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Lipoprotein I, a TLR2/4 Ligand Modulates Th2-Driven Allergic Immune Responses

Hilde Revets,1* Gwenda Pynaert,† Johan Grooten,† and Patrick De Baetselier*

Asthma is an inflammatory lung disease that is initiated and directed by Th2 and inhibited by Th1 cytokines. Microbial infections have been shown to prevent allergic responses by inducing the secretion of the Th1 cytokines IL-12 and IFN-γ. In this study, we examined whether administration of lipoprotein I (OprI) from Pseudomonas aeruginosa could prevent the inflammatory and physiological manifestations of asthma in a murine model of OVA-induced allergic asthma. OprI triggered dendritic cells to make IL-12 and TNF-α, with subsequent IFN-γ production from T cells. OprI stimulation of dendritic cells involved both TLR2 and TLR4. Intranasal coadministration of OprI with OVA allergen resulted in a significant decrease in airway eosinophilia and Th2 (IL-4 and IL-13) cytokines and this effect was sustained after repeated allergen challenge. The immediate suppressive effect of OprI (within 2 days of administration) was accompanied by an increase in Th1 cytokine IFN-γ production and a significant, but transient infiltration of neutrophils. OprI did not redirect the immune system toward a Th1 response since no increased activation of locally recruited Th1 cells could be observed upon repeated challenge with allergen. Our data show for the first time that a bacterial lipoprotein can modulate allergen-specific Th2 effector cells in an allergic response in vivo for a prolonged period via stimulation of the TLR2/4 signaling pathway. The Journal of Immunology, 2005, 174: 1097–1103.

The TLR play an essential role in innate immune responses in mammals (1, 2). Innate immunity provides an immediate and direct response in which pattern recognition receptors, such as TLRs, recognize and respond to various pathogen-associated molecular patterns by eliciting direct antimicrobial pathways (3) and by inducing NO (4) and phagocytosis (5). In addition, TLR signaling can induce the production of proinflammatory cytokines and up-regulate expression of costimulatory molecules, thereby activating not only innate but ultimately also adaptive immune responses and induction of Th1 effector responses (1, 6, 7).

Allergen-induced asthma is a disease in which the CD4 Th2 immune response plays a pivotal role and is characterized by high circulating levels of IgE, pulmonary eosinophilic inflammation, and airway hyperreactivity to bronchoconstrictive stimuli (8). Thus, an effective treatment for asthma should inhibit one or more of these processes. An interesting aspect of TLR biology is the possibility of exploiting the Th1 adjuvant properties of TLR signaling to down-regulate or dampen the characteristic Th2 response in atopic diseases such as asthma. Evidence from the literature has shown that treatment of allergen-sensitized mice with TLR9 ligand CpG DNA, before or after airway challenge, redirects the immune response from a Th2-like response toward a Th1-like response, leading to a reversal of established airway eosinophilia and bronchial airway hyperreactivity (9, 10). Moreover, direct conjugation of CpG to allergen reduced the dose of CpG required to cause this response (11) and as such reduced the risk of exacerbation of inflammatory responses, an adverse effect of CpG administration (12). Recent data also demonstrated that LPS signaling through TLR4 suppresses airway Th2 responses via NO synthase 2 activity (13) and independently of IL-12 (14).

Our laboratory has been examining the immunomodulatory activity of lipoprotein I (OprI),2 a N-terminal triacylated lipoprotein from Pseudomonas aeruginosa (15). We found that OprI acts as a natural adjuvant that induces long-lived Th1 immune responses against heterologous Ags/peptides when fused to its C-terminal or admixed and provided protection upon subsequent challenge with the pathogen (16).

In this study, we analyzed the molecular mechanism by which OprI exerts its adjuvant effect and examined whether transmucosal administration of OprI could alter the immunological and physiological manifestations of asthma using a mouse model of OVA-induced allergic asthma (17). We found that OprI affects the function of APCs by enhancing their ability to trigger naive T cells and confer to them the capacity to induce the development of Th1 cells. TLR signaling is implicitly involved in the response of APCs and mechanistic studies suggest that the primary site of action of OprI is both TLR2 and TLR4. Concomitant mucosal administration of OprI with allergen in presensitized mice efficiently inhibited the Th2 cell-mediated allergic response in parallel with the improvement of eosinophilic lung inflammation. This beneficial effect remained after a second allergen challenge, indicating a sustained effect. The results highlight the potential use of TLR2/4 ligands as adjuvants in the modulation of allergic inflammation.

Materials and Methods

Mice

C3H/HeN and C3H/HeJ mice (6–8 wk old) were purchased from Harlan. C3H/HeN/TLR2−/− and C3H/HeJ/TLR2−/− mice were obtained from Tularik. C57BL/6 mice were purchased from IFFA Credo CR Broekman.

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2 Abbreviations used in this paper: OprI, lipoprotein I; BM, bone marrow; DC, dendritic cell; BMDC, BM-derived DC; BAL, bronchoalveolar lavage; RT-QPCR, real-time quantitative PCR.

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and were housed under specific pathogen-free conditions in microisolation units.

**Antigen**

OVA (grade V) and LPS (Escherichia coli, strain 055:B5) were purchased from Sigma. OprI was purified by continuous electrophoresis from outer membrane preparations as described previously (18). Endotoxin activity was <1 endotoxin unit/µg protein as determined by the Limulus amebocyte lysate assay (BioWhittaker).

**Generation of bone marrow (BM)-derived dendritic cells (DC)**

DC culture medium (RPMI 1640; Invitrogen Life Technologies) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/100 µg/ml streptomycin, 5% FCS (Fetaclove II; HyClone), and 20 ng/ml recombinant mouse GM-CSF (a kind gift from Prof. K. Thieleman, Vrije Universiteit Brussel, Brussels, Belgium) was used to generate bone marrow-derived dendritic cells (BMDC) as described previously (19). After RBC lysis, bone marrow cells were resuspended at 2 × 10³/µl in DC medium. Cells were seeded (2 × 10⁶) in tissue culture grade petri dishes (100 mm). At day 3, 10 ml of fresh DC medium was added. On days 5 and 7, 10 ml of each plate was centrifuged and resuspended in 10 ml of fresh DC medium.

**Stimulation and Ag pulsing of DC**

At day 9, DC were stimulated by the addition of 1 µg/ml OprI or 20 ng/ml LPS. After 24 h, IL-12 and TNF-α concentrations in the supernatants were measured using standard sandwich ELISA protocols. Furthermore, the cells were stained with biotinylated mAbs for 20 min at 4°C and developed using the appropriate background fluorescence.

**Cytokines production**

C57BL/6 mice were anesthetized with avertin (2.5% w/v in PBS-low endotoxin). BAL was performed as described elsewhere (20) with 3 × 1 ml of Ca²⁺⁻ and Mg²⁺⁻free HBSS (Invitrogen Life Technologies) supplemented with 0.05 mM EDTA. After centrifugation of the BAL fluid, cells were counted in a hemacytometer. Differential cell counts were determined on cytospin preparations stained with May-Grünwald-Giemsa (Sigma-Aldrich). Primers for QPCR analysis of cytokine and chemokine expression

**Bronchoalveolar lavage**

Bronchoalveolar lavage (BAL) was performed 48 h after the last challenge. In a second protocol, all mice received 5 days after challenge with OprI and OVA or free OVA two additional intranasal challenges with 10 µg OVA.

**Cytokines production**

Concentrations of IFN-γ, IL-4 (Endogen), TNF-α, CXCL-1, and CXCL5 (R&D Systems) in BAL fluids were determined by sandwich enzyme immunoassay. Levels of IL-12 and TNF-α (R&D Systems) were determined by ELISA in DC culture supernatants. Levels of IL-4, IFN-γ (BD Pharmingen), and IL-13 (R&D Systems) were determined by ELISA in supernatants from lymph node cultures.

**Lang tissue CD4⁺ T cell isolation**

Lungs were minced and incubated for 30 min at 37°C in RPMI 1640 medium containing 150 U/ml collagenase II (Sigma-Aldrich), 0.02 mg/ml DNase I (Roche Molecular Biochemicals), and 10% FCS (Invitrogen Life Technologies). After washing the cells, CD4⁺ T cells were isolated by the CELLection Biotin Binder kit according to the manufacturer’s protocol (Dynal).

**Real-time quantitative PCR (RT-QPCR)**

RNA isolation was performed using an RNAeasy kit (Qiagen). cDNA was synthesized using a Superscript II Reverse Transcription Reagent kit (Roche Molecular Systems). RT-QPCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems) using a qPCR Core kit for SYBR Green I (Eurogentec) or on an iCycler apparatus using iQ SYBR Green Supermix (Bio-Rad). Gene expression was normalized using ribosomal protein S12, HMBS, or Rp113a. The primer sequences are reported in Table I.

**Statistical analysis**

Values are expressed as mean ± SD unless otherwise indicated. Comparison of the means between different groups was performed using the Mann-Whitney U test or the two-tailed unpaired t test. Values of p ≤ 0.05 are considered to be statistically significant.

**Results**

OprI matures/activates immature DC and enhances their capacity to prime Ag-specific T cells in vivo

We have previously shown in a murine *Leishmania* model that OprI acts as a natural adjuvant that induces long-lasting Th1-type immune responses against heterologous Ags in vivo and provides protection against subsequent challenge with the given pathogen (16). Since bacterial lipoproteins are molecules that stimulate innate immune cells to produce proinflammatory cytokines, we analyzed the ability of lipoprotein OprI to activate and to mature immature DC. To this end, BM-derived immature DC from C57BL/6 were incubated with OprI. Culture supernatant was assessed for the presence of IL-12p70 and TNF-α. As shown in Fig. 1A, IL-12 and TNF-α secretion was induced in BMDC stimulated with OprI, whereas only marginal levels of IL-12 and TNF-α were produced in nonstimulated BMDC. During OprI-mediated activation, the production of cytokines was accompanied by the up-regulation of MHC class II and class I molecules and of CD80, CD86, and CD40 costimulatory molecules on the surface of BMDC (Fig. 1B), demonstrating the ability of OprI to activate and mature immature DC.

Table I. Primers for QPCR analysis of cytokine and chemokine expression

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>CXCL1</td>
<td>5’-AAC GAC GAA AGA AGA CAG ACT G-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GAC GAG ACC AGG AAC AAG AG-3’</td>
</tr>
<tr>
<td>CXCL2</td>
<td>5’-CCC CCT GGT TCA GAA AAT CAT CC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCC CCA GTC TCT TCT ACT GT-3’</td>
</tr>
<tr>
<td>CXCL5</td>
<td>5’-AGC TCC CCA TTC ATG CGG ATG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTA TTG AAC ACT GGC CTT CCT-3’</td>
</tr>
<tr>
<td>CCL2</td>
<td>5’-TCA GCC AGA TGC AGT TAA CG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGC TGC TGA AGA CCT TAG GGG-3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5’-GCCAAGGCGTCTAGCTG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCAGTGAAATGGATCTACCAAGTTCAACATTC-3’</td>
</tr>
<tr>
<td>HMBS</td>
<td>5’-GAAACTCTGCTTCCTGCGCAT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TGCACCATCTTTCATCAGTGTTAG-3’</td>
</tr>
<tr>
<td>IL-4</td>
<td>5’-CCATGCTTGGAGAAAGAATCTACAGGTTG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GACTCTCATGCTGGACCTGTTAC-3’</td>
</tr>
<tr>
<td>IL-13</td>
<td>5’-TCAGCCATGAAATTTAACCATTGTTTGTT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTTGGAGTGTAAAGCCAGTCTTCTG-3’</td>
</tr>
<tr>
<td>Rpl113a</td>
<td>5’-CCCTGCTGCTCTCAGAGTTGT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TGGTTGCTACCTGCTGCTGTTAC-3’</td>
</tr>
<tr>
<td>S12</td>
<td>5’-CCCTGACTGACATCTGTCGCTGAG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGAAGGCTAGATGCTGAGTGTTG-3’</td>
</tr>
</tbody>
</table>
Since activation and maturation of BMDC, resulting in the production of proinflammatory cytokines and up-regulation of co-stimulatory molecules, may in turn trigger the development of adaptive immune responses, we wondered whether OprI-treated BMDC were able to prime Ag-specific T cells in vivo, in particular Th1 cells. Hereto, immature BMDC were incubated with OVA in the presence or absence of OprI and injected into the footpads of syngeneic C57BL/6 mice. Five days later, popliteal lymph node cells were harvested and cultured in the presence of OVA. Culture supernatants were assayed for IFN-γ, IL-4, and IL-13. Results express mean values ± SD of three mice and are representative of one of three separate experiments.

OprI mediates APC activation via TLR2 and TLR4

It has previously been shown that lipopeptides induce cytokine secretion and maturation of DC via TLR2 (4, 21, 22). Therefore, we wondered whether activation of adaptive immune responses and induction of Th1 effector responses by OprI requires Toll-mediated recognition and signaling. To this end, we investigated the secretion of the proinflammatory cytokine TNF-α by BMDC lacking functional TLR2 or TLR4 molecules or both in response to OprI and LPS. As shown in Fig. 3, stimulation of BMDC from TLR2- and TLR4-deficient mice with OprI led to an impaired response for TNF-α as compared with the TNF-α production from OprI-stimulated wild-type BMDC. No response at all for TNF-α was observed in BMDC from TLR2/TLR4 double-deficient mice. Production of TNF-α in response to stimulation with LPS, in contrast, was strongly induced in BMDC from TLR2-deficient mice (Fig. 3), whereas no response was seen in TLR4 and TLR2/TLR4 double-deficient mice, in line with previous reports (23, 24). To exclude whether the minute amounts of LPS present in the OprI preparations (<1 endotoxin unit/μg OprI) partially accounted for the induction of TNF-α, we also incubated BMDC with OprI in the presence of polymyxin B, an antibiotic that binds and neutralizes LPS. Polymyxin B clearly abrogated TNF-α production of LPS-stimulated BMDC but did not modify the effect from OprI (Fig. 3). The same results were obtained for IL-12 (data not shown). Taken

FIGURE 1. OprI activates and induces maturation of immature BMDC. Immature BMDC were incubated with OprI. A, IL-12p70 and TNF-α were assayed in culture supernatants. Results express mean values ± SD of three mice. *p ≤ 0.05 compared with DC alone. B, Surface expression of MHC class I and class II molecules and of CD80, CD86, and CD40 costimulatory molecules (thick lines) was determined. Solid profiles represent fluorescence distribution of unstimulated immature BMDC stained with the respective Abs. Data are representative of one of three separate experiments.

FIGURE 2. OprI-treated BMDC prime Ag-specific Th1 cells in vivo. Immature BMDC were incubated overnight with OVA in the presence or absence of OprI and injected into the footpads of syngeneic C57BL/6 mice. Five days later, popliteal lymph node cells were harvested for IFN-γ, IL-4, and IL-13. Results express mean values ± SD of three mice and are representative of one of three separate experiments.

FIGURE 3. OprI activates BMDC through TLR2 and TLR4. Immature BMDC derived from C3H/HeN, C3H/HeJ, C3H/HeN/TLR2−/−, and C3H/HeJ/TLR2−/− mice were incubated with OprI or LPS in the presence or absence of polymyxin B (PxB). TNF-α production was assayed in 24-h culture supernatants. Results express mean values ± SD of triplicate cultures of three mice and are representative of one of three separate experiments.
together, the results indicate that immune stimulation by OprI is TLR2/4 dependent.

**Coadministration of OprI and allergen to mice sensitized to develop Th2-type inflammation represses eosinophilic inflammation in the airways**

The initial acquired immune response that is responsible for the development of allergic diseases such as asthma is the generation of allergen-specific Th2 cells that produce IL-4, IL-13, and IL-5, which promote airway eosinophilia. One approach to treat asthma might be to use TLR-activating ligands to dampen the predominantly Th2-driven inflammation or to shift it into a more protective Th1 response. In this study, we examined the transmucosal effects of OprI on eosinophilic inflammation in the airways. Airway eosinophilia was induced in OVA/alum-sensitized C57BL/6 mice by challenging with two doses of OVA via an intranasal route. Analysis of the BAL revealed reduced airway eosinophilia in OVA-sensitized mice treated with OprI at the time of intranasal Ag challenge. However, the total cell number in the BAL fluid was not reduced. In fact, intranasal OprI administration induced a strong macrophage and neutrophil airway recruitment (Fig. 4A). To examine the duration of OprI activity, the OVA and OprI/OVA groups received two subsequent OVA challenges. As shown in Fig. 4B, repeated challenge resulted in a substantial rise in total BAL cell numbers, especially in the OVA group where a strong eosinophilia was observed. In contrast, eosinophilic inflammation in the airways of the OprI/OVA group was significantly suppressed. Moreover, challenge with OprI in combination with OVA reduced the total cell numbers and the amount of macrophages in the BAL fluid after repeated OVA challenges. In contrast with the neutrophilic inflammation observed immediately after coadministration of OprI and OVA, no neutrophilic inflammation was observed after a secondary OVA challenge (Fig. 4B). Together, these results document that local OprI administration at the time of Ag challenge rapidly induces strong airway inflammation with predominance of macrophages and neutrophils. In contrast, coadministration of OprI and OVA before a secondary OVA challenge significantly reduced total cellular and eosinophilic inflammation and no neutrophilic inflammation was observed.

**OprI administration suppresses airway-type 2 cytokine production**

To verify whether reduced eosinophilic airway inflammation was paralleled with diminished Th2 cytokine levels, BAL cells were cultured and stimulated with anti-CD3 and anti-CD28 mAb. In comparison to BAL cell cultures from OVA-challenged mice, which exhibited airway eosinophilia, mice challenged with OVA plus OprI showed greatly reduced levels of IL-4 (Fig. 5A). In contrast, the level of IFN-γ was enhanced upon treatment with OprI. To see whether this particular cytokine pattern prevailed after a secondary OVA challenge, the in situ activation state of recruited Th effector cells was determined. Therefore, cytokine expression levels in lung tissue CD4+ T cells were measured by RT-QPCR (Fig. 5B). Treatment of sensitized mice with OprI/OVA before...
receiving two additional allergen challenges resulted in greatly reduced IL-13 mRNA and IL-4 mRNA levels (5- and 10-fold, respectively) as compared with the OVA group. In contrast to the decreased levels of Th2-type cytokines, IFN-γ expression levels remained stable (Fig. 5B). Thus, intranasal administration of OprI down-regulated type 2 cytokine production and airway eosinophilia and this beneficial effect was sustained after repeated allergen challenges.

**Intranasal administration of OprI induces C-X-C and C-C chemokines involved in early neutrophil recruitment**

In mice treated with OprI/OVA, there was an increase in total BAL cell number compared with the OVA-treated mice (72 × 10⁶ in comparison to 36 × 10⁶ cells) 2 days after administration. This increase was mainly due to neutrophil recruitment as evidenced by BAL cell differential count (Fig. 4A). Neutrophil percentage progressively returned to baseline at day 7 (Fig. 4B). In fact, massive infiltration of cells occurred 6 h after OprI administration (data not shown).

Lung inflammation was evaluated by the measurement of cytokine and chemokine production. In lung homogenates, mRNA expression for CXCL1 and CXCL5 were transiently increased at 6 h after OprI administration, whereas high levels of CXCL2 and CCL2 mRNA were maintained for 24 h, after which they decreased (Fig. 6). Expression of CXCL1 mRNA was associated with the concomitant release of this cytokine in the BAL fluids at 6 h; CXCL5 production was also produced but remained present for at least 24 h in the BAL fluids (Fig. 7). In addition to chemokine production, administration of OprI also triggered the secretion of the inflammatory cytokine TNF-α (Fig. 7).

**Discussion**

Bacterial lipoproteins activate cells of the innate immune system, eliciting a signaling cascade resulting in NF-κB activation (4) and inflammatory cytokine production (25). The adjuvant activity of lipoproteins has been shown to require the expression of costimulatory proteins and inflammatory cytokines from APCs and results in the activation of adaptive Th1 responses (26).

In the present study, we identified the molecular mechanism of the adjuvant effect of OprI, a lipoprotein derived from *Pseudomonas aeruginosa*. We found that OprI adjuvant, adaptive Th1 responses by enhancing APC function. OprI triggered the maturation and activation of DC as evidenced by up-regulation of MHC and costimulatory molecules and the secretion of IL-12 and TNF-α.

Since TLR2 and TLR4 have been implicated in the recognition of bacterial cell wall products and possibly bridge innate and acquired immunity, we investigated the possible involvement of TLR2 and TLR4 in the immunostimulating activity of OprI. BMDC lacking functional TLR2 or TLR4 molecules still produced TNF-α and IL-12 upon OprI stimulation, although to a significantly lesser extent than OprI-stimulated BMDC from wild-type littermates. In contrast, proinflammatory cytokine production was totally impaired in BMDC lacking both functional TLR2 and TLR4 molecules upon OprI stimulation. Moreover, OprI-induced maturation of these BMDC was also impaired (data not shown), demonstrating the requirement of both TLR2 and TLR4 signaling pathways in the OprI-mediated APC maturation and activation. These results at first conflict with the current view that lipoproteins activate innate immune cells only via TLR2 signaling (4, 24, 27).

Recently, Duesberg et al. (28) showed that synthetic lipopeptides of the hepatitis C virus core protein, but not their corresponding free peptides, can activate cells via both TLRs 2 and 4. This indicates that activation of TLR2 and TLR4 does not only depend on the lipid moiety but also seems to require “recognition” of the amino acid sequence. In line with these findings, it may be hypothesized that interaction of TLR2 or TLR4 with the amino acid sequence...
moiety of OprI involves recognition of unknown motifs or three-dimensional structures, respectively. Further studies should be performed to precisely define the exact structural requirements for lipopolysaccharides or lipid-coupled peptides to activate TLR2 and TLR4. Since OprI has the ability to instruct APCs to induce Th1 immune responses, we investigated its potential to down-regulate or dampen a Th2 immune bias. Since Th2 cells are critical in the generation of allergen-induced airway inflammation because they release a particular set of cytokines (IL-4, IL-5, IL-13) that promote airway eosinophilia (8, 29, 30), we studied the effects of OprI on allergen-induced airway eosinophilia. Mucosal administration of OprI at the time of allergen challenge. Th1-related cytokines such as IFN-γ increase in the lungs. These C-X-C chemokines CXCL1, CXCL2, and CXCL5 in mRNA and protein levels in the lungs and BAL fluids, respectively. In addition, elevated mRNA levels of CXCL2 and CCL2 were present in the lungs. These CXC chemokines CXCL1, CXCL2, and CXCL5 and the C-C chemokine CCL2 have been implicated in the recruitment of neutrophils in response to TNF-α (32) and, therefore, are likely to have mediated the important but transient accumulation of neutrophils in BAL fluids upon OprI instillation.

In parallel with reduced eosinophilic inflammation, a decrease in IL-4 production was observed in BAL cells after primary challenge with OprI/OVA. A decrease in IL-4 mRNA levels was sustained in lung tissue CD4+ T cells after two subsequent allergen challenges in the OprI/OVA group. Concomitant with decreased IL-4 mRNA levels, reduced IL-13 mRNA levels were also observed as compared with the levels in the OVA group, indicating a diminished activation of locally recruited Th2 cells. This modification of the airway inflammatory response may result from the production of the Th1 cytokine IFN-γ observed following administration of OprI at the time of allergen challenge. Th1-related cytokines such as IFN-γ have been shown to inhibit IL-4 signaling (33) along with airway eosinophilia (34–36). In summary, this study demonstrates for the first time the potential of bacterial lipoprotein OprI as adjuvant to attenuate existing Th2 immune responses by enhancing maturation of APCs and their capacity to prime naive T cells in vivo via the TLR2/4 signaling pathway. Detailed analysis of the immunological parameters involved in the modulatory effect of OprI may further increase our understanding on how TLR signaling can be exploited in therapies for allergic diseases through efficient mechanisms of immunotherapy.

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