Outer Membrane Protein A (OmpA) of *Shigella flexneri* 2a Links Innate and Adaptive Immunity in a TLR2-dependent Manner and Involvement of IL-12 and Nitric Oxide*

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We determine that OmpA of *Shigella flexneri* 2a is recognized by TLR2 and consequently mediates the release of proinflammatory cytokines and activates NF-κB in HEK 293 cells transfected with TLR2. We also observe that in RAW macrophages TLR2 is essential to instigate the early immune response to OmpA via NF-κB activation and secretion of cytokines and NO. Consistent with these results, TLR2 knockdown using siRNA abolishes the initiation of immune responses. Processing and presentation of OmpA depend on TLR2; MHCII presentation of the processed antigen and expression of CD80 significantly attenuated in TLR2 knockdown macrophages. The optimum production of IFN-γ by the macrophage:CD4⁺ T cells co-culture depends on both TLR2 activation and antigen presentation. So, TLR2 is clearly recognized as a decisive factor in initiating host innate immune response to OmpA for the development of CD4⁺ T cell adaptive response. Furthermore, we demonstrate in vivo that intranasal immunization of mice with OmpA selectively enhances the release of IFN-γ and IL-2 by CD4⁺ T cells. Importantly, OmpA increases the level of IFN-γ production in Ag-primed splenocytes. The addition of neutralizing anti-IL-12p70 mAb to cell cultures results in the decreased release of OmpA-enhanced IFN-γ by Ag-primed splenocytes. Moreover, coinubcation with OmpA-pretreated macrophages enhances the production of IFN-γ by OmpA-primed CD4⁺ T cells, representing that OmpA may enhance IFN-γ expression in CD4⁺ T cells through the induction of IL-12 production in macrophages. These results demonstrate that *S. flexneri* 2a OmpA may play a critical role in the development of Th1 skewed adaptive immune response.

Shigellosis, or bacterial dysentery, is a highly contagious and severe inflammatory diarrhea caused by bacteria of the genus *Shigella*, of which *Shigella flexneri* is the predominant species. Each year, over 164 million cases occur worldwide, with the majority occurring in children in developing countries, and 1.1 million cases result in death (1). Although control and treatment of shigellosis outbreaks with antibiotics is possible, the constant emergence of antibiotic-resistant *Shigella* species, even to the newest antibiotics (2), makes development of an effective vaccine essential to help in the control of shigellosis. Toward vaccine approach our previous study has shown that an outer membrane protein (OMP)³ of *S. flexneri* 2a with a molecular mass of 34 kDa possesses all the attributes of an ideal vaccine candidate (3). Furthermore, it has been observed that the protein commences innate immune response through up-regulation of TLR2, adaptor protein MyD88, p38 MAP kinase, NF-κB, production of type-1 cytokines and chemokines, as well as T cell costimulatory molecules (MHC II, CD40, and CD80) in macrophages (4). Recently the 34-kDa OMP has been identified as OmpA of *S. flexneri* 2a (5). Moreover, it has been observed that OmpA of *S. flexneri* 2a elicits strong protective immunity in mice against shigellosis, which may involve a Th1-directed cell-mediated response (5). OmpA of *Klebsiella pneumoniae* has been shown to bind to and activate human macrophages and immature monocyte-derived dendritic cells in a TLR2-dependent manner (6, 7). It has also been found that OmpA of *Escherichia coli* efficiently stimulates cytokine production by dendritic cells (8). However, the role of *S. flexneri* 2a OmpA in linking the innate and adaptive immune responses remains unexplored. In the light of our previous report that OmpA activates an innate immune response through TLR2 (4), it is crucial to determine whether OmpA of *S. flexneri* 2a is specifically recognized by TLR2 to initiate the innate response and can contribute to the development of appropriate adaptive immune responses. Our earlier studies have also shown that the OMP...
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boosts the induction of NO (3) and IL-12p70 (4) by macrophages. NO up-regulates macrophage phagocytosis and killing, especially in bacterial infections (9). Recent studies demonstrate that NO plays a pivotal role in antigen processing and presentation (10) and IL-12 appears to be critical for the development of Th1 cells and initiation of cell-mediated immune responses (11). The present study has therefore been undertaken to elucidate whether OmpA of S. flexneri 2a is recognized by TLR2 at the onset of the innate immune response and ultimately mold it toward the adaptive immune system through activation of CD4\(^{+}\) T cells as well as the involvement of IL-12p70 and NO in the mechanism of action.

The cells of the monocyte/macrophage lineage play a key role in the induction and regulation of polarized innate and adaptive responses. The cells act by promoting adaptive responses in type I and type II directions, as well as by expressing specialized and polarized effector functions (12, 13). This is initiated by innate recognition of pathogen-associated molecular patterns by the pattern recognition receptors of the host, such as Toll-like receptors (TLRs), on macrophages or other mononuclear phagocytes. Binding of bacterial pathogen-associated molecular patterns to their respective TLRs activates signaling pathways that require adaptor proteins such as myeloid differentiation primary-response gene 88 (MyD88) and Toll/IL-1 receptor domain-containing adapter protein (14, 15). These engage downstream signaling cascades that culminate in the increased production of different cytokines and chemokines, which are key players of the host immune response to bacterial infections (16, 17). Two major TLR-mediated signaling pathways have been described in detail: the mitogen-activated protein kinases (MAPKs) family and the Rel family transcription factor NF-\(\kappa\)B (18–20). Another protein suggested to play a role in TLR signaling is PKC. Several PKC isoforms have been identified with their distinct function (21). A recent study demonstrates the role of PKC\(\alpha\) in regulating the MyD88-dependent TLR/IL-1R-induced cytokine production in both human and mouse dendritic cells (22). The involvement of PKC\(\alpha\) in the induction of nitric-oxide synthase in RAW264.7 cells through the activation of NF-\(\kappa\)B has also been found (23).

The adaptive immune system has evolved diverse responses to defend the host against a myriad of different pathogens. In response to intracellular microbes or viruses, CD4\(^{+}\) T cells are the crucial performer. CD4\(^{+}\) Th cells can be classified into functionally distinct subsets (Th1 and Th2) based on the profiles of cytokines they produce (11). The differentiation of Th1 and Th2 cells from the naive precursor CD4\(^{+}\) T cells is dependent on a combination of host genetic factors, the local cytokine milieu, and the type and dose of antigen encountered (11). Cytokines can either positively or negatively regulate the development of the Th subsets. IL-12 and IFN-\(\gamma\) are thought to drive the polarization of naive T cells toward Th1 cells, whereas IL-4 and IL-13 are important for the induction of Th2 responses (24). With regard to T lymphocyte biology, chemokines and their corresponding cellular receptors are also involved in intrathymic T cell development as well as in differentiation of effector T cells (25). Coordinated expression of chemokine receptors has been associated with functionally distinct T lymphocyte subsets. Th1 cells preferentially express CC chemokine receptor 5 (CCR5), CCR7, and CXC chemokine receptor 3 (CXCR3) (24), whereas Th2 cells predominantly express CCR3, CCR4, and CCR8 (26).

In the present study we demonstrate the S. flexneri 2a OmpA induced activation and type-1 polarization of CD4\(^{+}\) T cells both in vivo and in vitro. To understand the profound molecular mechanism of activation of adaptive immunity, we demonstrate in vitro that recognition of OmpA by TLR2 is essential for transcriptional activation of NF-\(\kappa\)B and production of cytokines and NO, which regulate the mounting of the Th1-mediated adaptive response to OmpA. Our experimental results indicate that OmpA-mediated augmentation of NO production from macrophages may result from the activation of NF-\(\kappa\)B and with the involvement of PKC\(\alpha\). Importantly, the elevated level of NO production occurring in OmpA-treated macrophages may result in the enhanced synthesis of IFN-\(\gamma\), a Th1 cytokine, in CD4\(^{+}\) T cells. Our study also reveals that IL-12p70 might act in combination with NO in the OmpA-induced secretion of IFN-\(\gamma\). The optimal production of IFN-\(\gamma\) by CD4\(^{+}\) T cells requires MHCII presentation of OmpA by macrophages. It has been observed that when OmpA is presented by TLR2 knockdown macrophages, macrophage surface expression of MHCII and CD80 as well as CD4\(^{+}\) T cell production of IFN-\(\gamma\) in the macrophages:CD4\(^{+}\) T cells co-culture was noticeably reduced.

EXPERIMENTAL PROCEDURES

Animal and Cell Line—BALB/c mice, originally obtained from Jackson Laboratories (Bar Harbor, ME), were bred and reared in the animal house facility at the National Institute of Cholera and Enteric Diseases, Kolkata, India. The experiments with animals were conducted in accordance with the Animal Ethical Committee guidelines of the National Institute of Cholera and Enteric Diseases, Kolkata, India.

Mouse RAW264.7 macrophage cell line, obtained from the American Type Culture Collection was maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml of penicillin, and 100 \(\mu\)g/ml of streptomycin. The HEK 293 cell line (ATCC) was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 10 \(\mu\)g/ml of ciprofloxacin (Sigma). The cells were grown in 5% CO\(_2\) at 37 °C to confluent monolayers in 24-well plates (BD Falcon).

siRNA Knockdown—The expression of TLR2 and PKC\(\alpha\) were blocked by transfection with siRNA (Santa Cruz Biotechnology) using the manufacturer’s protocol. The RAW264.7 macrophages were transiently transfected with TLR2 and PKC\(\alpha\) siRNA for 48 h. The transfected cells were then used for subsequent assays.

Immunogen—OmpA of S. flexneri 2a was expressed in E. coli BL21(DE3) and purified as described previously (5). The lipopolysaccharide present in the recombinant OmpA was removed by passing the protein through Detoxi-Gel endotoxin-removing resin and S3A peptide affinity gel columns, respectively. Absence of traces of LPS in the OmpA was confirmed by the Limulus amebocyte lysate chromogenic assay with Kinetic-QCL® (Lonza).
Preparation of Murine Peritoneal Macrophage (Mφ)—Mφ of BALB/c mice were isolated as described earlier (3). Briefly, peritoneal washings containing the macrophages were collected on sterile Petri dishes and incubated at 37 °C in 5% CO₂ for 2 h. The cells of the monocyte macrophage lineage adhered on the surface of the Petri dishes to form a confluent cell monolayer. The nonadherent peritoneal cells were removed by repeated washing of the plates with cold PBS. The adherent peritoneal cells were removed from the surface with a rubber scraper. The cells were washed thoroughly by suspending in PBS and subsequent centrifugation at 400 × g for 5 min. The cell pellet obtained was suspended in RPMI 1640 medium. Cells were seeded per well at a concentration of 1 × 10⁶ cells/ml in 96-well flat-bottomed tissue culture plates (BD Falcon). Mφ were cultured at 37 °C in a humidified 5% CO₂ atmosphere, in the absence and presence of OmpA of *S. flexneri* 2a in RPMI 1640 supplemented with 5 units/ml of penicillin G, 5 μg/ml of streptomycin, 0.1% gentamycin, 2% fetal bovine serum, and 0.1% insulin/transferrin/selenium. The integrity of the monolayer was monitored by inverted light microscopy (Olympus).

Isolation of CD4⁺ T Cells—T cells were isolated from spleens of BALB/c mice. Spleens were stained over 70-μm nylon cell strainers. The resulting single cell suspension of splenocytes containing CD4⁺ T cells were layered over Histopaque® and mononuclear cells were isolated via differential centrifugation. The CD4⁺ T cells were then purified on CD4⁺ T cell enrichment columns (R&D System). The purity of the cell preparations was determined by FACS analysis with phycoerythrin-conjugated anti-CD4 antibody (BD Pharmingen). Routinely, the purity of the cell preparations was >95%. The cells were then washed in RPMI 1640 containing 2% FCS, 5 units/ml of penicillin G, 5 μg/ml of streptomycin, and 0.1% gentamycin.

Peritoneal Macrophage and CD4⁺ T Cell Co-culture—CD4⁺ T cells (2 × 10⁶) were cultured with 10⁵ macrophages in a final volume of 200 μl in round-bottomed 96-well plates in the presence of antigen and inhibitor drug. Preliminary dose-response experiments were performed to determine the optimal concentration of the drug, i.e. the lowest concentration that inhibited the antigen processing and presentation and was not toxic for macrophages. For inhibition experiments, 1 μg/ml of chloroquine was supplemented to the macrophage:T cell co-culture simultaneously with the antigen.

Immunoblotting—Control and OmpA-stimulated peritoneal macrophages and RAW264.7 cells were washed twice with ice-cold phosphate-buffered saline and then homogenized in buffer consisting of 20 mM Tris-HCl, 2 mM EDTA, 10 mM EGTA, 1 mM PMSF, 20 μM leupeptin, 0.1% 2-mercaptoethanol, pH 7.5. The homogenates were centrifuged at 500 × g for 5 min at 4 °C to separate nuclei. The resulting supernatant was centrifuged at 35,000 × g for 35 min at 4 °C to separate the soluble and membrane fractions. After protein quantification, samples from cell homogenates and their equivalent cytosolic and membrane fractions were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and then probed using anti-PKCα antibody. To monitor equal loading of protein, Western blot analysis using antibody directed against actin was done for each experiment, as shown in the lower panels of Figs. 3 and 4B.

Immunization of Mice—The ability of the recombinant OmpA to promote the type-1 skewed immune response was ascertained in BALB/c mice. Immunization of mice was performed as described previously (5). Briefly, 7-week-old BALB/c female mice weighing ∼25 g were sedated by intramuscular injection of a mixture of 0.3 mg of xylazine hydrochloride and 1.0 mg of ketamine hydrochloride in 50 μl of saline. Each mouse was immunized intranasally with 3 μg of OmpA of *S. flexneri* 2a on days 0, 14, and 28. A total antigen volume of 25 μl was delivered in five to six small drops applied to the external nares with a micropipette. Control animals were inoculated intranasally with 0.9% saline.

Transient Transfection and NF-κB Luciferase Reporter Assay—HEK 293 cells were plated in 6-well tissue culture plates (4 × 10⁵ cells/well). Using TransFast™ transfection reagent (Promega), the cells were transfected with pNFkB-MetLuc2-Reporter vector (Clontech) and followed by human TLR2 FLAG cloned in pFlag-CMV-1 (Addgene). The empty vector pFlag-CMV-1 was used as a control and to normalize the DNA concentration for transfection reactions. RAW264.7 cells were transiently transfected with either 1.0 μg of the pMetLuc-control vector or the pNFkB-MetLuc2-Reporter vector containing the NF-κB promoter element by TransFast™ transfection reagent (Promega). Twenty-four hours after transfection, cells were stimulated with OmpA and RAW264.7 using the Ready-To-Glow™ Secreted Luciferase Reporter Assay System (Clontech), according to the manufacturer’s instructions.

Flow Cytometry—RAW macrophages were incubated for 6 h with either 5 μg/ml of OmpA or culture media. Cells were then harvested and incubated with FITC-conjugated mAbs to cell surface markers MHC-II and CD80 for 20 min at 4 °C. CD4⁺ T cells were isolated from the spleens of OmpA immunized and non-immunized mice and stained at 4 °C in the dark for 20 min with one of the FITC-conjugated anti-mouse CCR3, CCR5, CXCR3, or IL-12Rβ2 receptors. Cells were fixed in 1% paraformaldehyde prior to analysis. Stained cells were analyzed on a FACScalibur using CELLQuest software or FACSaria (BD Biosciences) by WinMDI software.

Intracellular cytokine staining was performed in macrophages and T cell co-cultures. At day 3, cells were collected, washed, and stimulated with phorbol myristate acetate (10 ng ml⁻¹) and ionomycin (1 μg ml⁻¹) for 6 h in the presence of brefeldin A. After blocking Fc receptors, the cells were stained for membrane-bound antigens (PerCP-conjugated anti-CD11b and antigen presenting cell-conjugated anti-CD4 antibodies for macrophages and CD4⁺ T cells respectively), washed with PBS, and fixed in 1% paraformaldehyde. Subsequently the cells were permeabilized and stained with phycoerythrin-conjugated anti-mouse IFN-γ antibody as well as an isotype control that included FITC-conjugated hamster anti-mouse IgG2a.

RT-PCR—Total RNA was prepared from CD4⁺ T cells, peritoneal macrophages, and RAW264.7 using the RNAqueous™, 4PCR kit (Ambion Inc.). The RNA was reverse-transcribed using the RETROscript™ kit (Ambion Inc.) and amplified by PCR using specific murine primers. The primer sequences used for PCR were as follows: mouse inducible NO synthase (iNOS) (forward, 5’-CAGCCCAACAATAACAAGATGACC-3’; reverse, 5’-CAGT-
RESULTS

TLR2-dependent Transcriptional Activation of NF-κB and Cytokine Production by OmpA of S. flexneri 2a—The observation that TLR2 mediates cellular responses to structures from numerous microbial cell wall constituents and may thus be central in host recognition of diverse bacterial pathogens (27) prompted us to investigate whether recognition of OmpA by TLR2 is critical for initiation of the innate immune response. To validate this phenomenon, NF-κB activation and cytokine secretion were performed in HEK 293 cells, which do not express endogenous TLR2. HEK 293 cells were transiently transfected with the TLR2 expression vector pFlag-CMV-1-hTLR2 and incubated with increasing concentrations of OmpA. The cell-free supernatants were assayed by ELISA. We found that in cells transfected with TLR2, OmpA stimulated IL-6, IL-12p70, and IL-1β production in a dose-dependent manner (Fig. 1A).

Because transcription factor NF-κB has been implicated in the expression of numerous cytokines and chemokines, we investigated the role of TLR2 in mediating NF-κB activation in HEK 293 cells treated with OmpA. HEK 293 cells were transiently co-transfected with the pNFκB-MetLuc2-Repoter vector and the human TLR2 FLAG plasmid or control vector, and the human TLR2 FLAG plasmid or control vector, respectively (Fig. 1B). The relative luciferase activities in cell supernatants were analyzed by the Ready-To-Glow Secreted Luciferase Reporter Assay System. All values represent the mean ± S.E. of at least three independent experiments. **, p < 0.001, relative to the control or vector control group.

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pFlag-CMV-1. Upon exposure of HEK-TLR2 cells to OmpA, NF-κB-mediated luciferase expression was detected and this activation was enhanced in a dose-dependent manner (Fig. 1B). In contrast, control cells that were transfected with pFlag-CMV-1 did not show any NF-κB-dependent luciferase activity. To further confirm the role of TLR2 in the induction of immune response, HEK 293-TLR2 cells were stimulated with synthetic triacylated lipopeptide Pam3CSK₄, a well known TLR2 ligand as well as LPS. LPS was used as a TLR4-dependent, TLR2-independent positive control. Our results indicated that Pam3CSK₄ was able to induce the release of IL-6 (Fig. 1C) and expression of the NF-κB luciferase reporter activity (Fig. 1D) in a dose-dependent manner. As a control LPS was unable to produce IL-6 and stimulate NF-κB in these cells. These data provide evidence that OmpA of S. flexneri 2a is recognized by TLR2 expressed on HEK 293 cells and subsequently activated downstream NF-κB signaling to produce IL-6, IL-12p70, and IL-1β.

**OmpA Induces iNOS Expression and NO Production in Peritoneal Macrophages and RAW 264.7**—Induction of NO production in macrophages by bacterial products is important for host defense against intracellular bacteria (28). RT-PCR analysis was performed to measure the iNOS mRNA levels over time. Expression of the iNOS gene was up-regulated in both peritoneal macrophages and RAW264.7 cells by stimulation with OmpA with a peak response at 4 h after treatment (Fig. 2A). To confirm that increased transcription correlates with NO production in peritoneal macrophages and RAW264.7, cells were incubated with the protein at different intervals. A time-dependent increase in the release of NO was observed in both cells (Fig. 2B).

**OmpA Triggers Phosphorylation of PKCα**—PKC isoforms regulate several signaling pathways, including innate immune responses induced by microbial products (29). PKC translocation to the plasma membrane has generally been considered the hallmark of activation. To explore the possibility that OmpA activates PKC through promoting PKC translocation, we performed immunoblotting analysis to test PKC in the cytosol and membrane. Stimulation of cells with OmpA of S. flexneri 2a for 30 min resulted in the translocation of PKC isoform α from the cytosol to membrane fractions, suggesting activation of this isoform (Fig. 3).

**TLR2 Is Essential in OmpA-stimulated PKCα Activation and NO Production**—In an attempt to confirm the prerequisite of TLR2 in the OmpA-induced activation of PKCα and NO release, we employed RAW264.7 cells, a mouse macrophage-like cell line that constitutively expresses murine TLR2. RAW264.7 cells were transiently transfected with TLR2 siRNA or scrambled siRNA, or remained nontransfected. Successful transfection was analyzed by TLR2 expression on the surface of RAW264.7 cells. Transfection with TLR2 siRNA resulted in a maximal decrease of surface TLR2 antibody staining (Fig. 4A, green-lined histogram) after 48 h after transfection than the untransfected control cells (Fig. 4A, blue-lined histogram) as measured by flow cytometry. In contrast, no change in basal TLR2 surface expression was observed on cells transfected with the scrambled siRNA (Fig. 4A, red-lined histogram). After 48 h of transfection with TLR2 siRNA, cells were treated in the presence or absence of OmpA, and cytosolic and membrane fractions of the cells were then prepared and subjected to immunoblot analysis to detect localization of PKCα. Interestingly OmpA-induced translocation of PKCα to the membrane was completely inhibited in TLR2 siRNA-transfected cells (Fig. 4B). Furthermore, it was also found that a significant reduction of OmpA stimulated NO production in TLR2 knockdown cells (Fig. 4C). Importantly it was observed that TLR2 siRNA-transfected RAW macrophages failed to produce NO when stimulated with the TLR2 agonist Pam3CSK₄, conversely the TLR4 agonist LPS strongly triggered the release of NO from these cells (Fig. 4D). These observations signify that OmpA is capable of inducing PKCα activation and secretion of NO by macrophages abso-
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FIGURE 3. OmpA stimulated translocation of PKCα to the plasma membrane. Both peritoneal macrophages and RAW264.7 macrophages were stimulated with OmpA for 30 min. Total protein from cell homogenates (H) as well as the cytosolic (C) and membrane (M) fractions was electrophoresed and immunodetected by Western blotting as described under “Experimental Procedures.” β-Actin was used as an internal control. Results are the mean ± S.E. for three experiments.

ultimately in a TLR2-dependent manner. TLRs play a crucial role in innate immune responses with the secretion of NO, and different cytokines and chemokines, which involve participation of many intracellular signaling molecules (30, 31) essential for host defenses against intracellular pathogens.

OmpA-mediated Release of NO Depends on Sequential Activation of PKCα and NF-κB Downstream of TLR2—Conventional PKCα has been implicated in the induction of nitric-oxide synthase in RAW264.7 with the involvement of NF-κB (23). Therefore, to investigate the involvement of PKCα in induction of NO production by OmpA, RAW macrophages were first transiently transfected with PKCα siRNA, followed by incubation in a medium containing OmpA. The levels of NO in cell culture supernatants were subsequently determined. As shown in Fig. 4C, knockdown of PKCα significantly inhibited the OmpA-mediated release of NO. To understand the molecular mechanism downstream of PKCα in the OmpA-induced secretion of NO, we examined the engaged transcription factor. Initially mouse RAW264.7 monocyte cells were transiently transfected with the pNFκB-MetLuc2-Repoter vector for 24 h and then treated with OmpA and assessed with regard to luciferase activity. Transfection of RAW264.7 macrophages with the reporter construct showed a strong time-dependent stimulation of NF-κB-mediated luciferase expression upon OmpA exposure (Fig. 5, A and B). To determine whether or not NF-κB activation was required for induction of NO by OmpA, we evaluated the effect of a specific NF-κB inhibitor on OmpA-induced luciferase activity and NO production in mouse macrophages. Pretreatment of cells with the specific NF-κB inhibitor, SN50 peptide (Santa Cruz Biotechnology Inc.), significantly decreased the luciferase response (Fig. 5C), as well as the production of NO by macrophages (Fig. 4C). These results demonstrate the involvement of NF-κB in the OmpA-activated release of NO. Finally, to confirm that PKCα activation is indispensable prior to NF-κB in the OmpA-mediated secretion of NO, we measured NF-κB reporter gene activity in PKCα knockdown RAW macrophages with regard to luciferase expression. A sharp decrease in the NF-κB luciferase response was noticed in the transfected cells (Fig. 5C), suggesting that PKCα appears to be upstream of NF-κB activation. Collectively these data identified a mechanism of OmpA-induced production of NO by macrophages, where TLR2 plays the central role in recognizing the antigen and transmits the signal to PKCα, which subsequently regulates activation of NF-κB to release NO.

OmpA of S. flexneri 2a Activates Both Innate and Adaptive Immune Responses Contributing to Antigen Presentation and CD4+ T Cell Activation in Vitro in TLR2-dependent Manner—TLR2-mediated activation of NF-κB results in the up-regulation of numerous molecules (e.g. NO, MHCII, and CD80) known to be important in antigen processing and presentation (32). In our earlier study it was observed that the OMP of S. flexneri 2a up-regulated the expression of MHCII, and CD80 on peritoneal macrophages (4). In the present study, we analyzed the role of TLR2 in regulation of these molecules on RAW macrophages stimulated in vitro with OmpA. RAW macrophages were transiently transfected with the TLR2 siRNA followed by stimulation with OmpA for 6 h. Cells were then analyzed by flow cytometry for expression of CD80 (B7-1) and MHCII. OmpA-induced wild type (WT) macrophages up-regulated the expression of CD80 and MHCII, which were 2.3-fold (2.25 ± 0.10, mean ± S.E., p < 0.05) and 3.3-fold (3.25 ± 0.12, mean ± S.E., p < 0.05) higher, respectively, as compared with control. TLR2 knockdown significantly prevented up-regulation of these molecules on macrophages (Fig. 6, A and B). These results signify that TLR2 is indispensable for the OmpA-mediated expression of MHCII and CD80 molecules on antigen presenting cells.

To determine the impact of TLR2 deficiency in antigen presenting cells on naive T cell activation, we measured IFN-γ production in cell culture supernatants of WT CD4+ T cells co-incubated with either WT RAW macrophages or TLR2 knockout macrophages. A considerable decrease in IFN-γ release was observed by CD4+ T cells, when OmpA was presented by TLR2-deficient macrophages (Fig. 6C). To confirm that the residual IFN-γ production resulting from OmpA was presented using the MHCII pathway and not from activation of an additional innate pathway, both WT- and TLR2-deficient macrophages were treated with chloroquine, a lysosomotropic agent that inhibits MHC class II antigen processing (33). In contrast with untreated co-cultures, those treated with chloroquine displayed considerably decreased IFN-γ production by CD4+ T cells when OmpA was presented by WT macrophages (Fig. 6C). Inhibiting the presentation by TLR2-deficient macrophages almost entirely abolished IFN-γ release (Fig. 6C), a Th1 cytokine, by CD4+ T cells. These data clearly illustrate that TLR2 is the critical factor in the OmpA-mediated link between the innate and adaptive immune responses.

OmpA-mediated IFN-γ Production in Immune CD4+ T Cells—We next examined Ag-specific Th1 and Th2 cytokine responses in 7-week-old mice. To determine the profile of cytokines released by CD4+ T cells in vivo, BALB/c mice were intra-nasally immunized with OmpA on days 0, 14, and 28. One week after the final immunization, spleens were excised from both immunized and nonimmunized mice and then CD4+ T cells were isolated. CD4+ T cells were stimulated in vitro with 2 μg/ml of plate-bound anti-mouse CD3ε and 1 μg/ml of soluble anti-CD28 for 2 days, after which the production of IFN-γ, IL-2, and IL-4 were determined by ELISA. In vitro re-stimulation of CD4+ T cells produced 310.23 pg/ml of IFN-γ (p < 0.05) and
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FIGURE 4. A, TLR2 knockdown with siRNA is detectable in mouse monocyte-derived macrophages using flow cytometry. RAW264.7 macrophages either remained untransfected or were transfected with siRNA against TLR2 or scrambled siRNA. After 48 h, cells were stained with FITC-conjugated TLR2 antibody. Cells transfected with TLR2 siRNA (red-lined histogram) had less TLR2 staining than the untransfected and scrambled siRNA-transfected cells (green-lined and blue-lined histograms, respectively). The data are representative of three independent experiments. B, OmpA induced PKCα activation depends on TLR2 expression. RAW264.7 macrophages were transiently transfected with either TLR2 siRNA or PKCα siRNA for 24 h separately followed by stimulation without or with OmpA for 30 min. Total protein from cell homogenates (H) as well as the cytosolic (C) and membrane (M) fractions were electrophoresed. Activation of PKCα was then checked by immunoblotting with anti-PKCα mAb. The figures are representative of three independent experiments having similar results. C, OmpA-induced NO production depends on TLR2, PKCα, and NF-κB. RAW264.7 cells were incubated in the absence and presence of OmpA or preincubated with either NF-κB inhibitor for 1 h or transiently transfected with TLR2 siRNA and PKCα siRNA for 48 h separately prior to addition of OmpA. The cell-free supernatants were assayed for NO accumulation after 72 h of incubation with the Griess reaction. Data represent mean ± S.E. of three independent experiments. *, p < 0.001, relative to the OmpA-stimulated group in the absence of NF-κB inhibitor, PKCα siRNA, or TLR2 siRNA. D, NO induction by TLR2 ligands is diminished in RAW264.7 cells with the siRNA targeted against TLR2. RAW macrophages were transfected with TLR2 siRNA or remained untransfected and 48 h post-transfection, cells were stimulated with medium, OmpA (5 μg/ml), Pam3CSK4 (1000 ng/ml), or LPS (1000 ng/ml). Nitrite production in the cell supernatants was measured at 72 h by the Griess reagent system. Data represent mean ± S.E. of three independent experiments, p < 0.01.

267.68 pg/ml of IL-2 (p < 0.05) (Fig. 7, A and B). In contrast, OmpA had no effect on the release of IL-4 (Fig. 7C), a Th2 cytokine, in the in vitro re-stimulated T cells. These results demonstrate that OmpA has the capacity to activate T cells in vivo to produce Th1 cytokines.

OmpA Induces Expression of Th1 Chemokine Receptors in Immune CD4+ T Cells—Chemokines are key regulators in the recruitment of appropriate effector cells to sites of inflammation. There is evidence that Th subsets differ in their migratory response to chemokines due to differential expression of chemokine receptors (34). Consequently, in addition to cytokines, we also analyzed the expression of different chemokine receptors in the immune CD4+ T cells by both RT-PCR and flow cytometry. Following intranasal immunization with OmpA up-regulation of CCR5, a signature type-1 chemokine receptor was observed in both gene and protein levels, which were 2.8-fold (2.76 ± 0.10, mean ± S.E., p < 0.05) and 2.2-fold (2.15 ± 0.11, mean ± S.E., p < 0.05) higher, respectively, with respect to nonimmunized control (Fig. 8, A and B). We did not find any change in the expression of CCR3, CCR4, and CXCR3 between immune and nonimmune CD4+ T cells (Fig. 8, A and B). This result correlates with our previous observation that the protein was incapable of up-regulating expression of CXCR3 ligand IP10 but induced the expression of CCR5 ligands RANTES, MIP-1α, and MIP-1β in peritoneal macrophages (4). These data suggest that in parallel to the cytokine profile, OmpA has the potential to exert Th1 adaptive immunity.

OmpA-treated Macrophages Enhanced IFN-γ Release in OMP-primed CD4+ T Cells through IL-12 Induction—To assess the type of OmpA-specific CD4+ T cells that were generated following intranasal immunization, production of IFN-γ and IL-4 were quantified in the macrophage:T cell co-culture supernatant by ELISA. For this, peritoneal macrophages of BALB/c mice were pretreated with OmpA. After 6 h the cells were washed and incubated with OmpA-primed CD4+ T cells. As shown in Fig. 9, A and B, immune T cells enhanced the production of IFN-γ (327.14 pg/ml, p < 0.05) when co-cultured with OmpA-pretreated macrophages but not IL-4. The levels of these cytokines were found to be baseline in the culture supernatants of nonimmunized T cells co-cultured with macrophages pre-treated with or without OmpA. These data clearly reveal that OmpA-specific CD4+ T cells are of the Th1 phenotype.

Because IL-12 is an important cytokine inducing IFN-γ from CD4+ T cells, it is likely that the levels of IFN-γ will be altered by reduced IL-12 levels in the macrophage:T cell co-culture and
A, involvement of NF-κB in the OmpA-mediated activation of macrophages. RAW264.7 were transiently transfected with either pMetLuc-control vector or pNFκB-MetLuc2-Reporter vector containing the NF-κB response element driving the expression of the sequence optimized secreted Metridia luciferase. 24 h after transfection, cells were incubated with or without OmpA (5 μg/ml). Samples of the media were removed at different time intervals and analyzed for Metridia luciferase activity. B, the results are expressed as induction over the values obtained with unstimulated RAW264.7 cells transfected with NF-κB reporter vector, which is assigned an arbitrary value of 1. The data are representative of three similar experiments. C, inhibition of OmpA-mediated NF-κB activation by NF-κB inhibitor, TLR2 siRNA, and PKCα siRNA in RAW264.7 cells. RAW264.7 cells were transiently transfected with the NF-κB reporter vector along with a control vector. At 24 h after the transfection, the cells were pretreated with either NF-κB inhibitor for 1 h or transfected again with TLR2 siRNA and PKCα siRNA for an additional 48 h followed by stimulation in the absence or presence of OmpA (5 μg/ml). Cell-free supernatants were harvested 8 h after OmpA stimulation, and luciferase activity was measured. Results are the mean ± S.E. for three experiments.

A TLR2 is critical for OmpA triggered expression of MHCII (A) and CD80 (B) on RAW264.7. RAW264.7 macrophages were incubated with and without OmpA or transiently transfected with TLR2 siRNA for 48 h prior to treatment with OmpA for 6 h. Cells were harvested and assayed for cell surface expression of MHCII and CD80. Representative data from three independent experiments are shown. C, involvement of both innate and adaptive immune responses in the OmpA-induced release of IFN-γ by CD4+ T cells. Wild type and TLR2 knockdown RAW264.7 macrophages were co-cultured with CD4+ T cells for 24 h and incubated without or with OmpA in the presence or absence of chloroquine. The cell-free supernatants were assayed for IFN-γ after 24 h of incubation. Data represent mean ± S.E. of three independent experiments, p < 0.005.
spleen cell cultures. Addition of anti-IL-12p70 mAb (100 pg/ml, BD Pharmingen) to cultures of the OmpA-treated macrophage:T cell co-culture and spleen cells resulted in a significant reduction of IFN-γ production (Fig. 9C).

To further confirm the role of macrophage-released IL-12 in the induction of IFN-γ by CD4+ T cells, intracellular staining of IFN-γ was performed by being gated on the CD4+ T cells in the macrophage:CD4+ T cell co-culture. Interestingly co-culture
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of OmpA-primed CD4+ T cells with OmpA-stimulated macrophages dramatically enhanced 34% (34.75 ± 1.15, mean ± S.E., p < 0.005) of the CD4+ T cells to express IFN-γ (Fig. 9D), as compared with the OmpA untreated co-culture. This enhancement was entirely IL-12 dependent; neutralization of IL-12p70 by addition of IL-12p70 mAb. Cell-free supernatants were collected 4 days later and assayed for IFN-γ production by ELISA. The data are the mean ± S.E. of one representative experiment of three performed, *, p < 0.001, relative to OmpA treated group in the absence of anti-IL-12p70 mAb. D, multicolor flow cytometric analysis of the intracellular IFN-γ expression after gating on CD4+ T cells from the macrophage:CD4+ T cell co-culture. Macrophages were cultured with and without OmpA or preincubated with neutralizing IL-12p70 mAb for 1 h separately prior to addition of OmpA followed by co-culture with OmpA-primed CD4+ T cells. In addition, OmpA-immunized and non-immunized spleen cells were restimulated with OmpA in the presence of neutralizing IL-12p70 mAb. Cell-free supernatants were collected 4 days later and assayed for IFN-γ production by ELISA. The results are presented as the mean ± S.E. of one representative experiment of three performed. The experiment was done three times with similar results.

DISCUSSION

We have previously reported that macrophage activation by the OMP of S. flexneri 2a participates in the development of innate immune response (4). In the present article we now demonstrate for the first time that the OmpA of S. flexneri 2a elicits the Th1-polarized adaptive immune response both in vivo and in vitro. We also depict for the first time that the OmpA of S. flexneri 2a is recognized by TLR2 to initiate the innate immune response and that the innate immunity mediated by TLR2 signaling is critical for mounting an optimal adaptive immune response to OmpA, leading to CD4+ T cell activation.

The family of Toll-like receptors function as membrane-bound sentinels that play a key role in the activation of the innate immune response by recognition of pathogen-associated microbial patterns (35). Numerous membrane components that are constituents of or are associated with the bacterial outer membrane and cell wall have been demonstrated to activate cells by a TLR-dependent mechanism (36). Recently several studies focused on the involvement of TLR2 in the initiation of the immune response to bacterial porin (37–41). Using HEK 293 cells as a model, we claim that the immune response elicited by the OmpA of S. flexneri 2a results from innate recognition through TLR2 by showing a dose-dependent transactivation of NF-κB and secretion of IL-6, IL-12p70, and IL-1β in the TLR2-transfected cells stimulated with OmpA. To further reinforce that TLR2 participates in the induction of the
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in innate immune response to OmpA and drive that response toward adaptive immunity we also successfully established a model that utilizes RNA interference in RAW264.7 macrophages. Transfection of RAW264.7 macrophages with TLR2 siRNA functionally knockdown the cellular response to OmpA by significant reduction in the activation of NF-κB and other factors known to modulate the adaptive immune response such as, macrophage presentation of MHCII and CD80 molecules, as well as production of NO by macrophages and IFN-γ, a signature Th1 cytokine, release in the macrophages:CD4⁺ T cells co-culture. Collectively these data confirm that TLR2 participates in the early innate immune response to OmpA and contributes to the development of type-1 restricted adaptive immunity.

Previously it has been found that macrophages treated with the OMP lead to the induction of proinflammatory cytokine IL-12p70, which depends on activation of TLR2 and NF-κB (4). In the present study we determined that OmpA significantly induces low levels of NO production by mouse macrophages in a time-dependent manner. The levels of NO mRNA expression have been significantly increased in the presence of OmpA, suggesting that induction of NO by the antigen occurs at the transcriptional level. NO is a key effector molecule for defense against intracellular pathogens, including bacteria, virus, and parasites (42, 43) as well as it has also been evident that low concentrations of NO preferentially activate Th1 cells (44).

The mechanism by which OmpA of S. flexneri 2a induces NO production in macrophages is not known. One possibility is that OmpA enhances the production of NO via phosphorylation of PKCα and consequent up-regulation of NF-κB activation. Knockdown of PKCα with siRNA significantly inhibits the OmpA-induced luciferase reporter activity and the release of NO. The PKC isoforms are important regulators of the TLR-mediated innate immune response against pathogens. Recently it has been documented that PKCα participates in MyD88-dependent TLR/IL-1R-induced immunomodulation by regulating the activation of NF-κB in both human and murine DC (22). Our findings indicate that silencing of TLR2 activation with siRNA results in the reduction of OmpA-mediated PKCα phosphorylation or activation, NF-κB luciferase activity (Fig. 5C), and production of NO, demonstrating that TLR2 activation may facilitate NO-dependent OmpA processing via up-regulation of NO production. These results clearly exhibit an important role of PKCα in the TLR2-mediated release of NO from murine macrophages by OmpA with the involvement of NF-κB.

Effective response to and control of microbial infection seems to require several levels of interactions between the innate and adaptive immune systems and for vaccination purposes, the induction of a strong CD4⁺ T cell response of the adaptive immune system is of great importance (45). Our results illustrate that the OmpA pre-treated macrophages induce antigen-primed CD4⁺ T cell activation with secretion of IFN-γ in vitro. The ability of OmpA to trigger CD4⁺ T cells for a polarized type-1 response has also been examined in vivo. It has been observed that re-stimulation of CD4⁺ T cells, isolated from intranasally OmpA immunized mice with anti-CD3e and anti-CD28 mAbs triggers a significant increase in the production of IFN-γ and IL-2 but not IL-4, the prototypic Th2 cytokine (46). Th1 cells secrete IL-2 and IFN-γ, which have been shown to regulate cellular immune responses by inducing Th1 differentiation (23, 47). This cytokine pattern suggests these T cells are CD4⁺ Th1 cells, indicating that the protein has the ability to trigger polarization toward the Th1 adaptive immune response. IFN-γ is a key player in shaping the adaptive immune response and is involved in directing Th1 cell-mediated immunity. In the present study the mechanism of IFN-γ secretion by OmpA-activated CD4⁺ T cells, whether through engagement of an innate pathway or with the classic adaptive immune signaling of MHCII-mediated antigen presentation has also been determined. Our findings reveal that chloroquine significantly inhibits IFN-γ release from T cells upon OmpA presentation by macrophages. It has been demonstrated that the TLR2-mediated signaling by macrophages may be involved in OmpA-induced IFN-γ production by T cells. Production of IFN-γ has been noticeably reduced in both naive and OmpA-primed CD4⁺ T cells following in vitro OmpA presentation by TLR2 knockdown macrophages compared with OMP presentation by wild type macrophages. These data indicate that the optimal induction of IFN-γ by CD4⁺ T cells relies on both arms of the immune response, i.e. depends on both TLR2 (innate arm) activation and antigen presentation (adaptive arm). This result corroborates with the findings of Wang et al. (48) that TLR2 plays a central role in the production of Th1 cytokine IFN-γ from dendritic cells:CD4⁺ T cell co-cultures in response to a Gram-negative bacterial carbohydrates. Furthermore, the OMP-stimulated induction of IL-12p70 secretion by macrophages, as has been shown by us previously (4), may result in enhanced IFN-γ production by OmpA-primed CD4⁺ T cells, as it is well known that IL-12 exhibits a remarkable ability to enhance IFN-γ and inhibit IL-4 in CD4⁺ T cells (49). Addition of inhibitory anti-IL-12p70 mAb efficiently inhibits IFN-γ producing CD4⁺ T cells and IFN-γ production by the activated CD4⁺ T cells, if IL-12p70 was present at the initiation of the cell cultures, demonstrating that the OmpA-induced release of IL-12p70 by macrophages is a critical mechanism, which results in the enhancement of Th1 cytokine production by CD4⁺ T cells. Taken together these results suggest that reduced CD4⁺ T cell activation might be associated with reduced IL-12 signaling to the CD4⁺ T cells from IL-12 inhibited and TLR2 knockdown macrophages as well as lessened presentation of OmpA by the MHCII pathway because of reduced secretion of NO and decreased expression of MHCII and CD80.

Chemokines/chemokine receptors have been recognized as critical elements for determining the trafficking of T cells to inflammatory sites (50). The polarization of T cells into Th1 and Th2 cells is associated with their expression of different subsets of chemokine receptors (51). The CD4⁺ T cells isolated from intranasally OmpA immunized mice express increased levels of CCR5, whereas there has been no significant induction in the expression of CCR3, CCR4, and CXCR3. The expression of the RANTES/MIP-1α/MIP-1β receptor CCR5 together with CCR1 and CXCR3 has been associated with polarized Th1 cells (26, 34, 52). The high level expression of CCR5 on CD4⁺ T cells in combination with strong induction of IFN-γ supports that
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OmpA has the ability to prime these cells toward the Th1 phenotype.

Our findings also reveal an increased surface expression of IL12Rβ2 on immune CD4+ T cells. Among the several factors reported to be important for inducing type-1 T cell differentiation, IL-12 clearly plays the dominant role. IL-12R is composed of two subunits, β1 and β2. Between these two subunits, IL12Rβ2 is detected selectively in cells responsive to IL-12 such as T cells. Recently it is evident that a low concentration of NO induces the expression of IL-12 receptor β2 in T cells (44). In correlation with this the low levels of NO produced by the OmpA-triggered macrophages may be involved in the enhanced IL12Rβ2 expression and consequently promoting Th1 cell differentiation.

In conclusion TLR2 is the critical regulatory molecule in initiating the host innate response required for optimal CD4+ T cell responses to OmpA. Moreover, OmpA of S. flexneri has been identified as a novel molecule coordinating the innate and adaptive immune responses, hence proving itself as an optimal vaccine candidate. Future studies are being conducted toward the generation of TLR2-deficient mice and evaluation of the role of TLR2 to the convergence of innate and adaptive responses stimulated by OmpA of S. flexneri 2a.

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Outer Membrane Protein A (OmpA) of *Shigella flexneri* 2a Links Innate and Adaptive Immunity in a TLR2-dependent Manner and Involvement of IL-12 and Nitric Oxide

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