Endotoxin tolerance attenuates airway allergic inflammation in model mice by suppression of the T-cell stimulatory effect of dendritic cells

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Abstract

Prior exposure of dendritic cells (DCs) and monocytes/macrophages to LPS causes unresponsiveness to subsequent LPS stimulation, a phenomenon called endotoxin tolerance (ET). ET impairs antigen presentation of these cells to T cells by down-regulating expression of MHC class II and co-stimulatory molecules such as CD86 and CD40. Some epidemiological studies have shown that endotoxin acts as a protective factor for allergic diseases. Accordingly, LPS has beneficial effects on the onset of airway allergic inflammation in model animals by Th1 skewing or induction of regulatory T cells. However, results derived from asthma model animals are controversial, probably due to the difficulty of handling LPS. We previously generated a monoclonal agonistic antibody against Toll-like receptor (TLR) 4, named UT12, which mimics the biological activities of LPS, exhibiting more potent and sustained ET than does LPS. In this study, we took advantage of UT12 to generate prolonged ET to explore the possibility that ET is involved in the inhibitory effects of the TLR4 signals on asthma model mice. Induction of ET by UT12 inhibited the capacity of DCs to expand ovalbumin (OVA)-specific T\textsubscript{h}2 and T\textsubscript{h}17 cells, without inducing T\textsubscript{h}1 cell or regulatory T-cell populations or producing inhibitory cytokines. Accordingly, administration of UT12 before the OVA sensitization significantly suppressed airway allergic inflammation by OVA inhalation. Taken together, these results demonstrate that ET induced by activating TLR4 signals attenuates airway allergic inflammation through direct suppression of the T-cell stimulatory effect of DCs in asthma model mice.

Keywords: bronchial asthma, hygiene hypothesis, lipopolysaccharide, T\textsubscript{h}2, Toll-like receptor 4

Introduction

Engagement of Toll-like receptor (TLR) 4 by LPS potently stimulates dendritic cells (DCs) and monocytes/macrophages and triggers activation of innate immunity, followed by induction of acquired immunity. However, prior exposure of these cells to LPS causes unresponsiveness to subsequent LPS stimulation and down-regulates expression of inflammatory cytokines in vitro or in vivo, which is called endotoxin tolerance (ET, LPS tolerance, refs. 1–3). ET acts to protect hosts against inflammation-induced injury. Moreover, monocytes/macrophages in the presence of ET decrease expression of MHC class II and co-stimulatory molecules such as CD86 and CD40, leading to inhibition of antigen presentation to T cells (4–7). Thus, ET impairs host immunity, including innate and acquired immune responses. In accordance with these characteristics of ET, monocytes in patients with endotoxin shock down-regulate the expression of MHC class II molecules (8). Furthermore, it has been recently demonstrated that ET is observed also in monocytes in patients with cystic fibrosis (7, 9) and with acute coronary syndrome (10), which indicates that ET may be more broadly related with various pathophysiological conditions than expected. However, the involvement of ET in our immune responses has been poorly understood.
Bronchial asthma is a complex disorder caused by a combination of genetic and environmental factors (11). Recently, the prevalence of allergic diseases, including bronchial asthma, has dramatically increased. The ‘hygiene hypothesis’ suggests that an important environmental factor might be recent improvements in hygiene, including reducing exposure to endotoxins (12, 13). Much attention has been paid to the protective role of such exposure, based on epidemiological studies showing that the level of exposure to endotoxin is inversely correlated with allergen sensitization (14–16) or occurrence of allergic diseases (14), although the results of several studies are controversial (17–19).

A number of studies aimed directly at examining the effects of LPS administration on asthma model animals have been performed; however, the results are conflicting (20–26). The difference of the in vivo effects of LPS may be attributed to the differences among the studies in the moieties or doses of LPS or the time or the route of its administration (27). Several mechanisms by which LPS might inhibit the onset of bronchial asthma have been proposed. Some reports demonstrate that TLR4 skewing in the TLR4/MD-2 complex induced by LPS leads to the inhibition of the onset of bronchial asthma (20, 26), although it has also been reported that the protective effects of LPS were not accompanied by increased IFN-γ production nor were they dependent on the existence of this cytokine (22, 28).

Some studies demonstrate that LPS or a killed Mycobacterium vaccae suspension induces regulatory T cells such as IL-10-expressing cells or CD4+CD125lo cells producing IL-10 and transforming growth factor (TGF)-β, which could inhibit allergic responses (29, 30). However, to the best of our knowledge, there is no report that proposes that ET constitutes a protective mechanism of LPS on airway allergic inflammation.

We previously established a monoclonal agonistic antibody against TLR4/MD-2 complex, named UT12. We found that this antibody mimics the biological activities of LPS both in vitro and in vivo, including activation of nuclear factor-kappa B, production of tumor necrosis factor (TNF)-α and IL-6 and induction of endotoxin shock (31). UT12 has several advantages over LPS, such as a longer half-life (7 days versus <8 min) in vivo and direct actions on the TLR4/MD-2 complex independent of LPS-binding protein (LBP) and CD14 (31). Furthermore, macrophages or DCs inactivate internalized LPS by removing fatty acyl chains by acylloxyacyl hydrolase, whereas UT12 continues to transduce the signals via TLR4 without being internalized into the cells (32). Consequently, UT12 induces more potent and sustained ET than does LPS (31).

In this study, we took advantage of UT12 to generate prolonged ET to explore the possibility that the presence of ET is involved in the inhibitory effects of TLR4 signals on asthma model mice. Administration of UT12 turned DCs non-functional in T-cell expansion in vivo, and thus, the mice showed impaired expansion of T12 and T17 cells. Accordingly, these mice showed alleviation of airway allergic inflammation. Taken together, these results demonstrate that in asthma model mice, ET induced by activation of TLR4 signals inhibits allergic inflammation through direct suppression of the T-cell stimulatory effect of DCs.

**Methods**

**Animals**

BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). Female mice 6–8 weeks old were used in all experiments. Experiments were undertaken following the guidelines for care and use of experimental animals of the Japanese Association for Laboratory Animals Science (1987).

**Antibodies**

An agonistic mAb against mouse TLR4, UT12, was prepared as described before (31). The subclass of UT12 is IgG3; therefore, mouse IgG3 (Y5606; Sigma–Aldrich, St Louis, MO, USA) was used as the control antibody.

**Assessment of the state of ET by LPS challenge**

As outlined in Fig. 1(A), for antigen presentation, 50 μg ovalbumin (OVA; Sigma–Aldrich) and 1 mg alum (LSL, Tokyo, Japan) in 500 μl PBS were intra-peritoneally injected on day 0. On day 12, 0.5 μg LPS (Sigma–Aldrich) along with 25 mg d-galactosamine hydrochloride (d-GalN; Sigma–Aldrich) in 200 μl PBS were intra-peritoneally injected for lethal LPS challenge. One or 24 h prior to the injections of OVA/alum and LPS/d-GalN, 5 μg of either UT12 or control antibody, or 1 μg of LPS, was intra-peritoneally injected. Two hours after LPS challenge, small volumes of blood (<100 μl) were

Fig. 1. UT12-induced ET in model mice. (A) The experimental protocol is depicted. Control (ctrl) antibody, UT12 or LPS was administered to mice (n = 5 per group) 1 or 24 h before OVA sensitization (day 0) and lethal LPS challenge (day 12). (B) Serum TNF-α concentrations in mice pre-treated with control antibody (open bars), UT12 (solid bars) or LPS (gray bars) and challenged with the lethal dose of LPS 1 h (left panel) or 24 h (right panel) before OVA sensitization. One representative experiment of two is shown. **P < 0.01; NS, not significant.
collected from tails, and sera were subjected to TNF-α measurement by ELISA.

**ELISA**

The serum level of OVA-specific IgE was measured by DS MOUSE IgE ELISA (OVA) (DS Pharma Biomedical, Suita, Japan). TNF-α was measured by Mouse TNF-α ELISA Ready-SET-Go! (eBiosciences, San Diego, CA, USA).

**Assessment of antigen uptake**

FITC-conjugated OVA (FITC-OVA) was prepared by mixing OVA with FITC (Sigma–Aldrich), followed by gel filtration chromatography using Sephadex G-50 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Mice were intra-peritoneally injected with 50 μg OVA and alum on day 0 and subsequently with either 2 mg OVA or FITC-OVA and alum on day 12 as outlined in Fig. 2(A). Five micrograms of either UT12 or control antibody was i.p. injected on day −1 and day 11. Three hours after injection of FITC-OVA, spleens were subjected to flow cytometry.

**Flow cytometry**

Flow cytometry was performed by FACSCalibur (BD Biosciences, San Jose, CA, USA). For flow cytometry of splenic DCs, after FcR blocking using anti-CD16/32 antibody, the cells were stained by PE-labeled anti-MHC class II (M5/114.15.2; Miltenyi Biotec, Bergisch Gladbach, Germany), allophycocyanin anti-CD11c antibody, biotinylated anti-CD40 antibody (1C10; eBioscience) or biotinylated anti-CD86 antibody (GL1; eBioscience), followed by PerCP-Cy5.5-conjugated streptavidin (BD Biosciences). Foxp3 staining in the purified CD4+ cells was performed using Allophycocyanin anti-Mouse/Rat Foxp3 Staining Set (eBioscience).

**OVA sensitization, ex vivo challenge and in vivo airway challenge**

Mice were sensitized by intra-peritoneal injections of 50 μg OVA and 1 mg alum on days 0 and 12. Five micrograms of either UT12 or control antibody or 1 μg of LPS was injected intra-peritoneally 1 or 24 h before the sensitization.

For ex vivo challenge, the mice were killed on day 15, spleens were removed and then splenocytes or CD4+ cells were incubated in the presence of bone marrow-derived dendritic cells (BMDCs) were incubated with OVA. For challenge of splenocytes, 1 × 10^7 cells were incubated in 5 ml RPMI1640 containing 10% FCS (Invitrogen, Carlsbad, CA, USA) in a 60-mm dish (Thermo Fisher Scientific, Waltham, MA, USA) in the presence of 50 μg ml−1 OVA for 48 h and then CD4+ cells

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Fig. 2. ET induced by UT12 down-regulates expression of co-stimulatory molecules on splenic DCs but not OVA uptake. (A) The experimental protocol is shown. (B) Mice (n = 3 per group) pre-treated with control (ctrl) antibody or UT12 were sensitized with OVA (first sensitization) and with OVA or FITC-OVA (second sensitization). Fluorescent signals of FITC-OVA are depicted in the CD11chigh fraction of splenocytes in control antibody- (left panel) or UT12-administered (right panel) mice. Gray and open areas represent OVA expression in FITC-OVA- and OVA-administered mice, respectively. One representative experiment of three is shown. (C) Mice (n = 3 per group) pre-treated with control antibody or UT12 were sensitized with OVA. Expression of MHC class II (left panel), CD86 (middle panel) and CD40 (right panel) on the CD11chigh fraction of splenocytes is depicted. Gray and open areas represent expression in UT12- and control-antibody-treated mice, respectively. One representative experiment of three is shown.
were purified. After FcR blocking using anti-CD16/32 antibody (Miltenyi Biotec), CD4\(^+\) cells were purified by negative selection using CD4 T-cell isolation kit (Miltenyi Biotec), followed by positive selection using PE-labeled anti-CD4 antibody (GK1.5; eBioscience)-bound anti-PE microbeads (Miltenyi Biotec). The purity was >92%. Splenic DCs were enriched by positive selection using CD11c microbeads (Miltenyi Biotec), followed by fluorescence-activated cell sorting using FACS Aria (BD Biosciences). The purity was >95%. For challenge of CD4\(^+\) T cells, 5 × 10\(^6\) CD4\(^+\) T cells and 1 × 10\(^5\) splenic DCs were incubated in 5 ml RPMI1640 containing 10% FCS in a 60-mm dish (Thermo Fisher Scientific) or 1 × 10\(^6\) CD4\(^+\) T cells and 1 × 10\(^5\) splenic DCs were incubated in 1 ml RPMI1640 containing 10% FCS in a well of a 24-well plate (Thermo Fisher Scientific), in the presence of 50 μg ml\(^{-1}\) OVA for 48 h.

For in vivo airway challenge, mice inhaled 0.1% OVA in saline for 1 h on days 22, 26 and 30, were assessed for airway hyperresponsiveness (AHR) on day 31 and were analyzed by bronchoalveolar lavage (BAL) on day 32. They were then killed for blood collection and lung histology as outlined in Fig. 6(A).

Preparation of BMDCs
Bone marrow cells were removed from femurs and tibias of BALB/c mice, and 1 × 10\(^7\) cells suspended in RPMI1640 containing 10% FCS were cultured with 20 mg ml\(^{-1}\) of granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA) for 1 week. DCs were purified by positive selection using PE-labeled anti-CD11c antibody (N418; eBioscience) and anti-PE microbeads.

Reverse transcription–PCR
Total RNA was extracted by TRizol Reagent (Invitrogen). Reverse transcription (RT) reaction primed with random hexamer was performed using QuantiTect Rev. Transcription Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR analysis was performed as previously described (33). Primer sequences and PCR conditions are available upon request.

Assessment of AHR
Airway responsiveness to methacholine was assessed with a four-chamber whole-body plethysmograph from Buxco Electronics (Wilmington, NC, USA) as previously described (33).

Analysis of BAL
After mice were anesthetized, lungs were lavaged with 500 μl of BAL liquid (0.1% BSA/50 μM EDTA/PBS), and bronchoalveolar lavage fluid (BALF) was collected. This procedure was repeated three times. BALF cell counts were determined with a hemocytometer (CDA500; Sysmex, Kobe, Japan). BALF cytospins were prepared, slides were stained with Diff-Quik (Sysmex) and then the numbers of eosinophils, neutrophils and mononuclear cells were counted.

Lung histology
Paraffin-embedded lung sections were prepared and stained with hematoxylin and eosin and periodic acid-Schiff.
for IgE and IgG1 induced by IL-4, also decreased in cultured splenocytes (Fig. 3C). On the other hand, the expression level of IFN-γ, the signature cytokine of Th1 responses, was induced only slightly in this system, excluding the possibility that UT12 induces Th1,1 skewing in the Th1/Th2 dichotomy. These results demonstrate that ET induced by UT12 down-regulates both Th1 and Th17 responses by allergen sensitization.

**Impaired expansion of allergen-specific T cells by UT12**

Next, we investigated whether down-regulation of Th1 responses in splenic CD4+ T cells in ET mice induced by UT12 is due to impaired expansion of OVA-specific CD4+ T cells. To do so, we incubated CD4+ T cells purified from splenocytes with BMDCs in vitro instead of splenic antigen-presenting cells in the presence of OVA, as outlined in Fig. 4(A). CD4+ T cells cultured in this condition expressed high amounts of IL-4, IL-13 and IL-17A. However, CD4+ T cells derived from the mice pre-treated with UT12 in vivo significantly decreased the expression of these cytokines, particularly IL-4 and IL-13, as well as in the total splenocyte culture (Fig. 4B).

To investigate whether UT12-treated DCs have a potency to impair T-cell expansion directly, we incubated splenic CD4+ T cells with splenic DCs in the presence of OVA and then analyzed expression of IL-4 and IL-13. Splenic CD4+ T cells were all from OVA-sensitized mice treated with control antibody and splenic DCs were derived from OVA-sensitized mice treated with either control antibody or UT12. Splenic CD4+ T cells cultured with splenic DCs from UT12-treated mice showed significant down-regulated expression of IL-4 and IL-13 (Fig. 4C). Thus, expansion of allergen-specific CD4+ T cells by splenic DCs is impaired by in vivo treatment of DCs with UT12. These results strongly support that ET induced by UT12 impairs the expansion of allergen-specific CD4+ T cells in vivo by inhibiting the function of splenic DCs for T-cell activation, leading to down-regulation of Th1 responses in these cells.

No augmented induction of inhibitory cytokines or expansion of regulatory T cells in ET mice induced by UT12

It is known that regulatory T cells play important roles in regulating the onset of bronchial asthma (34, 35). Accordingly, it was reported that LPS and *M. vaccae*, thought to activate TLR signals, inhibited asthmatic phenotypes in model mice by inducing IL-10 and TGF-β (29, 30). Although it was strongly suggested that UT12 impairs expansion of allergen-specific T cells, we could not exclude the possibility that UT12 induces inhibition of T-cell responses, for example, by inducing inhibitory cytokines or expanding regulatory T cells. Therefore, we investigated whether administration of UT12 induces expression of IL-10 and/or TGF-β to inhibit Th1 responses in splenic T cells. *Ex vivo* OVA stimulation, represented in Fig. 3(A), induced significant expression of IL-10 in CD4+ T cells. Injection of UT12 had a tendency to down-regulate expression of IL-10 as well as of IL-4, IL-13 and IL-17A (Fig. 3B). The expression level of TGF-β was very low in these experiments, and UT12 did not enhance it, even in splenocytes (data not shown).

We then analyzed the effects of UT12 on the ratio of the population of TReg, defined as Foxp3+ cells in spleen, as outlined in Fig. 5(A). The Foxp3+ cell population in spleen CD4+ T cells was about 14–18%, comparable with a previous report (34), and this was not affected by *ex vivo* OVA challenge or treatment with UT12 (Fig. 5B). We also observed that the expression level of CTLA-4, which is constitutively
expressed in Foxp3+ T<sub>reg</sub> cells and plays an important role for the suppressive activity of T<sub>reg</sub> cells (36), did not change in CD4<sup>+</sup> T cells in spleen (data not shown). These results strongly support that the blockage of T<sub>H</sub>2 responses by administration of UT12 is not due to induction of inhibitory cytokines or expansion of inducible regulatory T cells or T<sub>reg</sub> in
spleen but due to impaired expansion of allergen-specific Th2 cells.

**Inhibition of airway allergic inflammation by administration of UT12 in allergen-challenged mice**

We then examined whether UT12-induced ET impairs the capacity of allergen-specific Th2 and Th17 cells in DCs to expand, resulting in attenuating airway allergic inflammation by allergen challenge. We injected UT12 intra-peritoneally 24 h before OVA sensitization followed by inhalation of OVA on days 22, 26 and 30 (Fig. 6A). OVA inhalation caused enhanced AHR and eosinophil-dominant inflammation in airways and tissues, decreased bronchial spaces and generation of goblet cells and elevated serum levels of OVA-specific IgE, whereas UT12 greatly diminished all these phenotypes (Fig. 6B–E). We confirmed that the inhibitory effects of UT12 on airway allergic inflammation were observed from 1 to 48 h before the sensitization but not 6 days before the sensitization and the inhibitory effects were stronger in 24 h than 1 h before the sensitization, consistent with the ET-inducible effects (Figs 1B and 6B and C; data not shown). When we administered up to 1 μg of LPS, AHR and eosinophil-dominant infiltration in BALF were decreased when administered 1 h before the sensitization but not 24 or 48 h before the sensitization (Fig. 6B and C; data not shown), indicating that the inhibitory effects of UT12 on airway allergic inflammation were more potent and sustained than LPS in parallel with induction of ET. Taken together, our results indicate that ET induced by UT12 alleviates airway allergic inflammation by allergen challenge in model mice.

**Discussion**

Although many studies have been carried out to examine the effects of LPS on asthma model animals, the results are conflicting (20–26). This inconsistency may be caused at least partially by the difficulties of handling of LPS—the short half-life of LPS in vivo (<8 min) and the complicated mechanism of transfer and recruitment of LPS to TLR4 via LBP and CD14 in vivo—as well as different LPS moieties among the LPS.
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studies. Furthermore, the duration of the LPS-mediated signal is transient because LPS is immediately inactivated by cellular acylxoyacyl hydrolase upon its entry into the target cells (32). To overcome these problems, in this study, we took advantage of UT12, an agonistic antibody against the TLR4/MD-2 complex. Pre-treatment with UT12 induced more potent and sustained ET than that of LPS, as we expected (Fig. 1). In accordance with this result, the inhibitory effect of UT12 on airway allergic inflammation was more significant and much more prolonged than LPS (Fig. 6). Thus, application of UT12 instead of LPS enabled us to analyze the pure effects of activated TLR4 signals on allergen sensitization or the onset of airway allergic inflammation, with the effects more stable and exaggerated than LPS.

Several mechanisms—TNF-α skewing in the Th1/Th2 dichotomy, induction of regulatory T cells producing IL-10 and TGF-β and T-cell anergy induced by IL-10 or via activation of CD137—have been reported to explain how LPS shows beneficial effects on asthma model animals (20, 26, 29, 30, 37, 38). We propose for the first time that the presence of ET leading to the impairment of antigen presentation in DCs can explain how the TLR4 signals inhibit allergen sensitization or the onset of bronchial asthma. ET has been demonstrated to impair antigen presentation in monocytes, possibly correlated with the pathogenesis of endotoxin shock (8), cystic fibrosis (7, 9) and acute coronary syndrome (10). It is reasonable to assume that impairment of antigen presentation by ET down-regulates the capacity of DCs to expand all T cells including allergen-specific Th2 and Th17 cells.

It is intriguing to apply our present finding, that ET inhibits allergen sensitization or the onset of bronchial asthma, to humans. Various epidemiological studies suggest that exposure to endotoxins is inversely correlated with allergen sensitization (14–16) or the occurrence of bronchial asthma (14). However, it has been obscure how the level of exposure to endotoxins affects the host immune responses and whether they are correlated with allergen sensitization or occurrence of bronchial asthma. Our present study suggests that down-regulated expression of MHC class II and co-stimulatory molecules including CD86 and CD40 on DCs and circulating monocytes may be good biomarkers reflecting the presence of ET in vivo. It has been reported that monocyte-derived DCs from asthma patients show higher CD86 expression than healthy donors (39) and that serum levels of soluble CD86 are up-regulated in asthma patients compared with healthy donors (40, 41). These reports may support an inverse relationship between the degree of ET and the susceptibility to bronchial asthma. Weak but persistent exposure to LPS may evoke a chronic state of ET, protecting against allergic diseases. Further studies are needed to clarify this point.

Because the presence of ET leads to impairment of antigen presentation, altering host immunity, it is highly possible that ET is correlated with various pathophysiological conditions other than bronchial asthma or allergic diseases. It is known that Th1- or Th17-type inflammatory diseases—type I diabetes mellitus, multiple sclerosis and Crohn’s disease—have also been on a dramatic increase in recent decades (42). Thus far, there is no evidence showing that ET is involved with these diseases; however, it is of great interest to explore this possibility.

Because of the inhibitory effects on airway allergic inflammation, the present study indicates the usefulness of the applying an agonistic antibody against human TLR4 as a therapeutic agent against bronchial asthma and, possibly, other diseases that may be associated with ET. UT12 has several advantages compared with other LPS-mimicking reagents because of its resistance against restoration from ET and a longer half-life than LPS. Thus far, there has been no trial to improve or prevent bronchial asthma using TLR4 agonists. Based on the findings that oligodeoxynucleotides containing unmethylated cytosine–guanine (CpG ODNs), an agonist for TLR9, improve allergic phenotypes in animal models, several human studies using CpG ODNs are underway, showing some beneficial effects (43). Our present study extends our understanding of how TLR4 signals inhibit the onset of bronchial asthma and suggests the therapeutic potential of an agonistic antibody against human TLR4 for bronchial asthma.

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