TLR1 or TLR6) could explain the association of PorB to 293-TNFR cells at 0°C. Interestingly, there are reports that other porins, like those from *Shigella dysenteriae*, require TLR6 in addition to TLR2 for induction of cell activation (35, 36). This hypothesis was examined using cells cotransfected with TLR2 and TLR1 or TLR6. Comparison of PorB binding to either cell type demonstrated that when TLR1 was over-expressed on the cell surface, PorB binding was enhanced. In contrast, although in this model TLR6 over-expression was higher than TLR1 (data not shown), it failed to increase PorB binding. Because the TLR2/TLR1 complex is thought to be preassembled on the cell surface in a ligand-independent manner (18, 21), one could speculate that the TLR2/TLR1 complex might contain a more accessible binding site for PorB. Conversely, once PorB is bound to TLR2 alone, the formation of a TLR2/TLR6 complex might not be favored. Based on this model, the presence of endogenous TLR1 could explain the moderate binding detected when PorB is incubated with 293 cells expressing only TLR2.

The ability of PorB to activate TLR2-transfected 293 cells has been shown (6). In this report, we have measured IL-8 secretion in response to porin as a measure of cell activation. PorB elicited increased levels of IL-8 in 293 cells expressing TLR1 and TLR2 compared with cells expressing TLR2 and TLR6 or TLR2 alone. However, low IL-8 secretion observed in 293-TLR2/6 and 293-TLR2/2 cells might be once again explained by the presence of low endogenous TLR1. The spike in IL-8 production induced by the highest concentration of PorB in 293-TLR2/TLR6 cells might be due to a non-TLR1-mediated cell activation. Upon saturation of the heterodimer formed by expressed TLR2 and endogenous TLR1, when PorB is used at high concentration, it could bind to the TLR2 component of the TLR2/TLR6 complex and induce non-specific cell activation. The same mechanism might explain the high levels of IL-8 detected in response to Pam3CSK4 in both cell types, if Pam3CSK4 has a high affinity for TLR2. The TLR2/TLR1 complex recognizes preferentially triacylated bacterial lipopeptide (21), although recent data have disputed its specificity (37). On the contrary, TLR2/TLR6 complex seems to have a higher selectivity for diacylated lipopeptides (29). Another example of bacterial products that can activate cells through a TLR2/TLR1 complex is OspA (38), a lipoprotein from *Borrelia burgdorferi*, although direct binding of OspA (or any strictly proteinaceous ligand) to TLR2 or TLR1 has not been demonstrated. In contrast, neisserial PorB does not contain any lipid moieties associated to its structure. These observations imply that different regions of TLR2, TLR1, and TLR6 might be involved in the ligand recognition. Further proof of the requirement of TLR1 for PorB activity is its immune-stimulating ability, which was examined in vitro in murine B cells. PorB signaling through TLR2 and MyD88 induces up-regulation of expression of the surface markers CD86 and MHC class II on B cells (6), dendritic cells (8), and other APCs. Our results show that TLR2 and TLR1 are required for murine B cells activation by PorB. In fact, upon incubation with PorB, the percent of B cells expressing high levels of CD86 is decreased in TLR1 knockout mice, demonstrating that its ability to activate the B cells is likely

mediated by the formation of a TLR2/TLR1 complex. In addition, preliminary studies on human peripheral blood B cells show that pretreatment with anti-TLR1 blocking Ab abrogates the ability of PorB to induce IL-6 and IL-8 secretion (L. Ganley-Leal and L. M. Wetzler, unpublished observation).

As mentioned, the requirement for TLR2/TLR6 heterodimerization for *S. dysenteriae* porin (39) signaling has been reported in mice; mRNA levels for TLR2 and TLR6 were increased upon murine peritoneal B-1 and B-2 cells exposure to *Shigella* porin, and up-regulation of surface expression of TLR2, CD80, and CD86 (40, 41) has been detected. An increased level of IL-6 and TNF- $\alpha$  production (42) was also noted. Although both PorB and *Shigella* porin belong to the porin protein superfamily, in a protein sequence alignment of Gram-negative bacterial porins, enteric and neisserial porins form two distinct families with only 31% homology (43). It is intriguing to speculate that maybe differences in their structure could account for the difference in TLR-recognition specificity.

In conclusion, we have shown that TLR2 is the primary mediator for PorB binding to the cell surface, and we have characterized a protein-protein interaction for TLR2 engagement. This interaction appears to be maximized in the presence of TLR1, which makes it a putative coreceptor for PorB interaction. Furthermore, the TLR2-mediated cellular activation induced by PorB, demonstrated in 293 cells and murine B cells, also requires the expression of TLR1.

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## Disclosures

The authors have no financial conflict of interest.

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