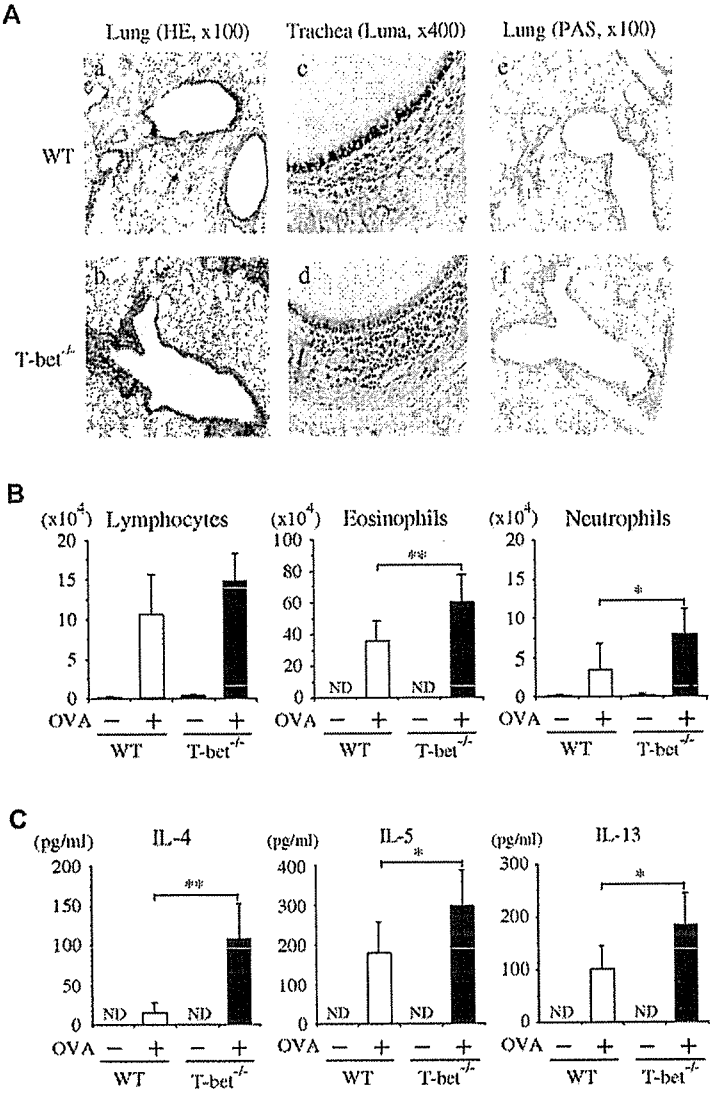

FIG 2. Antigen-induced eosinophil and neutrophil recruitment into the airways is enhanced in T-bet$^{-/-}$ mice. **A-C,** OVA-sensitized T-bet$^{-/-}$ mice and WT mice on a BALB/c background were challenged with the inhalation of OVA. *A,* At 36 hours after the OVA inhalation, lung and trachea were removed and sections were stained with HE, PAS, or Luna (n = 5). The number of lymphocytes, eosinophils, and neutrophils *(B)* and the levels of IL-4, IL-5, and IL-13 *(C)* in the BALF were evaluated at 36 hours after the OVA inhalation. Data are means ± SDs (n = 8 for *B* and 5 for *C*, respectively). *$P < .05$; **$P < .01$. *ND,* Not detectable.

significantly affected by the absence of STAT6 (Fig 3, *A*), suggesting that the mechanism underlying the neutrophil recruitment enhanced by the absence of T-bet is different from that underlying the enhanced eosinophil recruitment.

Differentiation of IL-13–producing CD4$^+$ T cells is increased in T-bet$^{-/-}$ CD4$^+$ T cells

To address the role of T-bet in antigen-induced T_H cell differentiation, antigen-induced T_H cell differentiation in DO11.10$^+$T-bet$^{-/-}$CD4$^+$ T cells was compared with that in DO11.10$^+$CD4$^+$ T cells. As shown in Fig 4, *A*, when splenocytes from DO11.10$^+$T-bet$^{-/-}$ mice were stimulated with antigenic peptide (OVA323-339) in nonpolarizing T_H0 condition, T_H1 cells (IL-4$^-$IFN-γ^+ cells) were decreased but T_H2 cells (IL-4$^+$IFN-γ^- cells) were increased compared with those in DO11.10$^+$ mice (n = 5 mice in each group). T_H1 cell differentiation was still significantly reduced in DO11.10$^+$ T-bet$^{-/-}$CD4$^+$ T cells even when IL-12 was added to induce T_H1 cell differentiation (T_H1 condition; n = 5; Fig 4, *A*). On the other hand, in T_H2 polarizing condition (in the presence of IL-4 and anti-IFN-γ antibody), the number of T_H2 cells (IL-4$^+$ IFN-γ^- cells) was similar between DO11.10$^+$T-bet$^{-/-}$ CD4$^+$ T cells and DO11.10$^+$CD4$^+$ T cells (Fig 4, *A*).

We also investigated IL-13 production in DO11.10$^+$ T-bet$^{-/-}$ CD4$^+$ T cells at single cell levels. Interestingly, in nonpolarizing T_H0 condition, CD4$^+$ T cells that produce IL-13 but not IL-4 were significantly

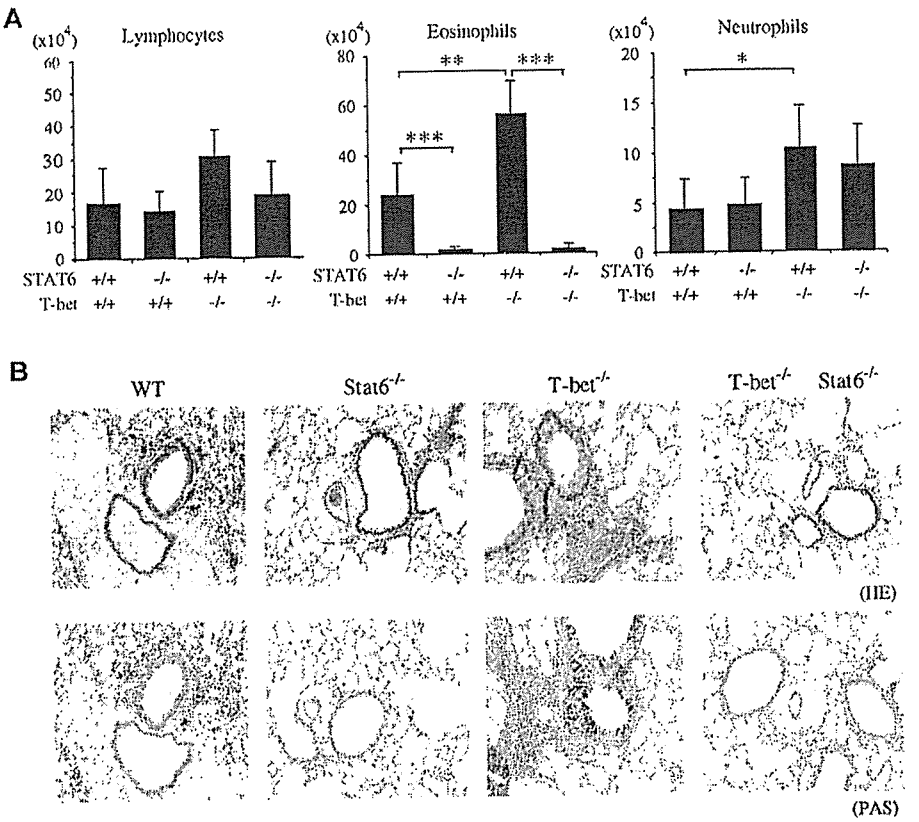

FIG 3. The enhanced antigen-induced eosinophil recruitment but not neutrophil recruitment into the airways of T-bet$^{-/-}$ mice depends on STAT6. **A and B,** OVA-sensitized T-bet$^{-/-}$ mice, STAT6$^{-/-}$ mice, T-bet$^{-/-}$STAT6$^{-/-}$ mice, and littermate WT mice were challenged with the inhalation of OVA. *A,* The number of lymphocytes, eosinophils, and neutrophils in the BALF was evaluated at 36 hours after the OVA inhalation. Data are means ± SDs for 5 mice in each group. *$P < .05$; **$P < .01$; ***$P < .001$. *B,* Representative photomicrographs of HE staining *(upper panels)* or PAS staining *(bottom panels)* of lung sections are shown (n = 5).

increased in DO11.10^{+}T-bet$^{-/-}$CD4^{+} T cells compared with those in DO11.10^{+}CD4^{+} T cells (Fig 4, *B*). The measurement of IL-13 levels in the supernatant of antigen-stimulated CD4^{+} T cells confirmed the enhanced IL-13 production in DO11.10^{+}T-bet$^{-/-}$CD4^{+} T cells (data not shown). These results suggest that in addition to the T-bet's role in inducing T_H1 cell differentiation, T-bet may be required for the downregulation of IL-13 production during antigen-specific CD4^{+} T-cell differentiation.

T-bet expression in CD4^{+} T cells is vital for the inhibition of antigen-induced eosinophil recruitment into the airways

It has been shown that T-bet is expressed and exhibits its function not only in CD4^{+} T cells but also in CD8^{+} T cells, B cells, and nonlymphoid cells.[17-19] To determine whether the expression of T-bet in CD4^{+} T cells is vital for the inhibition of antigen-induced allergic airway inflammation, we performed the adoptive transfer experiments of T-bet$^{-/-}$CD4^{+} T cells. To eliminate the influence of endogenous immune response during the sensitization and the inhaled antigen challenge as well as the influence of T-bet expression in nonlymphoid cells in recipient mice, we used T-bet$^{-/-}$Rag-2$^{-/-}$ mice as recipient mice. CD4^{+} T cells from DO11.10^{+}T-bet$^{-/-}$ mice or littermate DO11.10^{+} mice were transferred to T-bet$^{-/-}$ Rag-2$^{-/-}$ mice, and these mice were immunized twice with OVA/alum and then challenged with the inhaled OVA. As shown in Fig 5, *A*, when CD4^{+} T cells from DO11.10^{+} mice were transferred to T-bet$^{-/-}$Rag-2$^{-/-}$ mice, eosinophil recruitment into the airways was induced at 36 hours after OVA inhalation. As expected, in the absence of the CD4^{+} T-cell transfer or the OVA inhalation, few eosinophils were detected in the BALF (data not shown), indicating that both the transferred CD4^{+} T cells and the OVA challenge are required for the eosinophil recruitment in this system. When T-bet$^{-/-}$Rag-2$^{-/-}$ mice were transferred with CD4^{+} T cells from DO11.10^{+} T-bet$^{-/-}$ mice, antigen-induced eosinophil recruitment into the airways was significantly increased compared with those transferred with CD4^{+} T cells from DO11.10^{+} mice (n = 5; $P < .005$; Fig 5, *A*). Moreover, IL-13 was increased but IFN-γ was decreased in the BALF in the mice transferred with DO11.10^{+}T-bet$^{-/-}$ CD4^{+} T cells (n = 5; $P < .005$; Fig 5, *B*). These results suggest that the expression of T-bet in CD4^{+} T cells is

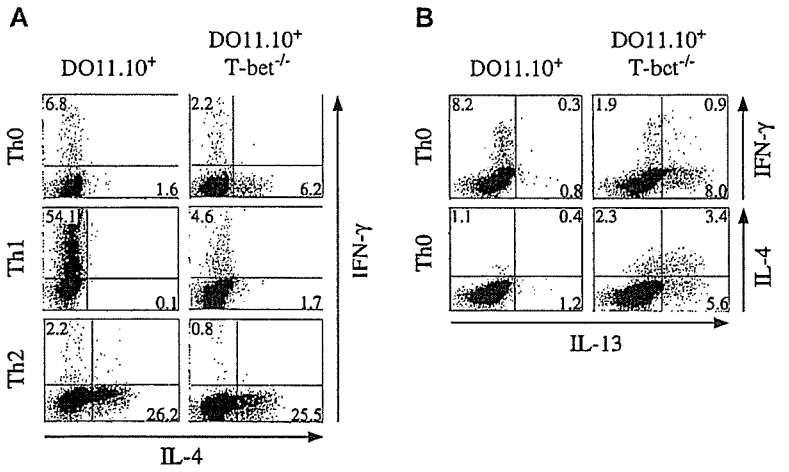

FIG 4. Differentiation of IL-13-producing $CD4^+$ T cells is increased in T-$bet^{-/-}$ mice. A, Splenocytes from $DO11.10^+$ T-$bet^{-/-}$ mice or littermate $DO11.10^+$ mice were stimulated with OVA323-339 peptide in T_H0, T_H1, or T_H2 condition and intracellular cytokine profiles (IL-4 vs IFN-γ) of $CD4^+$ T cells were determined. Shown are representative FACS profiles (n = 5). B, Similar to *A*, intracellular cytokine profiles (IL-13 vs IFN-γ and IL-13 vs IL-4) were determined on $CD4^+$ T cells in T_H0 condition (n = 5).

vital for the inhibition of antigen-induced T_H2 cytokine production and eosinophil recruitment in the airways.

T-bet expression in $CD4^+$ T cells is vital for the downregulation of antigen-induced IL-17 production and neutrophil recruitment in the airways

Interestingly, in addition to antigen-induced eosinophil recruitment, antigen-induced neutrophil recruitment was significantly enhanced in the mice transferred with $DO11.10^+$T-$bet^{-/-}CD4^+$ T cells (Fig 5, *A*). Moreover, the levels of IL-17, a representative cytokine that induces neutrophil-rich inflammation,[20] in the BALF were profoundly increased in mice transferred with $DO11.10^+$ T-$bet^{-/-}CD4^+$ T cells (n = 5; $P < .005$; Fig 5, *B*). To examine whether IL-17 is involved in the enhanced neutrophil recruitment in the mice transferred with $DO11.10^+$T-$bet^{-/-}CD4^+$ T cells, a neutralizing antibody against IL-17 was administered to the mice at 12 hours before the inhaled OVA challenge. As shown in Fig 5, *C*, antigen-induced neutrophil recruitment but not eosinophil recruitment in the airways was significantly inhibited by the administration of anti–IL-17 antibody (n = 4; $P < .05$). These results suggest that the expression of T-bet in $CD4^+$ T cells is involved in the suppression of IL-17 production and subsequent neutrophil recruitment in the airways on antigen inhalation.

T-bet inhibits the differentiation of IL-17–producing $CD4^+$ T cells

It has recently been suggested that IL-17–producing $CD4^+$ T cells (T_H17 cells) are a distinct lineage from T_H1 cells and T_H2 cells,[21] and that IFN-γ and T-bet can repress the differentiation of T_H17 cells.[22-24] Therefore, we finally examined the role of T-bet in the regulation of IL-17–producing $CD4^+$ T cells by using $DO11.10^+CD4^+$ T cells. As shown in Fig 6, when splenocytes from $DO11.10^+$ T-$bet^{-/-}$ mice were stimulated with OVA323-339 in nonpolarizing T_H0 condition, T_H17 cells (IL-17^+IFN-γ^- cells) were consistently increased compared with those in $DO11.10^+$ mice (n = 5 mice in each group). In T_H1 polarizing condition, the number of T_H17 cells in $DO11.10^+$T-$bet^{-/-}CD4^+$ T cells was also significantly higher than that in $DO11.10^+CD4^+$ T cells. These results suggest that T-bet is involved in the inhibition of antigen-induced T_H17 cell differentiation.

DISCUSSION

In the current study, we show that the expression of T-bet in $CD4^+$ T cells is crucial not only for the inhibition of T_H2 cell–mediated eosinophilic inflammation in the airways but also for the inhibition of T_H17 cell–mediated neutrophilic inflammation in the airways. We found that antigen-induced eosinophil recruitment, goblet cell hyperplasia, and T_H2 cytokine production in the airways were enhanced in BALB/c T-$bet^{-/-}$ mice (Fig 2). However, in the absence of STAT6, T-bet deficiency could not induce the antigen-induced eosinophilic airway inflammation (Fig 3). Adoptive transfer of T-$bet^{-/-}$ or T-$bet^{+/+}$ $CD4^+$ T cells to T-$bet^{-/-}$Rag-$2^{-/-}$ mice revealed that the expression of T-bet in $CD4^+$ T cells was vital for the inhibition of antigen-induced eosinophil recruitment into the airways (Fig 5). Interestingly, we also found that antigen-induced, IL-17–mediated neutrophil recruitment into the airways was enhanced in T-$bet^{-/-}$ mice (Figs 2, 3, and 5) and that T-$bet^{-/-}CD4^+$ T cells preferentially differentiated into T_H17 cells that mediated neutrophilic inflammation in the airways (Figs 5 and 6). Taken together, these results indicate that the expression of T-bet in $CD4^+$ T cells is crucial for the inhibition of the differentiation of both T_H2 cells and T_H17 cells and thereby for the downregulation of T_H2 cell–mediated eosinophilic

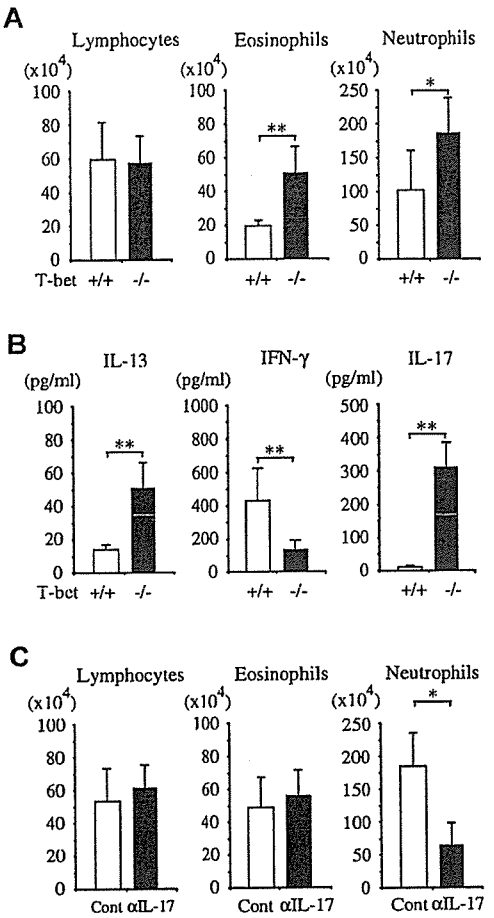

FIG 5. Expression of T-bet in $CD4^+$ T cells is vital in the inhibition of antigen-induced eosinophil and neutrophil recruitment into the airways. **A and B,** Purified $CD4^+$ T cells from $DO11.10^+$ $T\text{-}bet^{-/-}$ mice or $DO11.10^+$ mice were transferred intravenously to $Rag\text{-}2^{-/-}$ $T\text{-}bet^{-/-}$ mice. These mice were immunized with OVA/alum and then challenged with the inhaled OVA. Thirty-six hours later, the number of lymphocytes, eosinophils, and neutrophils *(A)* and the levels of IL-13, IL-17, and IFN-γ *(B)* in the BALF were evaluated. Data are means ± SDs (n = 5). $*P < .05$; $**P < .005$. IL-4 and IL-5 were not detected in both groups. **C,** Similar to *A*, $DO11.10^+$ $T\text{-}bet^{-/-}CD4^+$ T cells were transferred to $Rag\text{-}2^{-/-}T\text{-}bet^{-/-}$ mice, and these mice were immunized with OVA/alum. At 12 hours before the inhaled OVA challenge, these mice were injected intraperitoneally with antimurine IL-17 antibody or control rat IgG_{2a}. The number of lymphocytes, eosinophils, and neutrophils in the BALF was evaluated at 36 hours after the OVA inhalation. Data are means ± SDs (n = 4). $*P < .05$. *Cont*, Control.

inflammation and T_H17 cell–mediated neutrophilic inflammation in the airways.

We demonstrate that T-bet inhibits antigen-induced T_H2 cell–mediated allergic airway inflammation, as evidenced by enhanced antigen-induced eosinophilic airway inflammation in BALB/c $T\text{-}bet^{-/-}$ mice (Figs 2 and 5). It has been shown that allergic eosinophilic airway inflammation is mediated by T_H2 cells and also that the T_H1/T_H2 cell balance is important for regulating allergic airway inflammation.[1,2,25] STAT6 is clearly a key transcription factor that induces antigen-induced eosinophilic airway

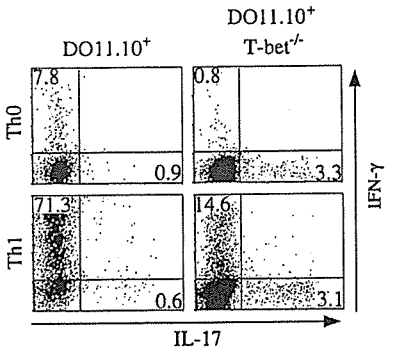

FIG 6. Differentiation of IL-17–producing $CD4^+$ T cells is increased in $T\text{-}bet^{-/-}CD4^+$ T cells. Splenocytes from $DO11.10^+T\text{-}bet^{-/-}$ mice or $DO11.10^+$ mice were stimulated with OVA323-339 peptide in T_H0 or T_H1 polarizing condition. Intracellular cytokine profiles (IL-17 vs IFN-γ) were determined on $CD4^+$ T cells. Shown are representative FACS profiles (n = 5).

inflammation[1,2,25] (Fig 3). T-bet is a T_H1-specific transcription factor that controls T_H1 cell differentiation through the transcriptional activation of the IFN-γ gene and upregulation of IL-12Rβ2 chain expression.[6,7,26] Moreover, T-bet represses T_H2 cytokine genes independently of IFN-γR signaling.[6] Thus, our results obtained by generating $T\text{-}bet^{-/-}STAT6^{-/-}$ mice indicate that Stat6 is essential for T_H2 cell–mediated allergic airway inflammation and dominates over T-bet–mediated suppression of antigen-induced allergic airway inflammation (Fig 3).

We also show that in addition to conventional T_H2 cells, $CD4^+$ T cells that produce IL-13 but not IL-4 are increased by the absence of T-bet (Fig 4). By using $DO11.10^+$ T-cell receptor transgenic system, we found that the frequency of $CD4^+$ T cells that produce IL-13 but not IL-4 was significantly increased in $DO11.10^+$ $T\text{-}bet^{-/-}CD4^+$ T cells compared with that in $DO11.10^+$ $CD4^+$ T cells (Fig 4). Recently, a substantial body of evidence points to a nonredundant role for IL-13 in the regulation of allergic airway inflammation and goblet cell hyperplasia.[25] More recently, it has been demonstrated that neutralization of IL-13 but not IL-4 results in the suppression of spontaneous eosinophilic airway inflammation in C57BL/6 $T\text{-}bet^{-/-}$ mice.[27] Further studies uncovering the mechanism by which T-bet suppresses IL-4 and IL-13 differently may provide a possible target for bronchial asthma.

We unexpectedly found that BALB/c $T\text{-}bet^{-/-}$ mice did not develop airway inflammation spontaneously (Fig 1), whereas we confirmed that C57BL/6 $T\text{-}bet^{-/-}$ mice, which were bred in the same conditions as BALB/c $T\text{-}bet^{-/-}$ mice, spontaneously developed airway inflammation with intense eosinophil and lymphocyte infiltrates[8] (Fig 1). Therefore, the difference in the environmental factors could not account for the absence of spontaneous airway inflammation in BALB/c $T\text{-}bet^{-/-}$ mice. BALB/c mice have been reported to exhibit predominantly T_H2 responses to exogenous antigens, whereas C57BL/6 mice exhibit T_H1 dominant responses to the same

stimuli.[28] Indeed, BALB/c mice exhibit significantly higher levels of antigen-induced eosinophil recruitment in the airways than C57BL/6 mice.[29] In addition, antigen-induced eosinophil recruitment into the airways was high in BALB/b (H-2^b) mice, like BALB/c mice, but low in B10.D2 (H-2^d) mice,[29] suggesting that the BALB background rather than the H-2 haplotype is vital for the enhanced antigen-induced eosinophil recruitment in the airways. Currently, the exact reason why C57BL/6 T-bet$^{-/-}$ mice but not BALB/c T-bet$^{-/-}$ mice develop spontaneous airway inflammation is unknown. We speculate that the inhibitory effect by T-bet on T_H2 cells may be stronger in T_H1-biased C57BL/6 mice than in T_H2-biased BALB/c mice, and thus the absence of T-bet may result in uncontrolled T_H2 cell activation even by the environmental antigens to induce spontaneous allergic airway inflammation in C57BL/6 T-bet$^{-/-}$ mice.

Second, we clearly show that T-bet regulates antigen-induced neutrophilic inflammation in the airways by inhibiting T_H17 cell development. We showed that antigen-induced neutrophil infiltration in the airways was significantly enhanced in T-bet$^{-/-}$ mice compared with that in WT mice (Figs 2 and 3). The neutrophil response was STAT6 independent as shown by the results of T-bet$^{-/-}$ STAT6$^{-/-}$ mice (Fig 3). We also showed that T-bet$^{-/-}$ CD4$^+$ T cells mediated the enhanced antigen-induced neutrophil infiltration and IL-17 production in the airways, and the enhanced response was canceled by anti–IL-17 antibody treatment (Fig 5). Moreover, the *in vitro* study revealed that antigen-stimulated T-bet$^{-/-}$CD4$^+$ T cells preferentially differentiated into IL-17–producing cells (Fig 6). Thus, T-bet potently inhibits not only T_H2 cell–mediated eosinophilic inflammation in the airways but also T_H17 cell–mediated neutrophilic inflammation in the airways.

Our results indicate that T-bet, but not Stat6, is an important regulator for T_H17 cell–mediated neutrophilic inflammation *in vivo*. It has been shown that T_H17 cell development is potently inhibited by a T_H1 cytokine, IFN-γ, and a T_H2 cytokine, IL-4.[22,23] Indeed, the blockade of IFN-γ and IL-4 is required for the development of a substantial number of T_H17 cells.[22,23] It has also been shown that the downstream signaling of STAT1/T-bet and STAT6, respectively, can inhibit IL-23–induced T_H17 cell differentiation from naive CD4$^+$ T cells.[22] However, we found that STAT6 had no significant inhibitory effect on antigen-induced neutrophil infiltration in the airways in the presence or absence of T-bet (Fig 3). This is consistent with the findings that in STAT6-deficient T cells, there was no difference in IL-17 production compared with that of WT T cells.[23] Regarding the mechanisms for T-bet–mediated inhibition of T_H17 cell development, it has been suggested that IFN-γ inhibits T_H17 cell differentiation by downregulating the IL-23 receptor expression.[22] IFN-γ has also previously been shown to inhibit TGF-β signaling, which is critical for T_H17 cell development,[21] by a STAT1-induced synthesis of the inhibitory Smad7.[30] Taken together, it is suggested that in addition to the inhibition of T_H2 cell differentiation in allergic inflammation, the inhibition of T_H17 cell development may be one of nonredundant functions of T-bet to prevent the development of neutrophilic inflammation as well as autoimmune pathology.[21]

Regarding the possible involvement of IL-17 in asthma, the ability of IL-17 to evoke migration of neutrophils[20] but not of eosinophils makes it likely that this cytokine is involved in the pathogenesis of severe asthma, in which accumulation of neutrophils in the airways is a hallmark of disease.[3] Indeed, it has been shown that IL-17 is expressed in the airways of patients with asthma[31] and that its expression is increased in patients with moderate-to-severe asthma compared with patients with mild asthma and normal controls.[32] In addition, we have shown here that T-bet is crucial for the suppression of antigen-induced IL-17 production and subsequent neutrophil recruitment in the airways (Fig 5). Taken together, it is suggested that the dysfunction of T-bet may be involved in the pathogenesis of severe asthma.

In conclusion, from the analysis of T-bet$^{-/-}$ mice, we have established 2 important points. First, the expression of T-bet in CD4$^+$ T cells is vital for the inhibition of antigen-induced T_H2 cell differentiation and thereby regulates antigen-induced, T_H2 cytokine-mediated eosinophil recruitment into the airways. Second, T-bet is also crucial for the inhibition of antigen-induced T_H17 cell differentiation and thereby regulates antigen-induced, IL-17–mediated neutrophil recruitment into the airways. These results provide new insights into the pathophysiology of allergic airway inflammation, especially in severe asthma.

We thank Dr L. Glimcher for T-bet–deficient mice, Dr S. Akira and Dr K. Takeda for STAT6-deficient mice, and Dr K. Murphy for DO11.10 mice.

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Prostaglandins & other Lipid Mediators 83 (2007) 42–49

PROSTAGLANDINS & other LIPID MEDIATORS

Quantification of leukotriene B4 glucuronide in human urine

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Received 31 July 2006; received in revised form 26 September 2006; accepted 28 September 2006
Available online 5 December 2006

Abstract

We have developed a method for measuring leukotriene B4 glucuronide, a marker of systemic leukotriene B4 biosynthesis, in human urine. This method involves the separation of two positional isomers of leukotriene B4 glucuronide by high-performance liquid chromatography, followed by hydrolysis with β-glucuronidase and then leukotriene B4 quantification by enzyme immunoassay after purification by high-performance liquid chromatography. One of two positional isomers of leukotriene B4 glucuronide was predominantly present in urine. The concentration of the isomer increased in urine from aspirin-intolerant asthma patients after aspirin challenge. Urinary leukotriene E4 and leukotriene B4 glucuronide concentrations in 13 normal healthy adults were 94.6 pg/mg-creatinine (median) and 22.3 pg/mg-creatinine, respectively. Urinary LTE4 concentration increased during the first 3 h after allergen inhalation in atopic patients. However, allergen-induced bronchoconstriction was not associated with an increased concentration of LTB4 glucuronide in urine. The method enabled us to precisely determine urinary leukotriene B4 glucuronide concentration.

Keywords: Leukotriene E4; Leukotriene B4; Bronchial asthma

1. Introduction

Leukotrienes (LT) are a group of bioactive compounds, which play important roles in immediate hypersensitivity reaction and inflammation. The biosynthesis of LTs is initiated by the oxygenation of arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid, which is subsequently converted to LTA4. This unstable epoxide LTA4 is enzymatically metabolized to LTC4, which is converted in turn to LTD4 and then to LTE4. LTB4, the alternative end product of the 5-lipoxygenase pathway, is produced from LTA4 by LTA4 hydrolase. Cysteinyl-LTs (LTC4, LTD4, and LTE4) are potent constrictors of bronchial smooth muscle and are also potent and specific chemoattractants for eosinophils. LTB4 is a potent chemoattractant for neutrophils and is considered to play a role in various inflammatory responses. There have been many studies showing that cysteinyl-LTs have considerable potential to contribute to the pathophysiological features of asthma. Significance of LTB4 in bronchial asthma is still controversial. As determined from the pharmacological properties of an LTB4 antagonist, LTB4 is considered to have no substantial impact on the pathophysiological features of bronchial asthma [1]. On the other hand, a study using LTB4 receptor knockout mice showed that LTB4 has a substantial impact on the development of Th2-type immunoresponses [2]. As mentioned above, the role of LTB4 in the development of the pathophysiological features of bronchial asthma has not been completely clarified.

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doi:10.1016/j.prostaglandins.2006.09.010

Urine has been found to be a useful biological fluid in monitoring the endogenous release of chemical mediators. If metabolic clearance systems including liver function were not altered in the disease state, urinary metabolite concentration provides an easily performed method to monitor whole-body production of the precursor. The major drawback of using urine is that a reliable metabolite of endogenous production of the mediator needs to be identified and urine analysis cannot provide any information on the cellular origin of mediators. The concentration of LTE4 in human urine is considered a good marker of LTC4 production in the human body. LTB4 itself is not a good marker of LTB4 production because of its rapid metabolism, and the metabolite that can be a good marker of LTB4 production has not been identified until very recently. Karin et al. suggested the possibility of using the concentrations of LTB4 glucuronide and 20-carboxy LTB4 in urine as markers of LTB4 whole-body production [3]. Studying the LTB4 production status in the body of asthmatic patients is useful for clarifying the clinical significance of LTB4. In a previous study, we measured LTB4 glucuronide concentration in urine and found that aspirin-intolerant asthmatic patients subjected to an aspirin tolerance test showed an increase in not only LTE4 concentration but also LTB4 glucuronide concentration in urine [4]. Because LTB4 glucuronide concentration was calculated by subtracting LTB4 concentration in urine after hydrolysis by β-glucuronidase from LTB4 concentration in urine before hydrolysis, no information on the concentration of the isomer of LTB4 glucuronide was obtained in that study. Moreover, relatively large amounts of urine samples are required for this measurement method and errors frequently occur in the evaluation of urine samples containing low concentrations of LTB4 glucuronide. The aim of this study is to develop a method of measuring the concentration of LTB4 glucuronide in human urine.

2. Materials and methods

2.1. Preparation of LTB4 glucuronide

Two isomers of LTB4 glucuronides were synthesized by reacting LTB4 with a mixture of UDP-glucuronosyltransferase cofactors containing uridine 5′-diphosphoglucuronic acid (BD Biosciences, Woburn, MA, USA) in the presence of human liver microsomes (BD Biosciences) according to manufacturer's instruction.

2.2. Aspirin challenge test

Three aspirin-intolerant asthma patients (two males and one female, 37–54 years old) received intravenous aspirin challenge. These three patients were in a clinically stable condition and all medications were stopped for at least 12 h prior to the challenge test. At the time of the study, FEV1.0 exceeded 80% of the predicted value. The challenge test was performed as reported previously [4]. Briefly, urine samples were collected at the beginning of the study. After the intravenous injection of 1 ml of saline, if FEV1.0 did not change by more than 10% from the prechallenge baseline, doubled doses of lysine aspirin (12.5, 25, 50, 100, and 200 mg equivalent of aspirin) were intravenously administered. FEV1.0 was recorded every 10 min after the administration and the time interval between administrations of increasing doses was 30 min. The challenge test was stopped when a positive reaction occurred, which was defined as a decrease in FEV1.0 by 20% or more from the baseline. Urine samples were collected for the measurement of LTB4 glucuronide and LTE4 concentrations during the following periods: the hour immediately prior to the provocation test and 0–3, 3–6, 6–9, and 9–24 h after the onset of bronchoconstriction. Urine samples were collected in polypropylene tubes containing 4-hydroxy-TEMPO and stored at −35 °C until analysis. Permission to conduct the study was obtained from the Sagamihara National Hospital Ethics Committee and all the subjects gave their informed consent.

2.3. Allergen inhalation test of atopic asthmatic patients

Bronchial provocations with cat, mite or house dust allergens were performed on seven patients on nine occasions. The patients were five female and two male, 21–73 years of age (mean, 33.9), with a mean forced expiratory volume in 1 s (FEV1.0) of about 90% of the predicted value. The patients inhaled an allergen solution in a Devilbiss nebulizer operated by air at a flow rate of 5 l/min. The nose was clipped and aerosols were inhaled through a mouthpiece during tidal breathing. After the measurement of baseline FEV1.0, patients inhaled saline for 2 min. When a change in FEV1.0 was not observed, allergen inhalation was performed. Allergen solution was inhaled for

2 min and FEV1.0 was measured 10 min after each inhalation. The starting concentration of the allergen extract for inhalation was determined from the threshold concentration on intradermal skin test. Allergen solutions at increasing concentrations were inhaled until a decrease in FEV1.0 of at least 20% was achieved. All asthma medications were withheld for at least 12 h before the provocation test. Urine samples were collected at the same interval as described above.

2.4. Measurement of urinary LTB4 glucuronide concentration

We used an Empore C18 disk cartridge (3 M, St. Paul, MN, USA) for extracting LTB4 glucuronide and LTB4, because the cartridge enabled the extract of these compounds from urine and the chromatographic effluent into a small volume of methanol with sufficient recovery. Siliconized glass tubes, polypropylene tubes and polypropylene pipettes were used throughout the study. After an aliquot of urine (2 ml) was loaded on an Empore C18 disk cartridge, the cartridge was washed with distilled water and LTB4 glucuronide was eluted with 0.5 ml of methanol. When using authentic LTB4 glucuronide, the rate of recovery of both isomers of LTB4 glucuronide from the Empore C18 disk cartridge was estimated to be more than 90%. The methanol extract was concentrated under a nitrogen stream. The residue was purified by high-performance liquid chromatography (HPLC) and the fraction corresponding to the retention times of both isomers of LTB4 glucuronide were collected. HPLC was performed using a NOVA-PAK C18 column (Waters, Milford, MA, USA) with a solvent mixture of methanol–distilled water–acetic acid (65:35:0.1, v/v/v) containing 0.1% EDTA (pH adjusted to 5.4 with ammonium hydroxide) at a flow rate of 1.0 ml/min at 37 °C. The methanol extract could be injected into the HPLC column every 20 min. After the addition of two volumes of distilled water to the effluent, LTB4 glucuronide was extracted using an Empore C18 disk cartridge. To liberate LTB4, LTB4 glucuronide was incubated with β-glucuronidase (200 U, G7646, Sigma, St. Louis, MO, USA) in 1 ml of 0.1 M phosphate buffer (pH 7.0) at 37 °C for 20 h. The solution was purified by HPLC using the same elution buffer as described above. The fraction corresponding to the retention time of authentic LTB4 was collected and LTB4 was extracted with an Empore C18 disk cartridge. LTB4 concentration was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). The concentrations were expressed as picogram per milligram of creatinine.

2.5. Cross-reactivity of each isomer of LTB4 glucuronide to the antibody used in the enzyme immunoassay

Cross-reactivity of each isomer to the antibody against LTB4 was determined by incubating with LTB4 tracer and LTB4 or each isomers at increasing concentrations.

2.6. Measurement of urinary LTE4 concentration

LTE4 was quantified using an enzyme immunoassay kit (Cayman Chemical) after purification by HPLC as reported previously [5].

2.7. Normal healthy subjects

The controls (6 male and 7 female, 30–55 years of age) were non-atopic healthy hospital staff members. None of them were taking any medication.

2.8. Analysis of data

Data are expressed as median values and range in bracket unless otherwise specified. Differences between groups were evaluated using the Wilcoxon *t*-test. The Kruskal–Wallis H-test, a non-parametric statistical test, was performed for time-course experiments. When the test showed a significant difference, pairwise comparisons were performed using the Mann–Whitney *U*-test with Bonferroni's correction. A value of $p < 0.05$ was considered statistically significant.

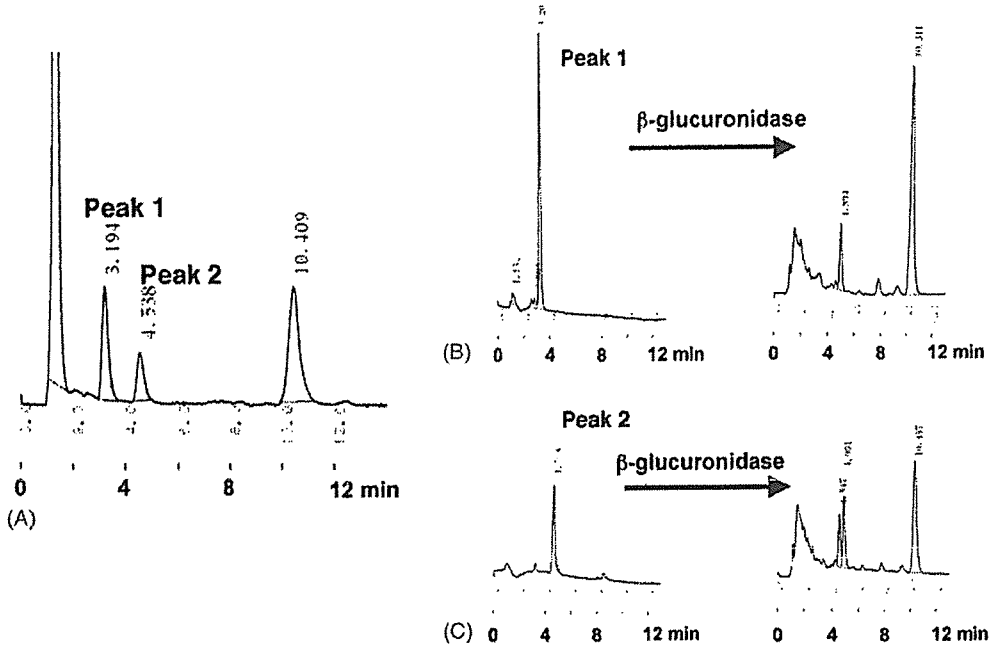

Fig. 1. The reaction mixture of LTB4, uridine 5′-diphosphoglucuronic acid and human liver microsomes was analyzed by HPLC (A). LTB4 was eluted at the retention time of about 10.4 min. LTB4 was generated by the incubation of the fraction corresponding to peak 1 (B) or that to peak 2 (C) with β-glucuronidase.

3. Results

3.1. Preparation of LTB4 glucuronide

After the incubation of LTB4 with uridine 5′-diphosphoglucuronic acid in the presence of human liver microsomes, the reaction mixture was analyzed by HPLC using the same solvent as that for LTB4 purification. Three distinct peaks appeared on the chromatogram (Fig. 1A). The peak of the fraction eluted at the retention time of approximately 10.4 min corresponds to LTB4. When the fractions for the two other peaks, which eluted at shorter retention times (3.2 and 4.5 min), were incubated with β-glucuronidase in 1 ml of 0.1 M phosphate buffer, both fractions generated LTB4, suggesting that these are isomers of LTB4 glucuronide (Fig. 1B and C).

3.2. Time course of hydrolysis of LTB4 glucuronide by β-glucuronidase

To determine the kinetics of the hydrolysis of LTB4 glucuronide, each isomer of LTB4 glucuronide was incubated with β-glucuronidase at 37 °C for various durations. LTB4 glucuronide of peaks 1 and 2 showed a weak cross-reactivity to the antibody against LTB4 in enzyme immunoassay (Fig. 2). The concentration at which each isomer reduced the binding of the LTB4 tracer by 50% was compared with the concentration of LTB4. When assessed on the basis of Fig. 2, the cross-reactivities of the isomers corresponding to peaks 1 and 2 were 0.15% and 1.8%, respectively. Since LTB4 glucuronide might coexist with LTB4 in the solution during a brief incubation, the concentration of LTB4 was measured by enzyme immunoassay after purification by HPLC. Fig. 3 shows that about 20 h was required for the completion of the hydrolysis of both isomers of LTB4 glucuronide.

3.3. Concentration of each isomer of LTB4 glucuronide

To examine the concentration of each isomer of LTB4 glucuronide, each isomer of LTB4 glucuronide was separately collected by HPLC. When the concentration of an isomer of LTB4 glucuronide was determined by enzyme immunoassay after hydrolysis with β-glucuronidase, the concentration of the fraction corresponding to peak 1, which eluted earlier

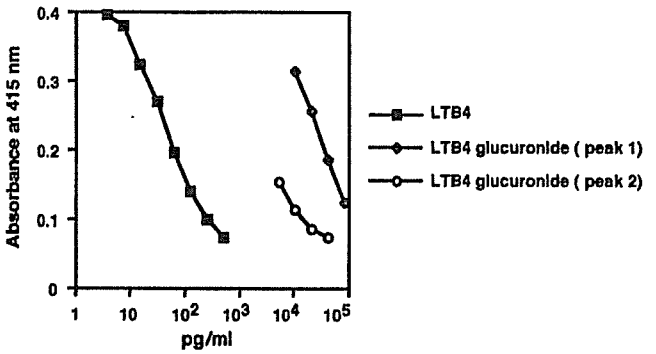

Fig. 2. Cross-reactivity of two isomers of LTB4 glucuronide to antibody against LTB4.

by HPLC, was significantly higher than that corresponding to peak 2 (20.9 pg/mg-creatinine, [4.7–76.7] versus 3.65 [0–9.6], $n = 10, p < 0.01$ analyzed by the Wilcoxon t-test). There was a significant correlation between the concentrations of both isomers ($r = 0.651, p = 0.04$).

3.4. *Quantification of urinary LTB4 glucuronide by enzyme immunoassay in combination with HPLC*

We do not know the precise concentration of LTB4 glucuronide because there has been no information about the molecular extinction coefficient of LTB4 glucuronide. Thus, we examined the recovery rate of the procedure from various aspects as follows. (1) After isolation of LTB4 glucuronide by HPLC, LTB4 glucuronide was incubated with β-glucuronidase. After extraction of LTB4 from the reaction mixture with an Empore C18 disk cartridge, LTB4 was quantified by enzyme immunoassay. Because the concentration of LTB4 was determined to be 2.8 pmol/ml, the concentration of LTB4 glucuronide in the starting solution was assumed to be 2.8 pmol/ml without considering the rate of hydrolysis with β-glucuronidase treatment. Five aliquots of LTB4 glucuronide (2.8 pmol/ml) were processed using all procedures, namely, hydrolysis with β-glucuronidase, LTB4 extraction with an Empore C18 cartridge, LTB4 purification by HPLC, and LTB4 extraction from effluent with an Empore C18 cartridge. The LTB4 concentration was determined to be 2.11 ± 0.31 pmol/ml by enzyme immunoassay, suggesting that the total recovery rate was $75.4 \pm 11.0\%$ (mean ± S.D., $n = 5$). (2) When LTB4 (600 pg) was injected into the HPLC column and the effluent containing LTB4 was extracted with an Empore C18 cartridge, the recovery rate was $90.7 \pm 4.2\%$ ($n = 5$), suggesting that LTB4 is recovered in high yield. (3) When four aliquots of an isomer (peak 1) of LTB4 glucuronide were hydrolyzed with β-glucuronidase, the concentration of LTB4 was 142 ± 9.93 ng/ml. The coefficient of variation was 7%, suggesting that LTB4 glucuronide can be reproducibly converted to LTB4 under our experimental conditions. Nevertheless, beside the recovery rate described above, LTB4 glucuronide concentration may be slightly underestimated because we do not know whether LTB4 glucuronide was fully converted to LTB4 by incubation with β-glucuronidase.

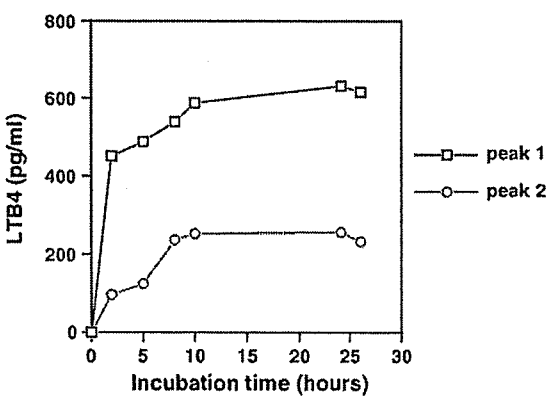

Fig. 3. Kinetics of hydrolysis of LTB4 glucuronide by incubation with β-glucuronidase; peaks 1 and 2 correspond to the fractions eluted at the retention times of 3.2 and 4.5 min shown in Fig. 1, respectively.

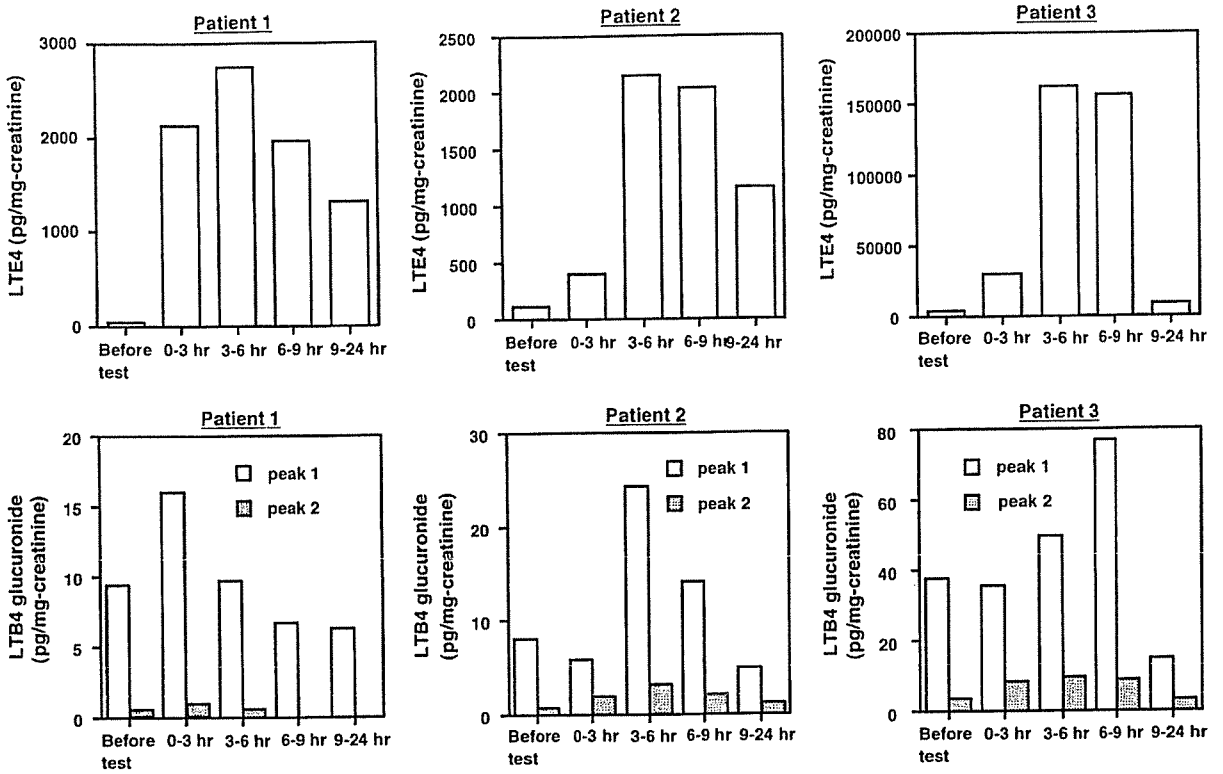

Fig. 4. Changes in concentrations of urinary LTE4 and two LTB4 glucuronide isomers in three patients with aspirin-intolerant asthma after intravenous aspirin challenge.

3.5. Concentrations of LTE4 and two LTB4 glucuronide isomers in aspirin-intolerant asthma patients after aspirin provocation

As shown in Fig. 4, a significant increase in the concentration of LTE4 was observed in urine from the three aspirin-intolerant asthma patients after the aspirin challenge. Of the two isomers of LTB4 glucuronide, a significant increase in the concentration of the isomer corresponding to peak 1 was observed. These results agreed with those in our previous report [5]. On the other hand, although the concentration of the isomer corresponding to peak 2 also increased, the increase was not apparent owing to the originally low concentration.

3.6. Concentrations of LTE4 and LTB4 glucuronide in normal healthy adults

In 13 normal healthy subjects, the concentrations of LTE4 and LTB4 glucuronide were 94.6 pg/mg-creatinine (median, range 21.4–159.9) and 22.3 pg/mg-creatinine (range 7.4–49.1), respectively. There was no correlation between LTE4 concentration and LTB4 glucuronide concentration in urine from normal healthy adults.

3.7. Concentrations of LTE4 and LTB4 glucuronide in atopic asthmatic patients after allergen inhalation

All patients developed isolated early asthmatic responses after allergen inhalation. The baseline FEV1.0 was 3.06 ± 0.861 (mean $\pm$ S.D.) and the mean percent decrease in FEV 1.0 after allergen inhalation was $67.8 \pm 17.4\%$ at 10 min. All the patients showed an increase in concentration of urinary LTE4 in the first 3 h after the challenge. There was a significant increase in urinary LTE4 concentration during the 3 h after FEV1.0 decreased by 20% or more from the baseline after allergen inhalation (96.1 pg/mg-creatinine, [61.7–346] versus 449 pg/mg-creatinine, [110–1546], $p < 0.05$). If LTB4 release is induced by allergens in atopic asthmatic patients, it is possible to detect an increase in urinary LTB4 glucuronide concentration after allergen inhalation. However, there was no significant change in urinary LTB4 glucuronide concentration after allergen inhalation (Fig. 5). These results suggest that cysteiny-LTs are

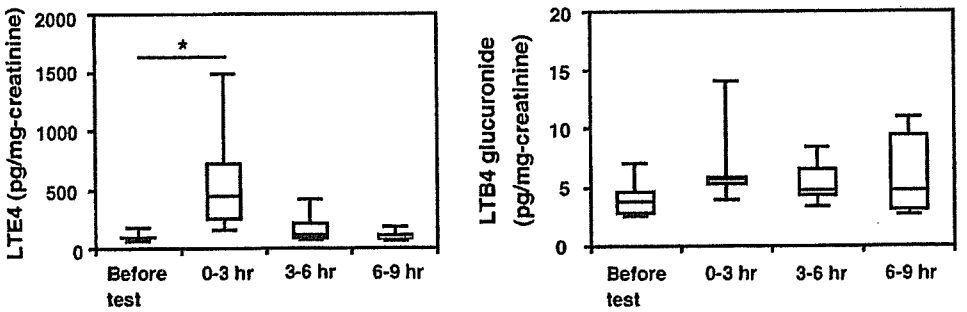

Fig. 5. Changes in urinary LTB4 glucuronide (right) and LTE4 (left) concentrations in atopic asthmatic patients after allergen inhalation. Data are presented as box plots showing medians and interquartile ranges. In the box plots, the lower boundary indicates the 25th percentile. The line within the box indicates the 50th percentile (median) and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles, respectively. *Significantly different from baseline concentration ($p < 0.05$).

involved in causing bronchoconstriction induced by inhalation challenge during early asthmatic responses. The absence of significantly increased concentrations of LTB4 glucuronide during bronchoconstruction suggests that LTB4 is not involved in the induction of early bronchial responses; alternatively, LTB4 is generated in amounts too small to be reflected in an increased concentration of urinary LTB4 glucuronide.

4. Discussion

When LTB4 and uridine 5′-diphosphoglucuronic acid were incubated in the presence of human liver microsomes, two new peaks appeared on the HPLC chromatogram (Fig. 1A), and this chromatogram agreed with that reported by Turgeon et al. [6]. When the fractions corresponding to the two peaks, which were distinctively eluted after the retention times of approximately 3.1 min and 4.5 min, were incubated with β-glucuronidase, both fractions generated LTB4, suggesting that these peaks correspond to LTB4 glucuronide. In human urine, the concentration of the isomer that was eluted by HPLC after a shorter retention time was significantly higher than that of the isomer that was eluted after a longer retention time. In an in vitro experiment using human liver microsomes, the height of peak 2 was approximately one-half that of peak 1 (Fig. 1A), suggesting that the isomer of LTB4 glucuronide corresponding to peak 2 was produced at a lower concentration than the isomer corresponding to peak 1. Although the distinct LTB4 glucuronide isomers show the same fragmentation pattern in LC–MS/MS analysis, the structure of LTB4 glucuronide, namely, the functional group of LTB4 to which glucuronic acid attaches, has not been clarified [6].

The possibility that the concentration of LTB4 was underestimated cannot be denied because we were not able to determine recovery rate of the entire experimental procedure, particularly the rate of hydrolysis of LTB4 glucuronide using β-glucuronidase. However, we examined the recovery rate of the procedure from various aspects and considered that there was no significant recovery loss.

The early asthmatic responses after allergen inhalation seem to be mainly induced by bronchoconstriction mediators released from cells, such as mast cells, within the airway lumen. Many studies showed that LTE4 concentration in urine increases in atopic patients following the inhalation of an allergen solution [7–11]. In our study, a significantly high concentration of LTE4 was observed in urine samples obtained during 3 h after bronchoconstriction as shown in Fig. 5. On the other hand, the concentration of LTB4 glucuronide generally did not significantly increase. This may be expected because human mast cells produce a large amount of LTC4 following IgE-dependent stimulation but produce only a small amount of LTB4 [12,13]. Moreover, LTB4 is produced by transcellular biosynthesis induced by cell-to-cell interaction [14]; however, the results shown in Fig. 5 suggest that allergen inhalation causes low, if any, LTB4 production induced by cell-to-cell interaction. Because it has been reported that only 0.2% of intravenously administered LTB4 is excreted in urine, a slight change in the concentration of LTB4 glucuronide may not be detected because of measurement errors. There is a possibility that changes in LTB4 concentration is more difficult to detect than those in LTE4 concentration except for considerable changes.

If we assume that the amount of creatinine produced in a human body is 1500 mg/day and that approximately 0.2% of LTB4 produced in a human body is excreted as LTB4 glucuronide in urine [3], the amount of LTB4 produced in the body of a healthy person can be calculated as approximately 16.7 μg/day (49.7 nmol/day), because the LTB4

glucuronide concentration in the urine sample from a healthy person was determined to be 22.3 pg/mg-creatinine in our study. On the other hand, because it was reported that approximately 5% of LTC4 is excreted as LTE4 in urine [15], the amount of LTC4 produced in the body of a healthy person can be similarly calculated as approximately 2.82 μg/day (4.5 nmol/day).

In conclusion, we established a method of measuring LTB4 glucuronide, which may be used as a marker of systemic LTB4 production. Using the method described in our previous report, we found that errors tended to occur when the concentration of LTB4 glucuronide was low because we calculated the concentration of LTB4 glucuronide from the difference between the concentration of LTB4 hydrolyzed and that not hydrolyzed. These errors rarely occur in the present method (data not shown); moreover, the advantage of this method is that it requires half the amount of a urine sample for analysis compared with that used in the previous method. By measuring the concentration of LTB4 glucuronide as well as that of LTE4, the condition of the human leukotriene pathway can be comprehensively evaluated.

Conflict of interest

The authors have no conflicting financial interests.

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Int. Arch. Allergy Immunol in press

BTLA Inhibits Antigen-induced Eosinophil Recruitment into the Airways

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ABSTRACT

BACKGROUND: Signaling through CD28 family co-receptors regulates activation of $CD4^+$ T cells positively and negatively. It has been shown that stimulatory co-receptors such as CD28 and ICOS play critical roles in the induction of allergic airway inflammation. However, the role of B and T lymphocyte attenuator (BTLA), an inhibitory co-receptor expressed preferentially in Th1 cells, in the regulation of allergic airway inflammation remains to be determined.

METHODS: We examined antigen-induced eosinophil recruitment and cytokine production in the airways in antigen-sensitized BTLA-deficient ($BTLA^{-/-}$) mice. We also examined antigen-induced cytokine production and cell proliferation of splenic T cells in antigen-sensitized $BTLA^{-/-}$ mice.

RESULTS: Antigen-induced eosinophil recruitment and IL-5 production in the airways was enhanced in antigen-sensitized $BTLA^{-/-}$ mice. On the other hand, antigen-induced Th1 and Th2 cytokine production as well as T cell proliferation of splenocytes was normal in $BTLA^{-/-}$ mice.

CONCLUSION: BTLA inhibits antigen-induced eosinophil recruitment into the airways by preventing IL-5 production from Th2 cells.

INTRODUCTION

Allergic airway inflammation is associated with intense eosinophil and T cell infiltration into the submucosal tissue of airways and the inflammatory cells cause epithelial damage and then airway

hyperreactivity (1, 2). In a murine model of allergic airway inflammation, it has been demonstrated that Th2 cells and their cytokines such as IL-4, IL-5, and IL-13 orchestrate the recruitment of eosinophils into the airways and airway hyperreactivity upon antigen exposure (3).

Activation and subsequent differentiation of naïve $CD4^+$ T cells is regulated not only by the strength of signals through T cell receptor but also by the signals derived from stimulatory and/or inhibitory co-receptors (4). Stimulatory co-receptors expressed on T cells include CD28 and ICOS and transduce signals necessary to fully activate T cells (4-6). Inhibitory co-receptors include CTLA-4, programmed death receptor-1 (PD-1), and B and T lymphocyte attenuator (BTLA) and transduce signals that inhibit T cell activation (4-6). The balance between positive and negative co-receptor signaling is thought to be critical not only for the maintenance of immune tolerance to self-antigens but also for the appropriate immune responses to exogenous antigens.

Recently, some of co-receptors have been demonstrated to be involved in the regulation of Th2 cell-mediated allergic airway inflammation. It has been shown that the inhibition of signaling through CD28 by CTLA-4 Ig results in the suppression of Th2 cell-mediated allergic airway inflammation and airway hyperreactivity (7, 8). B7-2 blocking exhibits similar effects (7, 9, 10), suggesting that interaction between B7-2 and CD28 is important in the induction of Th2 cell-mediated allergic airway inflammation. Moreover, it has been shown that ICOS is also involved in the initiation and maintenance of allergic airway inflammation (11). However, the role of BTLA, an inhibitory co-receptor expressed preferentially in Th1 cells and B cells (12), in the regulation of allergic airway inflammation is still largely unknown.

In this study, we examined the regulatory role of BTLA in allergic airway inflammation using BTLA-deficient ($BTLA^{-/-}$) mice. We found that antigen-induced eosinophil recruitment and IL-5 production in the airways was enhanced in $BTLA^{-/-}$ mice. We also found that antigen-induced Th1 and Th2 cytokine production of splenic T cells was normal in $BTLA^{-/-}$ mice. Our results indicate that BTLA negatively regulates antigen-induced eosinophil recruitment into the airways by inhibiting IL-5 production from Th2 cells.

MATERIALS and METHODS

Mice and genetic analysis

BTLA-deficient ($BTLA^{-/-}$) mice (12) were backcrossed to BALB/c mice (Charles River Laboratories, Kanagawa,

Japan) for at least 8 generations and littermate wild-type (WT) mice were used as controls. Mice were housed in microisolator cages under pathogen-free conditions. All experiments were performed according to the guidelines of Chiba University.

Antigen-induced eosinophil and T cell recruitment into the airways of sensitized mice

Antigen-induced allergic airway inflammation was induced as described previously (13, 14). In brief, BTLA$^{-/-}$ mice (age 7-8 weeks) and littermate WT mice were immunized intraperitoneally twice with 4 μg of ovalbumin (OVA) (Sigma Chemical Co., St. Louis, MO) in 4 mg of aluminum hydroxide (alum) at a two-week interval. Twelve to 14 days after the second immunization, the sensitized mice were challenged with aerosolized OVA dissolved in 0.9% saline by a nebulizer for 20 minutes. As a control, 0.9% saline alone was administered by the nebulizer. Forty-eight hours after the OVA inhalation, bronchoalveolar lavage (BAL) was performed with 2 ml of phosphate-buffered saline containing 1% bovine serum albumin (Sigma Chemical Co.). Bronchoalveolar lavage fluid (BALF) was centrifuged at 400 g for 5 minutes at 4°C and differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution. A fraction of the cells were subjected to a flow cytometric analysis for the lymphocyte surface phenotyping of CD4 and CD8 using a FACScalibur (Becton Dickinson, Mountain View, CA) and CELLQuest software.

Antigen-induced goblet cell hyperplasia in sensitized mice

OVA-sensitized BTLA$^{-/-}$ mice and littermate WT mice were challenged with the aerosolized OVA by a nebulizer as described above. Forty-eight hours after the OVA inhalation, a sagittal block of left lung was excised, fixed in 10% buffered-formalin, and embedded in paraffin. Lung sections (3 μm thick) were stained with periodic acid-Schiff (PAS) according to standard protocols. The number of goblet cells was counted on PAS-stained lung sections as described elsewhere (15).

Antigen-induced proliferation and cytokine production of splenocytes

Spleen was removed from OVA-sensitized BTLA$^{-/-}$ mice and littermate WT mice and a single cell suspension of splenocytes was prepared. Splenocytes (2 x 10^5) were then suspended in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (MBL, Nagoya, Japan), 10 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were cultured in triplicate in the

absence or presence of OVA (100-400 μg/ml) in a 96-well microtiter plate for 72 hours at 37°C. In some experiments, splenocytes (2 x 10^5) were cultured for 72 hours in a microtiter plate coated with 5 μg/ml of anti-CD3ε mAb (145-2C11, BD PharMingen, San Diego, CA). The culture supernatant was collected and the amounts of IL-4, IL-5, and IFN-γ were determined as described below. Splenocytes (2 x 10^5) were also cultured in the same conditions and the proliferation of cells was evaluated using CellTiter-Glo reagent (Promega, Madison, WI) as described previously (16).

ELISA for cytokines

The amounts of IL-4, IL-5, and IFN-γ in the BALF and the culture supernatant were determined by the enzyme immunoassay using murine IL-4, IL-5, and IFN-γ ELISA kits from BD PharMingen. The assays were performed in duplicate according to the manufacturer's instruction. The amounts of IL-13 in the BALF were determined by an ELISA kit from R&D systems (Minneapolis, MN). The detection limits of these assays were 15 pg/ml of IL-4 and IL-13, 30 pg/ml of IL-5, and 50 pg/ml of IFN-γ.

Data Analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the unpaired t-test. P values <0.05 were considered significant.

RESULTS

Antigen-induced eosinophil recruitment into the airways is enhanced in BTLA$^{-/-}$ mice

Given that CD4$^+$ T cells and their cytokines play an important role in regulating antigen-induced allergic airway inflammation (1-3) and that BTLA is expressed on CD4$^+$ T cells and attenuates cytokine production and proliferation of CD4$^+$ T cells (12), we examined whether BTLA is involved in the regulation of antigen-induced allergic airway inflammation using BTLA$^{-/-}$ mice. OVA-immunized BTLA$^{-/-}$ mice and littermate WT mice were challenged with aerosolized OVA and the number of eosinophils and CD4$^+$ T cells recovered in bronchoalveolar lavage fluids (BALF) was evaluated. As shown in Fig. 1A, antigen-induced eosinophil recruitment into the BALF at 48 hours after the inhalation was significantly enhanced in BTLA$^{-/-}$ mice by 47% as compared with WT mice (WT mice: 20.7 ± 6.6 vs. BTLA$^{-/-}$ mice: 30.4 ± 12.1, x10^4/mice, mean ± SD, n = 10 mice in each group, $p<0.05$). Antigen-induced eosinophil recruitment into the BALF at 72 hours after antigen inhalation was also enhanced in BTLA$^{-/-}$ mice (data not shown).

Antigen-induced neutrophil recruitment into the BALF at 48 hours after antigen inhalation tended to be enhanced in $BTLA^{-/-}$ mice, but the enhancement did not reach statistical significance (Fig. 1A). FACS analysis of BALF cells revealed that the number of $CD4^+$ T cells recovered at 48 hours after antigen inhalation was similar in $BTLA^{-/-}$ mice and WT mice (n = 10) (Fig. 1A). The levels of IL-5 but not IL-4 or IL-13 in the BALF at 48 hours after antigen challenge were also increased in $BTLA^{-/-}$ mice (n = 10, $p<0.05$) (Fig. 1B). On the other hand, IFN-γ was undetectable in the BALF in both $BTLA^{-/-}$ mice and WT mice at 48 hours after antigen inhalation (data not shown). In addition, no significant difference was detected in goblet cell hyperplasia between $BTLA^{-/-}$ mice and WT mice (n = 10) (Fig. 1C).

Antigen-induced T cell proliferation and cytokine production are indistinguishable between $BTLA^{-/-}$ mice and WT mice

We next examined the immune status of OVA-sensitized $BTLA^{-/-}$ mice. $BTLA^{-/-}$ mice and littermate WT mice were immunized with OVA/alum and OVA-induced proliferation and Th1 and Th2 cytokine production of splenocytes were evaluated. As shown in Fig. 2A, OVA-induced proliferation of splenocytes in OVA-sensitized $BTLA^{-/-}$ mice was similar to that in OVA-sensitized WT mice (n = 5). OVA-induced production of IL-4, IL-5, and IFN-γ was also similar in OVA-sensitized $BTLA^{-/-}$ mice and WT mice (n = 5) (Fig. 2B). Polyclonal T cell activation with anti-CD3ε mAb also induced similar levels of T cell proliferation and IL-4, IL-5, and IFN-γ production in $BTLA^{-/-}$ mice and WT mice (Fig. 2A and 2B). These results suggest that BTLA has no significant role in controlling Th1 or Th2 cell differentiation.

DISCUSSION

In this study, we show that BTLA plays an important role in the attenuation of antigen-induced allergic airway inflammation. BTLA has originally been identified as a gene preferentially expressed in Th1 cells and has been described to inhibit T cell responses (12). Therefore, we speculated that Th2-type immune responses including allergic airway inflammation would be suppressed by an uncontrolled activation of Th1 cells in $BTLA^{-/-}$ mice. However, we found that antigen-induced eosinophil recruitment and IL-5 production in the airways, a representative Th2-type immune response in mice, was rather enhanced in $BTLA^{-/-}$ mice on a Th2-biased BALB/c background (Fig. 1A and 1B). We also found that antigen-induced IFN-γ production from splenocytes was

not enhanced in BTLA$^{-/-}$ mice (Fig. 2B). These results suggest that BTLA inhibits antigen-induced eosinophil recruitment into the airways by preventing IL-5 production from Th2 cells. Thus, BTLA not only regulates primary T cell activation (17), but may also regulate the magnitude of Th2 cell-mediated allergic inflammatory responses in the tissues.

At present, the precise mechanism underlying the enhanced IL-5 production in the airways of BTLA$^{-/-}$ mice is still unclear. As noted above, the lacking of BTLA signaling in Th2 cells themselves may result in the enhanced IL-5 production upon antigen stimulation in vivo. Because BTLA is also expressed on CD25^{+}CD4^{+} regulatory T cells (18), it is possible that the dysfunction of CD25^{+} CD4^{+} regulatory T cells in BTLA$^{-/-}$ mice may result in the enhanced IL-5 production from antigen-stimulated Th2 cells in the airways. Further investigation is needed to determine the cell population that is responsible for the enhanced IL-5 production in the airways in BTLA$^{-/-}$ mice.

Recently, Deppong et al. have shown that BTLA$^{-/-}$ mice on a Th1-biased C57BL/6 background show persistent eosinophilic inflammation and goblet cell hyperplasia in the airways even at day 15 following the intranasal antigen challenge, whereas the wild-type mice have complete resolution at this time point (19), indicating that BTLA is critical for resolving allergic airway inflammation. In addition, we have shown here that BTLA$^{-/-}$ mice on a BALB/c background exhibit enhanced IL-5 production and eosinophilic inflammation in the airways at 2 days after the antigen inhalation (Fig. 1). These results suggest that BTLA is critical not only for the resolution of allergic airway inflammation but also for the suppression of the induction of allergic airway inflammation.

On the other hand, we found that in vitro antigen-induced IL-4, IL-5 or IFN-γ production from splenocytes was not significantly different between BTLA$^{-/-}$ mice and WT mice (Fig. 2B), suggesting that BTLA has no significant effect on T helper cell differentiation to induce either Th1 cells or Th2 cells. The lack of an inhibitory effect of BTLA on the in vitro cytokine production from splenocytes might be due to the optimal sensitization and abundant generation of both Th1 and Th2 memory cells in our experimental protocols that would be relatively resistant to the BTLA inhibition, since it was shown that the T cell inhibition by BTLA was small when T cells were stimulated with large amounts of antigens (17).

It has recently been shown that in addition to mature T cells, B cells and CD11c^{+} dendritic cells express BTLA (12, 18, 20). TNF receptor-family member herpesvirus entry mediator