

CUTTING EDGE

Cutting Edge: Activation of Toll-Like Receptor 2 Induces a Th2 Immune Response and Promotes Experimental Asthma¹

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Recognition of microbial components by APCs and their activation through Toll-like receptors (TLR) leads to the induction of adaptive immune responses. In this study, we show that activation of TLR2 by its synthetic ligand Pam3Cys, in contrast to activation of TLR9 by immunostimulatory DNA (ISS-ODN), induces a prominent Th2-biased immune response. Activation of APCs by Pam3Cys resulted in the induction of Th2-associated effector molecules like IL-13, and IL-1 β , GM-CSF and up-regulation of B7RP-1, but low levels of Th1-associated cytokines (IL-12, IFN α , IL-18, IL-27). Accordingly, TLR2 ligands aggravated experimental asthma. These data indicate that the type of TLR stimulation during the initial phase of immune activation determines the polarization of the adaptive immune response and may play a role in the initiation of Th2-mediated immune disorders, such as asthma. The Journal of Immunology, 2004, 172: 2739–2743.

Efficient immune responses depend on the interaction between the innate and adaptive immune system. Immune responses against invading pathogens are initiated by Toll-like receptors (TLR)³ that recognize distinct structurally conserved components of pathogens. Probably with the exception of TLR3, the receptor for dsRNA, cell activation by all TLR family members is largely dependent on the adaptor molecule myeloid differentiation factor 88 (MyD88). Stimulation of the TLR leads to recruitment of MyD88, engagement of IL-1R-associated kinase and TNFR-associated factor 6 and activation of transcription factors such as NF- κ B and AP-1 (1, 2). This ultimately results in up-regulation of costimulatory molecules, secretion of cytokines, and enhanced uptake and presentation of Ag. Both TLR-dependent activation of APC and processing and presentation of Ag are necessary for the induction of

adaptive T and B cell responses. Polarization toward a Th1 or Th2 phenotype is crucial for the defense against pathogens, but can also be associated with the induction of autoimmune disease (Th1) or asthma (Th2). Although it is well known that all TLR stimuli lead to activation of APCs, their particular influence on the subsequent induction of adaptive immunity in vivo has only been partially delineated. In this study, we indicate that TLR2 and TLR9 ligands elicit two disparate adaptive immune responses.

Materials and Methods

Materials

Mice. C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) and 129/SvEv (B&K Universal, East Yorkshire, U.K.) mice were 6- to 8-wk-old.

Reagents. ISS-ODN (1668: TCCATGACGTTTCCTGATGCT) was synthesized by TIB MOLBIOL (Adelphi, NJ). OVA was purchased from Worthington (Lakewood, NJ) and the synthetic lipopeptide Pam3Cys was obtained from EMC Microcollections (Tübingen, Germany).

Tissue culture. Cells were cultured in RPMI 1640 (Cellgro; Mediatech, Herndon, VA) supplemented with 10% FCS (Life Technologies, Gaithersburg, MD), 2 mM L-glutamine (Cellgro), and 100 U/ml penicillin-100 μ g/ml streptomycin (Pen/Strep; Cellgro). Mouse bone marrow-derived dendritic cells (BMDCs) were cultured as previously described (3).

Immunization protocols

C57BL/6 mice were immunized with 50 μ g of OVA alone or in combination with ISS-ODN (50 μ g) or Pam3Cys (5–500 μ g) s.c. on days 0 and 14. On day 39, mice received an i.v. boost of 20 μ g of OVA. At day 42, mice were sacrificed and total splenocytes were restimulated for secondary CTL and cytokine assays as described (4, 5). Proliferation of splenocytes was determined by [³H]thymidine uptake.

Experimental asthma

129/SvEv were immunized s.c. with 50 μ g of OVA alone or in combination with ISS-ODN (50 μ g) or Pam3Cys (50 μ g) on days 0 and 7. Mice were intranasally challenged with 5 μ g of OVA 7 days and 1 day before sacrifice. At day 21, the mice were tested for airway responsiveness to methacholine (3–24 mg/ml; Sigma-Aldrich, St. Louis, MO) and a bronchoalveolar lavage for the differential lung cell count was performed as previously described (6, 7). Mediastinal lymph nodes (LN) were digested with DNase I/collagenase VII (Boehringer Mannheim/Roche, Indianapolis, IN/Sigma-Aldrich) and restimulated with OVA for T cell cytokine analysis and used for FACS stains.

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³ Abbreviations used in this paper: TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; BMDC, bone marrow-derived dendritic cell; LN, lymph node; AHR, airway hyper-reactivity.

ELISA

OVA-specific IgG2a, IgG1, and IgE levels were measured from serum samples collected by retro-orbital eye bleeds (8). IFN- γ , IL-5 (BD PharMingen, San Diego, CA), and IL-13 (RD Biosystems, Minneapolis, MN) were determined from supernatants of splenocytes that were restimulated with OVA *in vitro* (5). The levels of IL-12p40, IL-12p70, IL-10, and IL-6 (BD PharMingen) and IL-13 (RD Biosystems) were determined by ELISA.

Bioassay (type I IFN)

Type I IFN levels in supernatants from BMDC 16 h after stimulation were measured using an antiviral protection assay as described (9).

Flow cytometry

BMDC and mediastinal LN were stained with Abs purchased from eBioscience (San Diego, CA). Surface marker expression was analyzed on a FACSCalibur flow cytometer using CellQuest (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (Tree Star, San Carlos, CA).

Real-time PCR

Quantitative real-time PCR was performed using the ABI Prism 7700 (Applied Biosystems, Foster City, CA). Primers were generated using the Primer3 software (Ref. 10; www.genome.wi.mit.edu/genome_software/other/primer3.html).

Results

TLR2 ligands bias the adaptive immune response toward a Th2 phenotype and can lead to aggravation of asthma

To determine the role of particular TLRs in the generation of adaptive immune responses, mice were immunized with different TLR ligands in combination with OVA as a model Ag. Production of Ig subclasses (IgG2a, IgG1, and IgE), secretion of cytokines from *in vitro*-restimulated splenocytes, CTL response, and the effect on a murine experimental model of asthma were analyzed. Because our preliminary results indicated that the TLR2 ligand (Pam3Cys) and TLR9 ligand (ISS-ODN) lead to the most distinctive immune responses, we concentrated on these two ligands in our current investigations. As shown in Fig. 1, A–C, ISS-ODN and Pam3Cys induced different Ab profiles. Immunization with ISS-ODN/OVA resulted in an Ag-specific IgG2a response, whereas immunization with Pam3Cys/OVA resulted in a pronounced IgG1 response and induction of IgE (Fig. 1C). While ISS-ODN primed CD4 T cells to produce IFN- γ , Pam3Cys induced IL-13 production (Fig. 1, D–E). Restimulation with medium alone did not induce any production of IFN- γ or IL-13. The IgG isotype bias and the production of IgE and IL-13 induced by Pam3Cys/OVA were abrogated in TLR2-deficient mice, proving that TLR2 is a critical receptor for Pam3Cys (data not shown). Immunization with peptidoglycan (100 μ g) as an adjuvant in combination with OVA was less potent than immunizations with Pam3Cys/OVA in regard to cytokine production and induction of Ab response, but also showed a preferential induction of IgG1 ($26,547 \pm 14,455$ U/ml for peptidoglycan/OVA vs $299,082 \pm 73,142$ U/ml for Pam3Cys/OVA, 3 wk after immunization) over IgG2a (not detectable for peptidoglycan/OVA vs 1473 ± 925 U/ml for Pam3Cys/OVA, 3 wk after immunization).

Both stimuli, ISS and Pam3Cys, led to induction of CTL activity (Fig. 1F); however, the induction of CTL activity by ISS-ODN was significantly more prominent than that induced by Pam3Cys. Titrating the amount of Pam3Cys over three orders of magnitude did not significantly change this low CTL activity, nor did it change the Th2 bias in regard to cytokine and Ab production.

Immunization with ISS-ODN/OVA and Pam3Cys/OVA induced a similar proliferative response in OVA-restimulated splenocyte cultures (Fig. 1G).

To test whether the Th1 (by ISS-ODN) and Th2 (by Pam3Cys) polarization observed above alters the propensity of the immunized animals to develop experimental asthma, Pam3Cys/OVA- or ISS-ODN/OVA-immunized mice were re-challenged with OVA intranasally at two occasions and airway hyper-reactivity (AHR), recruitment of eosinophils to the lung, and cytokine release of *in vitro*-restimulated bronchial LN cells were evaluated. Priming with ISS-ODN/OVA improved AHR, decreased the number of eosinophils in the lung and induced IFN- γ , whereas priming with Pam3Cys/OVA aggravated the AHR, increased the number of eosinophils and led to the production of Th2 cytokines (Fig. 1, H–J).

Taken together, these data show that both ISS-ODN and Pam3Cys induce significant Ag-dependent immune responses. However, ISS-ODN polarizes the immune response toward a Th1 phenotype, whereas Pam3Cys leads to Th2-specific cytokine and Ig production and only a modest CTL response. The data further suggests that the opposing Th1/Th2 polarization induced by ISS-ODN and Pam3Cys can have an effect on the development of Th2-associated diseases such as experimental asthma.

TLR2 ligands differentially induce Th2-associated cytokines and B7RP-1

Costimulatory molecules and the cytokine production by DC play a crucial role in the differentiation of naive CD4 T cells. To explore whether these factors may explain the differential Th polarization induced by ISS-ODN and Pam3Cys, BMDC were stimulated with ISS-ODN or Pam3Cys and the expression of costimulatory molecules and the production of cytokines were determined. As shown in Fig. 2A, both ISS-ODN and Pam3Cys induced up-regulation of the costimulatory molecules CD40, B7-1 and B7-2, whereas only Pam3Cys led to an up-regulation of B7RP-1. The up-regulation of B7RP-1 was even more pronounced in mature DC from mediastinal LN after immunization with OVA and Pam3Cys (Fig. 2B), whereas ISS-ODN showed less effect.

We next analyzed production of IFN $\alpha\beta$, IL-12 p40, IL-12 p70, IL-6, IL-10, and IL-13 (Fig. 2C), and a panel of mRNAs known to be involved in Th1/Th2 polarization (Fig. 2D) by BMDC. There was a clear difference in the pattern of cytokines induced. Whereas ISS-ODN induced primarily cytokines associated with a Th1 phenotype like IL-12, IL-18, IL-27, and IFN $\alpha\beta$, Pam3Cys preferentially induced Th2-associated cytokines like IL-13, GM-CSF, and IL-1 β . Other genes like TNF- α and I κ B α were comparably induced. A similar pattern of cytokine production with Pam3Cys inducing Th2-associated cytokines was observed in primary CD11c⁺ DC isolated from the spleen. Again, the cytokine response and gene induction by Pam3Cys in BMDC was dependent on TLR2 (data not shown).

Discussion

In this study, we analyzed the effect of specific TLR stimuli on Th polarization and experimental asthma. Preliminary experiments indicated that ISS-ODN (TLR9) and Pam3Cys (TLR2) induced the most contrasting immune responses. When used as an adjuvant, Pam3Cys induced a Th2 polarization as reflected

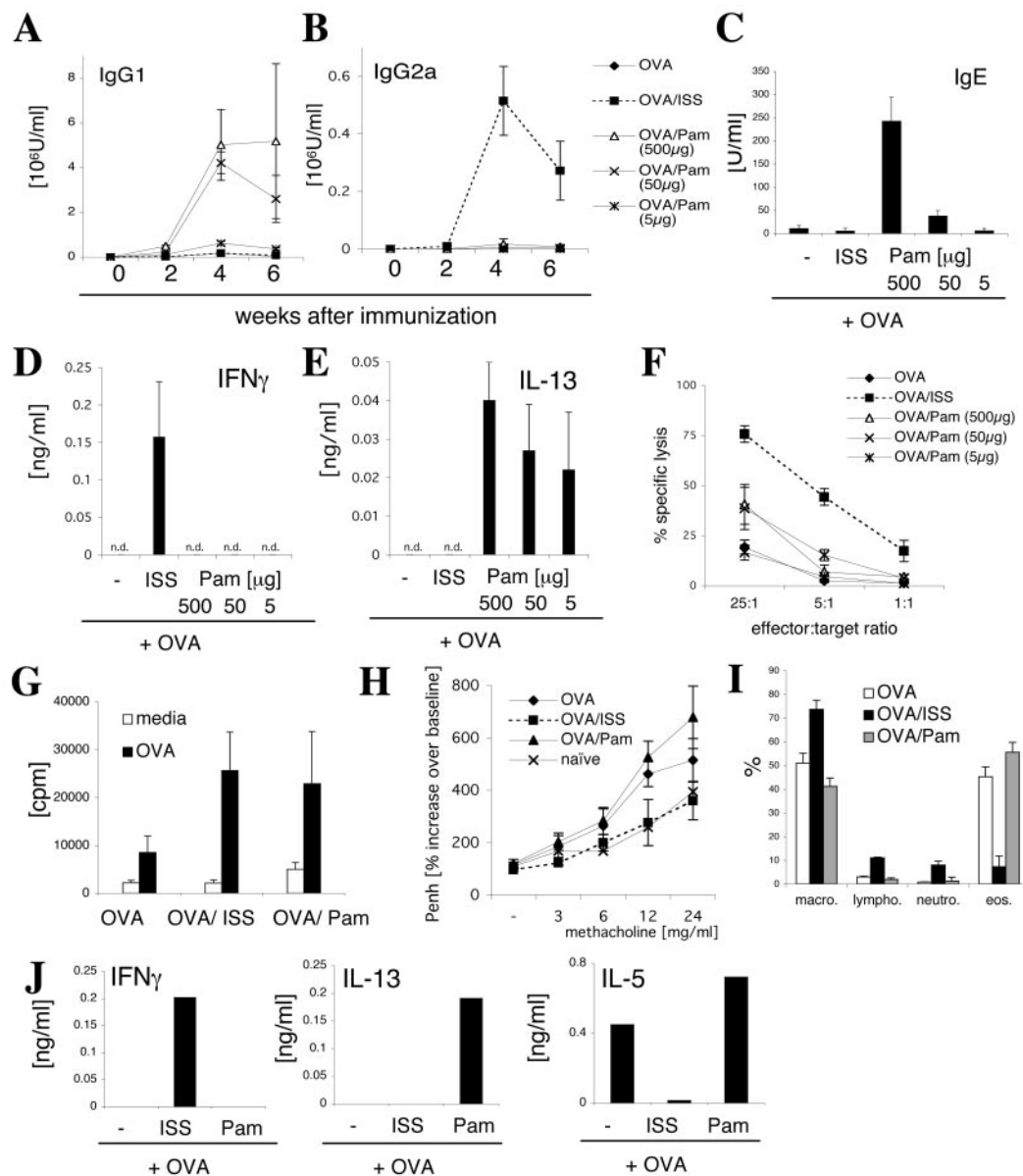


FIGURE 1. Differential immune responses induced by ISS-ODN- and Pam3Cys-based immunizations. *A–G*, Mice were immunized s.c. with OVA (50 μ g) either alone or in combination with ISS-ODN (50 μ g) or Pam3Cys (500, 50, or 5 μ g) at days 0 and 14 and rechallenged i.v. with 20 μ g of OVA 3 days before sacrifice. *A–B*, Ab isotypes (IgG1, IgG2a) were determined by ELISA from serum samples taken before immunization and at weeks 2, 4, and 6. *C*, IgE was determined by ELISA from serum samples at week 4. *D–F*, Total splenocytes were analyzed for the secondary CTL activity and OVA-specific cytokine secretion. *G*, Proliferative response of OVA-restimulated splenocytes was determined by [³H]thymidine uptake. *H–J*, Mice were immunized s.c. with OVA (50 μ g) either alone or in combination with ISS-ODN (50 μ g) or Pam3Cys (50 μ g) twice and rechallenged intranasally with 5 μ g of OVA 7 days and 1 day before testing. *H*, Airway responsiveness to aerosolized methacholine was tested. *I*, Percentage of macrophages (macro.), lymphocytes (lympho.), neutrophils (neutro.), and eosinophils (eos.) from the BAL was determined. *J*, OVA-specific cytokine secretion of mediastinal LN cells was measured. Data are shown as mean $\pm$ SEM, n = 5 per group (n = 8 per group for the AHR and eosinophil count), ISS = ISS-ODN, Pam = Pam3Cys, n.d. = not detectable.

in the cytokine profile and the production of specific IgG subclasses and IgE, whereas ISS-ODN induced a Th1 polarization. As expected, the effector functions induced by Pam3Cys were dependent on TLR2. Similar Th2 biasing effects were seen after immunization with peptidoglycan, which also activates cells via TLR2. Consistent with our data a recent study showed that different TLR ligands induce distinct Th cell responses in human DC with Pam3Cys inducing a Th2 polarization (11). LPS from *Porphyromonas gingivalis*, which in contrast to LPS from other species activates cells via TLR2, has also been described to induce T cell cytokines associated with a Th2 phenotype (12). In

a different study, it was shown that the Th1/Th2 polarizing effect of LPS from *Escherichia coli*, which triggers cells via TLR4, was concentration-dependent (13). At low concentrations LPS induced a Th2 bias, whereas at higher concentrations a Th1 phenotype was reported. In contrast, Pam3Cys-induced Th2 polarization appeared not to be concentration dependent. Even a 10-fold higher amount of Pam3Cys than required for maximum induction of IL-13 and IgG1 production did not induce Th1-specific parameters like IFN- γ or IgG2a production. Whether the differences in concentration dependence between LPS and Pam3Cys are due to different signaling pathways or

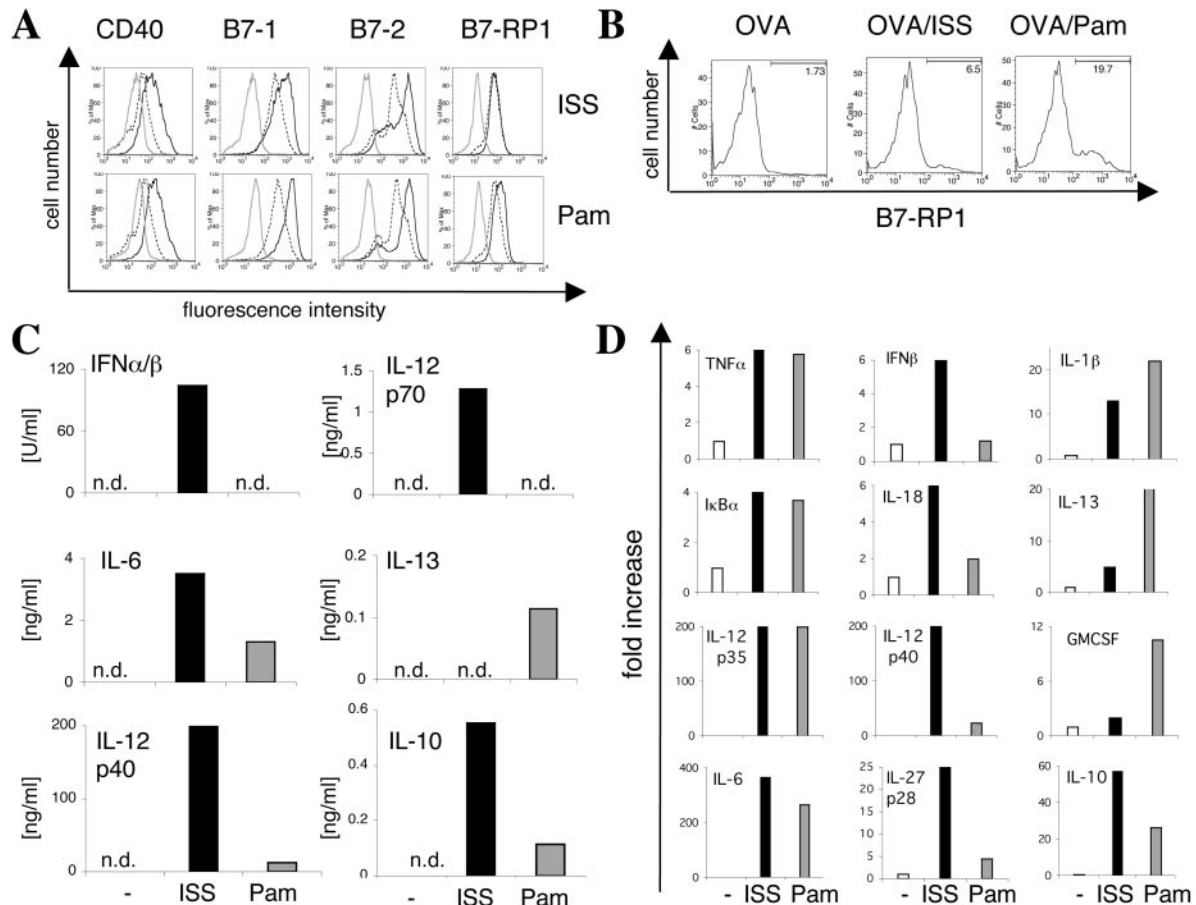


FIGURE 2. Activation of BMDC by ISS-ODN and Pam3Cys. *A*, Expression of costimulatory molecules in vitro. BMDC were cultured in the absence or presence of ISS-ODN (1 μ M) or Pam3Cys (5 μ g/ml) for 16 h. Surface expression of CD40, B7-1, B7-2, and B7RP-1 were measured by flow cytometry. Data shown are from one representative experiment of six experiments and are gated on the live CD11c⁺ population. Gray lines: isotype control, dashed lines: unstimulated control, solid lines: ISS-ODN or Pam3Cys (Pam) stimulated cells. *B*, Expression of B7RP-1 on DC from mediastinal LN cells in vivo. Mice were immunized as described in Fig. 1, *G–H*. Mediastinal LN were harvested and surface expression of B7RP-1 determined by flow cytometry. Data shown are pooled cell preparations from three mice per group and are gated on the live CD11c⁺/MHC class II high population. *C*, Concentration of IL-12p40, IL-12p70, IL-13, IL-10, and IL-6 were measured by ELISA from the culture supernatant of BMDC after stimulation with ISS-ODN (1 μ M) or Pam3Cys (5 μ g/ml) for 16 h. IFN- α was determined by bioassay. Data from one representative experiment of four experiments is shown. *D*, Expression of TNF- α , I κ B α , IL-12p35, IL-6, IFN β , IL-18, IL-12p40, IL-27p28, IL-1 β , IL-13, GM-CSF, and IL-10 mRNA were determined by quantitative real-time PCR. BMDC were cultured in the absence (control) or presence of ISS-ODN (1 μ M) and Pam3Cys (5 μ g/ml) for 6 h. Data from one representative experiment of five experiments normalized to the expression of CPH are shown and expressed as fold increase over control. ISS = ISS-ODN, Pam = Pam3Cys.

whether different cell types contribute to the phenomenon observed needs to be investigated.

At the cellular level, stimulation of BMDCs with ISS-ODN or Pam3Cys showed distinct activation profiles. Up-regulation of costimulatory molecules like CD40, B7-1 and B7-2 was comparable, whereas up-regulation of B7RP-1, which was shown to support Th2 responses (14), seemed more pronounced after Pam3Cys stimulation in vitro and in vivo. IL-13, IL-1 β , and GM-CSF, which support Th2 differentiation or allergy (15–18), were preferentially induced by Pam3Cys. In contrast, Th1-associated cytokines like IL-12, IFN β , IL-18, and IL-27 (17, 19) were greatly diminished in comparison to stimulation with ISS-ODN. This effect was also seen with IFN-dependent genes (data not shown).

The signaling pathways of TLR2 and TLR9 share common molecules. It was shown that signaling through the adaptor molecule MyD88 plays an important role in the induction of Th1-associated immunity (20). ISS-ODN, a TLR ligand that is believed to be completely dependent on MyD88, was shown to

induce a strong Th1 bias (21). In contrast, TLR2 uses at least one additional molecule called Toll-IL-1R domain-containing adaptor protein (22, 23). Whether this or further signaling molecules might explain the diversity seen with those two TLR ligands remains to be elucidated.

In summary, we found that the immune response induced by TLR ligands can play an important role in the initiation or prevention of Th2-associated diseases. Immunization with Ag in the context of TLR2 ligands, in contrast to the protective effects of TLR9, can result in experimental asthma. There have been reports suggesting that bacterial infections can be associated with asthma. Interestingly, chlamydia and mycoplasma, bacteria that are strongly associated with the onset of asthma, as well as some viral infections and air pollution particles were shown to elicit some of their effects via TLR2-mediated mechanisms (24–27). Our study demonstrates that the type of TLR stimulation is able to regulate the development of Th1/Th2-polarized adaptive immune responses. Therefore, it seems reasonable to consider TLR2 and TLR2-dependent signaling pathways as

possible inducers of the immune deviation that results in asthma in humans.

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