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FcεRI-mediated thymic stromal lymphopoietin production by interleukin-4-primed human mast cells

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ABSTRACT: A significant increase of mRNA expression of thymic stromal lymphopoietin (TSLP) has been reported in the bronchial mast cells (MCs) of asthmatic subjects; however, the mechanism underlying the upregulation of TSLP mRNA and protein remains unknown.

FcεRI-mediated activation of human MCs upregulated TSLP mRNA expression by 5.2±2.9-fold, while activation of the MCs using lipopolysaccharide and polyriboinosinic:polyribocytidylic acid failed to upregulate TSLP. Stimulation of MCs with interleukin (IL)-4 alone did not affect the TSLP mRNA expression, while pre-incubation of MCs with IL-4 for 48 h significantly enhanced the FcεRI-mediated TSLP mRNA expression (by 53.7±15.9-fold; $p<0.05$) and the amount of TSLP in the cell pellets increased significantly from 23.4±4.3 pg·mL^{-1} to 121.5±3.7 pg·mL^{-1} ($p<0.0001$). However, the released TSLP was rapidly degraded by proteases that were released by MCs. We identified the population of cells expressing TSLP in the lungs of 16 asthmatic and 11 control subjects by immunohistochemistry. The percentage of TSLP-positive MCs in the total population of MCs was significantly increased in asthmatic airways ($p<0.0001$).

Thus, MCs are able to store TSLP intracellularly and to produce TSLP following aggregation of FcεRI in the presence of IL-4.

KEYWORDS: Asthma, FcεRI, human, interleukin-4, mast cells, thymic stromal lymphopoietin

Bronchial asthma is characterised by airway inflammation, with predominant infiltration by eosinophils and CD4+ T-lymphocytes [1]. The pulmonary CD4+ cells of asthmatic patients predominantly produce T-helper cell (Th) type 2 cytokines, including interleukin (IL)-4, IL-5, IL-9 and IL-13, which play essential roles in the pathogenesis of asthma by enhancing the growth, differentiation and recruitment of eosinophils, basophils, mast cells (MCs) and immunoglobulin (Ig)E-producing B-cells, and by directly inducing airway hyperreactivity [2]. Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that supports the growth and differentiation of B-cells and the proliferation of T-cells [3, 4]. TSLP has recently drawn attention because it has been shown to be capable of triggering dendritic cell (DC)-mediated Th2 inflammatory responses [4, 5]. Lung-specific overexpression of TSLP in mice is associated with airway inflammation and hyperreactivity characteristic of that induced by Th2 cytokines, and increased IgE [6], and TSLP receptor knockout mice fail to mount inflammatory lung responses to inhaled antigens [7]. In humans, the number of cells expressing TSLP mRNA in the bronchial epithelium and submucosa has been found to be significantly increased in asthmatic subjects in comparison with that in healthy controls [8]. Human TSLP has been shown to be produced by airway epithelial cells through the mediation of Toll-like receptor (TLR)3 [9]. TSLP activates CD11c+ DCs and induces Th2-attracting chemokines, such as thymus- and activation-regulated chemokines (also known as CC chemokine ligand (CCL)17) and macrophage-derived chemokines (CCL22) [5]. TSLP-activated DCs prime naïve Th2 cells to produce IL-4, IL-5, IL-13 and tumour necrosis factor (TNF)-α but not IL-10. TSLP also induces human DCs to express the OX40 ligand but not IL-12 [10]. It conditions the DCs to support homeostatic proliferation of central memory Th2 cells [11]. Therefore, TSLP produced by human bronchial mucosal epithelial cells not only triggers DC-mediated allergic inflammation but also maintains and further polarises central memory Th2 cells in allergic diseases.

MCs are known to be the primary cells involved in allergic reactions, and are, for the most part, activated by cross-linking of the high-affinity IgE

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receptor, FcεRI, expressed on their cell surface [12, 13]. After activation, the MCs exert their biological effects by releasing preformed as well as *de novo*-synthesised mediators, such as histamine, leukotrienes and various other cytokines/chemokines [13]. Furthermore, human MCs have been reported to express functional TLRs, including TLR2, TLR3 and TLR4 [14–16]. A significant increase in the number of MCs expressing TSLP mRNA has been shown in the bronchial epithelium and submucosa of asthmatic subjects in comparison with that in healthy controls [8]. However, the mechanism underlying the upregulation of TSLP mRNA expression under these conditions remains unknown. Whether the MCs themselves produce the TSLP protein is also unknown.

We therefore examined the mechanisms underlying the upregulation of TSLP mRNA and protein expression in human MCs *in vitro*, and compared the TSLP protein expression level in the bronchial mucosa between asthmatic and control subjects.

MATERIALS AND METHODS

Generation of adult peripheral blood-derived MCs

Human peripheral blood mononuclear cells (PBMNCs) were separated from venous blood samples of normal volunteers. All the human subjects enrolled in the present study provided written informed consent for participation, and the study was conducted with the approval of the ethical review board of each hospital (Nihon University Hospital, Tokyo, Japan, and National Research Institute for Child Health and Development, Tokyo, Japan). The PBMNCs were isolated by centrifugation on a Ficoll–Isopaque density gradient (Nycomed, Oslo, Norway). Lineage-negative MNCs were selected from the PBMNCs and cultured in serum-free Iscove methylcellulose medium (Stem Cell Technologies Inc., Vancouver, BC, Canada) and Iscove's modified Dulbecco's medium (IMDM) containing 200 ng·mL^{-1} recombinant human stem cell factor (rhSCF), 50 ng·mL^{-1} recombinant human (rh)IL-6 (PeproTech EC Ltd, London, UK) and 1 ng·mL^{-1} rhIL-3 (Intergen, Purchase, NY, USA), as previously described [14]. On day 42 of culture, the methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in IMDM containing 100 ng·mL^{-1} rhSCF, 50 ng·mL^{-1} rhIL-6 and 2% fetal calf serum. About 93–99% of MCs cultured at 13 weeks old expressed FcεRI on their surface. The MCs also showed expression of 2×10^5 copies of tryptase mRNA and 3×10^3 copies of chymase mRNA per ng of total RNA. About 35–50% of the MCs expressed chymase.

Activation of the human MCs

The MCs were sensitised by incubation at 37°C for the indicated time period with 1 μg·mL^{-1} rh myeloma IgE (CosmoBio, Tokyo, Japan), in the presence or absence of IL-4 (10 ng·mL^{-1}; R&D Systems Inc., Minneapolis, MN, USA). The cells were then washed and challenged with rabbit anti-human IgE antibody (Ab; 3 or 15 μg·mL^{-1}; Dako, Kyoto, Japan), a calcium ionophore A23187 (10^{-6} M; Sigma–Aldrich, St Louis, MO, USA), or culture medium alone at 37°C for the indicated time period. After incubation, the cell-free culture supernatants and cell pellets were harvested for measurement of the cytokine protein levels by ELISA and of the TSLP mRNA level by reverse transcriptase (RT)-PCR, respectively. For all the experiments, the cells were suspended in complete IMDM containing rhSCF and rhIL-6.

Real-time quantitative RT-PCR

The changes in the TSLP mRNA expression level in the MCs were measured by TaqMan (Applied Biosystems, Foster City, CA, USA) analysis, as previously described [17]. Human gene-specific primers and probe sets for TSLP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using the Assays-on-Demand^{TM} service (Applied Biosystems). The changes in the TSLP mRNA expression level normalised to GAPDH mRNA level were monitored at the indicated time point. Triplicate samples of cells were collected at each time point. Real-time PCR was performed on the corresponding cDNA synthesised from each sample. Threshold cycle (Ct), which has been shown to be inversely correlated with the target mRNA expression level, was defined as the cycle number at which the reporter fluorescence emission increased above the midpoint along the amplification curve. The data were analysed using an equation (amount of target gene=$2^{-\Delta\Delta C_t}$) [18]:

$$\Delta\Delta C_t = (C_{t,TSLP} - C_{t,GAPDH})Time_x - (C_{t,TSLP} - C_{t,GAPDH})Time_0$$

$Time_x$ is any time point and $Time_0$ represents the 1× expression of TSLP mRNA level normalised to GAPDH mRNA level.

Effect of protease inhibitors on MC supernatant-mediated TSLP degradation

rhTSLP (R&D Systems Inc.) was incubated for 16 h with 100 μL of medium alone, the supernatant of unstimulated MCs, or the supernatant of stimulated MCs in the presence or absence of protease inhibitors (Sigma–Aldrich). The supernatant of unstimulated MCs was obtained from resting MCs (1.5×10^5·mL^{-1}) cultured for 16 h, and the supernatant of stimulated MCs was obtained from MCs (1.5×10^5·mL^{-1}) that had been activated with anti-IgE for 16 h.

Preparation of MC lysates

For TSLP ELISA, the cells were resuspended in ice-cold lysis buffer (1% NP-40) containing protease inhibitors and kept on ice for 5 min prior to centrifugation at 20,800 × g to remove the cell debris.

ELISA

Human I-309 and TSLP were measured with ELISA kits purchased from R&D Systems, Inc. The sensitivities of the assays for human I-309 and TSLP were 15.625 pg·mL^{-1} and 7.8 pg·mL^{-1}, respectively. A standard curve for TSLP (7.8–500 pg·mL^{-1}) was constructed using computer software capable of generating a four-parameter logistic curve-fit. The data were linearised by plotting the logs of the TSLP concentrations (7.8–500 pg·mL^{-1}) against the logs of the optical density values.

Flow cytometric analyses

For intracellular TSLP or TNF-α staining, after incubation with BD GolgiStop^{TM} (BD Biosciences, Tokyo, Japan) according to the protocol, the MCs were fixed, permeabilised, and suspended in blocking medium, as previously described [14]. The MCs were then stained for 20 min at 4°C in the dark with anti-TSLP Ab (R&D Systems Inc.), phycoerythrin (PE)-conjugated anti-TNF-α monoclonal (m)Ab (BD PharMingen, Tokyo, Japan), or the

isotype control Ig (sheep IgG or PE-conjugated mouse IgG1) in the blocking medium. The cells were then washed and incubated with Alexa555-conjugated donkey anti-sheep IgG (1/200 dilution; Invitrogen, Tokyo, Japan), and washed again and analysed using the FACScalibur (BD Biosciences) and Cell Quest software (BD Biosciences). The mean fluorescence intensities of the MCs stained with the respective Abs and control Ab were determined.

Study population

The study was conducted with the approval of the Ethics Committee of Dokkyo Medical University School of Medicine (Tochigi, Japan), and written informed consent for participation was obtained from each of the subjects, in accordance with the Helsinki Declaration of the World Medical Association. In total, 16 asthmatic subjects (10 requiring step-2 asthma management, four requiring step-3 asthma management, and two requiring step-4 asthma management), in whom the disease severity was judged based on a combination of the severity of asthma symptoms and the frequency of occurrence of the symptoms, according to the criteria of the Japanese Society of Allergology (Tokyo) [19], and 11 healthy controls without asthma were studied. The demographic characteristics of the asthmatic subjects and healthy controls are shown in table 1. None of the subjects had experienced bronchial or respiratory tract infections during the month preceding the test. None of the asthmatic subjects had developed acute disease exacerbation within 3 months prior to his/her entry into the study. None of the subjects was a current smoker, and none had smoked during the previous 2 yrs.

Procedures of the human experiments

All of the asthmatic patients and healthy controls underwent blood tests, pulmonary function tests, measurement of airway responsiveness and fibreoptic bronchoscopy. Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which the airway resistance began to increase during continuous inhalation of acetylcholine in incremental concentrations [17]. Bronchial tissue samples were collected from the subcarinal region between the right lower lobe and middle lobe bronchi (origin of the right B6 bronchus) in the asthmatic subjects using a pair of standard forceps during fiberoptic bronchoscopy, as previously described [17]. Each biopsy specimen was immediately placed in optimal cutting temperature medium, snap-frozen in liquid nitrogen, and stored at -80°C until cryostat sectioning. The cells were dual-stained with fluorescein isothiocyanate-conjugated anti-tryptase mAb (clone AA1; Dako) and biotinylated sheep anti-human TSLP Ab (R&D Systems Inc.). TSLP+ cells were visualised by incubation with streptavidin. Sections were stained with Mayer's haematoxylin and Hansel's stain. TSLP+ cells were counted in at least six high-power fields of each sample by three independent observers.

Statistical analysis

Differences between paired groups were analysed by an unpaired t-test and considered significant at $p<0.05$. Correlations were analysed by calculation of Pearson's correlation coefficients. Values are expressed as mean±SEM.

RESULTS

IL-4 upregulated IgE-mediated TSLP mRNA expression in human MCs

Our initial microarray analysis of human cultured MCs revealed a very low level of TSLP transcripts in resting MCs (mean±SEM 1.7±0.4% relative to the GAPDH mRNA expression level, n=4) and upregulation of the TSLP transcript level following FcεRI aggregation (approximately three-fold, data

TABLE 1 Characteristics of the asthmatic subjects and controls

	Step 2	Step 3	Step 4	Control
Cases	10	4	2	11
Age yrs	35±18.2	42±13.4	42±27.6	50±14.3
Male:female	6:4	1:3	1:1	7:4
Clinical history month	53±72.3	101±188.9	150±127.3	NA
Atopic	6	3	2	0
Nonatopic	4	1	0	11
FEV_1 mL	2949±1197	1550±405	1385±765	2838±247
FEV_1 %	81.0±12.4	59.7±10.9	51.4±0.2	85.4±2.9
Airway responsiveness to acetylcholine μg·mL^{-1}	6218.8±2471.4	2031.5±115.7	174.3±277.0	⩾2000¶
Subjects	9	3	2	9
Inhaled corticosteroids#	10	4	2	0
Oral corticosteroids#	0	0	1	0
Long-acting β-agonists#	3	1	2	0
Leukotriene receptor antagonists#	0	1	2	0
Theophylline#	9	4	2	0
IgE U·mL^{-1}	332±189.1	215±71.0	1276±1024.5	79±45.5
Subjects	9	4	2	4

Data are presented as n or mean±SD, unless otherwise stated. FEV_1: forced expiratory volume in 1 s; Ig: immunoglobulin; NA: not available; #: medication taken by the subjects; ¶: the maximum value of the airway responsiveness to acetylcholine was 2000 μg·mL^{-1}.

not shown). We measured the TSLP mRNA level in the mRNA extracted from resting and IgE/anti-IgE-activated human MCs that had or had not been exposed to IL-4 for 48 h. Although the expression level of TSLP mRNA varied from donor to donor, FcεRI-mediated activation of human cultured MCs resulted in only a 5.2±2.9-fold increase of TSLP mRNA expression in the absence of pre-incubation with IL-4 (n=3; fig. 1a), while pre-incubation of the cells with IL-4 significantly increased the FcεRI-mediated TSLP mRNA expression by 53.7±15.9-fold ($p<0.05$, n=3; fig. 1a). We previously reported the expression of TLR4 in human lung MCs [14]. Human peripheral blood-derived cultured MCs have also been reported to express TLR3 [16]. Recent studies demonstrated the induction of TSLP production by primary human airway epithelial cells in response to polyriboinosinic:polyribocytidylic acid (polyI:C) [9, 20], we also examined the effect of lipopolysaccharide and polyI:C on the TSLP mRNA upregulation in interferon-γ-treated human cultured MCs; however, our results revealed that neither of these TLR ligands induced TSLP mRNA expression (data not shown).

We next analysed the kinetics of TSLP mRNA expression induced by FcεRI or stimulation with a calcium ionophore in IL-4-treated MCs (fig. 1b). After activation of the IL-4-pretreated MCs with anti-IgE, the TSLP mRNA expression level appeared to reach its peak at 6 h (donor a) or 8 h (donor b), and to wane by 16 h. Following stimulation with the calcium ionophore, the TSLP expression level increased gradually, peaked at 8 h, and remained detectable until 24 h. To clarify the effect of IL-4 alone on the TSLP mRNA expression level and the optimal incubation time with IL-4 for priming of the MCs for FcεRI-mediated TSLP mRNA expression, MCs were sensitised with IgE for 10 days and incubated in the presence of IL-4 for the final 0, 1, 5 or 7 days, or for all of the 10 days. After incubation with IgE and IL-4, the MCs were washed and challenged with anti-IgE. As shown in figure 1c, incubation with IL-4 alone, for any length of time, did not affect the TSLP mRNA expression level. After preincubation of MCs with IgE alone for 10 days (IL-4 incubation time, 0 day), the FcεRI cross-linking was clearly associated with upregulation of the TSLP mRNA expression. The FcεRI-mediated TSLP mRNA expression level varied from donor to donor and peaked after 5–10 days of exposure to IL-4. Thus, IL-4 clearly upregulated the FcεRI-mediated TSLP mRNA expression.

Next, we measured the TSLP protein levels in the supernatants and cell pellets of the activated MCs. Human MCs were pretreated with IL-4 and IgE for 2 days, and then challenged with anti-IgE for 16 h. Figure 1d shows the TSLP content of the cell pellets from three donors. After FcεRI aggregation, the TSLP content in the cells increased significantly from 23.4±4.3 to 121.5±3.7 $pg \cdot mL^{-1}$ ($p<0.0001$). However, no TSLP was detected in the supernatants of either the resting or anti-IgE-challenged MCs from any of the three donors in the present study (data not shown).

TSLP secretion from MCs following aggregation of FcεRI

As some cytokines released by MCs, including IL-6 and IL-13, have been reported to be cleaved by proteases that are also released by the MCs [21], we hypothesised that proteases produced by the activated MCs might degrade the TSLP in the cell supernatants. To confirm this hypothesis, rhTSLP was incubated with the supernatants of unstimulated and stimulated MCs in the presence or absence of protease inhibitors, and reduced immunoreactivity for TSLP was found in both the supernatants of the unstimulated MCs and the activated MCs (fig. 2a). Dimethyl sulfoxide (DMSO) was used as the vehicle in the negative controls, in place of the protease inhibitors. The protease inhibitors inhibited the reduction of TSLP immunoreactivity in the MC supernatants, although only partially. We found that the majority of the proteases were released within 1 h after FcεRI aggregation (data not shown). To detect immunoreactive TSLP produced by the activated MCs, we washed the MCs 1 h after FcεRI aggregation and resuspended the cells in a solution containing protease inhibitors (fig. 2b). We confirmed that some cytokines were degraded by proteases released by MCs (data not shown). Since I-309 was most susceptible, we measured the level of I-309 in the supernatant as a positive control. The level of I-309 in the protease inhibitor-treated supernatants of the IgE/anti-IgE-activated MCs was significantly increased as compared with that in the DMSO-treated supernatants of the IgE/anti-IgE-activated MCs (fig. 2c). When the cells were washed following incubation for 1 h with or without anti-IgE, and then incubated with protease inhibitors for 15 h, TSLP was detected in the supernatants of both the resting and activated MCs in four of the seven donors examined. Figure 2d shows the mean±SEM for the four donors in whom TSLP was detected in the supernatants of the MCs. When the cells were washed following incubation for 1 h with anti-IgE and resuspended in a solution without protease inhibitors, TSLP was still detected in the supernatants of the activated MCs, suggesting that some other factor(s) produced by the MCs within 1 h of activation may also be responsible for the degradation of TSLP. We tried to measure the TSLP in the supernatants of long-term (5- to 7-day) IL-4-pretreated MCs following FcεRI aggregation; however, there was no significant increase in the TSLP immunoreactivity (data not shown), presumably because the TSLP was degraded by proteases and some other factors released by the activated MCs; as a result, it was difficult to measure the precise concentration of TSLP released by the MCs. Therefore, we assessed the TSLP production by IgE/anti-IgE-activated MCs using monensin. The addition of BD GolgiStop™, a protein transport inhibitor containing monensin, to IgE/anti-IgE-activated MCs would block the intracellular protein transport process, which would result in the accumulation of cytokines in the Golgi complex. The percentage of TNF-α+ MCs, measured as the positive control, increased from 12.9 to 44.1% following cross-linking of FcεRI (fig. 3). We confirmed that the percentage of TSLP+ MCs also increased from 6.1 to 21.1% following cross-linking of FcεRI (fig. 3).

Upregulation of FcεRI-mediated TSLP mRNA expression by IL-4

As IL-4 upregulated FcεRI-mediated TSLP mRNA expression in human MCs, we determined whether this might be due to enhanced cell-surface expression of FcεRI on the MCs. We compared the cell surface expression level of FcεRI between MCs treated with IgE for 7 days, and MCs treated with IL-4 for 7 days and incubated with IgE for the final day, by fluorescence-activated cell sorter (FACS; fig. 4a). The FcεRI

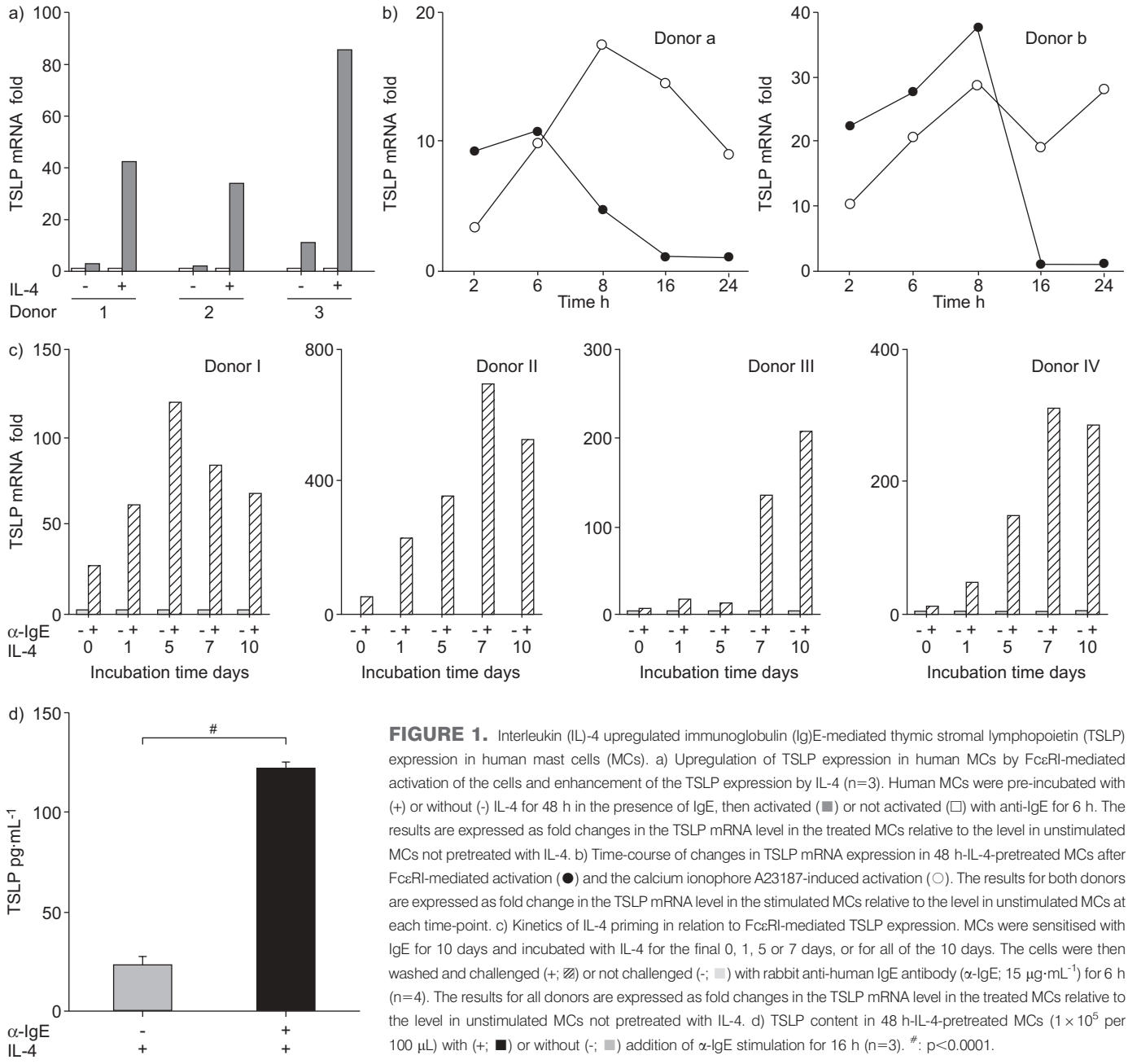

FIGURE 1. Interleukin (IL)-4 upregulated immunoglobulin (Ig)E-mediated thymic stromal lymphopoietin (TSLP) expression in human mast cells (MCs). a) Upregulation of TSLP expression in human MCs by FcεRI-mediated activation of the cells and enhancement of the TSLP expression by IL-4 (n=3). Human MCs were pre-incubated with (+) or without (-) IL-4 for 48 h in the presence of IgE, then activated (■) or not activated (□) with anti-IgE for 6 h. The results are expressed as fold changes in the TSLP mRNA level in the treated MCs relative to the level in unstimulated MCs not pretreated with IL-4. b) Time-course of changes in TSLP mRNA expression in 48 h-IL-4-pretreated MCs after FcεRI-mediated activation (●) and the calcium ionophore A23187-induced activation (○). The results for both donors are expressed as fold change in the TSLP mRNA level in the stimulated MCs relative to the level in unstimulated MCs at each time-point. c) Kinetics of IL-4 priming in relation to FcεRI-mediated TSLP expression. MCs were sensitised with IgE for 10 days and incubated with IL-4 for the final 0, 1, 5 or 7 days, or for all of the 10 days. The cells were then washed and challenged (+; ▨) or not challenged (-; ▩) with rabbit anti-human IgE antibody (α-IgE; 15 μg·mL^{-1}) for 6 h (n=4). The results for all donors are expressed as fold changes in the TSLP mRNA level in the treated MCs relative to the level in unstimulated MCs not pretreated with IL-4. d) TSLP content in 48 h-IL-4-pretreated MCs (1×10^5 per 100 μL) with (+; ■) or without (-; ▩) addition of α-IgE stimulation for 16 h (n=3). #: p<0.0001.

expression level on MCs treated with IgE for 7 days was higher (donor 1) than or almost the same as (donor 2) that on the MCs treated with IL-4 for 7 days and incubated with IgE for the final day (fig. 4b). However, the FcεRI-mediated upregulation of the TSLP mRNA expression level in the MCs treated with IL-4 for 7 days and incubated with IgE for the final day was significantly higher than that in the MCs treated with IgE for 7 days (fig. 4c), suggesting that the FcεRI-mediated upregulation of TSLP mRNA by IL-4 is not simply due to increased expression of FcεRI on the MCs.

TSLP expression in the bronchial mucosal MCs of asthmatic subjects

To determine whether the TSLP+ cells included MCs and whether the number of TSLP+MCs in the bronchial mucosa was increased in asthmatic subjects compared with that in the healthy controls, we conducted dual-staining of biopsy specimens obtained from the asthmatic and healthy control subjects with anti-TSLP and anti-tryptase Abs. Bronchial MCs in the biopsy specimens obtained from the asthmatic subjects were clearly positive for TSLP (fig. 5a–c), whereas the bronchial biopsy specimens from the healthy controls showed little immunoreactivity for TSLP (fig. 5d). In the bronchial mucosa of both the asthmatic and the healthy control subjects, 90% of the TSLP+ cells were MCs and the remaining 10% were airway epithelial cells (fig. 5e). We then counted the absolute number of tryptase+ cells in the mucosal specimens (fig. 5f). The number of MCs was significantly higher in the asthmatic subjects than that in the healthy controls (p<0.0001; fig. 5f). The number of TSLP+ MCs was also significantly higher in the

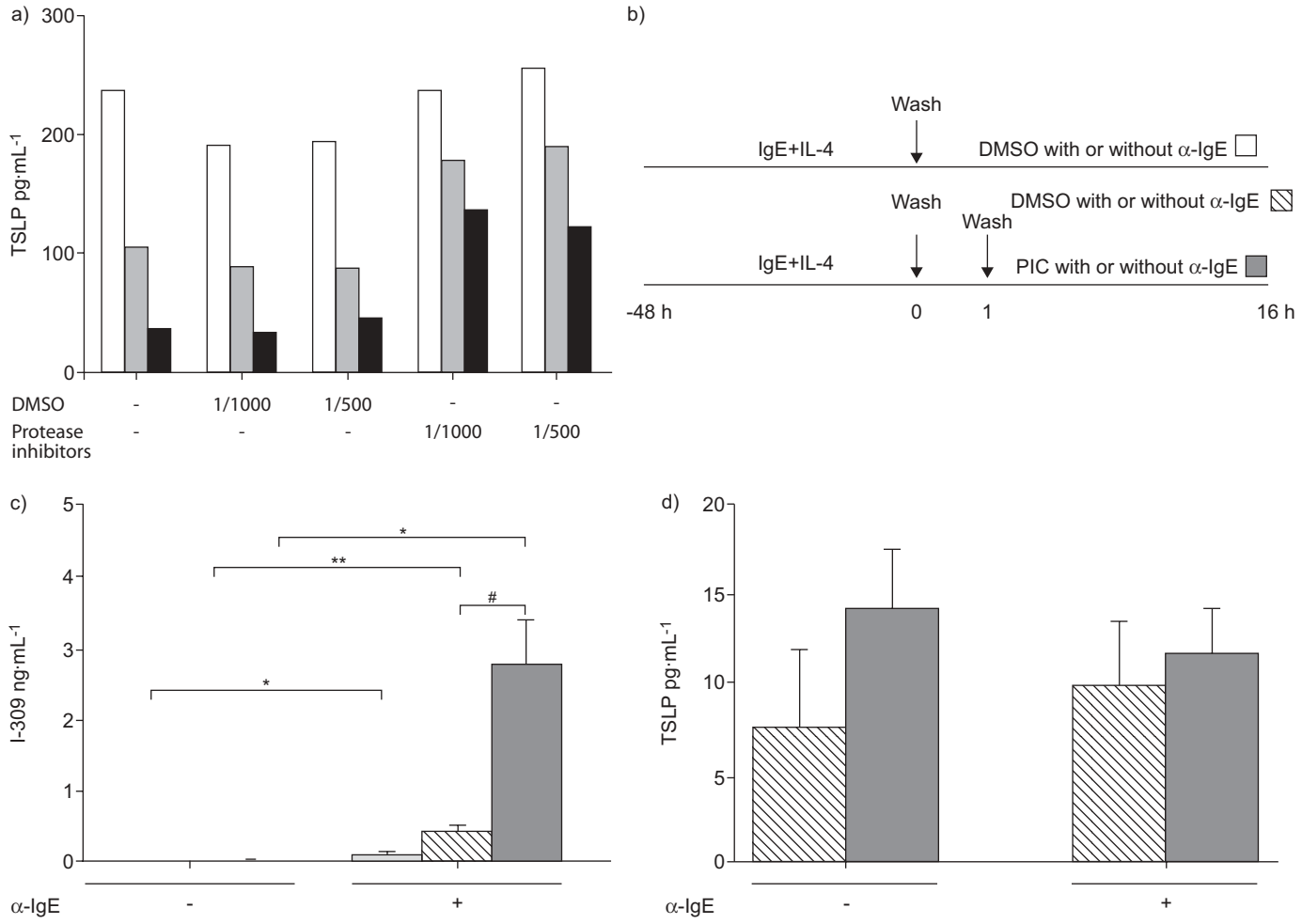

FIGURE 2. Thymic stromal lymphopoietin (TSLP) secretion from mast cells (MCs) following aggregation of FcεRI. a) Effect of protease inhibitors on MC-supernatant-mediated TSLP degradation. Recombinant human TSLP 250 pg·mL^{-1} was incubated for 16 h with medium alone (□), the supernatant of unstimulated MCs (▩), or the supernatant of stimulated MCs (■) in the presence or absence of dimethyl sulfoxide (DMSO; 1/500 or 1/1,000 dilution of the stock concentration) and protease inhibitors (1/500 or 1/1,000 dilution of protease inhibitor cocktail). The results are representative of three separate analyses with similar results. b) Protocol for detection of TSLP in the supernatant of the activated MCs. MCs were pretreated with immunoglobulin (Ig)E (1 μg·mL^{-1}) and IL-4 (10 ng·mL^{-1}) for 48 h and then incubated for 16 h with (+) or without (-) anti-IgE (α-IgE), without any washing after incubation for 1 h in the presence or absence of anti-IgE (▩). The MCs were washed after incubation for 1 h in the presence or absence of anti-IgE and resuspended for 15 h in culture medium containing either DMSO (1/1,000 dilution of stock concentration; ▨) or protease inhibitors (1/1,000 dilution of a protease inhibitor cocktail (PIC); ■). c) Effect of protease inhibitors on I-309 secretion by 48 h-IL-4-pretreated MCs (1 × 10^5 per 100 μL) after anti-IgE stimulation (α-IgE), using the above described protocol (b). Data are expressed as means±SEM (n=3). *: $p<0.05$; **: $p<0.01$ for the difference in the I-309 concentration in the cell supernatant between unstimulated and stimulated MCs; #: $p<0.05$ for the difference in the I-309 concentration in the cell supernatant between the protease inhibitor-treated MCs and the control MCs. d) Effect of protease inhibitors on the TSLP secretion by 48 h-IL-4-pretreated MCs (1 × 10^5 per 100 μL) after α-IgE using the above described protocol (b). Data are presented as mean±SEM for four different donors whose MCs released detectable levels of TSLP.

asthmatic subjects than in healthy controls ($p<0.0001$; fig. 5g). In addition, the percentage of TSLP+ MCs in the total population of MCs was significantly higher in the asthmatic subjects than in healthy controls ($p<0.0001$; fig. 5h). The percentage of TSLP+ MCs in the total population of MCs was also significantly higher in atopic asthmatic subjects than in nonatopic asthmatic subjects ($p<0.05$; fig. 5i). However, there was no significant difference in the percentage of TSLP+ MCs in the total population of MCs as a function of the disease severity, *i.e.* between asthmatic subjects requiring step 2 *versus* step 3 or 4 management (data not shown). We further conducted immunohistochemical analysis with anti-chymase mAb and anti-TSLP Ab to identify differences in the expression levels of TSLP among various MC subtypes, *i.e.* MCs containing tryptase only (MCT) *versus* MCs containing both tryptase and chymase; the results revealed that TSLP was mainly localised in MCT (data not shown).

Correlation between the percentage of TSLP+ MCs in the total population of MCs and various markers of bronchial asthma

We next investigated the relationship between the percentage of TSLP+ MCs in the total population of MCs and various clinical markers in asthmatic subjects and healthy controls (fig. 6). The results showed a significant correlation between the percentage of TSLP+ MCs in the total population of MCs and the serum IgE level ($p<0.05$, $r^2=0.131$). There were also

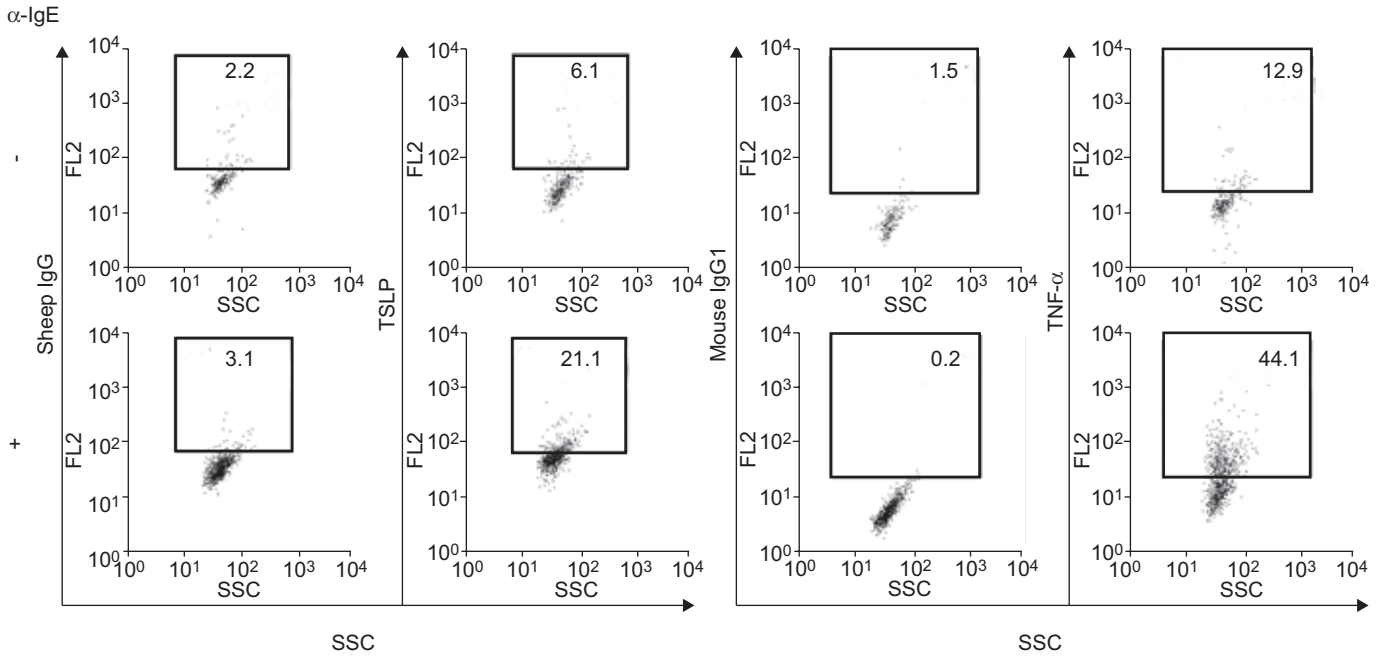

FIGURE 3. Effect of monensin on the thymic stromal lymphopoietin (TSLP) immunoreactivity in interleukin (IL)-4-treated mast cells (MCs) following aggregation of FcεRI. MCs were incubated with IL-4 and immunoglobulin (Ig)E for 7 days and washed and challenged with anti-IgE (α-IgE; +) or medium alone (-). Monensin (BD GolgiStop™; BD Biosciences, Tokyo, Japan) was added to the MC suspension, which was followed by incubation for 12 h. Intracellular staining of the MCs was performed using anti-TSLP antibody (Ab) or anti-tumour necrosis (TNF)-α monoclonal Ab. The side scatter (SSC) and the fluorescence (FL2) intensity were plotted on the *x-axis* and *y-axis*, respectively. The MCs were gated from the values of the forward scatter and SSC. The numbers in the figures indicate the percentage of TSLP+ or TNF-α+ MCs. These data are representative of the data obtained from three different donors. Similar data were obtained the other two donors.

significant correlations of the percentage of TSLP+ MCs in the total population of MCs to the degree of airway hyperresponsiveness to acetylcholine ($p<0.05$, $r^2=0.252$), the peripheral blood eosinophil count ($p<0.05$, $r^2=0.243$) and the sputum eosinophil count ($p<0.05$, $r^2=0.437$).

DISCUSSION

In this study, we have demonstrated that IgE-mediated TSLP mRNA expression in human MCs is upregulated by IL-4 exposure (fig. 1). MCs stored TSLP, and the amount of TSLP in the MCs increased following aggregation of FcεRI. MCs spontaneously produced TSLP, the protein was released from the cells following FcεRI aggregation, but the TSLP was also degraded by MC-derived proteases (figs 2 and 3). Furthermore, we also demonstrated a higher proportion of TSLP+ MCs among the human bronchial mucosal MCs of asthmatic subjects than among those of healthy controls (fig. 5). We thus confirmed that the bronchial MCs stored TSLP and that the protein level was increased in the bronchial MCs of asthmatics. The percentage of TSLP+ MCs in the total population of MCs was significantly correlated with the serum IgE level, suggesting that IgE-mediated activation increased the production of TSLP by the MCs (fig. 6). In addition, the percentage of TSLP+ MCs in the total population of MCs was significantly correlated with the degree of bronchial hyperresponsiveness and the peripheral blood and sputum eosinophil counts, suggesting that MC-derived TSLP may play an important role in the pathogenesis of asthma.

FcεRI-mediated TSLP mRNA expression in the MCs was significantly upregulated by IL-4 priming. In a previous study, our group reported that IL-4 profoundly increased FcεRI-mediated production of macrophage inflammatory protein (MIP)-1α, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human MCs [22]. IL-4-induced priming of human intestinal MCs for enhanced survival and Th2 cytokine generation has been reported to be associated with increased activity of extracellular signal-regulated kinase 1/2 and c-Fos [23]. Using a DNA array, IL-4 has also been demonstrated to favour the induction of Th2 cytokine expression in human cord blood-derived cultured MCs following FcεRI aggregation [24]. IL-4 has been reported to upregulate FcεRI expression on human MCs [25]. However, we found that the upregulation of FcεRI-mediated TSLP mRNA by IL-4 is not simply due to increased expression of FcεRI on the MCs (fig. 4).

It was difficult to measure the precise amount of TSLP released from activated MCs because of the simultaneous release of MC-derived proteases. Even when we used protease inhibitors to prevent the degradation of TSLP, the level of TSLP production by the activated MCs appeared to be no greater than that by the control cells (fig. 2d); in fact, the TSLP level in the supernatants obtained from the activated MCs of some donors was below the limit of detection. The reason is unclear but the degree of induction of FcεRI-mediated TSLP mRNA upregulation by IL-4 varied from donor to donor (fig. 1); it is possible that the amount of proteases released from the MCs also varies from donor to donor. Since protease inhibitors prevented degradation of only half of the rhTSLP by the MC supernatants (fig. 2a), it was considered that the MCs may also produce other factor(s) that might degrade TSLP and that the

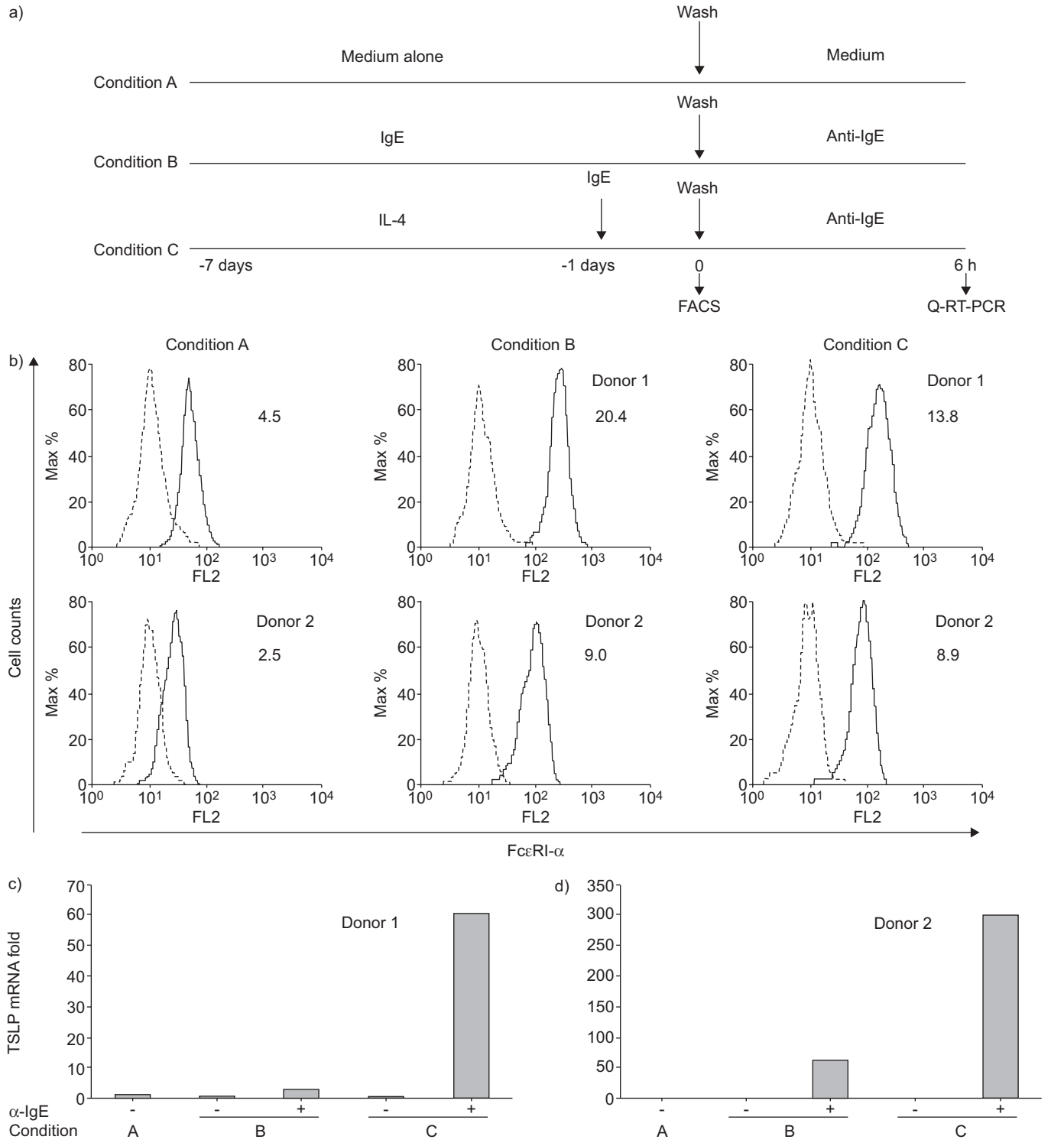

FIGURE 4. Upregulation of FcεRI-mediated thymic stromal lymphopoietin (TSLP) mRNA by interleukin (IL)-4 was not simply due to increased expression of FcεRI on the mast cells (MCs). a) Protocol of the experiments. Condition A: MCs were incubated with culture medium for 7 days. MCs were then washed and incubated with medium alone for 6 h. Condition B: MCs were incubated with immunoglobulin (Ig)E for 7 days; cells were washed and challenged or not challenged with anti-IgE for 6 h. Condition C: MCs were incubated with IL-4 and IgE was added for the final day; cells were washed and challenged or not challenged with anti-IgE for 6 h. b) Fluorescence-activated cell sorter (FACS) analysis for FcεRI-α-chain-expressing MCs under each condition on day 0. Two donors' results are shown as histograms overlaid with control staining (- - -). Numbers in figures indicate MFI ratio (FcεRI-α-chain expression value/isotype control value). c and d) TSLP mRNA expression level in MCs in donors 1 (c) and 2 (d) under each condition following FcεRI aggregation. MCs were treated under each condition (A, B and C) and challenged (+) or not challenged (-) with anti-IgE (αIgE) for 6 h. Results are expressed as fold change in the TSLP mRNA level in the treated MCs relative to the unstimulated MCs under condition A. FL2: fluorescence.

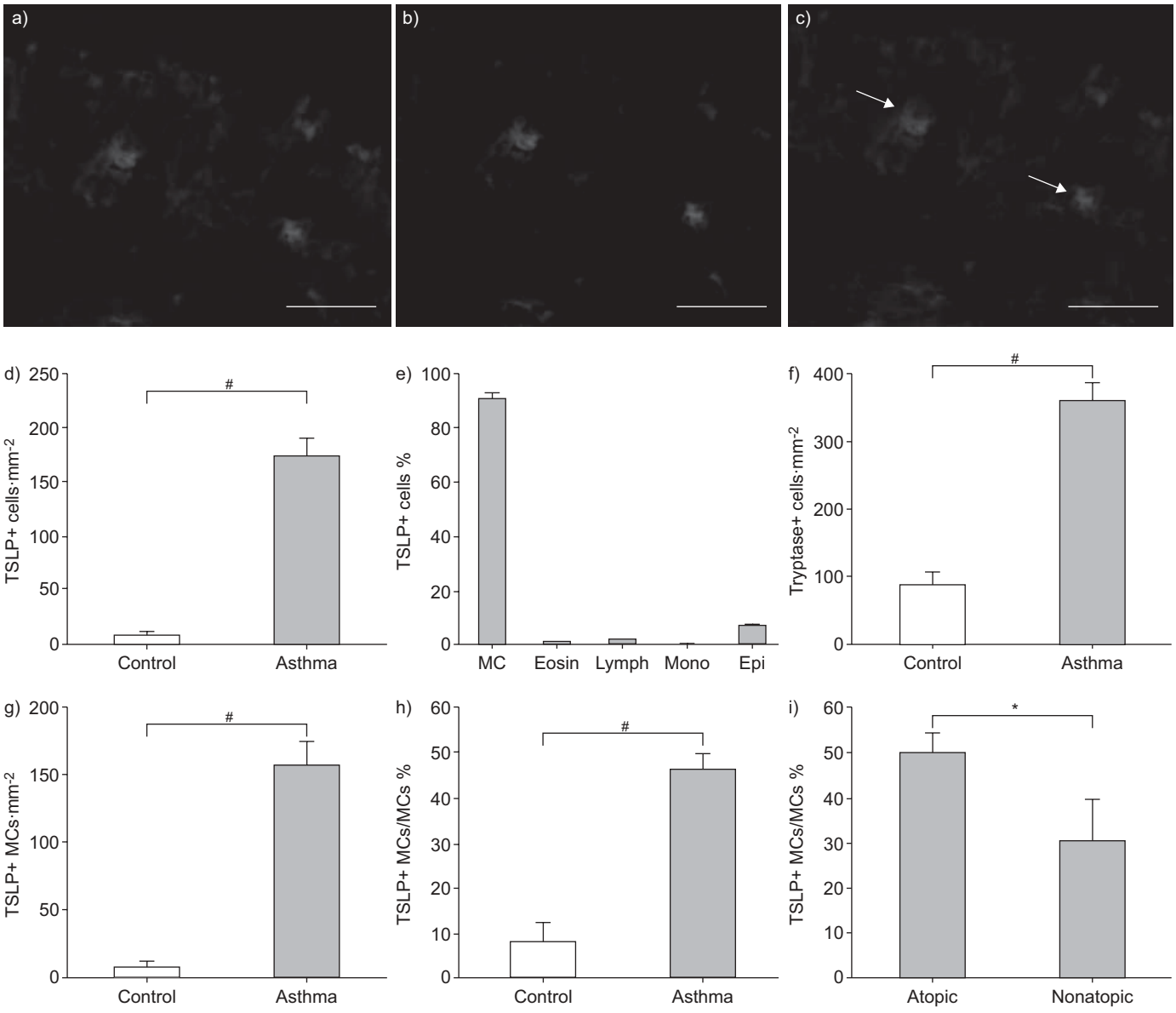

FIGURE 5. Significant increase of thymic stromal lymphopoietin (TSLP) expression in the human bronchial mucosal mast cells (MCs) of asthmatic subjects (n=16) in comparison with that in healthy controls (n=11). a–c) Colocalisation of TSLP (a) in tryptase+ MCs (b); merged cells (arrows) are shown in c. Scale bars=50 μm. d, f, g and h) Significant difference in the number of TSLP+ cells·mm^{-2} (d), tryptase+ cells·mm^{-2} (f), TSLP+ MCs (g), and percentage of TSLP+ MCs in the total population of MCs (h) of the bronchial mucosa of healthy controls and asthmatic subjects. e) Percentages of MCs, eosinophils (Eosin), lymphocytes (Lymph), monocytes (Mono) and epithelial cells (Epi) among the total number of TSLP+ cells in the bronchial specimens from asthmatic subjects and healthy controls. i) Significant difference in the percentage of TSLP+ MCs in the total population of MCs in the bronchial mucosa of atopic asthmatic patients and nonatopic asthmatic patients. *: $p<0.05$; #: $p<0.0001$.

addition of protease inhibitors alone may be insufficient to prevent the degradation of TSLP by the MC supernatants. However, TSLP was detected in the cell pellets in all of the 10 donors in the present study (data not shown), and the addition of the protease inhibitors to the supernatants did not affect the amount of TSLP in the cell pellets measured after aggregation of FcεRI (data not shown). Therefore, we confirmed the increase in the intracellular TSLP immunoreactivity of monensin-treated MCs after IgE-mediated activation (fig. 3). These findings suggest that MCs store TSLP and release it both spontaneously and following aggregation of FcεRI, and that the released TSLP is rapidly degraded by MC proteases and possibly by other as-yet-unknown factors. Thus, TSLP produced by the MCs may exert its effect in the microenvironment around the MCs. Furthermore, our findings suggested that the increased expression of TSLP in the MCs obtained from the bronchial mucosa of asthmatic subjects was due to the significant upregulation of FcεRI-mediated TSLP production by IL-4.

YING *et al.* [8] previously reported that ~40% and ~20% of TSLP mRNA+ cells in the bronchial mucosa of asthmatic subjects were MCs and epithelial cells, respectively. In the present study, we found that 90% of the bronchial mucosal

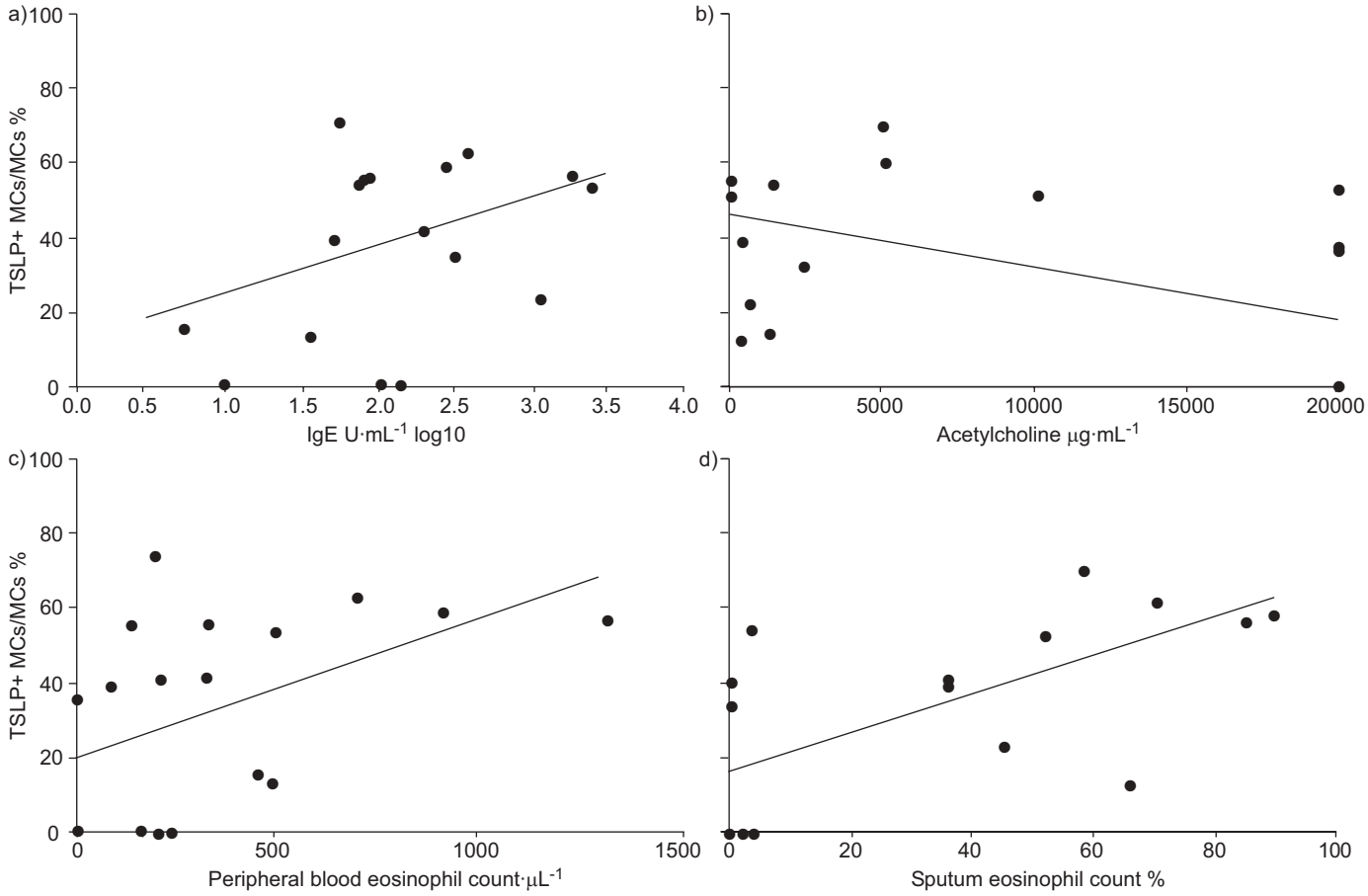

FIGURE 6. Correlation between the percentage of thymic stromal lymphopoietin (TSLP)+ mast cells (MCs) in the total population of MCs and various markers of bronchial asthma. Correlation between the percentage of TSLP+ MCs in the total population of MCs and: a) the serum immunoglobulin (Ig)E levels (asthma n=15, control n=4; r^2=0.131, p=0.048); b) the degree of bronchial hyperresponsiveness (asthma n=14, control n=9; r^2=0.252, p=0.024); c) the peripheral blood eosinophil count (asthma n=16, control n=8; r^2=0.243, p=0.045); and d) the sputum eosinophil count in asthmatic patients and healthy controls (asthma n=13, control n=8; r^2=0.437, p=0.024).

TSLP protein+ cells were MCs. This discrepancy may be due to the difference in the expression level between TSLP mRNA and TSLP protein. MCs are known to store cytokines in their cytoplasm. The level of TSLP in epithelial cells is synergistically increased by a combination of IL-4 and double-stranded RNA, suggesting that respiratory viral infection and the recruitment of Th2 cytokine-producing cells may amplify Th2 cytokine-mediated inflammation *via* the induction of TSLP in the airways of asthmatic subjects [9]. However, no evidence has been accumulated suggesting that allergens might directly induce TSLP production in the airways of asthmatics. Our *in vivo* and *in vitro* studies suggest that MCs are one of the main sources of TSLP in bronchial asthma; this is highly relevant to our understanding of allergic asthma. TSLP, which is released by MCs following aggregation of FcεRI, is capable of triggering DC-mediated Th2 inflammatory responses by induction of Th2-attracting chemokines, such as CCL17 and CCL22 [5], and by priming naïve Th2 cells to produce IL-4, IL-5, IL-13 and TNF-α, but not IL-10 [4, 5]. MC-derived TSLP plays an important role in the pathogenesis of bronchial asthma by facilitating cross-talk with DCs. It has been reported that TSLP stimulates the production of Th2 cytokines by human MCs synergistically with IL-1 and TNF-α [20], suggesting an autocrine/paracrine effect of MC-derived TSLP. Therefore, MC-derived TSLP in bronchial asthma may induce and aggravate allergic inflammation.

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STATEMENT OF INTEREST

None declared.

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