Regulation of allergic airway inflammation through Toll-like receptor 4-mediated modification of mast cell function


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Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, December 19, 2005 (received for review April 19, 2005)

In a mouse experimental asthma model, the administration of bacterial lipopolysaccharide (LPS), particularly at low doses, enhances the levels of ovalbumin (OVA)-induced eosinophilic airway inflammation. In an effort to clarify the cellular and molecular basis for the LPS effect, we demonstrate that the OVA-induced eosinophilic infiltration in the lung is dramatically increased by the administration of LPS in wild-type mice, whereas such increase was not observed in mast-cell-deficient mice or Toll-like receptor (TLR4)-deficient mice. Adoptive transfer of bone-marrow-derived mast cells (BMMCs) from wild-type, but not from TLR4-deficient, mice restored the increased eosinophilic infiltration in mast-cell-deficient mice. Wild-type BMMCs pretreated with LPS in vitro also reconstituted the eosinophilic infiltration. Moreover, in vitro analysis revealed that the treatment of BMMCs with LPS resulted in NF-κB activation, sustained up-regulation of GATA1 and -2 expression, and increased the capability to produce IL-5 and -13. Dramatic increases in the expression of IL-5 and -13 and Eotaxin-2 were detected in LPS-treated BMMCs after costimulation with LPS and IgE/Ag. Overexpression of GATA1, but not GATA2, in MC9 mast cells resulted in increased transcriptional activity of IL-4, -5, and -13. Furthermore, the levels of transcription of Th2 cytokines in BMMCs were decreased by the introduction of small interfering RNA for GATA1. Thus, mast cells appear to control allergic airway inflammation after their activation and modulation through TLR4-mediated induction of GATA1 and subsequent increase in Th2 cytokine production.

Conflict of interest statement: No conflicts declared.

Ferre available online through the PNAS open access option.

Abbreviations: BAL, bronchoalveolar lavage; BMMC, bone-marrow-derived mast cell; DC, dendritic cell; H.E., hematoxylin and eosin; KO, knockout; LPS, lipopolysaccharide; OVA, ovalbumin; PMMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; TLR, Toll-like receptor.

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Adaptive Transfer of Wild-Type (+/+ ) BMMCs Reconstitutes LPS-Mediated Enhancement of Eosinophilic Airway Inflammation in W/Wv Mice. To further investigate the requirement of mast cells in enhanced eosinophilic inflammation induced by OVA plus LPS, wild-type mast cells were adaptively transferred into W/Wv mice. BMMCs were generated by culturing bone marrow cells with IL-3 for 4 weeks. A summary of infiltrating cells in the BAL fluid is presented in Fig. 2A, and representative photographic views of infiltrated cells (May–Giemsa staining) in each group are shown in Fig. 10, which is published as supporting information on the PNAS web site. The administration of +/+ BMMCs resulted in the dramatic eosinophilic infiltration in the BAL fluid of W/Wv mice immunized OVA and LPS when compared with mice not given wild-type (+/+ ) BMMCs (see Fig. 10h). No apparent effect was observed in the numbers of macrophages, neutrophils, and lymphocytes with the +/+ BMMC transfer.

Concurrently, histological analysis showed that the transfer of +/+ BMMCs as described above resulted in the dramatic increase in the levels of eosinophilic infiltration in the airway of W/Wv mice (Fig. 2B). There were also moderate increases in the numbers of total cells. Collectively, these results clearly indicate that mast cells play a critical role in LPS-mediated enhancement of eosinophilic inflammation.

TLR4 on Mast Cells Is Required for LPS-Mediated Enhancement of Eosinophilic Inflammation. The results thus far indicated that mast cells are critical for LPS-mediated enhancement of allergic airway eosinophilic inflammation. Because TLR4 is a known receptor for LPS (30), we next examined the involvement of TLR4 molecules on mast cells in the LPS-mediated enhancement of airway inflammation. BMMCs prepared from TLR4 KO mice showed a normal surface phenotype, e.g., the expression of c-kit and FcεRI (data not shown). Ten million BMMCs prepared from wild-type or TLR4 KO mice were transferred into W/Wv mice. As anticipated, the levels of eosinophilic infiltration were enhanced by the administration of wild-type (+/+), but not in the case of TLR4 KO, BMMCs (Fig. 2C; and see Fig. 11, which is published as supporting information on the PNAS web site). Also, LPS-mediated enhancement in the total numbers of infiltrating leukocytes was marginal in the TLR4 KO BMMC transfer group. It would appear that TLR4 on mast cells is crucial for LPS-mediated enhancement of eosinophilic inflammation.

To further investigate the involvement of TLR4 on mast cells, wild-type and TLR4 KO BMMCs were first treated with LPS (10 μg/ml) for 7 days in vitro and then transferred into W/Wv mice. No immunization was performed. Two days after the challenge with OVA, infiltrated cells in the BAL fluid were assessed. As shown in Fig. 2D, although the levels were modest, a significant increase in the number of eosinophils was detected with the administration of wild-type BMMCs pretreated with LPS. Representative photographic views of infiltrated cells (May–Giemsa staining) are shown in Fig. 12, which is published as supporting information on the PNAS web site.
PNAS web site. Moreover, TLR4 KO mice were challenged directly with/w/o LPS and OVA to assess the LPS-mediated enhancement of airway inflammation, and no enhancing effect of LPS on the eosinophilic infiltration was observed in TLR4 KO mice (Fig. 2E; and see Fig. 13, which is published as supporting information on the PNAS web site). The LPS-induced increase in the number of other cell types, including macrophages and neutrophils, was modest, and no increase was seen in TLR4 KO mice.

Treatment with LPS Modulates Cytokine Production Profiles of Mast Cells. To analyze the molecular changes induced in mast cells after LPS treatment, BMMCs were cultured with or without LPS treatment (10 μg/ml) for 1 week in vitro. The expression levels of FceRI, c-kit, I-K, CD54, and RIPA/B were similar between wild-type and TLR4 KO BMMCs before and after LPS treatment (data not shown). The LPS-treated BMMCs were then restimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, and the ability to produce various cytokines (IL-5, -13, -6, and -4 and TNF-α) was assessed by ELISA. The levels of IL-5 and -13 were substantially increased after LPS treatment, whereas the production of IL-6 and TNF-α was only slightly increased (Fig. 3A). IL-4 was not detected with or without LPS treatment (data not shown).

Next, we assessed the mRNA expression of IL-4, -5, -13 and -13 and Eotaxin-2 after IgE/AE stimulation in wild-type and TLR4 KO BMMCs cultured with LPS. The mRNA expression of IL-4, -5, and -13 and Eotaxin-2 was increased dramatically for all of these cytokines in wild-type BMMCs. In the case of TLR4 KO BMMCs, however, the increase was seen in the case of IL-4 and -13 but not in the case of IL-5 and Eotaxin-2 (Fig. 3B). More specifically, the induction in IL-5 was barely detectable, indicating that the Ag/IgE-induced expression of IL-5 was most sensitive to the effect of LPS/TLR4-mediated modification of BMMC function.

To assess more directly whether LPS/TLR4-mediated signaling synergizes with IgE/AE-dependent responses in BMMCs, BMMCs were stimulated with combinations of LPS and IgE/AE in vitro. As shown in Fig. 3C, clear synergistic effects were observed in the expression of IL-5 and -13 and some effect on Eotaxin-2 but much less in the case of IL-4.

LPS Treatment Induces NF-κB Activation and Increases Expression of GATA1 and -2 in BMMCs. Because NF-κB is activated through TLR4 after LPS ligation (18), we wanted to test whether LPS treatment induces NF-κB activation in mast cells. A gel-shift assay for NF-κB was performed with BMMCs treated with LPS. A significant activation of NF-κB was detected in wild-type BMMCs but not in TLR4 KO BMMCs (Fig. 3D Left). In an AP-1 gel-shift assay, a modest increase was detected after LPS treatment, but no difference was observed between wild-type and TLR4 KO groups (Fig. 3D Right).

Although GATA3 is reported to be a downstream target of NF-κB activation (31) and is critical for chromatin remodeling of the Th2 cytokine gene loci (32–34) and transcription of the IL-5 and IL-13 genes in Th2 cells (35–37), GATA3 is not expressed in BMMCs (7). In contrast, GATA1 and -2 play a key role in mast-cell differentiation (38, 39). We examined the levels of protein expression of GATA1 and -2 in BMMCs treated with LPS. As shown in Fig. 3E, the levels of GATA1 and -2, but not -3, were substantially increased in the BMMCs after LPS treatment for 3 days.
LPS-induced increase in the levels of GATA1 and -2 protein was not detected in TLR4 KO BMMCs (Fig. 3F), but increases were detected in STAT6 KO BMMCs (data not shown). The increase in GATA1 and -2 protein was also observed after 7-day cultures (data not shown). Thus, TLR4-mediated signaling is critical for GATA1 and -2 up-regulation in BMMCs upon LPS treatment.

**GATA1 Controls the Expression of Th2 Cytokines in Mast Cells.** Finally, we studied whether GATA1 and -2 can play a functional role in transcription of IL-5, -13, and -4 in mast cells. We used a MC9 mast-cell line for a reporter gene assay. The introduction of GATA1, but not -2 or -3, into MC9 cells resulted in substantial induction of the reporter activities of IL-4 and -5 promoters (Fig. 4A). When GATA1 was introduced by retrovirus into MC9 cells, the mRNA expression of IL-4, -5, and -13, but not of Eotaxin-2, was increased significantly (Fig. 4B). The increase in Th2 cytokine mRNA was not observed with GATA2 overexpression. Furthermore, we tested whether the inhibition of GATA1 expression in BMMCs would result in decreased expression of Th2 cytokines. BMMCs cultured with LPS were transfected with small interfering (si)RNA specific for GATA1 during the treatment with LPS. As expected, the mRNA levels of IL-4, -5, and -13 were all decreased significantly (Fig. 4C). These results suggest that GATA1 controls the expression of Th2 cytokines in mast cells, such as MC9 and BMMCs, with LPS stimulation.

**Discussion**

The results presented here indicate that mast cells and their TLR4 molecules are crucial for LPS-mediated enhancement of allergic airway eosinophilic inflammation. After LPS treatment, BMMCs acquired an increased ability to produce Th2 cytokines, such as IL-5 and -13. Clear synergistic effects in the expression of IL-5 and -13 were detected in LPS-treated BMMCs after costimulation with LPS and IgE/Ag. GATA1 appeared to be important for the transcription of Th2 cytokines in mast cells. These findings suggest that LPS-induced TLR4 signaling modulates mast-cell function and regulates allergic airway inflammation in vivo.

Dendritic cells (DCs) are well recognized to play a central role in inflammatory reactions elicited by LPS (16). When DCs are activated by LPS through TLR4, they become mature and acquire an increased ability to prime T cells (40). In particular, mature DCs produce increased levels of IL-1 and -12 and TNF-α that lead to the promotion of Th1-skewed responses (41). However, it is also well recognized in humans that LPS is a risk factor for asthma (42, 43). In some mouse models, LPS was reported to elicit airway inflammation (14, 22, 44). To investigate the molecular basis underlying the LPS-induced mast-cell activation and regulation of allergic eosinophilic airway inflammation, we used an experimental model with a low-dose LPS administration, in which Th2-dependent experiments were performed, and similar results were obtained. (C) Wild-type BMMCs were stimulated with combinations of LPS and IgE/Ag. Transcriptional levels of IL-4, -5, and -13 and Eotaxin-2 were determined by real-time RT-PCR analysis. Two independent experiments were performed, and similar results were obtained. (D) EMSAs for NF-κB and AP-1. Nuclear extracts of wild-type and TLR4 KO BMMCs were cultured with LPS for 1 week and then stimulated with IgE/Ag. Transcriptional levels of IL-4, -5, and -13 and Eotaxin-2 were detected by real-time RT-PCR analysis. Two independent experiments were performed, and similar results were obtained. (E) The levels of protein expression of GATA1 and -2 in LPS-stimulated BMMCs. BMMCs treated with LPS for 3 days and CD4 T cells cultured under Th2-skewed conditions for 3 days were prepared. Nuclear extracts were used for immunoblotting with specific mAbs specific for GATA1, -2, and -3. Arbitrary densitometric units normalized with the band intensity of tubulin α are shown under each band. Three independent experiments were performed, and similar results were obtained. (F) GATA1 and -2 expression in LPS-treated BMMCs from wild-type and TLR4 KO mice. Two experiments were performed, and similar results were obtained.
cosinophilic airway inflammation is selectively induced (14). As for a mechanism, Eisenbarth et al. (14) suggested the importance of LPS-induced DC activation for the induction of eosinophilic airway inflammation. From the studies presented here, we propose a different mechanism, whereby mast cells, another innate immunity cell type, play a crucial role for regulating eosinophilic airway inflammation. LPS-induced mast-cell activation and modulation with increased production of Th2 cytokines, such as IL-5 and -13, appear to control the severity of eosinophilic airway inflammation.

It is known that IL-5 and -13 play crucial roles in the induction and the severity of eosinophilic airway inflammation in the lung (45). The levels of Th2 cytokines, such as IL-5 and -13, produced by mast cells were increased dramatically after LPS treatment (Fig. 3A). We detected decreased levels of IL-4, -5, and -13 and Eotaxin-2 in BAL fluid cells from mast-cell-deficient W/Wv mice as compared with +/+ mice (Fig. 6). The IgE/Ag-induced IL-5 expression in BMMCs was minimal in TLR4 KO BMMCs (Fig. 3B). Moreover, obvious synergetic effects in the expression of IL-5 and -13 were detected in LPS-treated BMMCs after cytokine modulation with LPS and IgE/Ag (Fig. 3C). Thus, although it is difficult to determine which Th2 cytokines produced by mast cells are most important for LPS-mediated enhancement of allergic eosinophilic inflammation, a direct effect of IL-5 in the activation and migration of eosinophils in the lung appears to be critical. Because BMMCs also produce increased TNF-α (~2-fold) after LPS treatment (Fig. 3A), it is conceivable that TNF-α produced from activated mast cells may also induce DC maturation and regulate eosinophilic airway inflammation. In addition, Eotaxin-2 expression induced by IgE/Ag stimulation in BMMCs (Fig. 3B and C) may also contribute to the inflammation by increasing eosinophil migration into the lung.

After in vitro LPS stimulation for a short period (<16 h), Supajatura V. et al. (21) reported that BMMCs produced TNF-α, IL-1β, -6, and -13 but not IL-4 or -5. Similarly Matsuda et al. (22) reported that LPS induced the production of IL-5,-10, and -13, but not IL-4, in BMMCs. The levels of cytokine production detected in those studies were much lower than those detected in this study (Fig. 3A), due, perhaps, to the different methods of LPS treatment. In our studies, to assess the changes in the ability to produce cytokines in BMMCs, the cells were treated with LPS for 1 week (Fig. 3A). We observed some increase in acetylation of histone H3/K9 at the Th2 cytokine gene loci, suggesting the occurrence of chromatin remodeling (M.Y. and T.N., unpublished observation). Although we do not know whether the LPS treatment induced a true “maturation” of BMMCs, it is clear that BMMCs acquired an ability to produce increased amounts of Th2 cytokines after LPS treatment for 1 week, reminiscent of the maturation process of DCs after LPS stimulation (40).

It has been reported that airway hyperreactivity to methacholine is modulated by the administration of high-dose LPS in rats (44) and mice (46). In the current experimental system, where a mild OVA sensitization protocol was used, we did not observe clear induction of airway hyperreactivity, and treatment with LPS/OVA did not change this situation significantly (M.Y. and T.N., unpublished observation). Further investigation will be needed to clarify the effect of LPS and the contribution of mast cells in the development of airway hyperreactivity.

In summary, in a mouse allergic asthma model, we found that mast cells play a crucial role for LPS-mediated enhancement of eosinophilic airway inflammation. Moreover, TLR4 molecules on mast cells were critical for LPS-induced mast-cell activation and functional modulation. Thus, a search for specific inhibitors acting on the TLR4-mediated signal transduction pathway could lead to an approach for the treatment of inflammation in patients with bronchial asthma, particularly during respiratory infection.

Materials and Methods

Animals. C57BL/6, WBB6F1 W/Wv mice (47) with (WBBxC57BL6)F1 background and WBB6F1 +/+ were purchased from Japan SLC (Shizuoka, Japan). STAT6-deficient and TLR4-deficient (TLR4 KO) mice backcrossed eight times with C57BL/6 mice were kindly provided by Dr. Shizuo Akira (Osaka University) (30, 48). All mice used in this study were maintained under specific-pathogen-free conditions. Animal care was in accordance with the guidelines of Chiba University.

Cell Cultures. BMMCs were generated as described in ref. 29.

Immunization and Challenge. Female mice (6–7 weeks old) were anesthetized with ketamine hydrochloride, xylazine, and fluntrazepam i.p. and sensitized intranasally with 10 μg of OVA [Grade V; Sigma Aldrich, LPS contamination <0.5 ng; measured by limulus amebocyte assay (Bio-Whittaker)] and 1 μg of LPS (Escherichia coli O55:B5, List Biological Laboratories, Campbell, CA) in 35 μl of PBS on days 0, 1, and 2. The sensitized mice were challenged on days 14, 15, 18, and 19 intranasally with 25 μg of OVA in 35 μl of PBS under anesthesia as described in ref. 14.
Collection and Analysis of BAL Fluid. Two days after the last challenge with OVA, BAL was prepared as described in refs. 28 and 29. One-hundred thousand viable BAL cells were cytogenotyped by using a Cytospin 3 (Shandon, Pittsburgh) and stained with a Grunwald-Giemsa solution (Merck) as described in ref. 9. Two hundred leukocytes were counted in each slide. Cell types were identified based on morphological criteria.

Lung Histology. On day 20, 1 day after the last OVA challenge, mice were killed by CO2 asphyxiation, and the lungs were infused with 10% (vol/vol) formalin for fixation and then subjected to H.E., Luna, or Masson-trichrome staining as described in refs. 28 and 29.

Treatment of BMMCs with LPS in Vitro. BMMCs were cultured with or without LPS (10 μg/ml) for 7 days. The LPS-stimulated BMMCs were activated with PMA (10 ng/ml) for 30 min with gentle agitation. The siRNA solution was added for 24 h. The BMMCs were stimulated with 10 ng/ml for 7 days before transfer, and no LPS was used for in vivo priming.

RT-PCR. Quantitative RT-PCR was performed by using Gene Expression assay (Applied Biosystems) and ABI prism 2000 as described in ref. 49. Hypoxanthine phosphoribosyltransferase was used for a control. The specific primers for detection of cytokines were described in ref. 34. Primer pairs for Eotaxin-2 and -3 were tagcct-ttgctgcatcttcc-3′ and 5′-taaacctgctgtctc-3′, respectively.

Immunoblot Analysis. Immunoblot analysis for GATA1, -2 and -3 expression assay (Applied Biosystems) and ABI prism 2000 as described in ref. 51. Immunoblot analysis for GATA1, -2 and -3 was performed as described in ref. 34. Anti-GATA1 (N1), anti-GATA2 (CG2-96) and anti-GATA3-HG3-31 (all from Santa Cruz Biotechnology) antibodies were used.

Luciferase Reporter Assay. Luciferase assay was performed by using Dual Luciferase Reporter instructions as described in ref. 32. A single copy of an IL-4 promoter (−766 bp) and IL-5 promoter (−1,200 bp) in the luciferase reporter plasmid PGL2 Basic (Promega) and MC9 (a mast-cell expressing some levels of GATA1 and -2 but not GATA3) cells were used.

EMSA EMSAs were performed by using Gel Shift Assay Systems (Promega) as described in ref. 51.

siRNA. Introduction of siRNA into BMMCs was performed as described in ref. 52. Predesigned siRNA for GATA1 was purchased from Ambion (#16704). In brief, 2 μl of TransIT-TKO transfection reagent (Mirus, Madison, WI) was diluted in 50 μl of serum-free/antibiotic-free RPMI medium 1640. Ten minutes later, 1 μl of 40 μM siRNA was added to the diluted transfection reagent and incubated for 30 min with gentle agitation. The siRNA solution was added for 24 h. BMMCs cultured with cells in 500 μl of medium per well in a 24-well plate. Two days after transfection, expression levels of GATA1 and IL-4, -5, and -13 mRNA were assessed by quantitative RT-PCR.

We thank Drs. Ralph T. Kubo and Kiyoshi Takeda for helpful comments and constructive criticism in the preparation of the manuscript and Ms. Kaoru Sugaya, Satoke Norikane, and Hikari Asou for excellent technical assistance. This work was supported by Ministry of Education, Culture, Sports, Science, and Technology (Japan) Grants 17016010 and 17047007, Grants-in-Aid for Scientific Research in Priority Areas, Scientific Research B 17390139 and Scientific Research C 16616003; Grants-in-Aid for Young Scientists 17790317 and 17790318; and Special Coordination Funds for Promoting Science and Technology, the Ministry of Health, Labor, and Welfare (Japan), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (Japan), the Japan Health Science Foundation, the Kanae Foundation, the Uehara Memorial Foundation, and the Mochida Foundation.