A bacterial carbohydrate links innate and adaptive responses through Toll-like receptor 2

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Commensalism is critical to a healthy Th1/Th2 cell balance. Polysaccharide A (PSA), which is produced by the intestinal commensal *Bacteroides fragilis*, activates CD4$^+$ T cells, resulting in a Th1 response correcting the Th2 cell skew of germ-free mice. We identify Toll-like receptors as crucial to the convergence of innate and adaptive responses stimulated by PSA. Optimization of the Th1 cytokine interferon-γ in PSA-stimulated dendritic cell–CD4$^+$ T cell co-cultures depends on both Toll-like receptor (TLR) 2 and antigen presentation. Synergy between the innate and adaptive responses was also shown when TLR2$^{-/-}$ mice exhibited impaired intraabdominal abscess formation in response to *B. fragilis*. Commensal bacteria, using molecules like PSA, potentially modulate the Th1/Th2 cell balance and the response to infection by coordinating both the innate and adaptive pathways.

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Abbreviations used: BMDC, bone marrow–derived DC; HEK, human embryonic kidney; iNOS, inducible NO synthase; MALP-3, macrophage-activating lipopeptide 3; MHCII, MHC class II; PAMP, pathogen-associated molecular pattern; PSA, polysaccharide A; SCC, sterilized cecal contents; SEA, staphylococcal enterotoxin A; Sp1, type 1 *Streptococcus pneumoniae* capsular polysaccharide; TLR, Toll-like receptor; ZPS, zwitterionic polysaccharide.

The gram-negative anaerobic bacterium *Bacteroides fragilis* is an integral component of the normal gastrointestinal flora. This organism colonizes the intestinal tract of essentially all mammalian species; in fact, *B. fragilis* has no known reservoir other than mammals. Genomic sequencing and biochemical analysis have revealed that in *B. fragilis*, an unprecedented proportion of the genomic DNA is dedicated to the production of capsular polysaccharides, which are important virulence factors in most extracellular bacterial pathogens. Loci for at least eight *B. fragilis* capsular polysaccharides have been identified (1), and at least two of these capsular polysaccharides possess a zwitterionic charge motif (2). Studies of *B. fragilis* colonization of the gastrointestinal tract in germ-free mice have identified one of the zwitterionic polymers, polysaccharide A (PSA), as the first bacterial product with a demonstrated ability to stimulate T cell lineage differentiation of the mammalian immune system (3). Indeed, it is the polysaccharide's dual-charge structural motif that appears to confer this ability (4, 5).

Toll-like receptors (TLRs) play a critical role in early innate immunity by sensing the presence of microbial pathogens and initiating a response to clear them (6, 7). These receptors, homologues of the *Drosophila* Toll gene, recognize the highly conserved structural motifs called pathogen-associated molecular patterns (PAMPs) (8). Among the pathogen-associated molecules encompassed by the designation PAMP are endotoxin (LPS), peptidoglycan, and other bacterial cell-wall components including flagellin, bacterial DNA, and viral double-stranded RNA. At least 11 TLRs and their distinct ligands have been identified so far in mammals (9). Among these receptors are TLR4, which is activated by most bacterial LPSes (10), and TLR2, which is activated by several bacterial products, including peptidoglycans, lipoproteins, mycobacterial lipoarabinomannan, and atypical LPSes such as those found in *Porphyromonas* (8, 11). TLRs signal primarily through an adaptor protein, MyD88 (12), although some TLRs can signal through non–MyD88-dependent pathways (13, 14); this signaling ultimately results in NF-κB–mediated gene transcription (15). In fact, all the signaling pathways induce transcription of many immunologically important genes, such as those encoding for major histocompatibility complex MHC molecules (16), co-stimulatory molecules (17), cytokines, chemokines, and adhesion molecules (18).

It is through activation of these molecules that TLRs mediate inflammatory responses, coordinate innate and adaptive immunity (19), prime naive T cells (20, 21), induce memory (22), and facilitate the elimination of pathogens (23).

Extensive research has been performed to elucidate the role of TLRs in innate and adaptive immunity and to identify ligands that can stimulate these receptors (24). Although many known TLR ligands contain carbohydrate moieties, it appears that the noncarbohydrate portion of these molecules (e.g., lipid A in LPS) is usually critical for TLR ligation and activation (25). The potential role of pure carbohydrates as ligands for TLRs remains largely uninvestigated, even though antibodies to TLR2 and TLR4 have been shown to inhibit B cell and macrophage activation induced by polysaccharide fractions isolated from cell cultures of *Acanthopanax senticosus* (26). Likewise, low molecular weight hyaluronic acid oligosaccharides produced during inflammation, fragmented heparin sulfate, and glucuronoxylomannan have been reported to induce maturation of DCs through TLR4 (27, 28).

We have previously identified a mechanism by which PSA can stimulate the adaptive immune response by activation of $CD4^+$ T cells through an MHC class II (MHCII)–dependent mechanism (29). Processing and presentation of PSA depend on the production of NO; MHCII presentation of processed polysaccharides does not occur in mice lacking the inducible NO synthase gene ($iNOS^{-/-}$). In addition, MHCII and the co-stimulatory molecule CD86 are up-regulated upon PSA-mediated activation of APCs (30). Many of the immunologic events leading to PSA-mediated T cell activation (such as iNOS expression, MHCII presentation, co-stimulation, and cytokine production) have been shown in other systems to be linked to TLR activation (16–18). Therefore, we sought to determine whether PSA can initiate the innate immune response in a TLR-dependent manner that is consistent with—or even complementary to—the role played by this polysaccharide (through MHCII) in the adaptive portion of the response.

In this paper we demonstrate that PSA activation of TLR2 not only mediates the release of proinflammatory cytokines and NO by APCs but also leads to NF-κB nuclear translocation in these cells. Consistent with this result, $TLR2^{-/-}$ and $MyD88^{-/-}$ bone marrow–derived DCs (BMDCs) fail to produce TNF-α when incubated with PSA. Optimal production of IFN-γ by $CD4^+$ T cells requires MHCII presentation of PSA by BMDCs. However, when PSA is presented by $TLR2^{-/-}$ BMDCs, $CD4^+$ T cell production of IFN-γ is considerably decreased. Furthermore, IFN-γ production is almost completely abolished when both the innate (TLR2) and adaptive (antigen-processing) arms of the immune response are rendered nonfunctional.

In vivo abscess formation after challenge with PSA or live *B. fragilis* has been shown to depend on a robust adaptive immune response (30). We now demonstrate the additional requirement for an intact innate signaling system for abscess formation, documenting an impaired abscess-forming ability in $TLR2^{-/-}$ mice. These data reveal a critical role for PSA signaling via TLR2 in coordinating the innate and adaptive immune responses.

RESULTS

PSA activates cytokine production by APCs via TLR2-dependent mechanisms

To begin elucidating the response of professional APCs to the *B. fragilis* polysaccharide antigen PSA, we examined the cytokine production profiles of various APCs upon stimulation with the carbohydrate PSA. PSA, *Escherichia coli* LPS (a TLR4 agonist), and the synthetic macrophage-activating lipopeptide 3 (MALP-3; a TLR2 agonist) were incubated with a mouse RAW 264.7 macrophage cell line or primary mouse BMDCs. As shown in Fig. 1 A, TNF-α production induced by PSA was comparable to that produced in response to MALP-3 and *E. coli* LPS in both cell types. Similarly, PSA induced substantial IL-12p40 secretion by BMDCs; however, macrophages failed to produce IL-12p40 in response to any of the agonists tested. Poor IL-12 production by macrophages was not a dose-related phenomenon: even very high doses of PSA did not induce IL-12 production in these cells (unpublished data). Induction of these cytokines by APCs appeared to be a unique function of PSA in that other polysaccharides—such as mannan, polygalacturonic acid, and type 1 *Streptococcus pneumoniae* capsular polysaccharide (Sp1)—failed to stimulate TNF-α or IL-12p40 production in either cell type (unpublished data). Results similar to those described with RAW 264.7 cells were obtained with fresh human monocytes, THP-1–derived macrophages, and fresh mouse peritoneal macrophages.

To establish a role for TLR2 signaling in PSA-induced activation of APCs, an inhibitory mAb to TLR2 was used in NF-κB translocation and cytokine production experiments. This mAb, raised against the extracellular domain of mouse TLR2, inhibits cell activation in experimental mouse models of infection and prevents TLR2-driven lethal shocklike syndromes (31). Macrophages were preincubated with the mAb before the addition of stimuli to ensure complete blockage, and cytokine production was assessed 15 h after stimulation. At 15 h, TNF-α production was significantly reduced in PSA-stimulated cells preincubated with the anti-TLR2 mAb compared with cells treated with an isotype control antibody ($P < 0.05$; Fig. 1 B). Studies with the TLR2 agonist MALP-3 showed a similar blockade of cytokine production by the anti-TLR2 mAb (31), whereas TNF-α production by LPS-stimulated cells was unaffected by this mAb. Likewise, PSA-mediated cytokine release was not reduced by preincubation with an anti-TLR4 mAb (unpublished data).

NF-κB is a critical nuclear transcription factor that regulates many immune genes and cytokine responses (32). Various inducers, such as LPS, result in the degradation of IκB protein and the release of NF-κB dimers that subsequently translocate to the cell nucleus and activate appropriate target genes. To determine whether PSA induces these events in activated APCs, we used a modified ELISA to measure

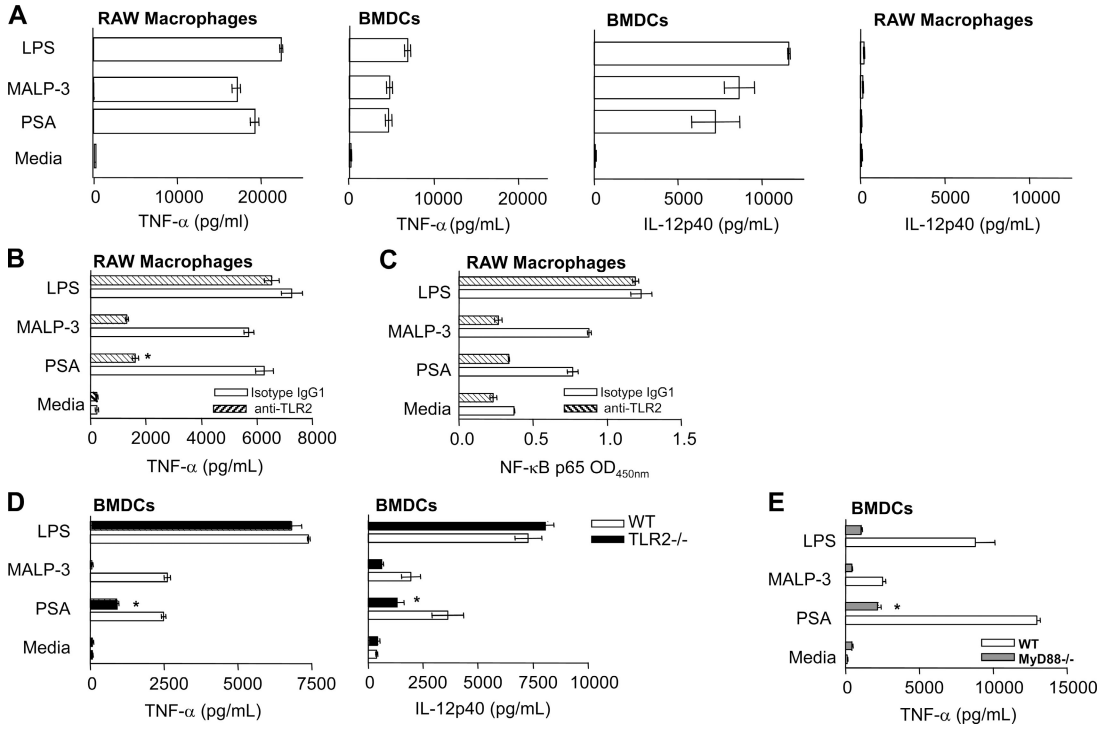

Figure 1. *B. fragilis* PSA induces inflammatory responses in APCs via TLR2-dependent signaling. (A) RAW macrophages and BMDCs were incubated for 15 h with medium control, 100 μg/ml PSA, 20 nM MALP-3, or 100 ng/ml *E. coli* LPS. Levels of TNF-α and IL-12p40 in the cell supernatants were measured by ELISA. (B) RAW macrophages were preincubated with 50 μg/ml anti–mouse TLR2 mAb (clone T2.5) or 50 μg/ml IgG1 isotype control for 1 h before the addition of the following stimulants: 100 μg/ml PSA, 20 nM MALP-3, or 100 ng/ml *E. coli* LPS. Production of TNF-α was assayed by ELISA after incubation for 15 h. (C) Preincubation of RAW macrophages with 50 μg/ml anti–mouse TLR2 mAb or 50 μg/ml isotype control was followed by incubation with 100 μg/ml PSA, 20 nM MALP-3, or 250 ng/ml *E. coli* LPS. NF-κB activation was measured in antigen-activated cell extracts at 1.5 h. (D) BMDCs isolated from WT or $TLR2^{-/-}$ mice were incubated with 100 μg/ml PSA, 20 nM MALP-3, or 100 ng/ml *E. coli* LPS. TNF-α and IL-12p40 production was measured by ELISA after incubation for 15 h. (E) In similar experiments, TNF-α production by $MyD88^{-/-}$ and WT BMDCs was examined. Data represent mean ± SEM. *, $P < 0.05$.

NF-κB p65 (RelA) protein levels after PSA activation. RAW 264.7 macrophages were incubated with PSA, MALP-3, LPS, and a medium control for various intervals, after which activation of NF-κB was measured. NF-κB p65 protein levels increased upon PSA stimulation, with peak activation at 1.5 h (unpublished data). Activation of NF-κB by PSA and MALP-3 was reduced back to the baseline level in the presence of the anti-TLR2 mAb, whereas LPS-induced activation of NF-κB was unaffected by blocking TLR2 activation (Fig. 1 C).

Engagement of TLRs by microbial products often results in TLR dimerization and subsequent recruitment of the adaptor molecule MyD88 (7); these events constitute one major pathway leading to NF-κB translocation and cytokine production. We further investigated the role of TLR2 and the adaptor protein MyD88 in PSA-mediated APC activation. BMDCs were isolated from WT, $TLR2^{-/-}$, and $MyD88^{-/-}$ mice. Upon PSA stimulation, $TLR2^{-/-}$ BMDCs produced lower levels of TNF-α and IL-12p40 than WT cells ($P < 0.05$; Fig. 1 D). Similar results were obtained with BMDCs isolated from $MyD88^{-/-}$ mice ($P < 0.0001$; Fig. 1 E). LPS-induced cytokine production was not affected by the absence of TLR2 but was significantly impaired in $MyD88^{-/-}$ cells, as this signal transduction protein is common to both TLR2 and TLR4 signaling pathways. These results establish a mechanism for PSA-induced activation of APCs through a TLR2-dependent mechanism, which requires intact MyD88.

Cytokine production is TLR2 dependent

In further cytokine production studies of the role of PSA in TLR2 activation, we used cells with known TLR expression patterns. Human embryonic kidney (HEK)–293 cells, which do not normally express TLR2 or TLR4, were cotransfected with the human *tlr2* or *tlr4* gene and incubated with the carbohydrate antigen (33, 34). PSA stimulation induced IL-8 production by TLR2-expressing cells but not by TLR4-positive cells (Fig. 2 A). The unresponsiveness of TLR4-transfected cells to PSA confirmed the absence of *E. coli* LPS contamination in the PSA sample. The human astrocytoma cell line U373 (35), which lacks the TLR2 receptor, failed to produce IL-6 when stimulated with either PSA or the known TLR2 agonist MALP-3, whereas the TLR4 agonist *E. coli* LPS strongly induced the release of IL-6 by this cell type (Fig. 2 B).

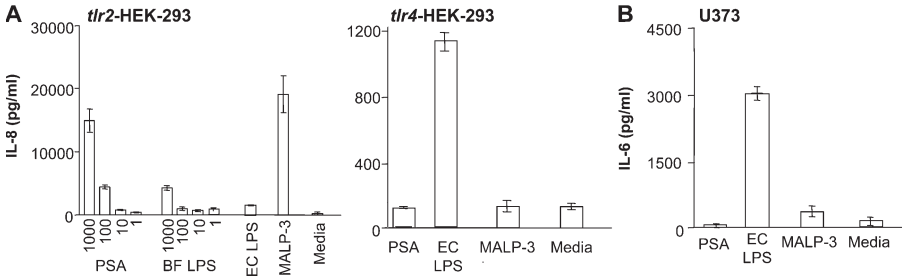

Figure 2. TLR2 is required for PSA activation. (A) HEK cells expressing TLR2 or TLR4/MD2 were incubated for 15 h with 1–1000 μg/ml PSA, 1–1000 μg/ml *B. fragilis* LPS (BFLPS), 20 nM MALP-3, or 250 ng/ml *E. coli* LPS (ECLPS). Cell supernatants were assayed for IL-8 by ELISA. (B) Human U373 astrocytoma cells were incubated with 1 mg/ml PSA, 20 nM MALP-3, or 250 ng/ml *E. coli* LPS, and IL-6 levels were measured by ELISA. Data represent mean ± SEM.

Using purified *B. fragilis* LPS, we confirmed that our observations with PSA were not due to contamination of the PSA preparation with this molecule. In experiments with TLR2-expressing HEK-293 cells, *B. fragilis* LPS at concentrations as low as 1 μg/ml (but not lower) results in IL-8 production. IL-8 release with *B. fragilis* LPS stimulation corresponds to that with PSA stimulation of 100 μg/ml (Fig. 2 A). We examined the PSA preparation by SDS-PAGE with ProQ staining, which detects LPS at low-nanomolar concentrations. No *B. fragilis* LPS was detected in lanes loaded with a 10-mg/ml sample of PSA, a result indicating that LPS exists at a concentration of <100 ng/ml in a 100-μg/ml solution of polysaccharide. This concentration of *B. fragilis* LPS is an order of magnitude below the minimal TLR activation concentration in these cells (Fig. 2 A).

BMDCs from TLR2$^{-/-}$ mice take up PSA into their endosomal compartment

BMDCs from WT and TLR2$^{-/-}$ mice were incubated with fluorescently labeled PSA for 3 h to allow fluorescence uptake by the APCs. Cells were then fixed and stained with MHCII-specific mAbs, and cellular localization was assessed. Both WT and TLR2$^{-/-}$ BMDCs efficiently endocytosed PSA (Fig. 3). PSA and MHCII colocalized within cells, a result indicating that PSA had entered the MHCII compartment within the traditional class II pathway (Fig. 3, arrows).

PSA expressing a zwitterionic charge motif induces NO production through TLR2

When cells encounter microbes or foreign antigens, both reactive oxygen species and reactive nitrogen species are produced. This oxidative environment not only facilitates bacterial killing and degradation (36) but also enables APCs to process T cell–dependent carbohydrate antigens such as PSA for presentation by MHCII (29). We have previously shown that T cells from iNOS$^{-/-}$ mice cannot respond to PSA because of a defect in carbohydrate antigen processing by their APCs. In these animals, PSA is not presented on the surface of the APCs in the presence of MHCII, and T cells are not activated. Consequently, abscess formation (which depends on T cell activation) does not occur in these animals (29).

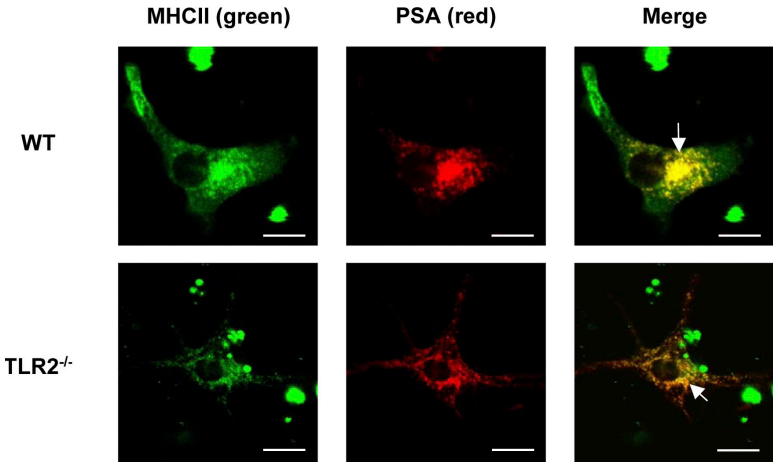

Figure 3. PSA is endocytosed by TLR2$^{-/-}$ BMDCs. BMDCs were incubated for 3 h with fluorescent PSA (red) molecules. The cells were fixed and stained with a mAb specific for MHCII protein (green). Both WT and TLR2$^{-/-}$ cells endocytosed PSA in a manner that resulted in colocalization (arrow, yellow) with MHCII protein. MHCII expression was not dependent on the presence of TLR2$^{-/-}$ under these conditions. Bars, 10 μm.

To establish a link between the adaptive arm of the immune response to PSA and our data demonstrating innate recognition of the same antigen, we examined whether PSA activation of TLR2 signaling can induce the expression of the iNOS gene. RAW macrophages were incubated with antigens for 8 h, and total mRNA was extracted and subjected to RT-PCR analysis with gene-specific primer sets. As shown in Fig. 4 A, transcription of the iNOS gene was substantially up-regulated in APCs after incubation with PSA. To confirm that increased transcription correlates with NO production, we monitored NO production by APCs in the presence and absence of blocking mAbs to TLRs using the Griess Reagent System to detect nitrite production. After stimulation for 15 h, the macrophages produced readily detectable levels of NO, but this production was significantly reduced in the presence of anti-TLR2 mAb ($P = 0.0002$; Fig. 4 B). NO production stimulated by *E. coli* LPS was not affected by this antibody.

It has been established that the positive charges from free amino groups in PSA are critical in PSA-induced T cell activation and abscess formation (2). To determine whether charge also plays a key role in the innate portion of the response to PSA, we conducted cytokine and NO production assays using *N*-acetylated PSA molecules containing only negative charges. The elimination of positive charges in PSA resulted in reduced IL-8 production in TLR2-expressing HEK-293 cells ($P = 0.0002$ at 62.5 μg/ml incubation concentration; Fig. 4 C). Similar results were observed with RAW macrophages and BMDCs (unpublished data). Elimination of positive charges also resulted in significant reductions in NO production by RAW macrophages ($P < 0.0001$; Fig. 4 D). However, zwitterionic charge alone is not sufficient for polysaccharide stimulation of TLR activity: Sp1, which is a zwitterionic polysaccharide and contains a positively charged free amino group, failed to stimulate cytokine production by RAW macrophages (unpublished data).

PSA activation of both innate and adaptive immune responses contributes to antigen presentation and CD4$^+$ T cell activation

TLR2 activation of NF-κB has been reported in other systems to result in the up-regulation of numerous molecules known to be important in PSA processing and presentation (e.g., NO, MHCII, and CD86) (9). We examined the role of TLR2 in the up-regulation of these molecules on BMDCs stimulated in vitro with PSA. PSA was incubated with BMDCs for 24 h, and CD11c$^+$ cells were analyzed by flow cytometry for expression of CD86 (B7-2) and MHCII (I-E/I-A). PSA-stimulated WT BMDCs up-regulated CD86 expression by 82% and MHCII expression by 29%. BMDCs from TLR2$^{-/-}$ mice did not up-regulate these molecules (Table I).

We next examined the impact of TLR2 deficiency in APCs on naive T cell activation, measuring IFN-γ production by WT CD4$^+$ T cells coincubated with BMDCs derived

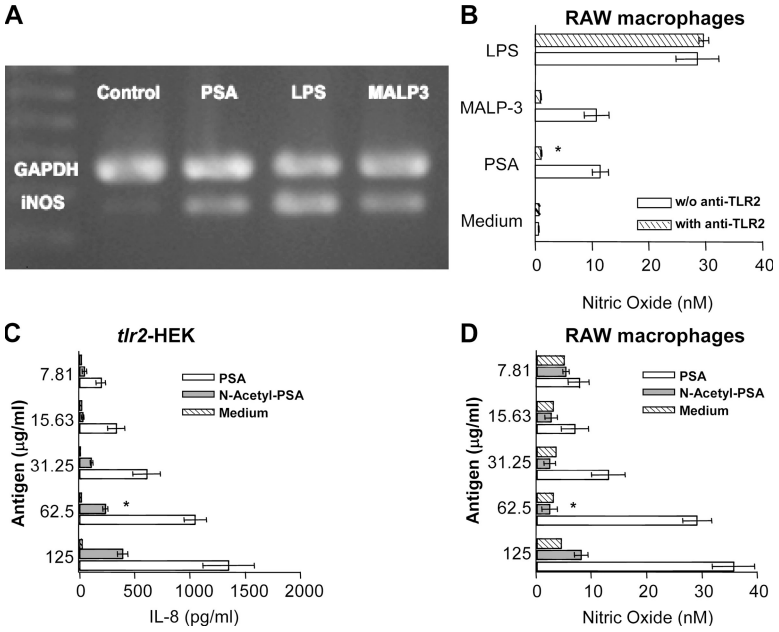

Figure 4. NO production is dependent on TLR2 activation by zwitterionic PSA. (A) RAW macrophages were incubated for 8 h with medium control, 100 μg/ml PSA, 200 nM MALP-3, or 100 ng/ml *E. coli* LPS. Total RNA was purified on an RNeasy column, and a 100-ng portion of each sample was subjected to RT-PCR using iNOS gene–specific primers. GAPDH was used as a standard to normalize gene amplification. (B) RAW macrophages were incubated with or without 50 μg/ml anti-TLR2 mAb for 1 h before incubation with antigens. Nitrite production in the cell supernatants was measured at 15 h by the Griess Reagent System. (C) TLR2-expressing HEK-293 cells were incubated with PSA or *N*-acetylated PSA at different concentrations, and IL-8 production was assessed at 15 h. (D) RAW macrophages were incubated with increasing concentrations of PSA or *N*-acetylated PSA for 15 h, and NO production was measured by the Griess Reagent System. Data represent mean ± SEM. *, $P < 0.05$.

Table I. TLR2-mediated enrichment of CD86 and MHCII expression by PSA-stimulated BMDCs

		Media	100 μg/ml PSA
WT BMDCs	CD86	18%	33%
	MHCII	32%	42%
TLR2$^{-/-}$ BMDCs	CD86	26%	24%
	MHCII	42%	40%

BMDCs derived from either wild-type or TLR2$^{-/-}$ C57BL/6J mice were incubated for 24 h with 100 μg/ml PSA or medium alone. The expression of the MHCII molecule and the co-stimulatory molecule CD86 was determined by flow cytometry. The percentage of cells expressing either of these surface molecules is reported.

from TLR2-sufficient and -deficient mice. When PSA was presented by TLR2$^{-/-}$ BMDCs, there was a considerable decrease in IFN-γ production by CD4$^+$ T cells (Fig. 5). To ensure that the remaining IFN-γ production resulted from PSA presented via the MHCII pathway and not from stimulation of another innate pathway, we treated both WT and TLR2$^{-/-}$ BMDCs with chloroquine, which prevents acidification of the MHCII compartment vesicle—a crucial step in the MHCII antigen-processing pathway that is required to free the peptide-binding site on the MHCII molecule before peptide–MHCII interaction (37, 38). Compared with untreated co-cultures, those treated with chloroquine exhibited substantially reduced IFN-γ production by CD4$^+$ T cells when PSA was presented by WT BMDCs. Blocking presentation by TLR2$^{-/-}$ BMDCs almost completely eliminated IFN-γ production by CD4$^+$ cells. Similar results were obtained using the drug cytochalasin D, which inhibits cytoskeleton polymerization—a process that is necessary for the endocytosis of molecules into the APC before processing by the MHCII pathway (unpublished data). The superantigen staphylococcal enterotoxin A (SEA) induced comparable IFN-γ production by CD4$^+$ cells after stimulation of either WT or TLR2$^{-/-}$ BMDCs; in addition, chloroquine treatment did not affect SEA-induced CD4$^+$ cell activation, because SEA does not require intracellular processing by the APC before presentation by MHCII molecules (Fig. 5). Similar experiments were performed using PSA-primed CD4$^+$ T cells isolated from mice that had received repeated administration of 50 μg PSA over a 2-wk period. IFN-γ production by these primed CD4$^+$ T cells was greater than that seen by the naive cells after in vitro PSA stimulation in the presence of WT BMDCs only. TLR2$^{-/-}$ BMDCs induced minimal IFN-γ production even by the PSA-primed CD4$^+$ T cells.

TLR2 is critical for PSA-dependent abscess formation

Abscess formation is a complex host response to bacterial infection that requires several cell types. The activation of CD4$^+$ T cells by zwitterionic polysaccharide (ZPS) antigens is one essential factor in the process (39). Using a model of intraabdominal abscess formation, we investigated whether this T cell–dependent process also depends on the initial interaction of PSA with TLR2. We administered PSA or live *B. fragilis* bacteria i.p., along with an adjuvant of sterilized cecal contents (SCC), to TLR4-hyporesponsive C3H/HeJ mice, TLR2$^{-/-}$ mice, and appropriate WT controls. WT and TLR4-hyporesponsive mice formed abscesses normally in response to challenge with either PSA or *B. fragilis* (Fig. 6). In contrast, similarly challenged TLR2$^{-/-}$ mice exhibited a considerably reduced ability to form abscesses.

DISCUSSION

Remarkably, each *B. fragilis* organism synthesizes at least eight distinct polysaccharides (PSA to PSH). The expression of seven of these polysaccharides is modulated by an intricate genetic mechanism that allows each polysaccharide to undergo reversible phase variation (1). Phase variation of these surface polysaccharides guarantees the existence of a population of organisms most fit to survive in the changing intestinal milieu by adapting to local conditions and stimulating local and systemic interactions with the host immune system.

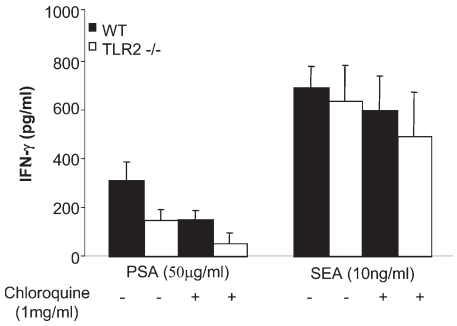

Figure 5. PSA stimulates IFN-γ production by CD4$^+$ T cells through activation of both innate and adaptive immune responses. BMDCs isolated from WT and TLR2$^{-/-}$ mice were co-cultured with WT CD4$^+$ T cells for 24 h and stimulated with either PSA or SEA in the presence or absence of chloroquine, an inhibitor of antigen processing. IFN-γ production by the CD4$^+$ T cells was measured by ELISA. Data represent mean ± SEM.

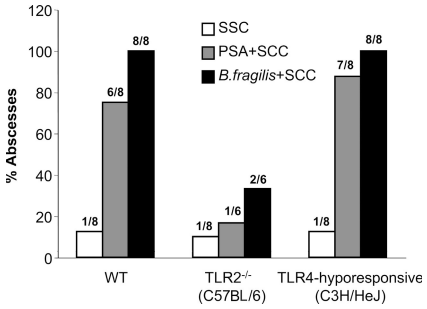

Figure 6. TLR2 signaling is required for abscess formation in vivo. Groups (n = 8) of WT, TLR2$^{-/-}$, or TLR4-hyporesponsive mice received an i.p. injection of sterilized cecal contents alone (SCC; 1:10 dilution, vol/vol) or in combination with 50 μg PSA per mouse or 10^8 CFU *B. fragilis* per mouse. 7 d later, the mice were examined for the presence of intraabdominal abscesses. The number of mice positive for abscesses in each group is shown.

The *B. fragilis* surface polysaccharides are involved in key interactions with the innate immune system. The innate immune system plays a critical role in the maintenance of intestinal epithelial homeostasis and in protection against intestinal injury and associated mortality (40). We now report that innate immunity mediated by TLR2 signaling is critical for an optimal adaptive immune response to intestinal microbes, leading to the T cell activation that is essential for the development of a proper Th1/Th2 cell balance in the mammalian immune system (3).

PSA is the most abundant polysaccharide of *B. fragilis*. Its zwitterionic charge motif is critical for its processing by APCs in a NO-dependent fashion through the MHCII pathway and its presentation to T cells (29). DCs and macrophages are two types of professional APCs that encounter and present antigens originating in the intestine, as well as exogenous antigens found in the early stages of infection. Because these cells play a critical role in the development of T cell–mediated immune responses, it is important to understand the APC–PSA interaction leading to the downstream signaling events that help to shape the host response to this carbohydrate.

We have identified TLR2 as a PSA receptor that plays a critical role in initiating the innate immune response through both the production of cytokines and the priming of the adaptive response to the polysaccharide. In this paper we show that PSA initiates a critical signaling cascade in APCs wherein the polysaccharide's agonist effect on APCs leads to several important biologic events, including (a) activation of the transcription factor NF-κB, (b) production of the proinflammatory cytokine TNF-α, and (c) production of cytokines and other molecules known to modulate the adaptive immune response (e.g., IL-12, CD86, MHCII, and NO). In studies eliminating TLR2 activation with antibodies or using $TLR2^{-/-}$ mice, we document reductions in PSA-induced NF-κB production and in the release of NO and proinflammatory cytokines. NF-κB activation by TLR2 is mediated via the MyD88 signaling pathway. These data suggest that TLR2 stimulation may facilitate NO-dependent polysaccharide processing through up-regulation of NO production. In conjunction with NO up-regulation, TLR2-mediated signaling results in up-regulation of MHCII and co-stimulatory molecule expression (CD86) by APCs, along with increases in the expression of the chemokine receptor CC chemokine receptor 7 (unpublished data). CC chemokine receptor 7 expression is associated with DC maturation and homing to the secondary lymphoid organs (41). Collectively, these TLR2-mediated events appear to be important in maximizing the adaptive immune response to PSA by enhancing DC maturation and antigen presentation.

IFN-γ is important in shaping the adaptive immune response and is associated with driving Th1 cell–mediated immunity. We therefore examined whether PSA could induce $CD4^+$ T cell activation and production of this cytokine either through activation of innate immune signals or via the more classic adaptive immune pathway of MHCII-associated antigen presentation. Clearly, optimal induction of IFN-γ by $CD4^+$ T cells depends on both arms of the immune system. PSA processing and presentation by the MHCII pathway was inhibited by treatment of APCs with either chloroquine or cytochalasin (29). Both drugs partially blocked IFN-γ production by T cells upon PSA presentation by DCs. These findings substantiate our earlier report that MHCII-deficient DCs fail to induce IFN-γ production by $CD4^+$ T cells in response to PSA (3). However, we now demonstrate the involvement of TLR2-mediated signaling by APCs in regulating IFN-γ production by T cells. Considerably less IFN-γ was produced by both naive and PSA-primed $CD4^+$ T cells after in vitro PSA presentation by $TLR2^{-/-}$ DCs than after PSA presentation by WT DCs. Presumably, this decreased ability to activate $CD4^+$ T cells is linked to a combination of reduced IL-12 signaling to the T cell from TLR2 knockout DCs and decreased presentation of PSA by the MHCII pathway because of decreased NO production and decreased expression of MHCII and CD86.

We have previously shown that PSA-induced IL-12 production by DCs is vital for optimal IFN-γ production by T cells (3). IL-12 is a heterodimer that is composed of two subunits—p35 and p40—and is secreted by activated DCs. IL-12 signals through the IL-12R protein, which couples to the JAK–STAT signaling pathway—or, more specifically, to transcription factors such as STAT4 (42). Recent experiments have shown that a critical stage in Th1 cell differentiation from naive $CD4^+$ T cells involves the transcription factor T-bet (T-box expressed in T cells). T-bet induces IL-12Rβ2 chain expression and allows IL-12/STAT4 signaling to optimize IFN-γ production, which facilitates the Th1 cell developmental process (43). In addition, we have shown that blocking IL-12 production by APCs stimulated with PSA can completely eliminate the production of IFN-γ by $CD4^+$ T cells (3). We now demonstrate that PSA induces IL-12p40 production in DCs and that the induction of IL-12p40 is substantially attenuated in $TLR2^{-/-}$ DCs. These data suggest that TLR2 is a critical receptor on DCs that can facilitate $CD4^+$ T cell priming through IFN-γ production in response to carbohydrate antigens, a process that ultimately leads to Th1 cell differentiation. Interestingly, confocal microscopy (Fig. 3) revealed that PSA is taken up well into the endosomal compartment of TLR2-deficient DCs. Therefore, TLR2 does not function as an uptake receptor for PSA despite its importance in stimulating T cell priming.

It is important to note that macrophages, unlike DCs, fail to produce the Th1 cell–stimulating cytokine IL-12 upon stimulation with PSA (Fig. 1 A). These cell type–dependent effector functions may point to differential expression of pattern recognition receptors and coreceptors, as well as different signal transduction cascades in the two types of APCs (44). The innate immune system reportedly uses a wide variety of microbial recognition receptors, including TLRs, lectins, scavenger receptors, and integrins, to identify potential antigens (45–47). The outcomes of receptor ligation include phagocytosis, the release of oxidative species and proinflammatory

cytokines, and the development of adaptive immunity; yet, not all antigens induce these responses equally. Recently, it has been shown that DCs can differentiate between self- and nonself-antigens through TLR engagement and that, on the basis of the antigen source, these cells can generate preferentially presented peptide–MHC complexes from the contents of phagosomes derived from microbes (48). It has been suggested that more than one receptor may be involved in antigen binding by TLRs (49, 50). For example, the C-type lectin dectin-1 cooperates with TLR2 to induce an inflammatory response to zymosan (51). Given the differential cytokine production by DCs and macrophages in response to PSA-mediated TLR2-dependent stimulation, it is reasonable to suggest that distinct patterns of expression of various coreceptors on individual cell types are involved in shaping the overall response to PSA. Stimulation of TLR2 by PSA appears to be partially charge dependent. Elimination of positively charged amine groups on PSA via *N*-acetylation reduces the production of IL-8, TNF-α, and NO by TLR2-expressing HEK cells, DCs, and macrophages (Fig. 4, C and D). These observations support previous reports that the charges on PSA are critical for polysaccharide-mediated T cell activation (2). However, one important issue related to antigen structure is that the TLR2-mediated response of the APCs tested was not solely dependent on zwitterionic charge. Sp1, another T cell–activating ZPS, failed to activate TLR2. Therefore, the zwitterionic charge motif of PSA is necessary but not sufficient to induce TLR2-mediated APC activation. Specific structure–function studies of PSA–TLR2 interaction are needed.

We show in this paper that both the innate and adaptive pathways of immunity are involved in at least one biologic response to PSA: intraabdominal abscess formation. TLR2-deficient mice display a substantially reduced ability to form intraabdominal abscesses after challenge with either PSA or live bacteria along with SCC (Fig. 6). We have previously shown that mice with defects in antigen processing or presentation by the MHCII pathway are likewise unable to form abscesses (29). Interplay of the two arms of the immune system may be required not only for an optimal response to PSA but also for other biologic functions of PSA, such as stimulation of cell lineage differentiation.

The identification of a critical role for TLR2 signaling in PSA-mediated activation of APCs adds an essential piece of information to our understanding of how this polysaccharide antigen stimulates the host immune response. On the basis of these data, we propose an integrated model for PSA-dependent activation of DCs and $CD4^+$ T cells that involves considerable interplay of the innate and adaptive arms of the immune system (Fig. 7). In the innate portion of this response, PSA stimulates APCs through a TLR2-dependent mechanism. TLR2 activation then results in NF-κB–dependent NO production, which is critical for PSA processing and presentation by MHCII and, thus, for initiation of the adaptive immune response. PSA-mediated TLR2 signaling also up-regulates MHCII and co-stimulatory molecule expression on the surface

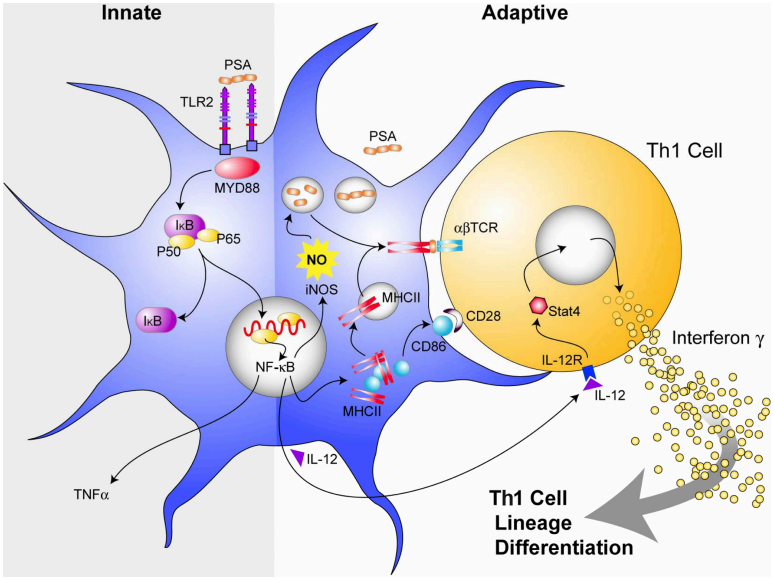

Figure 7. Proposed model for the intersection of innate and adaptive immunity during polysaccharide-induced abscess formation in the peritoneal cavity. The innate response begins with TLR2 recognition of PSA and the subsequent stimulation of the MyD88-mediated pathway inside the APC. NF-κB is translocated into the nucleus, stimulating the transcription of several important genes, including TNF-α. TNF-α is secreted by the APC and up-regulates intracellular adhesion molecule 1 on the mesothelium. Meanwhile, NF-κB translocation also leads to enhanced iNOS expression as well as up-regulation of MHCII and CD86, thus facilitating PSA processing and presentation by MHCII proteins. Presentation of PSA on the cell surface by MHCII leads to adaptive $CD4^+$ T cell activation and T cell secretion of IFN-γ. IFN-γ production by the T cell is optimized by TLR2-mediated IL-12 production by the dendritic cell. This figure illustrates the interplay among innate signals and the shaping of the adaptive response for a common purpose: IFN-γ production.

of APCs, further linking the innate and adaptive responses to this antigen. Upon antigen presentation by MHCII and recognition by αβ T cell receptors (along with co-stimulation), PSA-activated $CD4^+$ T cells secrete IFN-γ. This process is enhanced by the production of the cytokine IL-12 by DCs, which occurs as a consequence of TLR2 stimulation. These experiments identify TLR2 as a major factor in initiating the host response required for optimal $CD4^+$ T cell responses to PSA. Moreover, the results of these studies define a novel system that identifies the crucial interplay between the innate and adaptive arms of the host response.

MATERIALS AND METHODS

Animals and cell lines. Male 4–8-wk-old C57BL/6j, C3H/OuJ, and C3H/HeJ mice were maintained in a pathogen-free facility. Animal experiments were performed in accordance with the guidelines of the Harvard Medical School Standing Committee on Animals.

HEK-293 cells were stably transfected with TLR2 or TLR4 (33, 34). Mouse RAW 264.7 macrophages and human THP-1 monocytes were obtained from the American Type Culture Collection. THP-1 cells were incubated with 100 ng/ml PMA for 3 d to obtain macrophages. Unless stated otherwise, cells were grown in a cell culture incubator at 37°C with 5% CO_2.

Primary cell culture. BMDCs were cultured by previously described methods with minor modifications (33). In brief, bone marrow was collected from femurs and tibias of 8-wk-old WT, $TLR2^{-/-}$, or $MyD88^{-/-}$ mice. Cells were resuspended in complete RPMI medium (American Type Culture Collection) containing 10 ng/ml GM-CSF (R&D Systems). After 4 d of culture, cells were placed in fresh medium containing GM-CSF. On day 7, adherent and nonadherent cells were collected and seeded into either 6- or 96-well culture plates, and experiments were performed the next day.

Bacteria and reagents. PSA was purified from a mutant strain of *B. fragilis* (Δ44) according to a previously published method (52). No LPS was detectable in the PSA preparation by gel electrophoresis, ^{1}H-NMR analysis, or endotoxin assay. PSA was modified with acetic anhydride in 0.1 M $NaHCO_3$ to obtain *N*-acetylated PSA.

E. coli LPS was purchased from Sigma-Aldrich and was further purified by phenol extraction A synthetic lipopeptide, MALP-3 (Pam3Cys-SKKKK), was purchased from EMC microcollections GmbH and was used as a TLR2 agonist. Polysaccharide controls included mannan, polygalacturonic acid, and ZPS from Sp1.

RT-PCR. RAW 264.7 cells were plated at 5×10^5 in 3 ml of DMEM on sixwell plates 24 h before assay. Different antigens (100 μg/ml PSA, 20 nM MALP-3, and 250 ng/ml LPS) were added to corresponding wells, and plates were incubated for another 8 h. Total RNA was extracted with RNeasy columns (QIAGEN) according to the manufacturer's protocol. RT-PCR was performed with gene-specific primer sets for the GAPDH, iNOS, and TNF-α genes. 100 ng of total mRNA was subjected to one-step RT-PCR (SuperScript III; Invitrogen) and amplified (iCycler; Bio-Rad Laboratories). The primer sequences and sizes of PCR products were as follows: GAPDH, GGTGTGAACCACGAGAAATA (forward) and CTGTTGCTGTAGCCGTATTC (reverse; 569 bp) (1); iNOS, ACGCTTGGGTCTTGTTCACT (forward) and GTCTCTGGGTCCTCTGGTCA (reverse; 468 bp) (2); and TNF-α, AGCACAGAAAGCATGATCCG (forward) and CAGAGCAATGACTCCAAAGT (reverse; 702 bp) (3).

NO detection. NO production was quantified by measurement of the accumulation of nitrite (NO_2^-, a stable end product of NO) in cell culture supernatants with the Griess Reagent System (Promega).

NF-κB detection. The production of NF-κB p65 (RelA) was measured with NF-κB assay kits (TransAm; Active Motif).

Confocal microscopy. Confocal microscopy was performed as previously described (29).

BMDC and $CD4^+$ T cell co-culture experiments. For analysis of IFN-γ production by $CD4^+$ T cells, T cells were isolated from spleens of C57BL/6J mice (The Jackson Laboratory) by centrifugation in Ficoll and were purified on $CD4^+$ T cell enrichment columns (R&D Systems). BMDCs were purified from the femurs of both C57BL/6J and $TLR2^{-/-}$ mice as described previously (33). For cytokine assays, 2×10^5 $CD4^+$ T cells were combined with 10^5 BMDCs in 96-well round-bottom plates, and antigens and inhibitor drugs were added. SEA and PSA were used at concentrations of 10 ng and 100 μg, respectively. Preliminary dose–response experiments were conducted to determine the optimal concentration of each inhibitor drug, i.e., the lowest concentration that inhibited the endosomal pathway and was not toxic for the BMDCs. For inhibition experiments, 1 μM cytochalasin D or 1 μg/ml chloroquine were added to the cells simultaneously with the antigens

Flow cytometry. BMDCs were incubated for 24 h with either 100 μg/ml PSA or culture media. Cells were then harvested and incubated with fluorescent-conjugated mAbs to the cell surface markers CD86 (clone GL1) and I-A/I-E (clone M5/114.15.2; BD Biosciences) for 30 min at 4°C. Cells were washed and analyzed on a flow cytometer (FC500; Beckman Coulter). Appropriate isotype controls were included in each experiment.

ELISA. All cytokine ELISA kits, except the one for IL-12p40 (BD Biosciences), were obtained from R&D Systems. 10^5 cells were seeded onto 96-well flat-bottom plates 1 d before stimulation. Different concentrations of antigens (e.g., PSA, MALP-3, and LPS) were added to wells in triplicate (with a medium control), and plates were incubated for 15 h.

Mouse model of intraabdominal abscess formation. The intraabdominal abscess model used in these studies was based on that described in a previous publication (53).

Statistical analysis. Results are expressed as mean ± SD. Data were analyzed, as appropriate, by unpaired *t* tests with Prism software for Windows (version 3.00; GraphPad Software). Differences were considered statistically significant at $P < 0.05$.

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