Cutting Edge: Immune Stimulation by Neisserial Porins Is Toll-Like Receptor 2 and MyD88 Dependent

Paola Massari, Philipp Henneke, Yu Ho, Eicke Latz, Douglas T. Golenbock and Lee M. Wetzler

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The immunopotentiating activity of neisserial porins, the major outer membrane protein of the pathogenic Neisseria, is mediated by its ability to stimulate B cells and up-regulate the surface expression of B7-2. This ability is dependent on MyD88 and Toll-like receptor (TLR)2 expression, as demonstrated by a lack of a response by B cells from MyD88 or TLR2 knockout mice to the porins. Using previously described TLR2-dependent reporter constructs, these results were confirmed and were shown to be due to induction of NF-κB nuclear translocation. This is the first demonstration of known vaccine adjuvant to stimulate immune cells via TLR2. The Journal of Immunology, 2002, 168: 1533–1537.

Neisserial porins, the major outer membrane proteins of the pathogenic neisserial species, are potential anti-neisserial vaccine candidates and are known to be immunogenic in humans and animals in the absence of exogenous adjuvants (1, 2). In addition, purified neisserial porins induce an enhanced immune response against poorly immunogenic Ags (e.g., peptides (3), and a T cell-dependent immune response to T-independent Ags (e.g., capsular polysaccharide (2, 4)). Neisserial porins have been used as adjuvants in various vaccine formulations such as anti-Haemophilus influenzae type b (5), anti-malaria (3), anti-pneumococcal polysaccharide conjugate vaccine (6), anti-meningococcal polysaccharide conjugate vaccines (7), anti-melanoma (8), and anti-group A streptococcus (9).

The mechanism of the adjuvanticity of neisserial porins in vaccine formulations correlates with their ability to up-regulate the expression of the costimulatory molecule B7-2 (CD86) on the surface of B cells and other APCs (4, 10). The increased surface expression of B7-2 induces augmented costimulation of T cells through the interaction with its counterreceptor, CD28 (10). The effect of neisserial porins on B cells, including increased B7-2 surface expression and B cell proliferation, is not due to LPS, as studies were performed using the LPS-nonresponsive mouse strain C3H/HeJ (4, 10, 11). The lack of response to LPS in these mice is due to a natural point mutation in the gene encoding the Toll-like receptor (TLR)2 (12), necessary for LPS-mediated signal transduction (13).

Toll proteins, responsible for the dorsoventral development in Drosophila (14), are involved in antifungal responses in the adult fly (15). The mammalian orthologs of Drosophila Toll, termed TLRs (16), are type I transmembrane proteins belonging to the pattern recognition receptor family. They are involved in the innate immune response by recognizing microbial conserved structures called pathogen-associated molecular patterns (PAMPs) (16), such as LPS, bacterial lipoprotein, peptidoglycan, lipoteichoic acid, bacterial unmethylated CpG DNA, mycobacterial lipoarabinomannan, and yeast mannans. The recognition of PAMPs by the pattern recognition receptor leads to the activation of various intracellular signaling cascades which modulate nuclear translocation of the transcription factor NF-κB (17), induction of cytokines, and expression of effecter molecules, such as the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) (18). In this way, it has been suggested that signaling by the TLRs bridges innate and adaptive immunity, allowing the host to more efficiently combat microbial infections.

The best-characterized TLRs, to date, are TLR2 (19) and TLR4 (20). TLR2 is involved in the recognition of Gram-positive bacteria and mycobacteria (21, 22) and bacterial products such as lipopeptides (23, 24). TLR4 mediates the effect of Gram-negative bacteria by LPS (13, 25) together with CD14 (26). Recently, another molecule, MD2, has been shown to interact with the ectodomain of TLR4 to confer LPS responsiveness, an essential prerequisite for TLR4 signaling (27). Engagement of TLRs by microbial products results in homodimerisation and recruitment of the adaptor molecule MyD88 (28), the functional homolog of Drosophila adaptor molecule Tube. In mammals, engagement of MyD88 leads to NF-κB nuclear translocation (19, 29).

Porins are of particular interest because they have been characterized as potent adjuvants and have great potential as a novel component of vaccines. Neisserial porins belong to the Gram-negative porin superfamily (30) and share significant structural similarities with other members of the family, resembling other

Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; PAMP, pathogen-associated molecular pattern; CHO, Chinese hamster ovary; HEK, human embryonal kidney.
PAMPs. Given the importance of mammalian TLRs in innate immunity, including their role in B7 up-regulation, and in the light of their ability to discriminate between different pathogens and bacterial products, their involvement in the response to neisserial porins was investigated. Thus far, the involvement of TLRs on the effect of immune adjuvants has only been theorized (16). However, one known immune adjuvant, unmethylated bacterial CpG DNA motifs, appears to induce its immunopotentiating effect through TLR9 (31).

Previous experimental evidence from studies using C3H/HeJ mice established that the immune response to neisserial porins is not abrogated in the absence of functional TLR4. Thus, the involvement of other TLRs in the cellular response to neisserial porins was investigated. Recent reports regarding the participation of TLR2 in response to Neisseria meningitidis (32, 33) suggested that outer membrane components of meningococcus can activate immune cells by engaging TLR2. However, the specific role of porins was not determined. B cells from TLR2 knockout mice (34) and MyD88 knockout mice (35) were used to elucidate the involvement of these molecules in the ability of the porin to stimulate B cells and induce increased surface expression of B7-2 and class II MHC. The data presented in this study directly demonstrate the importance of TLR2 in the adaptive immune response induced by potential vaccine adjuvants.

Materials and Methods

Animal strains

C57BL/6 wild-type mice and TLR2 knockout mice (34) or C57BL/6 and MyD88 knockout mice (35) were used.

Lymphocyte isolation, cell lines, and constructs

B lymphocytes were purified from splenocytes as described (10). A Chinese hamster ovary (CHO-K1) reporter cell line, clone 3E10 (36), stably transfected with human CD14 and an NF-κB-driven reporter construct, which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22). Derivatives of 3E10 were used: clone 7.19 (27) and clone 7.19-TLR2, which expresses which regulates the surface expression of Tac (CD25) Ag (22).

Neisserial porins and reagents

PorB was purified from N. meningitidis strain H44/76 lacking both PorA and RmpM (10, 37) as described (1). Purified neisserial LPS (kindly provided by Dr. M. Apicella, University of Iowa Medical Center, Iowa City, IA) was depleted of contaminating lipoproteins by phenol extraction method (38). The LPS inhibitor, compound E5564, was provided by Esai Research Institute (Andover, MA; patent reference no. WO-9639411-A1). The following anti-murine mAbs were used for flow cytometric analysis: anti-rat IgG, anti-CD86 (B7-2), anti-class II MHC (IAb), and anti-CD25, all FITC conjugated (BD PharMingen, San Diego, CA).

Cell incubations and flow cytometric analysis

B lymphocytes (5 × 10⁶/ml) were incubated with 10 μg/ml PorB or 100 ng/ml N. meningitidis LPS in the presence or absence of 10 μg/ml E5564. CHO reporter cell lines (10⁵/ml) were incubated with 20 μg/ml PorB or with 5 ng/ml IL-1β. The expression of surface Ags was examined by flow cytometric analysis (10, 36).

NF-κB luciferase reporter assay

HEK/TLR2 cells (5 × 10⁶/well) were incubated for 16 h with 0.1, 1, and 10 μg/ml PorB, heat-killed Listeria monocytogenes, or 5 ng/ml IL-1β. Cell activation was determined by measuring luciferase activity of the total cellular lysate using an assay kit from Promega (Madison, WI) according to the manufacturer’s instructions. The data are reported as the mean of triplicate determinations ± SD.

Results

Neisserial porin stimulation of B cells is MyD88 dependent

Purified splenic B cells from C57BL/6 wild-type and MyD88 knockout mice were incubated with purified meningococcal PorB or with medium alone. After 24 h, the levels of surface expression of B7-2 and class II MHC were measured by flow cytometry (Fig. 1A). PorB failed to mediate up-regulation of B7-2 or class II MHC on B cells from MyD88 knockout mice. B cells from C57BL/6 wild-type mice are responsive to LPS, due to the expression of intact TLR4 on their surface. In previous studies by our group (10) we have found that neisserial porin preparations do not appear to contain a significant amount of LPS, which may account for the induction of B7-2 surface expression. However, B cells from C57BL/6 wild-type mice and MyD88 knockout mice were also incubated with PorB in the presence of E5564, a TLR4 antagonist (39). E5564 abrogated the effect of LPS on B cells from C57BL/6 wild-type mice but did not modify the effect of neisserial porins, as shown in Fig. 1. A and B. Controls for LPS-mediated activation of B cells were performed by incubating the cells with N. meningitidis LPS, which could not induce expression of B7-2 or class II MHC in MyD88-deficient B cells (Fig. 2A). Similar results were obtained using purified Escherichia coli LPS (data not shown). These data demonstrate that B cell activation by neisserial porins is MyD88 dependent.

Dendritic cells (DCs) from MyD88 knockout mice have been shown to respond to LPS with delayed kinetics (40); however, no data are available on the behavior of purified splenic B cells from MyD88 knockout mice in response to LPS or other bacterial products. Therefore, longer incubations with neisserial porins were performed to determine whether a similar MyD88-independent stimulation pathway exists for neisserial porins. Purified B cells were incubated for 48 h with PorB or meningococcal LPS in the presence or absence of E5564, and the up-regulation of expression of B7-2 and class II MHC was measured by flow cytometric analysis. The histograms in Fig. 1B show that PorB was not able to induce increased surface expression of B7-2 or class II MHC in MyD88-deficient B cells after 48 h of incubation. Incubation with LPS for...
PorB induces TLR2-mediated NF-κB nuclear translocation

To investigate the interaction of neisserial porins with TLR2, two different cellular reporter constructs were used to measure the TLR2-mediated NF-κB nuclear translocation induced by neisserial porins. CHO cells, normally unresponsive to LPS, were transfected with CD14 (36) and a reporter gene encoding CD25 (Tac) (22). Among such transfectants, clone 7.19 (27), which had a negligible response to LPS (36), was transfected with a plasmid containing human \( \text{tr}2 \) gene, and 7.19/TLR2 cells were incubated for 16 h with PorB or IL-1β as a TLR2-independent control. Fig. 3A shows the level of CD25 surface expression, determined by flow cytometric analysis using anti-CD25 FITC-labeled Ab. PorB induced increased expression of CD25 on the surface of TLR2 transfected cells, while it failed to induce CD25 expression in the cells that were not transfected with TLR2.

HEK-293 cells, which do not normally express TLR2, TLR4, or MD-2 (41), were transiently cotransfected with an NF-κB-dependent luciferase reporter plasmid (13) and with the human \( \text{tr}2 \) gene. Cells were incubated with different concentrations of PorB and, as a TLR2-dependent positive control, with heat-killed \( L. \) monocytogenes. IL-1β incubation was also used as a control, and the naked vectors were used as a negative control. Cell lysates were obtained and incubated in the presence of luciferin as described. As shown in Fig. 3B, PorB induced a dose-dependent increase in luminescence only in cells transfected with TLR2. Increasing concentrations of meningococcal LPS (up to 1 μg/ml) failed to stimulate those cells, while TLR4/MD-2-expressing cells were stimulated by LPS but were not stimulated by PorB (data not shown). These latter data confirm that the presence of LPS in the porin proteosomes preparations was minimal and its effect on these experiments was negligible.

**TLR2 is essential for neisserial porin up-regulation of B7-2 and class II MHC in B cells**

B cells were isolated from spleens of TLR2 knockout mice (34) in a C57BL/6 background and from C57BL/6 wild-type mice, and were incubated with PorB or medium alone. After 24 h, the level of surface expression of B7-2 and class II MHC was determined by flow cytometric analysis. As shown in Fig. 4A, PorB failed to up-regulate the surface expression of B7-2 or class II MHC on B cells from TLR2 knockout mice, as compared with B cells from wild-type mice, already shown in Figs. 1 and 2. This indicates that the up-regulation of B7-2 and class II MHC surface expression was dependent on the normal expression of TLR2 on the B cell. B cells from TLR2 knockout mice were also incubated with PorB in the presence of E5564, which did not affect the expression of B7-2 or class II MHC in porin-treated cells (Fig. 4A), indicating that B cell activation was not mediated by engagement of TLR4. These data have been confirmed in another LPS-sensitive mouse strain, C3H/OuJ, in which the up-regulation of B7-2 and class II MHC induced by neisserial porins was not affected by the presence of this LPS inhibitor (data not shown).

As a control for the involvement of TLR4-mediated LPS stimulation, B cells from TLR2 knockout mice were incubated with
purified LPS from *N. meningitidis* in the presence or absence of E5564. Up-regulation of B7-2 expression and class II MHC on the B cell surface of TLR2 knockout mice induced by incubation with LPS was inhibited by the cotreatment with E5564 (Fig. 4B). Virtually identical results were obtained when *E. coli* LPS was used to stimulate the B cells (data not shown). These data indicate that the TLR4 pathway was not involved in the effect of neisserial porins on B7-2 or class II MHC expression in B cells but is solely involved in the effect of LPS on the expression of these ligands.

The increased surface expression of B7-2 and class II MHC on B cells from TLR2 knockout mice or C57BL/6 wild-type mice involved in the effect of LPS on the expression of these ligands. The possibility that TLR2 might be involved in response to neisserial porins was investigated, because this TLR appears to have an extraordinarily broad repertoire of ligands. All the lines of evidence suggested a central role for TLR2 in porin recognition.

TLR2 is known to be a receptor for bacterial lipopolysaccharides and their interaction has been shown to induce B7-2 surface expression on B cells (16). Outer membrane preparations may contain variable amounts of lipopolysaccharides. Biochemical analysis and in vitro studies comparing neisserial porin preparations with purified neisserial lipopeptide indicated that the effect of porin was not related to the presence of lipopeptide, supporting our hypothesis that the TLR2-mediated up-regulation of B7-2 and class II MHC on B cells surface by neisserial porins mediates their adjuvant activity on the immune response.

In conclusion, this work demonstrates that the effect of neisserial porins on the surface expression of B7-2 and class II MHC on B cells, which mediates the effect of these proteins, is dependent on the interaction of porins with TLR2 on the cell surface. Also, the presence of the effector molecule MyD88 is required for the activity of the porin. Furthermore, we have shown that the effect of neisserial porin on B cells is dependent on MyD88, and that a delayed response to neisserial porins or LPS was not detectable in B cells, as compared with previous reports regarding the effect of LPS on MyD88-deficient DCs (40). As we have demonstrated that the adjuvant activity of neisserial porins is specifically related to their ability to induce increased B7-2 surface expression on APCs, this work suggests that their adjuvant effect is therefore, dependent on porin-mediated TLR2 signaling events.

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