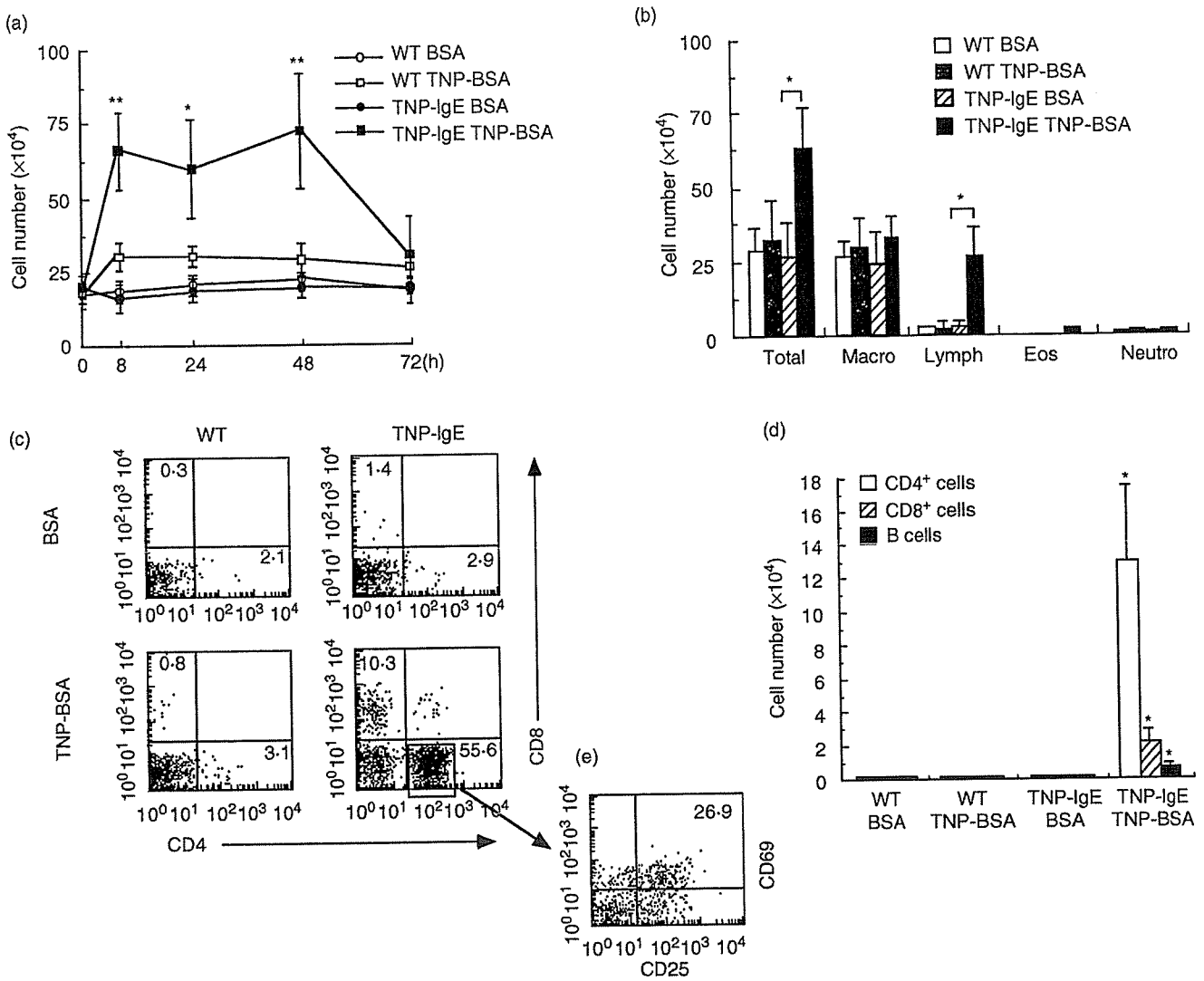

Fig. 1. IgE cross-linking induces CD4⁺ T cell recruitment into the airways. (a) Kinetics of antigen-induced airway inflammation in TNP–IgE mice. TNP–BSA or BSA (as a control) was administered intranasally to TNP–IgE mice and the littermate WT mice. At indicated times after the administration, bronchoalveolar lavage (BAL) was performed and the number of cells in BAL fluid (BALF) was counted. Data are means ± s.d. for eight mice in each group. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, significantly different from the mean value of the corresponding control response. (b) Cellular components in BALF. The number of lymphocytes, eosinophils, neutrophils and macrophages in BALF was evaluated 48 h after TNP–BSA or BSA administration by counting 500 cells stained with Wright–Giemsa solution. $n = 8$ mice in each group, $^{*}P < 0{\cdot}01$. (c) CD4 *versus* CD8 staining of BALF cells. BALF cells were subjected to FACS analysis 48 h after TNP–BSA or BSA administration. Shown is a representative staining of CD4 *versus* CD8 (gating on lymphocyte population) from five independent experiments. (d) The number of CD4⁺ cells, CD8⁺ cells and B cells in BALF 48 h after the challenge. $n = 8$ mice in each group, $^{*}P < 0{\cdot}01$, significantly different from the mean value of the corresponding control response. (e) CD25 *versus* CD69 staining of CD4⁺ T cells. BALF cells were subjected to FACS analysis 48 h after TNP–BSA administration. Shown is a representative CD25 *versus* CD69 staining of CD4⁺ T cells from five independent experiments.

administration in TNP–IgE mice (TNP–BSA $28{\cdot}0 \pm 9{\cdot}8$ *versus* BSA $2{\cdot}1 \pm 1{\cdot}0 \times 10^4$ at 48 h, $n = 8$ mice in each group, $P < 0{\cdot}01$) (Fig. 1b). In contrast, the number of eosinophils, neutrophils or macrophages was not significantly increased in TNP–IgE mice and WT mice at 48 h after TNP-BSA administration (Fig. 1b). FACS analysis revealed that the majority of lymphocytes in BALF of TNP–BSA-administered TNP–IgE mice were CD4⁺ T cells ($60{\cdot}2 \pm 10{\cdot}4\%$, $n = 6$) (Fig. 1c,d). The number of CD8⁺ T cells and B cells was also slightly increased in TNP–BSA-administered TNP–IgE mice (Fig. 1c,d). Although approximately 25% of CD4⁺ T cells exhibited an activated phenotype (CD25⁺ CD69⁺) (Fig. 1e), the levels of IL-4, IL-5 and IFN-γ in the BALF were still undetectable after TNP–BSA administration in TNP–IgE mice (data not shown). Histological analysis showed that the intranasal administration of TNP–BSA also induced lymphocyte recruitment in the perivascular and peribronchial spaces of the lung in TNP–IgE mice (data not shown).

IgE cross-linking does not enhance airway reactivity to methacholine

Next, we examined the effect of IgE cross-linking on airway reactivity to methacholine in TNP–IgE mice. TNP–BSA or BSA was

administered intranasally to TNP–IgE mice and WT mice and, 48 h later, the airway reactivity to aerosolized methacholine was measured by whole body plethysmograph. The intranasal administration of TNP–BSA did not increase airway reactivity to methacholine in TNP–IgE mice as compared with BSA administration (n = 5 in each group) (Fig. 2). As anticipated, TNP–BSA did not increase airway reactivity to methacholine in WT mice and the airway reactivity was comparable to that in TNP–BSA-administered TNP–IgE mice (data not shown). These results indicate that IgE cross-linking is not sufficient for the induction of airway hyperreactivity in this system.

Cyclooxygenase inhibitor prevents IgE-induced CD4⁺ T cell recruitment into the airways

We then determined which mediators are involved in IgE-induced CD4⁺ T cell recruitment into the airways. Because it has been suggested that prostaglandin D_2 (PGD_2) from mast cells is involved in CD4⁺ T cell recruitment [26], we examined the effect of acetylsalicylic acid on the IgE-induced CD4⁺ T cell recruitment in TNP–BSA-administered TNP–IgE mice. As shown in Fig. 3, the number of CD4⁺ T cells in BALF in TNP–BSA-administered TNP–IgE mice was significantly decreased by acetylsalicylic acid (acetylsalicylic acid (6 mg) $3{\cdot}9 \pm 1{\cdot}0$ *versus* saline $18{\cdot}3 \pm 5{\cdot}4 \times 10^4$, n = 5, $P < 0{\cdot}01$). The number of CD8⁺ T cells in BALF tended to be decreased by acetylsalicylic acid but the differences were not statistically significant (Fig. 3b). On the other hand, a cysteinyl leukotriene 1 receptor antagonist pranlukast did not decrease the number of CD4⁺ T cells and CD8⁺ T cells in BALF in TNP–BSA-administered TNP–IgE mice (data not shown). In addition, the administration of neutralizing antibody to TNF-α did not decrease the number of CD4⁺ T cells and CD8⁺ T cells in the BALF in TNP–BSA-administered TNP–IgE mice (data not shown). Taken together, these results suggest that prostaglandins are involved in IgE-induced CD4⁺ T cell recruitment into the airways.

IgE cross-linking enhances Th2 cell-mediated eosinophil recruitment into the airways

Finally, we studied whether IgE-dependent mast cell activation contributed to Th2 cell-mediated eosinophil recruitment into the airways by the adoptive transfer system of antigen-specific Th2 cells to TNP–IgE mice. OVA-specific Th2 cells prepared from DO11·10 mice were transferred to TNP–IgE mice or WT mice, and 2 days later the mice were challenged with the inhaled OVA or saline (as a control) for 20 min. TNP–BSA or BSA was then administered intranasally to the mice and the number of eosinophils and OVA-specific CD4⁺ T cells (KJ1-26⁺ CD4⁺ cells) in BALF was counted at 48 h after TNP–BSA or BSA administration. Without the cell transfer of OVA-specific Th2 cells, the inhaled OVA did not significantly induce eosinophil recruitment into the airways in TNP–IgE mice or WT mice (data not shown). When OVA-specific Th2 cells were transferred to WT mice and TNP–IgE mice, the inhaled OVA similarly induced eosinophil (Fig. 4a) and OVA-specific CD4⁺ T cell (Fig. 4b) recruitment into

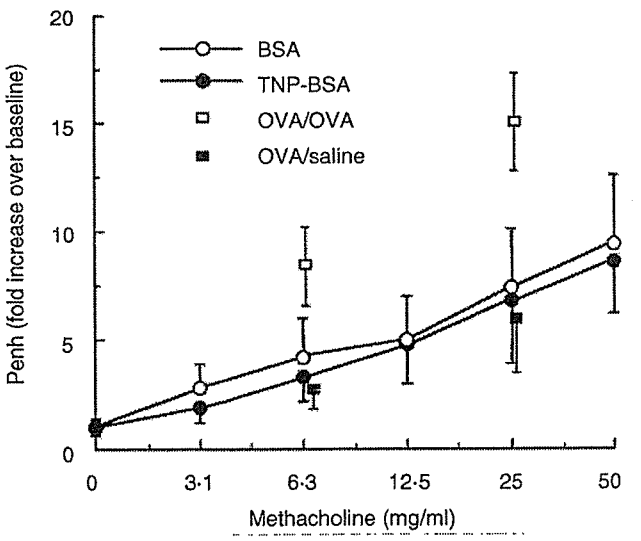

Fig. 2. IgE cross-linking does not induce airway hyperreactivity. TNP–BSA or BSA was administered intranasally to TNP–IgE mice. Forty-eight hours after TNP–BSA or BSA administration, airway reactivity was measured using a Buxco system where mice were exposed to increasing concentrations of aerosolized methacholine (3·1–50 mg/ml). Airway reactivity was expressed as enhanced pause (Penh) values for each concentration of methacholine over baseline response. As controls, OVA-sensitized BALB/c mice were challenged three times with the inhaled OVA or saline, and 24 h later airway reactivity to aerosolized methacholine was measured. Data are means ± s.d. for five mice in each group.

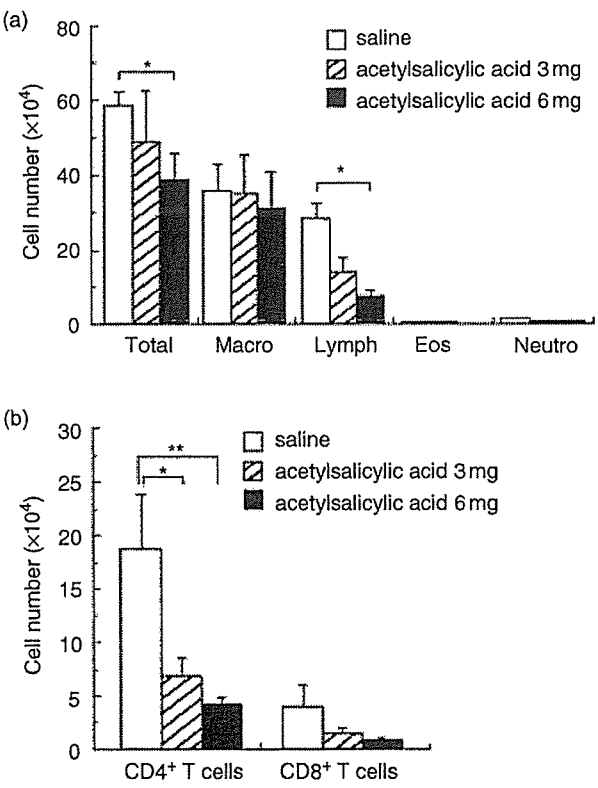

Fig. 3. Acetylsalicylic acid inhibits IgE-induced CD4⁺ T cell recruitment into the airways. TNP–IgE mice were injected intraperitoneally with acetylsalicylic acid (3 mg or 6 mg/mouse) or saline (as a control), and 30 min later TNP–BSA was administered intranasally to the mice. The number of lymphocytes, eosinophils, neutrophils and macrophages in BALF was counted 48 h after the TNP–BSA administration (a). The number of CD4⁺ and CD8⁺ T cells in BALF was also analysed by FACS (b). Data are means ± s.d. for five mice in each group, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

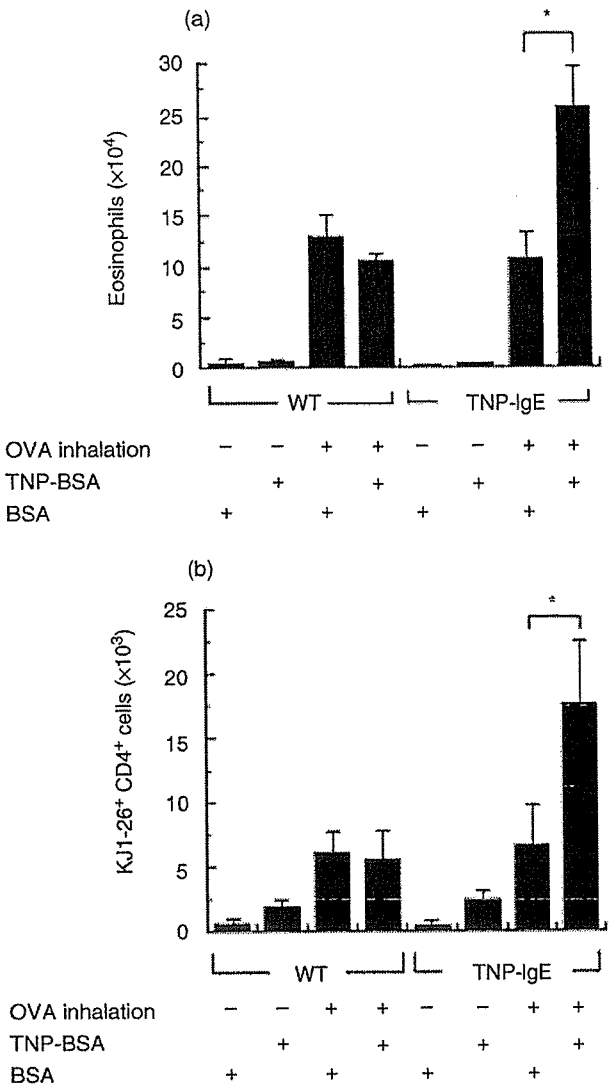

Fig. 4. IgE cross-linking enhances Th2 cell-mediated eosinophil recruitment into the airways. OVA-specific Th2 cells were prepared and transferred to TNP–IgE mice or WT mice as described in the Methods. Two days later, the mice were challenged with the inhaled OVA or saline (as a control) for 20 min. TNP–BSA or BSA was then administered intranasally to the mice, and the number of eosinophils (a) and OVA-specific $CD4^+$ T cells ($CD4^+$ KJ1-26^+) (b) in BALF was counted 48 h after TNP–BSA or BSA administration. Data are means ± s.d. for eight mice in each group, $^*P < 0{\cdot}01$.

the airways in both mice. On the other hand, intranasal administration of TNP–BSA alone did not induce eosinophil recruitment into the airways in TNP–IgE mice or WT mice even when Th2 cells were transferred to these mice (Fig. 4a). Interestingly, TNP–BSA administration significantly enhanced OVA-induced eosinophil ($n = 8$, $P < 0{\cdot}01$) (Fig. 4a) and OVA-specific $CD4^+$ T cell ($n = 8$, $P < 0{\cdot}01$) (Fig. 4b) recruitment into the airways in TNP–IgE mice but not in WT mice. FACS analysis revealed that the majority of OVA-specific $CD4^+$ T cells in the BALF exhibited an activated phenotype (data not shown). These results suggest that IgE-dependent mast cell activation enhances Th2 cell-mediated allergic airway inflammation by recruiting Th2 cells into the airways.

DISCUSSION

In this study, we show that using IgE transgenic mice without antigen sensitization, IgE cross-linking by a relevant antigen directly induces $CD4^+$ T cell recruitment into the airways in a prostaglandin-dependent manner (Figs 1 and 3). We also show that, although IgE cross-linking alone does not induce eosinophil recruitment into the airways, IgE cross-linking significantly enhances Th2 cell-mediated eosinophil recruitment into the airways (Fig. 4). Therefore, these results indicate that IgE-dependent mast cell activation enhances Th2 cell-mediated allergic airway inflammation by recruiting Th2 cells into the airways.

In a previous study [22], we showed that mast cells in ear skin of TNP–IgE mice were heavily loaded with TNP-specific IgE as detected by immunohistochemical staining. In contrast, such IgE-loaded mast cells were undetectable in WT mice even though the comparable numbers of mast cells existed in ear skin of TNP–IgE mice and WT mice. We also found that the epicutaneous application of picryl chloride carrying a TNP group induced an immediate cutaneous reaction in TNP–IgE mice but not in WT mice. Moreover, using peritoneal mast cells, we found that IgE bound to FcεRI on c-kit^+ mast cells in TNP–IgE mice. Therefore, it is suggested that intranasal administration of TNP–BSA induces mast cell activation through the cross-linking of FcεRI in TNP–IgE mice. However, it is still possible that FcεRI on basophils and eosinophils [27,28] as well as other IgE receptors including CD23 and Fcγ receptors [29] may be involved in TNP–BSA-induced $CD4^+$ T cell recruitment in TNP–IgE mice.

We show that IgE cross-linking principally induces $CD4^+$ T cell recruitment into the airways and thus enhances Th2 cell-mediated eosinophilic airway inflammation by recruiting Th2 cells into the airways. This implies that both antigen-specific IgE antibody on mast cells and antigen-specific Th2 cells cooperate synergistically to induce antigen-induced eosinophilic airway inflammation in asthma. Our findings are consistent with the previous observations that using mast cell-deficient mice, the role of mast cells in antigen-induced eosinophil recruitment into the airways can be detected only in the situation in which mice were weakly sensitized and challenged with antigens and thereby subsequent Th2 cell-mediated eosinophil recruitment was modest [19].

We demonstrate that, however, IgE-dependent mast cell activation alone is not sufficient for the induction of eosinophil recruitment into the airways (Fig. 1) or the induction of AHR (Fig. 2). In contrast to the convincing function of IgE and mast cells in the early phase reaction [30], the roles of IgE in allergic airway inflammation and AHR in the late phase are still controversial. In the previous study using IgE-deficient mice, it was demonstrated that the features of asthma, including eosinophil infiltration into the airways and AHR in the late phase, can be elicited in the absence of IgE [14], suggesting that IgE is not essential for the induction of allergic airway inflammation. On the other hand, in a previous study with the mice sensitized passively with antigen-specific IgE followed by the corresponding antigen challenge, it has been reported that antigen-induced mast cell activation induces eosinophil recruitment into the airways and induces AHR [31]. Interestingly, in their study it was reported that the repeated antigen challenges are required for the induction of eosinophilic airway inflammation and AHR in the passively sensitized mice [31]. Therefore, it is possible that antigen-specific T cells may be activated during the period of antigen

challenges and that these activated T cells may contribute to the induction of eosinophilic airway inflammation and AHR. This notion is in agreement with our finding that IgE cross-linking by antigens significantly induces eosinophilic airway inflammation only when antigen-specific Th2 cells are activated simultaneously by antigens (Fig. 4).

We have also found that IgE-induced $CD4^+$ T cell recruitment into the airways is significantly decreased by a cyclooxygenase inhibitor acetylsalicylic acid (Fig. 3) but not by a cysteinyl leukotriene 1 receptor antagonist pranlukast (data not shown), suggesting that prostaglandins are involved in IgE-induced $CD4^+$ T cell accumulation in the airways. Moreover, our findings that $CD4^+$ T cells are accumulated preferentially into the airways (Fig. 1d) suggest that the IgE-induced $CD4^+$ T cell recruitment is not due simply to an increase of vascular permeability. In this regard, it has been shown that PGD_2 is the major cyclooxygenase metabolite produced by mast cells in response to antigen challenge [32]. In addition, the importance of PGD_2 in allergic airway inflammation has recently been demonstrated by using mice deficient in PGD_2 receptor, DP [33]. More recently, it has been shown that PGD_2 also induces chemotaxis of Th2 cells through a novel PGD_2 receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [26]. Therefore, it is suggested that PGD_2 may be involved in IgE-induced $CD4^+$ T cell recruitment into the airways.

On the other hand, because it has also been shown that thromboxanes are involved in the accumulation of lymphocytes in the airways of a guinea pig asthma model [34], other prostanoids such as thromboxanes might be involved in IgE-induced $CD4^+$ T cell recruitment in TNP–IgE mice. It is also possible that acetylsalicylic acid may directly decrease the $CD4^+$ T cell recruitment by inhibiting adhesion of T cells to the endothelium [35].

Although it has been shown that mast cell mediators induce short-term AHR [36], our results suggest that IgE cross-linking alone does not significantly induce persistent AHR. A previous study also showed that anti-IgE antibody treatment of sensitized mice prevented systemic anaphylactic reactions, but failed to affect the development of persistent AHR associated with airway inflammation [37]. On the other hand, some studies revealed that IgE and mast cells were necessary for AHR associated with airway inflammation 24 h after antigen challenge [18,19]. The differences in the role of mast cells in the development of AHR may be explained by the differences in the relative contribution to AHR of activated T cells and their cytokines such as IL-13 [2] and eosinophils [7,37]. In addition, in the cell transfer experiments, we found that WT and TNP–IgE mice that had received OVA-specific Th2 cells and subsequent inhaled OVA challenge showed no significant increase in airway reactivity to methacholine even after TNP–BSA administration. It is consistent with the previous findings that AHR associated with mild airway eosinophilia induced by passive sensitization with IgE or exclusive airway sensitization and challenges with antigens could be detected only by *in vitro* airway smooth muscle contraction to electrical field stimulation but not by *in vivo* hyperresponsiveness to inhaled methacholine [31,38].

In summary, we have shown that IgE cross-linking by antigens of mast cells induces $CD4^+$ T cell recruitment into the airways and consequently enhances Th2 cell-mediated eosinophil recruitment into the airways. Although the molecular mechanisms underlying this phenomenon remains to be determined, our results show a novel relationship between IgE-dependent mast cell activation and Th2 cell-mediated allergic inflammation in the late-phase allergic airway responses.

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Tyk2 Is Essential for IFN-α-Induced Gene Expression in Mast Cells

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Key Words
Mast cells · Tyk2 · IFN-α · Stat1 · Immunity, innate

Abstract
Mast cells are recognized not only as the major effector cells of type I hypersensitivity reactions but also as an important player of innate immune response against bacterial infection. Type I IFNs are also involved in the response against bacterial infection. However, the role of type I IFNs and their associated Janus kinase Tyk2 in mast cell functions remains to be determined. In this study, we addressed this issue using Tyk2-deficient ($Tyk2^{-/-}$) bone marrow-derived mast cells (BMMCs). When BMMCs from wild-type (WT) mice were stimulated with IFN-α, they expressed mRNA for IFN-γ-inducible protein 10 (IP-10) and monocyte chemoattractant protein-5 (MCP-5). Interestingly, IFN-α-induced expression of IP-10 and MCP-5 was severely decreased in $Tyk2^{-/-}$ BMMCs. In addition, IFN-α-induced Stat1 phosphorylation was decreased in $Tyk2^{-/-}$ BMMCs. On the other hand, IFN-α-induced Stat1 phosphorylation and IP-10 and MCP-5 expression were normal in $Tyk2^{-/-}$ fibroblasts. These results indicate that IFN-α induces the expression of TNF-α and the chemokines IP-10 and MCP-5 in mast cells and thatTyk2 plays a nonredundant role in IFN-α signaling in mast cells.

Introduction

Mast cells are recognized as the major effector cells of type I hypersensitivity reactions by virtue of possessing the high affinity receptors for IgE and are known to play a critical role in allergic diseases such as atopic rhinitis, asthma, and atopic dermatitis [1, 2]. Recently, a number of studies have revealed that mast cells also play important roles in innate immune responses especially against gram-negative bacteria by recruiting neutrophils and monocytes into the inflammatory site through the production of proinflammatory cytokines such as TNF-α [3, 4]. Some of the bacterial components, including lipopolysaccharide (LPS), directly activate mast cells through their surface receptors [5, 6]. However, the precise mechanisms of mast cell activation in innate immune responses are still largely unknown.

Type I IFNs (IFN-α/β), key immunoregulatory cytokines produced by macrophages and plasmacytoid dendritic cells after the exposure to pathogens, modulate innate and adaptive immune responses [7]. Although the function of type I IFNs is principally associated with the protection against viral infections, recent studies have revealed that type I IFNs are also involved in the immune response against other pathogens [8, 9]. In this regard, it has been shown that preceding IFN-α treatment sensitizes the mice for an enhanced production of TNF-α upon LPS stimulation [10]. It has also been shown that type I IFNs

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are rapidly produced by macrophages upon LPS stimulation [11]. These findings suggest that type I IFNs may participate in immune responses against bacterial infection through the production of TNF-α.

Two Janus kinases (JAKs), Tyk2 and Jak1, are associated with IFN-α/β receptor components IFNAR1 and IFNAR2, respectively [12, 13]. Upon ligand binding, Jak1 and Tyk2 are activated and the activated JAKs phosphorylate Stat1 and Stat2 [12, 13]. Subsequently, these activated STATs associate to form either Stat1 homodimers or Stat1/Stat2 heterodimers, which then translocates to the nucleus to induce gene expression [14, 15]. Recently, the physiological function of Jak1 and Tyk2 in type I IFN signaling has been determined using mice lacking Jak1 or Tyk2 [16–19]. These studies have revealed that, whereas Jak1 is essential for responding to type I IFNs in most cell types [16], the requirement of Tyk2 in type I IFN signaling differs depending on cell types [17–19]. It has been demonstrated that Tyk2 is essential for IFN-α signaling in IL-7-dependent B cells [19] but not in fibroblasts [17, 18]. However, the role of Tyk2 in IFN-α signaling in mast cells is unknown.

In this study, we determined whether Tyk2 is essential for IFN-α signaling in mast cells. Our findings have clearly demonstrated that using Tyk2-deficient mice, Tyk2 is required for IFN-α-induced Stat1 phosphorylation and subsequent gene induction in mast cells.

Methods

Mice and Cytokines

Tyk2-deficient ($Tyk2^{-/-}$) mice [17] were backcrossed for more than four generations onto BALB/c mice (Japan SLC, Shizuoka, Japan). The mice were genotyped by PCR as described previously [17] 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 the Chiba University. Recombinant murine IFN-α and IL-12 were purchased from R&D systems (Minneapolis, Minn., USA).

Culture of Bone Marrow-Derived Mast Cells

Primary culture of IL-3-dependent bone marrow-derived mast cells (BMMCs) was prepared from 8- to 12-week-old WT or $Tyk2^{-/-}$ mice and maintained as described previously [20]. BMMCs obtained after 4 weeks of culture were >99% mast cells.

Culture of Fibroblasts

Skin fibroblasts were prepared from WT mice or $Tyk2^{-/-}$ mice and maintained as described previously [21].

Flow-Cytometric Analysis

BMMCs were stained and analyzed on FACScalibur (Becton Dickinson, San Jose, Calif., USA) using CELLQuest software. Anti-CD117 (c-kit) antibody (2B8) was purchased from BD PharMingen (San Diego, Calif., USA). Prior to staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BD PharMingen).

RT-PCR Analysis for IP-10, MCP-5, IFNAR1, IFNAR2, and Jak1

BMMCs or fibroblasts from WT mice or $Tyk2^{-/-}$ mice were stimulated with IFN-α (1,000 U/ml) at 37 °C for 3 h. Total cellular RNA was prepared using Isogen solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. The first-strand complementary DNA (cDNA) was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Pharmacia Biotech, Buckinghamshire, UK). cDNAs encoding IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein-5 (MCP-5), IFNAR1, IFNAR2, and Jak1 were amplified by PCR using the following primer pairs:

IP-10 5′-GAGATCATTGCCACGATGAA-3′ and 5′-CACTGGGTAAAGGGGAGT-3′, MCP-5 5′-AATCACAAGCAGCCAGTG-3′ and 5′-GGGAACTTCAGGGGGAAATA-3′, IFNAR1 5′-CCTGCTGAATAAGACCAGCAACTTC-3′ and 5′-GTGCTTTACTTCTACAGCGACCGTG-3′, IFNAR2 5′-CAAGCCTCTGCAACAAACCTCTGAC-3′ and 5′-GATTTCTCAGATGACCCATCTTCAG-3′, Jak1 5′-CTGCTAGCATGATGAGACAGGTTTC-3′ and 5′-TTGGAGTCTTCAACACACTCAGGAG-3′. β-Actin was used to normalize the cDNA amount to be used.

Immunoblotting

Preparation of whole cell extracts and immunoblottings were performed as described previously [20]. The following antisera were used: anti-phospho-Stat1 (New England Biolabs, Beverly, Mass., USA) and anti-Stat1 (Upstate Biotechnology, Lake Placid, N.Y., USA).

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 and Discussion

Development of IL-3-Dependent BMMCs Is Normal in $Tyk2^{-/-}$ Mice

To determine the role of Tyk2 in mast cell development, bone marrow cells from WT mice or $Tyk2^{-/-}$ mice were cultured in the presence of IL-3 and the number of mast cells were evaluated every 7 days. As shown in figure 1a, the number of mast cells recovered from the culture was indistinguishable between WT and $Tyk2^{-/-}$ mice. Over 99% of cells obtained after 4 weeks of culture were morphologically mast cells in $Tyk2^{-/-}$ mice as well as in WT mice (data not shown). In addition, $Tyk2^{-/-}$ BMMCs expressed comparable levels of c-kit to WT BMMCs (fig. 1b). These results suggest that Tyk2 is not required for the development of IL-3-dependent mast cells.

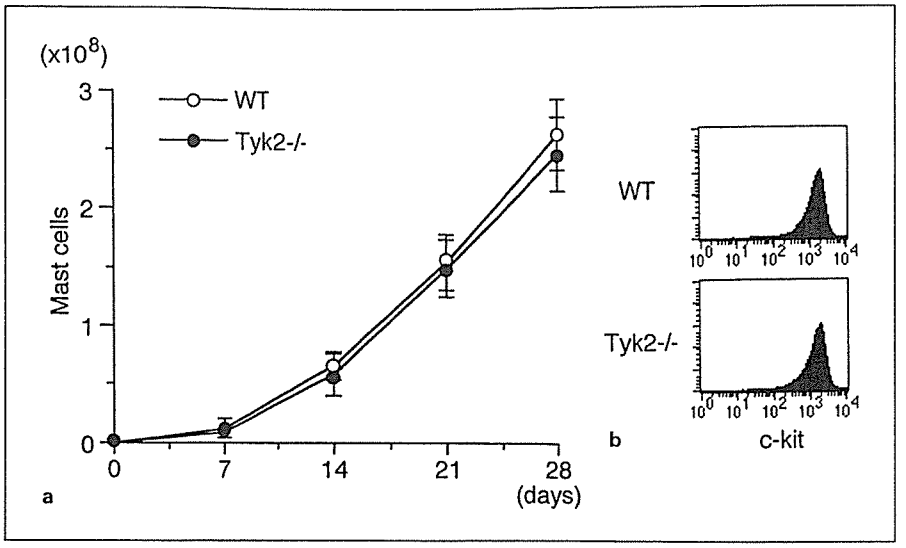

Fig. 1. Development of IL-3-dependent BMMCs is normal in Tyk2$^{-/-}$ mice. **a** Bone marrow cells from WT mice or Tyk2$^{-/-}$ mice were cultured in the presence of IL-3. Indicated days later, the number of BMMCs was evaluated. Data are means ± SD of 5 experiments for each genotype. **b** BMMCs from WT mice or Tyk2$^{-/-}$ mice were stained with anti-c-kit APC and analyzed on FACS. Representative FACS profiles for c-kit staining from five independent experiments are shown.

IFN-α-Induced Expression of IP-10 and MCP-5 Is Diminished in Tyk2$^{-/-}$ BMMCs

Jak1 and Tyk2 are associated with receptors for type I IFNs [12–15]. Using Jak1-deficient mice, it has been shown that Jak1 is essential for biological responses in IFN-α/β signaling [16]. On the other hand, it has been demonstrated that the requirement of Tyk2 in IFN-α/β signaling differs depending on cell types [17–19]. To examine the role of Tyk2 in IFN-α-mediated functions in mast cells, BMMCs from WT mice or Tyk2$^{-/-}$ mice were stimulated with IFN-α and the expression of IFN-responsive genes was analyzed at mRNA levels. As shown in figure 2, IFN-α-induced expression of IP-10 and MCP-5, which play important roles in the host defense to pathogens [22, 23], was severely decreased in Tyk2$^{-/-}$ BMMCs as compared with that in WT BMMCs. On the other hand, Tyk2$^{-/-}$ fibroblasts expressed mRNA for IP-10 and MCP-5 at a level comparable to that in WT fibroblasts (fig. 2). These results suggest that Tyk2 is essential for IFN-α-mediated gene expression in mast cells but not in fibroblasts. On the other hand, IL-12, another cytokine that utilizes Tyk2 as a signaling molecule [24] and augments innate immune responses [25], exhibited no significant effects even on WT BMMCs because of the absence of functional IL-12R (data not shown).

IFN-α-Induced Phosphorylation of Stat1 Is Diminished in Tyk2$^{-/-}$ BMMCs

IFN-α-mediated gene induction was diminished in Tyk2$^{-/-}$ BMMCs (fig. 2). Because most IFN-α-induced responses depend on Stat1 activation [26, 27], we next

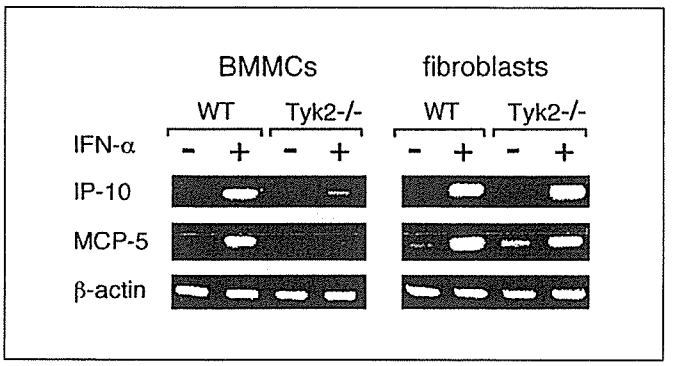

Fig. 2. IFN-α-induced gene expression is diminished in Tyk2$^{-/-}$ BMMCs. BMMCs or fibroblasts from WT mice or Tyk2$^{-/-}$ mice were stimulated with IFN-α (1,000 U/ml) for 3 h. The expression of IP-10 and MCP-5 mRNA was determined by RT-PCR. Representative data from 5 independent experiments are shown.

examined IFN-α-induced phosphorylation of Stat1 in Tyk2$^{-/-}$ BMMCs. As shown in figure 3, IFN-α-induced Stat1 phosphorylation was severely decreased in Tyk2$^{-/-}$ BMMCs as compared with that in WT BMMCs (fig. 3). In contrast, consistent with previous reports [17, 18], IFN-α-induced Stat1 phosphorylation was similarly observed in Tyk2$^{-/-}$ fibroblasts and WT fibroblasts (fig. 3). These results suggest that Tyk2 is essential for IFN-α-induced Stat1 phosphorylation and then for Stat1-dependent gene expression in mast cells.

Because IFN-α-induced Stat1 phosphorylation was diminished in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibro-

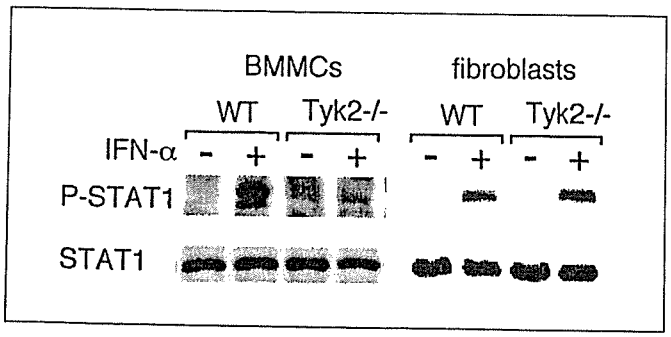

Fig. 3. Tyk2 is essential for IFN-α-induced Stat1 phosphorylation in BMMCs but not in fibroblasts. WT BMMCs, Tyk2$^{-/-}$ BMMCs, WT fibroblasts, or Tyk2$^{-/-}$ fibroblasts were stimulated with or without IFN-α (1,000 U/ml) for 30 min. Whole cell extracts were subjected to Western blotting with anti-phospho-Stat1 antibody or anti-Stat1 antibody. Representative data from 5 independent experiments are shown.

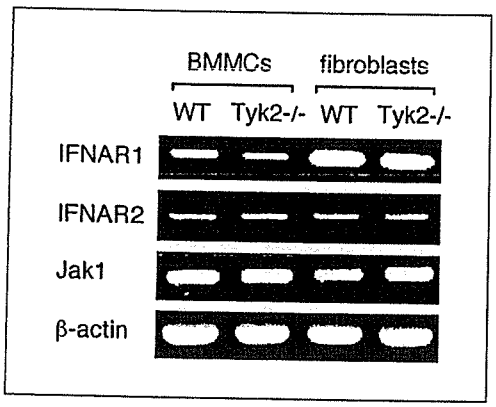

Fig. 4. Expression of IFNAR1 is lower in BMMCs than that in fibroblasts. Total cellular RNA was prepared from WT BMMCs, Tyk2$^{-/-}$ BMMCs, WT fibroblasts, or Tyk2$^{-/-}$ fibroblasts. Expression of IFNAR1, IFNAR2, and Jak1 transcripts was examined by RT-PCR analysis. Representative data from 5 independent experiments are shown.

blasts, we next compared the expression of IFN-α receptor components (IFNAR1 and IFNAR2) and Jak1 in BMMCs and fibroblasts. As shown in figure 4, regardless of the presence or absence of Tyk2, the expression of IFNAR1 was significantly lower in BMMCs than that in fibroblasts. On the other hand, the expression of IFNAR2 and Jak1 was comparable between BMMCs and fibroblasts (fig. 4). These results suggest that the limited expression of IFNAR1 in mast cells may account for the requirement of Tyk2 in IFN-α signaling.

Concluding Remarks

In the present study, we show that IFN-α induces the expression of the chemokines IP-10 and MCP-5 in mast cells and that Tyk2 is essential for IFN-α-induced gene expression in mast cells but not in fibroblasts. We found that IFN-α induced mRNA expression of IP-10 and MCP-5, important chemokines for innate immune responses [22, 23], in WT BMMCs, but the chemokine expression was diminished in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibroblasts. In addition, we found that IFN-α-induced Stat1 phosphorylation was decreased in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibroblasts. These results suggest that Tyk2 is required for IFN-α-induced Stat1 phosphorylation and subsequent gene expression in mast cells but not in fibroblasts.

Recent studies using Tyk2$^{-/-}$ mice have revealed that Tyk2 regulates both acquired and innate immune responses. It has been shown that Tyk2 is essential for IL-12-mediated T cell function, including IFN-γ production and Th1 cell differentiation [17, 18]. Tyk2 is also required for the downregulation of Th2 cell-mediated allergic inflammation in murine models of allergic asthma [28]. In addition, it has been demonstrated that Tyk2 plays an important role in endotoxin shock as a component of type I IFN signaling [29]. These findings suggest that Tyk2 is involved not only in acquired immune responses but also in innate immune responses. Our findings that Tyk2 is required for the IFN-α-induced expression of IP-10 and MCP-5 in mast cells also suggest the important roles of Tyk2 in innate immune responses.

We have shown here that Tyk2 is essential for IFN-α signaling in mast cells but not in fibroblasts. Although further studies are required, our data suggest that the expression levels of IFNAR1 may account for the different requirement for Tyk2 in IFN-α signaling between mast cells and fibroblasts.

Acknowledgments

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SHORT REPORT

Involvement of eicosanoids and surfactant protein D in extrinsic allergic alveolitis

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ABSTRACT: **The pathophysiology of extrinsic allergic alveolitis (EAA) involves oxidative lung damage as well as interstitial and alveolar inflammation. Macrophages and mast cells are inflammatory components of EAA that produce both leukotrienes (LTs) and prostaglandin D_2 (PGD_2). In addition, PGD_2 is also produced by the free-radical-catalysed peroxidation of arachidonic acid during oxidative stress. Urinary 8-iso prostaglandin $F_2\alpha$ (8-isoPGF$_2\alpha$) and serum surfactant protein D (SP-D) are considered appropriate biomarkers of oxidative stress and interstitial lung disease activity, respectively. The present study aimed to assess the association of these biomarkers with the pathophysiology of EAA.**

Two cases of acute EAA caused by the inhalation of fungi spores were reported. Eight asthmatic patients and six healthy control subjects were also enrolled in the current study.

The serum SP-D and urinary eicosanoid (LTE_4, PGD_2 metabolite (9α,11βPGF$_2$), 8-isoPGF$_2\alpha$) concentrations markedly increased during the acute exacerbation phase. These concentrations decreased following corticosteroid therapy in the EAA patients. There was a significant correlation between serum SP-D and urinary 9α,11βPGF$_2$ concentrations in the EAA patients.

In conclusion, although the present study proposes that serum surfactant protein-D and urinary eicosanoids are new biomarkers involved in the various immunological responses in extrinsic allergic alveolitis, further large-scale studies are needed to investigate the role of these compounds, not just as biomarkers, but also as biological potentiators of extrinsic allergic alveolitis.

KEYWORDS: **Extrinsic allergic alveolitis, 8-iso prostaglandin $F_2\alpha$, prostaglandin D_2, surfactant protein D**

Acute extrinsic allergic alveolitis (EAA) is characterised by oxidative lung damage [1]. During oxidative stress, prostaglandin D_2 (PGD_2) is nonenzymatically produced by the free-radical-catalysed peroxidation of arachidonic acid (isoprostane pathway) [2]. Briefly, isoprostanes are a unique series of PG-like compounds formed by the random oxidation of tissue phospholipids by oxygen radicals [2]. Thus, isoprostanes contain racemic mixtures of E-, D-, F-type and thromboxane-type prostane rings [3, 4]. The racemic D-ring isoprostane (12-isoPGD$_2$) subsequently undergoes rapid epimerisation to racemic PGD_2 [2].

In contrast, alveolar macrophages and mast cells produce cysteinyl-leukotrienes (CysLTs) and cyclooxygenase-dependent PGD_2 [5, 6]. Alveolar macrophages play a key role in acute EAA [7]. There is also a persistent increase in the number of alveolar mast cells in EAA patients [8]. EAA is categorised as a T-helper1-type disease and interferon (IFN)-γ plays a pivotal role in granuloma formation in EAA [7]. Interestingly, mast cells, which express the Fcγ receptor I after incubation with IFN-γ, can produce PGD_2 and CysLTs even in response to immunoglobulin (Ig)G stimulation [9].

Urinary leukotriene E4 (LTE_4) is now considered to be the most reliable analytical parameter for monitoring the endogenous synthesis of CysLTs [10, 11]. Similarly, urinary 9α,11β prostaglandin F_2 (9α,11βPGF$_2$) is a relatively stable PGD_2 metabolite and an appropriate indicator of mast cell activation [5]. Of the isoprostanes, 8-iso prostaglandin $F_2\alpha$ (8-isoPGF$_2\alpha$) is the best-characterised isomer and urinary 8-isoPGF$_2\alpha$ is considered the most accurate indicator of oxidant stress [2]. Taking this into account, it was

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hypothesised that patients with acute EAA show increased concentrations of urinary eicosanoids (CysLTs, 9α,11βPGF$_2$ and 8-isoPGF$_2$α).

Surfactant protein D (SP-D), which is produced by alveolar type II and Clara cells [12], is an important regulatory molecule in both pulmonary surfactant homeostasis and first-line defence mechanisms against microbial or allergen challenges [13]. Krebs von den Lungen-6 (KL-6) is also produced by alveolar type II cells [14]. The measurements of serum SP-D and KL-6 concentrations contribute to the early diagnosis of interstitial lung disease (ILD) [12, 14]. EAA is an acute ILD [7]. Previous studies have demonstrated significant increases in SP-A and KL-6 concentrations in the bronchoalveolar lavage fluid (BALF) of acute EAA patients [15]. Although plasma SP-D concentration is one of the most appropriate prognostic parameters of acute respiratory distress syndrome (ARDS) [16], knowledge of the serum SP-D profile of acute EAA patients is limited [17].

The present study aims to assess the association of these biomarkers with the pathophysiology of EAA.

METHODS

Case reports

Case 1

A 61-yr-old, nonsmoking female suffered from summer-type EAA caused by *Trichosporon asahii*, the most prevalent cause of EAA in Japan [18]. Cell counts revealed that 58.9% of total BALF cells were lymphocytes and the CD4/CD8 ratio of lymphocyte surface markers was 0.6. In addition, transbronchial lung biopsy specimens exhibited lymphocytic alveolitis with granulomas. The subject was diagnosed positive for precipitin to *T. asahii* by double immunodiffusion analysis. The positive findings were confirmed during a provocation test, following which the patient was allowed to return home.

Case 2

A 48-yr-old, nonsmoking female suffered from occupational EAA caused by *Aspergillus niger*, predominantly isolated from house dust in her workplace (a linen room). In addition to being strongly positive for precipitins to both house dust and *A. niger* extract, determined by double immunodiffusion analysis, the patient was also positive for a serum-specific IgG antibody against *A. niger* (10.8 mg·dL^{-1}). High-resolution computed tomography of the patient's chest revealed supportive radiographic findings [7]. The results from both cases are shown in table 1.

Both EAA patients fulfilled the American-European Consensus Conference criteria for ARDS (table 1) [19]. Intensive corticosteroid treatment (*i.v.* administration of 1,000 mg·day^{-1} methylprednisolone for 3 days, followed by oral administration of 0.5 mg·kg^{-1} prednisone) was tapered over the 7-week period, resulting in gradual improvements of both clinical symptoms and radiographic findings.

Control subjects

Eight (six female) stable asthmatic patients (mean age (range) 58 (33–73) yrs) were enrolled as diseased control subjects. Six (three female) healthy control subjects (44 (29–58) yrs) were also enrolled for comparative analysis of urinary eicosanoid data. All the subjects were nonsmokers. Permission to conduct the study was obtained from the Ethics Committee of the National Sagamihara Hospital (Japan) and all participating subjects gave informed consent.

Measurements

Serum and spot urine samples were collected between 09:00–11:00 h. In the case of subjects with acute EAA, urine samples were collected on admission and following therapy. The samples were analysed for KL-6, SP-D and eicosanoid concentrations by methods previously described [10, 12, 17, 20]. Double immunodiffusion analysis of precipitating antibodies against 18 different fungal species was performed according to the Ouchterlony method. A specific IgG antibody against *A. niger* was performed utilising the liquid-phase immunoassay AlaSTAT microplate system (Diagnostic Products Corporation, Los Angeles, USA).

Data analysis

Serum and urinary data were expressed as mean and median, respectively. Relationships were analysed using Spearman's rank correlation test. A p-value <0.05 was regarded as statistically significant.

RESULTS

Serum KL-6 and SP-D concentrations were markedly higher in the acute EAA patients than in the eight asthmatic patients (table 1). As shown in figure 1, urinary eicosanoid concentrations in the acute EAA patients (LTE$_4$: 420 and 185 pg·mg^{-1}-creatinine; 9α,11βPGF$_2$: 658 and 382 pg·mg^{-1}-creatinine; 8-isoPGF$_2$α: 393 and 537 pg·mg^{-1}-creatinine) were markedly higher than in the healthy control subjects (LTE$_4$: 45 pg·mg^{-1}-creatinine; 9α,11βPGF$_2$: 43 pg·mg^{-1}-creatinine; 8-isoPGF$_2$α: 187 pg·mg^{-1}-creatinine). Median values

TABLE 1 Serum Krebs von den Lungen-6 (KL-6) and surfactant protein D (SP-D) concentrations on admission

Subject	Causative organism	$Pa,O_2/Fi,O_2$ ratio	Serum	
			KL-6 U·mL^{-1}	SP-D ng·mL^{-1}
Case 1	*Tricosporon asahii*	91.4	6090	428
Case 2	*Aspergillus niger*	200	5770	396
Asthma group#			252±70	39±14

Data are presented as mean±SD. Pa,O_2: partial pressure of arterial oxygen; Fi,O_2: inspiratory oxygen fraction. #: n=8.

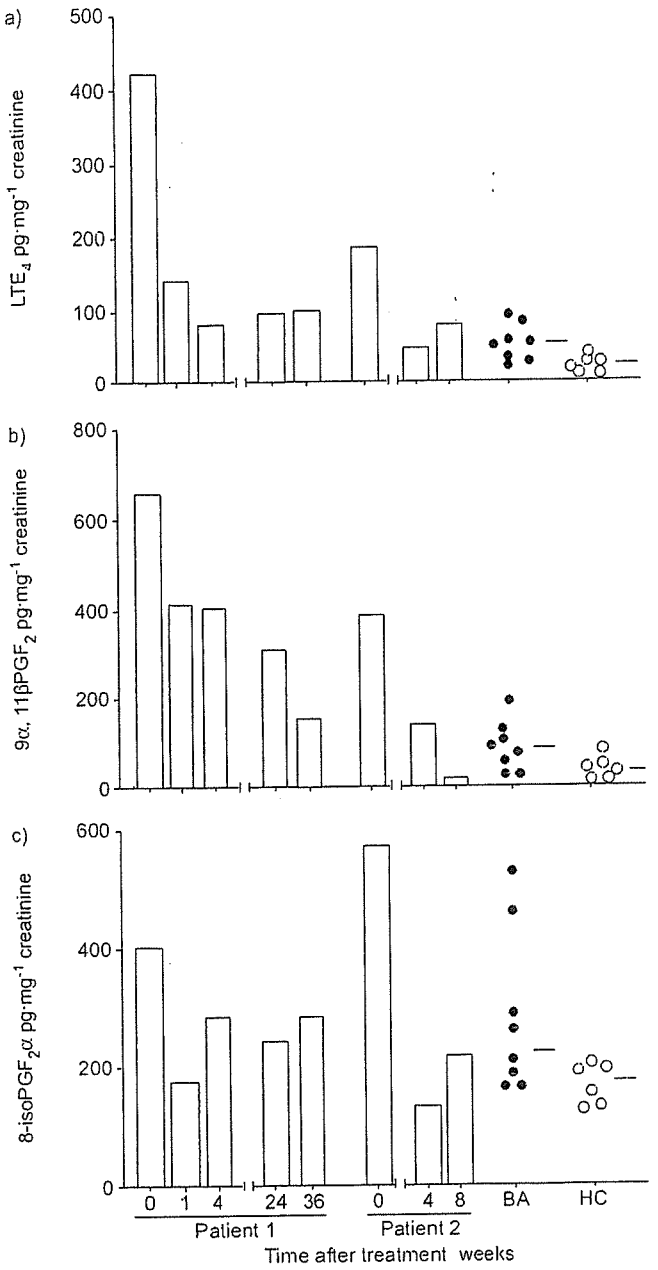

FIGURE 1. a) Urinary leukotriene E_4 (LTE_4), b) 9α,11β prostaglandin F_2 (9α,11βPGF$_2$), and c) 8-iso prostaglandin F$_2$α (8-isoPGF$_2$α) concentrations in the extrinsic allergic alveolitis patients. BA: asthmatic patients; HC: healthy control subjects. Horizontal bars indicate median values.

of urinary LTE_4, 9α,11βPGF$_2$ and 8-isoPGF$_2$ concentrations in the asthmatic patients were 55, 79 and 235 pg·mg^{-1}-creatinine, respectively. The serum SP-D, KL-6 and urinary eicosanoid concentrations decreased following corticosteroid therapy in the EAA patients (figs 1 and 2). There was a significant correlation between serum SP-D and urinary 9α,11βPGF$_2$ concentrations in the EAA patients ($p<0.05$, $rs=1$; fig. 2).

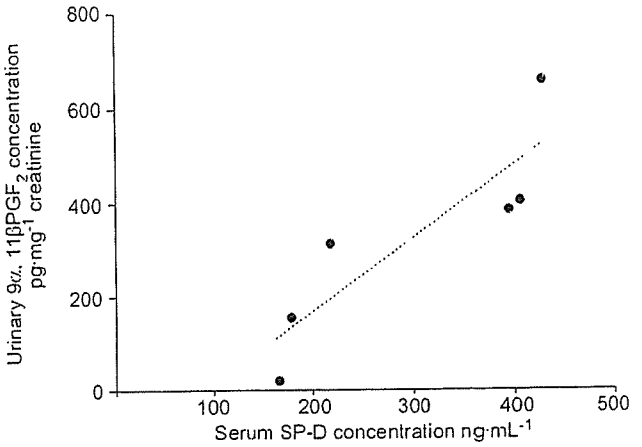

FIGURE 2. A significant correlation between serum surfactant protein D (SP-D) and urinary 9α,11β prostaglandin F$_2$ (9α,11βPGF$_2$) concentration was found in the extrinsic allergic alveolitis patients. $rs=1$; $p<0.05$.

DISCUSSION

The present authors demonstrated, for the first time, that acute EAA is characterised by eicosanoid overproduction. Although increased urinary LTE_4 concentration has been reported in patients with ARDS [21], to the current authors' knowledge this is the first report demonstrating a PGD_2 overproduction in patients with ARDS or even acute EAA. The CysLT and PGD_2 overproduction may well be associated with the increased cyclooxygenase activity of both alveolar macrophages [6] and mast cells [5]. However, the higher urinary 9α,11βPGF$_2$ concentrations in EAA patients are mainly considered to be a reflection of mast cell activation [5, 22]. Interestingly, mast cells activated by aggregated IgG, following IFN-γ-induced up-regulation of Fcγ receptor I, can produce both PGD_2 and CysLTs [9], which is consistent with the pathophysiology of EAA.

8-isoPGF$_2$α is an accurate biomarker of oxidative stress *in vivo* [2]. The present study demonstrated that urinary 8-isoPGF$_2$α concentration is increased in acute EAA patients, suggesting a central role for oxidant stress in the pathogenesis of acute EAA. The isoprostane pathway also contributes to the PGD_2 overproduction in acute EAA [2], although the current approach was unable to ascertain its relative contribution to PGD_2 production. It has recently been discovered that isoprostanes containing D- or E-type prostane rings are excreted into the urine as conjugates with *N*-acetyl cysteine sulfoxide, suggesting that these metabolites may be used as biomarkers to estimate whole-body production of D- or E-type isoprostanes [23]. Future experiments using this methodology will hopefully provide even more answers.

Increased 8-isoPGF$_2$α concentrations have been reported in the breath condensate of patients with ARDS [24], ILD [25] and asthma [26]. Recently, WOOD *et al.* [27] demonstrated that despite high variability, sputum 8-isoPGF$_2$α concentrations were significantly increased in patients with severe persistent asthma. The present study also demonstrated similar findings in that the two asthmatic patients with extremely high urinary 8-isoPGF$_2$α concentrations (458 and 525 pg·mg^{-1}-creatinine,

respectively) were characterised by severe persistent asthma and hypereosinophilia (11.2 and 17.2%, respectively).

These eicosanoids are also known to possess various other biological activities, such as being potent constrictors of pulmonary vascular smooth muscle and causing plasma exudation [4–6]. Although the full extent of the biological activity of the eicosanoids in acute EAA remains to be determined, the various components of the eicosanoid metabolic pathways may become therapeutic targets in acute EAA.

Consistent with a previous case report by TANAKA *et al.* [17], serum SP-D concentrations in the acute EAA patients were markedly increased. In contrast, serum SP-D concentrations in the asthmatic patients were low, which is in accordance with data by KOOPMANS *et al.* [28] Interestingly, the serum SP-D concentrations subsequently decreased and showed a significant correlation with urinary $9\alpha,11\beta PGF_2$ concentrations in the EAA patients. Serum SP-D is a biomarker of ILD activity [12] and SP-D plays a protective role in pulmonary inflammation [13]. PGD_2 and its metabolite, 15-d-PGJ_2, have the potential to serve as downregulators of lung injury induced by bleomycin [29]. Taken together, these findings suggest that both SP-D and PGD_2 appear to be important regulatory factors in the pathophysiology of EAA.

In conclusion, the present study proposes that serum surfactant protein D and urinary eicosanoids are new biomarkers involved in various immunological responses in extrinsic allergic alveolitis. Further large-scale studies are needed to investigate the role of these compounds, not just as biomarkers, but also as potentiators of extrinsic allergic alveolitis.

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ORIGINAL INVESTIGATION

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Functional promoter polymorphism in the *TBX21* gene associated with aspirin-induced asthma

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Abstract Asthma is a phenotypically heterogeneous disorder with many etiologic factors and clinical characteristics. T-bet, a Th1-specific transcription factor of T-box family, has been found to control interferon-γ (IFN-γ) expression in T cells. Mice lacking the T-bet gene (*tbx21*) demonstrate multiple physiological and inflammatory features reminiscent of human asthma. In order to examine whether polymorphisms in the candidate gene, *TBX21*, located on chromosome 17q21.32, are related to the risk of human asthma phenotypes, we have searched for genetic variations in the human *TBX21* gene and identified 24 single nucleotide polymorphisms (SNPs), including five novel SNPs, by direct sequencing in Japanese subjects. Among asthma phenotypes, a promoter −1993T → C SNP, which is in linkage disequilibrium with a synonymous coding 390A → G SNP in exon 1, is significantly associated with a risk of aspirin-induced asthma (AIA; $P=0.004$, $P_c=0.016$). This association has also been confirmed in additional independent samples of asthma with nasal polyposis ($P=0.008$), regardless of aspirin hypersensitivity. Furthermore, our data indicate that the −1993T → C substitution increases the affinity of a particular nuclear protein to the binding site of *TBX21* covering the −1993 position, resulting in increased transcriptional activity of the *TBX21* gene. Thus, in addition to the antigen-driven excess Th2 response, increased T-bet (and subsequent IFN-γ)

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production in human airways of individuals with the −1993T → C polymorphism could contribute to the development of certain asthma-related phenotypes, such as AIA.

Introduction

Asthma is defined as a chronic inflammatory lung disease that is characterized by airway hyperreactivity, eosinophil inflammation, and mucus hypersecretion resulting in intermittent airway obstruction (Busse and Lemanske 2001). A considerable increase has been noted in the incidence of allergic diseases including asthma in industrialized societies over the past three decades (Bach 2002; Woolcock and Peat 1997). The etiology of asthma is complex and multifactorial; development of the disease is controlled by both host genetic factors and a variety of environmental exposures. Although environmental influences, particularly a decrease in infections because of improved hygiene, might have increased allergic diseases, at least a dozen polymorphic genes have been calculated to regulate asthma, by controlling the inflammatory response, immunoglobulin E (IgE), cytokine, and chemokine production, and airway remodeling (Cookson 1999; Fahy et al. 2000; Umetsu et al. 2002).

Asthma is thought to arise from an imbalance in T helper type 1 (Th1)-Th2 immune regulation, resulting in the driving of the development of Th2-biased immune responses and the overproduction of cytokines such as interleukin 4 (IL-4), IL-5, IL-9, and IL-13, which mediate allergic inflammation (Renauld 2001; Umetsu et al. 2002). In contrast, Th1-type cytokine interferon-γ (IFN-γ) is essential for macrophage activation in cellular defense mechanisms, and IFN-γ-producing Th1 cells have been suggested to protect against allergic responses by dampening the activity of Th2 effector cells (Renauld 2001). However, the evidence from other in vivo studies of asthma conflicts with this hypothesis, suggesting a contribution of IFN-γ to asthmatic airway inflammation (Busse and Lemanske 2001; Salvi et al. 2001).

T-bet is a member of the T-box family of transcription factors that has been found to be expressed in IFN-γ-producing Th1, but not in Th2, cells. T-bet is a transcriptional regulator essential for the lineage commitment of Th1 cells by directly activating Th1-associated genetic programs and repressing Th2 cytokine production (Szabo et al. 2000). Recently, evidence has shown decreased numbers of $CD4^+$ T cells expressing T-bet in the airways of patients with allergic asthma, relative to control subjects (Finotto et al. 2002). Furthermore, deletion of the T-bet gene, *tbx21*, in mice results in airway eosinophilia, Th2 cytokine production, airway hyperresponsiveness (AHR), and changes of airway remodeling without allergen sensitization and challenge. Thus, T-bet-deficient mice demonstrate multiple physiological and inflammatory features reminiscent of human asthma (Finotto et al. 2002).

The human T-bet gene (*TBX21*) is located on chromosome 17q21.32, a region near to that linked with asthma in a genome screen for asthma and skin tests (Dizier et al. 2000; Zhang et al. 1999). Moreover, the region on mouse chromosome 11 that is syntenic to human chromosome 17q12-q22 has been linked to AHR (Zhang et al. 1999). So far, to our knowledge, there have been no reports showing disease-related polymorphism(s) in the *TBX21* gene. Based on these observations, we propose that genetic polymorphism contributes to susceptibility to human asthma and/or related phenotypes. To test this hypothesis, we have searched for polymorphisms in *TBX21* and then conducted a genetic association study in the Japanese population. Finally, we have investigated the functional consequences of disease-related polymorphisms.

Materials and methods

Subjects

We recruited 361 patients with childhood asthma (mean age 9.7 years, range 4–15 years, mean total serum IgE level, 1021 U/ml; 92% of whom were atopic), 313 adult patients with atopic asthma (mean age 49 years, range 20–81 years; mean total IgE, 775.7 U/ml), and 88 adult patients with non-atopic asthma (mean age 59 years, range 42–75 years; mean total IgE, 174.8 U/ml) from the Osaka Prefectural Habikino Hospital and the Miyatake Asthma Clinic. Patients with aspirin-induced asthma (AIA; mean age 53 years, range 24–73 years; 54% of whom were atopic; ≥50% had nasal polyposis) were recruited from the National Sagamihara Hospital. All patients with asthma were diagnosed according to the criteria of the National Institutes of Health, with minor modifications (National Heart, Lung, and Blood Institute, National Institutes of Health, 1997, http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.htm). The diagnosis of atopic asthma was based on the positive immunoassay test to common allergens (at least one of the following: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Aspergillus fumigatus*) or a higher total serum IgE of ≥400 kU/l, as used in our previous study (Mao et al. 1996). The criteria for a diagnosis of non-atopic asthma was a total serum IgE of < 400 kU/l and the absence of allergen-specific IgE (≤ 0.35 kU/l). All patients with AIA were documented to have histories of asthmatic attacks, such as severe bronchoconstriction and nasal symptoms, following the ingestion of more than two different kinds of non-steroidal anti-inflammatory drugs (NSAIDs) or to have had a positive reaction to aspirin systemic challenge. Of 72 AIA patients (58%), 42 were diagnosed on the basis of the aspirin challenge test, as previously described (Kawagishi et al. 2002; Mita et al. 2001). We also

independently recruited 42 asthmatic patients with nasal polyposis (AS/NP; mean age 54 years, range 23–75 years; 73% of whom were atopic). The NP was diagnosed on the basis of history, including nasal symptoms, clinical examination, nasal endoscopy, and sinus computerized tomography scanning. All adult asthmatics, except AIA patients, had no past history of aspirin hypersensitivity. Controls were 640 randomly selected healthy individuals with an age range 18–83 years from the same geographic areas who had neither respiratory symptoms nor history of asthma-related diseases and aspirin hypersensitivity. All subjects in this study were ethnically Japanese and gave written informed consent to participate in the study (or, for individuals less than 16 years old, their parents gave consent), according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama.

Screening for polymorphisms and genotyping

To identify single-nucleotide polymorphisms (SNPs) in the human *TBX21* gene, we sequenced all six exons, including a minimum of 200 bases of flanking intronic sequence, 2.2 kb of the 5′ flanking region, and 2.5 kb continuous to the 3′ flanking region of the sixth exon from 24 asthmatic subjects (12 unrelated children and 12 adults). Eighteen primer sets were designed on the basis of the *TBX21* genomic sequence from the GenBank database (accession number AC003665; Table 1). For each polymerase chain reaction (PCR), 5 ng genomic DNA was amplified in a total reaction volume of 10 μl containing 12.5 pmol each primer, 3.9 mM $MgCl_2$, 1.25 mM each dNTP, 0.5 U *Taq* polymerase. Cycling conditions were an initial 95°C for 2 min, followed by 37 cycles at 94°C for 30 s, 58°C or 60°C for 30 s, and 72°C for 3 min, with a final extension of 7 min at 72°C. Each fragment amplified by PCR was sequenced by using the BigDye Terminator (Applied Biosystems, Foster City, Calif., USA) on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The sequences were analyzed, and polymorphisms were identified by using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Mich., USA). Four selected SNPs, viz., −1993T → C, 99C → G, 1298T → C, and 7725T → C, were genotyped by three methods: PCR-RFLP (PCR-restriction fragment lenght polymorphism; for −1993T → C and 1298T → C), Invader assay (for 99C → G), and direct sequencing (for 7725T → C). For PCR-RFLP analysis, we used mismatched primers for the −1993T → C SNP (5′-GGTCTTACTGAAAGCTCTCA-3′ and 5′- TCTCCTCCCCAACACCTTACGC-3′) and for the 1298T → C SNP (5′-GGCTAGTGCAGTAAAGCTTG-3′ and 5′-GGTTTTCACTGGACCAGCCGC-3′) where the changed nucleotides are underlined. The amplified products were digested with *Hha*I (−1993T → C) or *BstU*1 (1298T → C) restriction enzymes (New England Biolabs, Bevery, Mass., USA) according to the manufacturer's instructions and were separated by electrophoresis on 4% agarose gels. Based on information available from the public JSNP database (http://snp.ims.u-tokyo.ac.jp), we generated the 99C → G SNP (IMS-JST000934) genotypes by using the Invader assay as previously described (Ohnishi et al. 2001). For the 7725T → C SNP, we performed direct sequencing with primers 5′-TTATCCAGGGTCATAGGGTAG-3′ and 5′-CCTCAGCCTTTAGAGAAGTTG-3′.

Luciferase assay

We generated luciferase reporter constructs, pGL3/−1993T and pGL3/−1993C, by cloning three concatenated copies of a 20-bp fragment of the *TBX21* gene into pGL3-Basic vector (Promega, Madison, Wis., USA) in the *Nhe*I site. The 20-bp primer sets carrying −1993T or −1993C alleles were 5′- CTAGCGGAGAAATGGTGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCACCCATTCCA AGATC-5′ (reverse) or 5′- CTAGC

Table 1 Primer sequences used in screening for SNPs of the human *TBX21* gene

F1	5′-TTTCCAGTAATAGCCGCTCCT-3′	R1	5′-CACAGCCTAGACACTGGTTC-3′
F2	5′-TTGCATAGTTACCATCCACCG-3′	R2	5′-GACCTTGGGATCCTTCACTAC-3′
F3	5′-AAGACTCCATTTGATCTTCAAC-3′	R3	5′-TTCACTCCACAAGGTGTCATG-3′
F4	5′-GTCAGGCTGGGACAGAAATG-3′	R4	5′-TGAGTTGGCTGCATCTTGTAG-3′
F5	5′-CTGGCTGCTGCTGATGCAG-3′	R5	5′-TGTCACTAGAGTCGCAGCGC-3′
F6	5′-AGTACTCGCCAAGAGCGTAG-3′	R6	5′-AAAAACAGACGAGACGTTCTTG-3′
F7	5′-TCGCGCTCAACAACCACCTG-3′	R7	5′-CTCAAAGTAAGACCGGAAAGG-3′
F8	5′-GGCTAGTGCAGTAAAGCTTG-3′	R8	5′-GACCAGAAGCTTGGGCTGTG-3′
F9	5′-CTCTGTTGTGTGGTCAGGAG −3′	R9	5′-TGAGAAGGTATGGAGGTAACC-3′
F10	5′-TTGAAGGAGGCAGTGGCTC-3′	R10	5′-AACACAGCTACCCAAAGTTATC-3′
F11	5′-TTATCCAGGGTCATAGGGTAG-3′	R11	5′-CCTCAGCCTTTAGAGAAGTTG-3′
F12	5′-TAACTTCCTCTACTTTTCCTGG-3′	R12	5′-AAACATCCTGTAGTGGCTGG-3′
F13	5′-TGCCTGGGCACTGTTGCAG-3′	R13	5′-GAAAAACGAACCTTCCTTCCTG-3′
F14	5′-CAACAATGTGACCCAGGTAG-3′	R14	5′-CAAGCTTTCCAACTCCAGTG-3′
F15	5′-GCCCTGTTTGTGCTGATACC-3′	R15	5′-CACAAGCAGAACCAGTCACC-3′
F16	5′-TGGGTTCAACTCAGCTTTGGT −3′	R16	5′-CTTTCATCATGTCATCTGCTC-3′
F17	5′-GCGAAGGAGACTCTAAGAGG-3′	R17	5′-TCTTGCTTCTTGAGATGTGGG-3′
F18	5′-CACGTATGTTATAACCATCAGC-3′	R18	5′-AGAGATAAAGGTGGAGGGCTG-3′

GGAGAAATGGCGGGTAAGGT T-3′ (forward) and 3′- GCCTCTTTACCGCCCATTCCA AGATC-5′ (reverse), respectively, where the added nucleotides for the *Nhe*I site are underlined. HEK293 or HeLa cells were cultured in growth medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Subconfluent cells cultured in 12-well plates were transiently co-transfected with 0.5 μg pGL3-Basic vector DNA or each reporter construct (pGL3/−1993T or pGL3/−1993C) and 10 ng pRL-TK vector DNA (Promega) as an internal control for transfection efficiency, by using 1.5 μl FuGENE six transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we then lysed the cells and measured firefly and *Renilla* luciferase activities in a luminometer by using the Dual-Luciferase Reporter Assay System (Promega).

Electrophoretic mobility shift assay

Nuclear extracts were prepared from HEK293 and HeLa cells as described previously (Dignam et al. 1983). Double-stranded oligonucleotides −1993T and −1993C were obtained by annealing three concatenated copies of 5′-GAAATGGTGGGTAAG-3′ and 5′-GAAATGGC-GGGTAAG-3′ with their respective complementary oligonucleotides. Electrophoretic mobility shift assay (EMSA) analysis was performed by using DIG gel shift kit (Roche). We prepared digoxigenin (DIG)-labeled double-stranded oligonucleotides corresponding to the sequence at position −2000 to −1986 of the *TBX21* promoter containing the −1993 polymorphism. For each binding reaction, we incubated DIG-labeled probes with nuclear extract (2–5 μg) in 1× binding buffer (20 mM HEPES, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitole, 30 mM KCl), 1 μg poly (dI-dC), and 0.1 μg poly L-lysine for 30 min on ice. For competition studies, we incubated unlabeled double-stranded oligonucleotide (100-fold molar excess) during preincubation. Reaction products were separated on 6% non-denaturing polyacrylamide gels in 0.3× TBE buffer (1× TBE buffer = 0.09 M TRIS-borate, 0.002 M EDTA, pH 8.3) and visualized by chemiluminescent detection. We scanned results into an LAS-3000 CCD camera system (Fuji Photo Film, Tokyo, Japan) and quantified each band intensity by using image analysis software Image Gauge Version 2.0 (Fuji Photo Film).

Statistical analysis

We calculated allele frequencies and tested agreement with Hardy–Weinberg equilibrium by using a χ^2 goodness of fit test at each locus. We then compared differences in allele frequencies and genotype distribution of each polymorphism between case and control subjects by using a 2×2 contingency χ^2 test with one degree of freedom or Fisher exact test and calculated odds ratios (ORs) with 95% confident intervals (95% CI). For multiple comparisons, *P*-values were corrected by the Bonferroni method. The linkage disequilibrium (LD) statistic *D′* was calculated by using the SNP Alyze statistical package (Dynacom, Chiba, Japan) as described elsewhere (Nakajima et al. 2002). Comparisons in reporter assays and EMSA experiments were performed with the Student's *t* test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Screening for common polymorphisms in TBX21

Direct DNA sequencing of the indicated regions in 12 asthmatic and 12 healthy subjects (total 24 subjects) identified 24 biallelic SNPs in *TBX21*: three in the 5′ flanking region, three in the coding region (one non-synonymous and two synonymous), three in the 3′ untranslated region, and 15 in the intron (Table 2, Fig. 1). Five of these 24 SNPs (532G → C, 729G → T, 2839G → A, 9408C → A, and 10143C → A) are novel, and another 14 have been reported recently in Korean (Chung et al. 2003) and Finnish (Ylikoski et al. 2004) populations. Nucleotide position one (+1) is the first adenine of the initiation codon (ATG), and the positions for other SNPs are relative to the ATG on genome contig AC003665. A graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene is given in Fig. 1. Since most of the SNPs were of relatively low frequency and in view of their location and LD with other sites, further genotyping and association studies in our asthma population focused on four SNPs: −1993T → C, 99C → G, 1298T → C, and 7725T → C. The distributions of all four SNPs were in Hardy–Weinberg equilibrium in the control group ($P > 0.05$). We calculated both *D′* and r^2 as statistical values for LD pair-wise between each SNP (Fig. 2). One of the three promoter SNPs (−1993T → C) and one synonymous coding SNP (390A → G, G130G) in exon one, were shown to be in strong LD.

TBX21 genotyping and association studies in asthma and related phenotypes

Initially, the association study was carried out on four clinical groups: child patients with asthma ($n = 361$), adult patients with atopic asthma ($n = 313$), adult patients with non-atopic asthma ($n = 88$), and adult patients with AIA ($n = 72$). Adult asthmatics, except AIA patients, had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Allele frequencies of each selected SNP were compared between the patients and the normal controls by using a χ^2 test with 1 d.f. (Table 3). After correction for the number of SNPs investigated (Bonferroni correction), we found a significant association between the promoter SNP at −1993 and AIA in our Japanese cohort ($P = 0.004$;

Table 2 Locations and allele frequencies of *TBX21* SNPs in Japanese (*UTR* untranslated region)

Number	SNP	Location	Amino acid substitution	Minor allele frequency (%)	Primers
1	−1993T → C[a]	Promoter	–	8.3	F3R3
2	−1514T → C	Promoter	–	7.1	F4R4
3	− 1499G → A	Promoter	–	2.4	F4R4
4	99C → G[a]	Exon 1	H33Q	6.3	F6R6
5	390A → G	Exon 1	G130G	8.3	F6R6
6	532G → C	Intron 1	–	2.2	F7R7
7	729G → T	Intron 1	–	4.2	F7R7
8	1298T → C[a]	Intron 1	–	16.7	F8R8
9	1662C → A	Intron 1	–	2.1	F8R8
10	2608G → A	Intron 1	–	2.1	F9R9
11	2839G → A	Intron 1	–	4.2	F9R9
12	7725T → C[a]	Intron 1	–	18.8	F11R11
13	8381A → T	Intron 1	–	16.7	F12R12
14	8762G → C	Intron 1	–	4.2	F12R12
15	9408C → A	Intron 2	–	2.3	F13R13
16	9882C → T	Intron 3	–	4.2	F14R14
17	9898C → T	Intron 3	–	4.2	F14R14
18	10143C → A	Intron 3	–	2.1	F14R14
19	10150T → C	Intron 3	–	4.2	F14R14
20	11268C → T	Intron 5	–	4.2	F16R16
21	11755G → A	Exon 6	P485P	2.1	F16R16
22	12077T → C	3′UTR	–	4.2	F17R17
23	12211G → A	3′UTR	–	2.1	F17R17
24	12403A → C	3′UTR	–	4.2	F17R17

[a]These SNPs were genotyped in a larger population

corrected P, $P_c = 0.016$). There was an increased risk for AIA associated with a C allele (OR = 1.93; 95% CI 1.22–3.06). Association analysis also demonstrated that a significant difference in allele frequency of −1993 SNP between AIA and other adult asthmatics who had no past history of aspirin hypersensitivity ($P = 0.001$). No other statistically significant association between disease status and genotype or any specific allele was detected from any of the other three case-control disease-association comparisons. We further analyzed the genotype and allele frequencies of the SNP at position 390, which was found to be in LD with SNP at −1993, in the AIA and control groups and then calculated the LD coefficient D' and r^2 between the −1993T → C and 390A → G SNPs ($D' = 0.92$; $r^2 = 0.85$). ORs of developing AIA at −1993T → C and 390A → G were 2.15 (95% CI 1.26–3.64) and 2.19 (95% CI 1.27–3.77), respectively, when the TC and TT (AG and GG) genotypes were compared with the wild-type TT (AA) genotype (Table 4). To determine whether these two SNPs in *TBX21* could also be associated with another AIA-related phenotype, we analyzed −1993T → C and 390A → G SNPs in 42 samples from independent adult AS/NP who either had a negative reaction to the aspirin challenge or no past history of aspirin hypersensitivity. Interestingly, comparison of the genotype and allele frequencies also revealed significant differences between the AS/NP group and the normal control group ($P = 0.008$ and 0.012, respectively). Furthermore, in the AIA case group, the C-allele frequency in AIA patients with NP tended to be much higher than that in AIA patients without NP (data not shown). Thus, although the sample size was small, we confirmed the *TBX21* SNP effect by using independent samples of AS/NP, regardless of aspirin hypersensitivity. We further analyzed two-loci haplotype distributions constituting the −1993T → C and 390A → G SNPs in the control, AIA, and AS/NP samples. Haplotype −1993T-390A was the

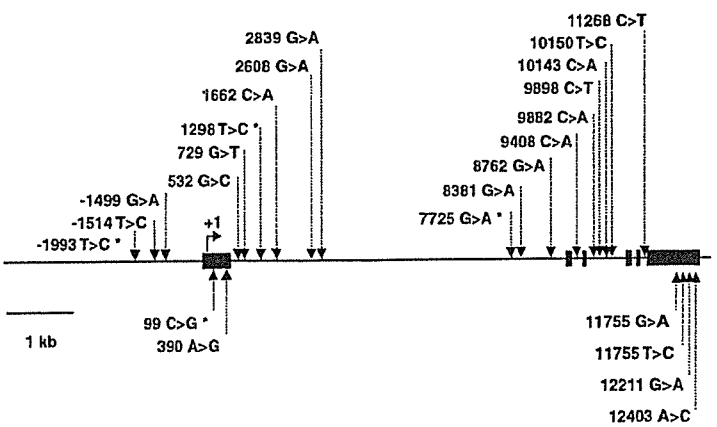

Fig. 1 Graphical overview of 24 SNPs identified in relation to the exon/intron structure of the human *TBX21* gene (*black boxes* five coding exons, *asterisks* SNPs genotyped in a larger population). Positions for SNPs are relative to the translation start site (+1)