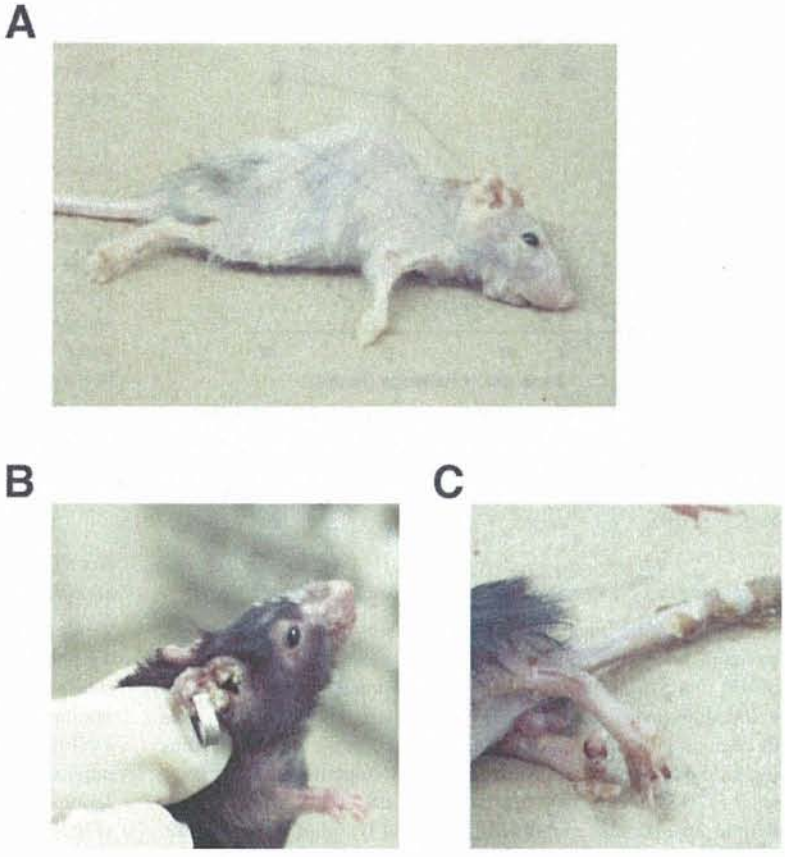

FIGURE 5. Tg mice develop dermatitis. In severe cases, individual Tg mice lost hair all over the body (*A*). In mild cases, the surface of the face, ear, foot, and tail showed redness and scaling (*B* and *C*).

analyses were also done. IL-4 production in cells from Tg and WT mice was similar and the percentage of positive cells was very low under all conditions (data not shown). These results demonstrate that T cells from T-bet Tg mice have a dominant Th1 differentiation pattern and suggest that overexpression of T-bet prevents Th2 differentiation. $CD8^+$ T cells from Tg mice also had higher levels of IFN-γ than WT mice in neutral condition, but not so markedly as that of $CD4^+$ T cells from Tg mice (Fig. 3*B*). We also determined the GATA-3 mRNA expression in the intracellular cytokine production analyses (Fig. 4). GATA-3 mRNA expression in Tg mice showed lower levels than those of wild mice, especially in a Th1 condition.

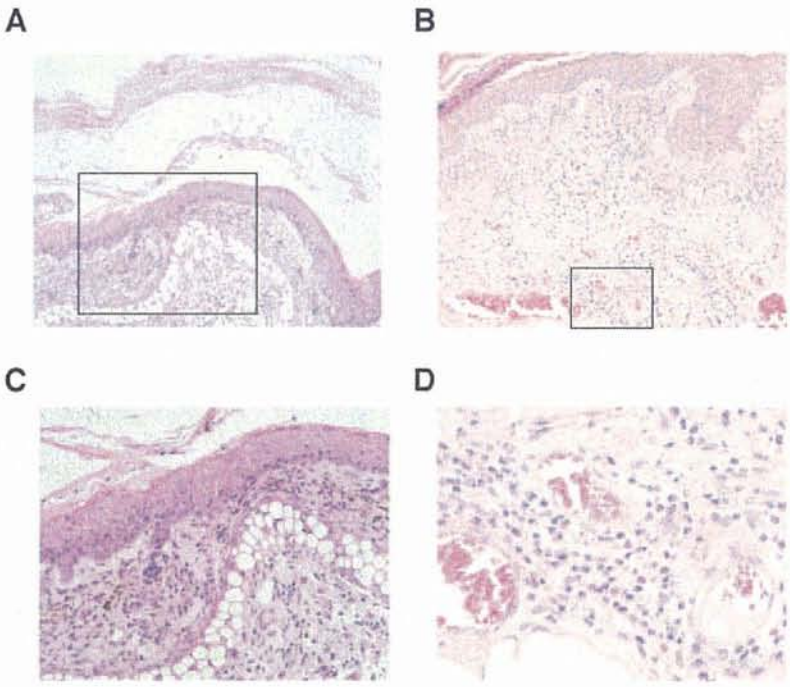

FIGURE 6. Histological appearance of the ear skin. *A* and *B*, In the histological analysis of ear skin, hyperkeratosis, acanthosis, broadening of the papillae, and infiltration of neutrophils lymphocytes, and melanophages are seen. *C and D*, At higher magnification of the squares from *A* and *B*, infiltration of mononuclear cells and neutrophils is observed. (H&E staining; magnification: ×100 (*A*), ×100 (*B*), ×200 (*C*), and ×400 (*D*).

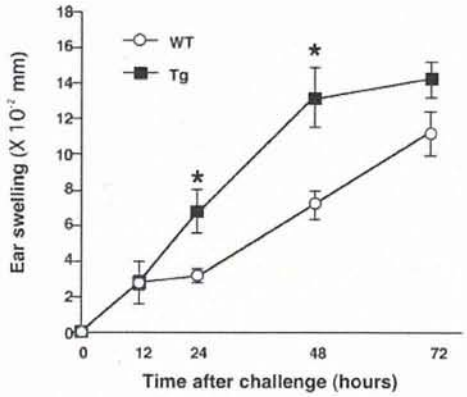

FIGURE 7. CHS reaction to DNFB in Tg and WT mice. The CHS response was determined by ear swelling at various times after hapten challenge. Time after challenge was 24 and 48 h; the ear swelling values in Tg mice (■, $n = 5$) were significantly higher than those in WT mice (○, $n = 5$). *, $p < 0.05$.

Development of contact dermatitis in T-bet Tg mice

During the initial analysis of the Tg cohorts we found that they developed dermatitis. At ~10 wk of age, ~28% of Tg line 725 (12 of 43) and 5% of Tg line 731 (3 of 61) mice spontaneously developed dermatitis characterized by swollen, flaky, and scaly skin in regions lacking body hair (e.g., tail or ears), which in some individuals progressed all over the body, together with alopecia (Fig. 5). Histological examination of the affected skin showed epidermal hyperkeratosis and neutrophil and lymphocyte infiltration similar to what is seen in contact dermatitis in humans (Fig. 6). To prove that the skin lesion was contact dermatitis although the Ag was not determined, we performed cell transfer experiment. We transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within one month. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone.

Augmentation of CHS reactions in Tg mice

To determine the role of T-bet overexpression in T cell subpopulations in CHS responses, 6-wk-old WT and Tg (Tg line 725) mice, which did not develop dermatitis, were sensitized with DNFB as described in *Materials and Methods*. Ear swelling responses to DNFB were significantly increased in Tg mice compared with WT mice (Fig. 7). The CHS response was significantly higher at 24 and 48 h after challenge. Histological analysis of hapten-treated WT and Tg ears showed characteristic features of CHS including dermal edema, mononuclear cell infiltration, and vascular enlargement (Fig. 8*B*). These histological changes were dramatically enhanced in hapten-treated Tg mouse ears (Fig. 8*B*).

Both $CD4^+$ and $CD8^+$ T cell lymph node cells (LNCs) produce significant amounts of IFN-γ in T-bet Tg mice

To determine IFN-γ production in skin-draining lymph nodes of Tg and WT mice, DNFB-primed LNCs were cultured under condition with medium alone. A significant increase of IFN-γ-producing cells was found among both $CD4^+$ and $CD8^+$ LNCs from DNFB-stimulated Tg mice (Fig. 9). In Tg mice, IFN-γ-producing cells were increased in $CD4^+$ LNCs even without DNFB stimulation. After stimulation, IFN-γ-producing cells increased further (Fig. 9*A*). Regarding $CD8^+$ LNCs, WT and Tg mice had the similar levels of IFN-γ-producing cells, but after DNFB stimulation the increase of IFN-γ-producing cells was greater in Tg mice (Fig. 9*B*). IL-10 production was also analyzed as a marker cytokine of

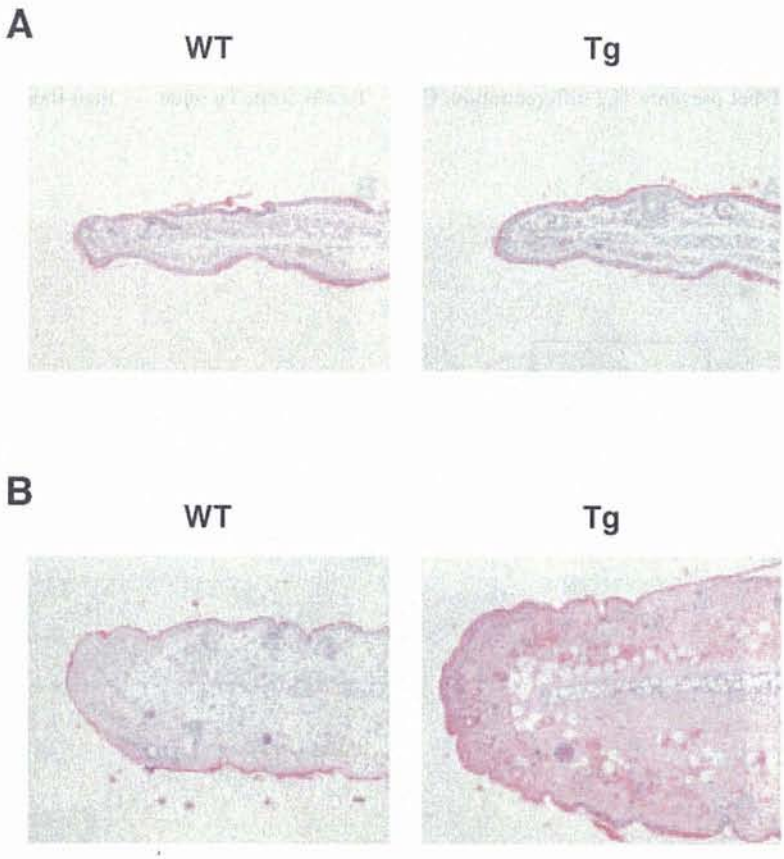

FIGURE 8. Representative results from histological analysis of CHS reactions. At 72 h after challenge, skin sections are shown from vehicle-treated mice (*A*) and hapten-treated mice (*B*) (H&E staining; magnification: ×100). *A*, Sections from vehicle-treated Tg mice exhibited normal histological features similar to those of vehicle-treated WT mice. *B*, Sections from hapten-treated WT and Tg mice displayed characteristic features of the CHS reaction including dermal edema, mononuclear cell infiltration, and vascular enlargement (*B*). The severity of the CHS reaction was greater in Tg mice.

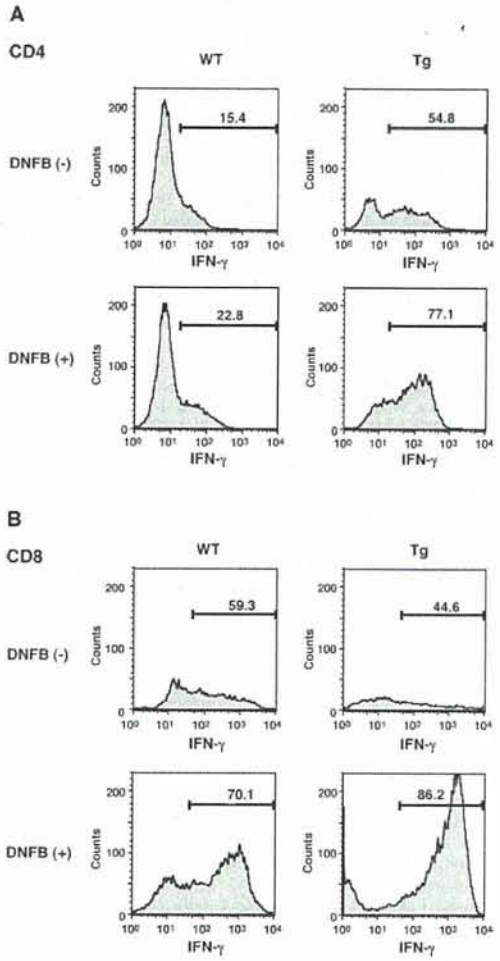

FIGURE 9. The number of IFN-γ-producing cells among $CD4^+$ and $CD8^+$ T cells was increased in CHS-sensitized Tg LNCs. LNCs from 72 h DNFB-sensitized or non-sensitized WT and Tg mice were cultured in medium alone and subjected to intracellular cytokine staining as described in *Materials and Methods*. Significantly higher numbers of IFN-γ-producing cells were detected in DNFB-sensitized Tg $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells. Results are representative of three independent experiments.

Th2 or Tc2. However, no significant differences between Tg and WT mice were found (data not shown).

Discussion

T-bet is known as a master regulator of Th1 development. It induces IFN-γ production and repression of Th2 cytokines in vitro (7). In this study, we generated T-bet-overexpressing mice characterized by Th1-dominant responses in vivo and in vitro. Thus, T-bet Tg mice showed Th1-dominant Ig production in vivo and Th1-dominant intracellular cytokine production in vitro. Because serum cytokines were below the level of detection, we analyzed serum Ig isotypes. IFN-γ promotes IgG2a class switching while IL-4 promotes IgG1 class switching, so concentrations of serum IgG2a and IgG1 reflect Th1 and Th2 responses in vivo (22–24). The higher IgG2a/IgG1 ratio present in the sera of Tg mice suggests that T-bet overexpression promotes IgG2a class switching and represses IgG1 class switching. At the same time, we analyzed intracellular cytokines and found that $CD4^+$ T cells from Tg mice produced higher levels of IFN-γ than WT mice under neutral, Th1- or Th2-promoting conditions. In contrast, IL-5 production in $CD4^+$ T cells from Tg mice was lower than that in $CD4^+$ T cells from WT mice under neutral or Th2-promoting conditions. The serological Ig findings are therefore consistent with data from $CD4^+$ T cell intracellular cytokine assays, emphasizing that these do indeed reflect the Th1/Th2 balance in vivo. T-bet has also been shown to regulate cytolytic effector mechanisms of $CD8^+$ T cells (8). Its expression is rapidly induced in $CD8^+$ T cells by signaling through the TCR and the IFN-γR, and it functions downstream of STAT1 (6, 9, 10). In the context of Ag-specific activation, T-bet is required for the differentiation of naive $CD8^+$ T cells into effector CTLs. In the present study, Tg mice also had a higher fraction of IFN-γ-producing $CD8^+$ T cells according to intracellular cytokine assays, but not as markedly as $CD4^+$ T cells. However, Tg mice showed higher response for IFN-γ-producing $CD8^+$ T cells in the CHS response. From the above results, we speculated that Tg mice not only had a Th1-dominant background but also a potential Tc1-dominant background.

In this study, spontaneous skin inflammation was observed in Tg mice, first occurring in regions lacking body hair such as the tail or ears, where the skin is easily in contact with external agents. Histological examination of affected skin showed epidermal hyperkeratosis, spongiosis, and neutrophil and lymphocyte infiltration. This is typical for contact dermatitis, a delayed-type hypersensitivity reaction. It is known that Th1 activity greatly contributes to the development of dermatitis (25, 26). It is shown here that a Th1-dominant response occurs in T-bet Tg mice. Tg mice expressing IFN-γ in the epidermis have also been investigated previously and found to have reddened skin, growth retardation, hair loss, and flaky skin lesions (25). Keratinocyte proliferation was increased and there was epidermal thickening with spongiosis and parakeratosis. The possible importance of several cytokines such as IL-2 (27), IL-6 (28), and IL-7 (29) in the development of contact dermatitis has also been investigated using Tg mouse models (30). In this study we used Tg mice overexpressing the T cell differentiation transcription factor T-bet, but not cytokine transgenes, to address contact dermatitis for the first time. The results from this study suggested that transcriptional regulation of T-bet might play an important role in contact dermatitis.

Contact allergens such as DNFB, oxazolone, and 2,4-dinitrochlorobenzene are used to induce CHS. In the present study we also showed that Tg mice responded significantly to DNFB. There have been major controversies on the respective roles of $CD4^+$ and $CD8^+$ T cells in the development of CHS inflammatory reactions (12). However, it has become clear that both $CD4^+$ and $CD8^+$ T cells can act as effector cells in this context (17). Wang et al. (17) showed that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells are crucial effectors in the CHS response to DNFB. Previous studies have also demonstrated that cytokines play important effector and regulatory roles in CHS responses. The type 1 cytokine IFN-γ promotes CHS, whereas type 2 cytokines down-regulate CHS responses (31–33). It has been demonstrated that type 1 cytokines can be produced by $CD8^+$ Tc1 cells as well as by $CD4^+$ Th1 cells in the same way that type 2 cytokines can be derived from Th2 and Tc2 cells (5, 34). Intracellular cytokine analyses showed that T-bet-overexpressing Tg mice had a large fraction of IFN-γ-positive cells within both $CD4^+$ and $CD8^+$ T cells. We also transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within 1 mo. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone. These results suggest that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells may be active as effector cells in CHS responses in this model. Furthermore, it is known that activated cells produce IFN-γ to activate skin resident cells, which produce cytokines and chemokines allowing the recruitment of the polymorphonuclear cell infiltrates characteristic of CHS. This effector phase of CHS takes

24 to 48 h in the mouse (12), coinciding closely with our results on 24- and 48-h DNFB hyperresponsiveness.

In contrast to T-bet, GATA-3 is known as a Th2 lineage commitment transcription factor (35, 36). We have previously shown that GATA-3 overexpression in Th1-dominant autoimmune disease can diminish autoimmune nephritis (20, 37) and, therefore, that therapy to regulate expression levels of transcriptional factors may be useful to control unbalanced Th1/Th2 activity in many diseases. The results of the present study also suggest that to control Th1 and Tc1 reactions via down-regulation of T-bet expression might be useful for alleviating contact dermatitis.

In conclusion, we have generated Th1- and Tc1-dominant mice that developed spontaneous skin inflammation very similar to contact dermatitis. These mice should be useful for revealing a link between some immune diseases and Th1/Th2 and Tc1/Tc2 dysbalance and may offer a valuable murine model of contact dermatitis.

Acknowledgment

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Disclosures

The authors have no financial conflict of interest.

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Functional Polymorphism in the *Suppressor of Cytokine Signaling 1* Gene Associated with Adult Asthma

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Suppressor of cytokine signaling (SOCS) 1 is an essential physiologic regulator of the IFN-γ signaling that is crucial to lead appropriate immune responses, and impaired IFN-γ production is considered a hallmark of atopic diseases. Recent study has shown that SOCS1 is also crucial in attenuating type 1 IFN signaling and in limiting the host response to viral infection. Clinical and experimental evidence suggest an important role for respiratory viral infections in the development of asthma. To assess genetic functional variants of *SOCS1* related to susceptibility and clinical phenotypes in adult asthma in a Japanese population, we conducted association and haplotype analyses of 462 subjects with adult asthma and 639 control subjects. After screening for polymorphisms, we identified a total of 13 variants and characterized the linkage disequilibrium (LD) mapping of the gene. Three variants were selected for genotyping with regard to the LD pattern, and we found a significant association between an *SOCS1* promoter polymorphism −1478CA > del and adult asthma ($P = 0.0063$). The three-locus haplotype of *SOCS1* using these three polymorphisms also showed a positive association with a haplotype T-C-del (−5388T, −3969C, and −1478 del; $P = 0.0097$). Furthermore, reporter gene analysis revealed that related promoter variant −1478 del enhanced the transcriptional level of *SOCS1* in human lung epithelial cells, and induced higher levels of protein expression of SOCS1 and lower phosphorylation of STAT1 stimulated with IFN-β. These findings suggest that the *SOCS1* gene might be involved in the development of adult asthma through functional genetic polymorphism.

Keywords: *SOCS1*; bronchial asthma; polymorphisms; haplotype; association study

CLINICAL RELEVANCE

Viral infections influence both the development and the severity of asthma. Our data suggest that the susceptible variant might affect the increased sensitivity to viral infection through higher SOCS1 production and contribute to asthma etiology.

Bronchial asthma is a complex disease caused by a combination of genetic and environmental factors (1, 2). Cytokines play an important role in chronic inflammation of the airways in asthma (3), and suppressor of cytokine signaling (SOCS)1, a member of the SOCS family of proteins, is rapidly transcribed after exposure of cells to cytokines (4, 5). SOCS1 interacts directly with the Janus kinases (JAK), essential intracellular mediators of immune cytokine action, and inhibits their tyrosine-kinase activity (4, 5).

SOCS1 is an essential physiologic regulator of the IFN-γ signaling that is crucial to lead appropriate immune responses (6, 7). Asthma is thought to arise from an imbalance in T helper type 1 (Th1)-Th2 immune regulation, and IFN-γ–producing Th1 cells have been suggested to protect against allergic responses by attenuating the activity of Th2 effectors cells (3). These findings implicated *SOCS1* as a candidate gene for involvement in asthma.

Clinical and experimental evidence suggests an important role for respiratory infections in the development of asthma (8, 9). Recent studies have shown that SOCS1 is an important *in vivo* inhibitor of type I interferon signaling (10). Cardiac myocyte-specific transgenic expression of *SOCS1* inhibits enterovirus-induced signaling of JAK-STAT, with accompanying increases in viral replication, cardiomyopathy, and mortality in coxsackievirus-infected mice (11). Inappropriate overexpression of *SOCS1* results in viral-mediated end-organ damage during the early stages of infection. Furthermore, SOCS1 inhibits expression of the antiviral proteins myxovirus resistance-A (MxA) and 2′-5′-oligoadenylate synthetase (2′-5′-OAS) (12). In addition, a recent study has shown that dsRNA induces SOCS1 expression, and SOCS1-negative feedback of STAT1 activation is a key pathway in the dsRNA-induced innate immune response (13). The interferons produced after viral infection or treatment with dsRNA stimulate Toll-like receptor (TLR)-3, interferon regulatory factor (IRF)-7, and monocyte inflammatory protein (MIP)-1α expression by activating the JAK-STAT pathway, which is inhibited by SOCS1 (13). These findings suggest that SOCS1 is a key protein regulating the antiviral response.

To discover genetic components in the pathogenesis of asthma, a large number of studies using polymorphic DNA markers, including linkage and association studies, have been performed (14–16). Although genetic studies have been conducted for the polymorphisms in IFN-γ and IFN-γ–related genes, the

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TABLE 1. PRIMERS FOR THE SNP SURVEY IN THIS STUDY

F1	TTGCGATACCCGACATTCTC	R1	TTCCCAGCTGCTGGGCTTTA
F2	TTCCCGACACATGGGTTAGA	R2	TGTTTGAAGCCCTAGTCCAG
F3	ACCAAAGCTGTCTGTCACTG	R3	GAAGATGCAGAACCTTGCCT
F4	TCACACACCTGACCTCTGGT	R4	ACCACAGGCTTCAGAGGAA
F5	TGCAAGCCATTGCAAATCCC	R5	GCCAGTCTTTTAAACCGGCT
F6	GTCCAGAAGAGAGGGAAACA	R6	TTCAGCCTCAGTGGACACAG
F7	AAGAGCTCCGCGGAGTACA	R7	ACACGGCATCCCAGTTAATG
F8	AGAACCTGGCTCGCATCC	R8	AGCAGATGTTTAGTGGACAGC

genetic influences of the polymorphisms and the haplotype of SOCS1 are unclear (17, 18). To test whether genetic variations of *SOCS1* contribute to asthma susceptibility or asthma-related phenotypes, we first performed LD mapping of the gene and conducted an association study and haplotype analyses with regard to the LD pattern. Furthermore, we performed functional analysis of the associated promoter variant −1478CA > del.

MATERIALS AND METHODS

Study Subjects

All subjects with asthma were diagnosed according to the American Thoracic Society criteria as described (19–21). The diagnosis of atopic asthma was based on one or more positive skin prick test responses to seven common aeroallergens in the presence of a positive histamine control and a negative vehicle control (22). The seven aeroallergens were house dust, *Felis domesticus* dander (Feld), *Canis familiaris* dander, *Dactylis glomerata*, Ambrosia, *Cryptomeria japonica*, and Alternaria. We recruited 462 adults with asthma (mean age, 50.1 yr [range, 20–75 yr]; male:female ratio = 1.0:1.35; atopic asthma 91.2%) and recorded the age, sex, serum total IgE level, eosinophil count, and clinical severity. The clinical severity of adult asthma was classified according to the criteria of the National Institutes of Health/Global Initiative for Asthma by physicians who were experts in allergic diseases, and was defined by symptoms at the time of entry into the study. The distribution of subjects was as follows: step 1, mild intermittent 3% (12 individuals); step 2, mild persistent 54% (246 individuals); step 3, moderate persistent 26% (117 individuals); and step 4, severe persistent 18% (83 individuals) (23). The serum IgE level was $\log_{10}$-transformed before analysis. In this study, "high IgE" and "high eosinophil count" levels were defined as those values in the 75th percentile or higher for total IgE and the eosinophil count (%). The mean of $\log_{10}$ (total IgE [tIgE] [IU/ml]) of patients was 2.34 (= $\log_{10}$ [218.8 IU/ml]). The 75th percentile value of $\log_{10}$ (tIgE) in patients was 2.71 (= $\log_{10}$ [512.9 IU/ml]), and that of eosinophils in patients was 8.0 (%). A total of 639 healthy individuals who had neither respiratory symptoms nor a history of asthma-related diseases (mean age, 43.5 yr [range, 20–75 yr]; male:female ratio = 2.67:1.0) were recruited by detailed physicians' interviews about whether they had been diagnosed with asthma, atopic dermatitis, and nasal allergies. No lung function or serum IgE data are available for this population. Estimation for population stratification in this study was done by genotyping 60 randomly selected single-nucleotide polymorphisms (SNPs), as suggested by Pritchard and Rosenberg (24). The global χ^2 value was 51.6, with a corresponding P value of 0.77, given 60 degrees of freedom. Genomic DNA was prepared in accordance with standard protocols. All individuals were Japanese and gave written informed consent to participate in the study in accord with the rules of the process committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN).

SNPs and Linkage Disequilibrium Mapping

To identify SNPs in the human *SOCS1* gene, we sequenced all exons, including a minimum of 200 bases of the flanking intronic sequence, 2 kb of the 5′ flanking region, and a 2 kb continuous 3′ flanking region of the last exon except for regions with interspersed repeats from 24 subjects with asthma. Primer sets were designed on the basis of genomic sequences from the GenBank database (accession number AC003665) (Table 1). The PCR product was reacted with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA). Sequences were assembled and polymorphisms were identified by the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, MI).

SNP Genotyping

For the −5388C > T and −3969C > T polymorphisms in *SOCS1*, genotyping was performed by the TaqMan allele-specific amplification (TaqMan-ASA) method. For −1478CA > del polymorphism in *SOCS1*, genotyping was performed by PCR-RFLP analysis using *Dde*I (Toyobo, Japan). Primers were 5′-TGTCGTCCAGCTGCACCTC and 5′-ACCA CAGGCTTCAGAGGAAC. The digestion products were visualized on 4% agarose gel stained with ethidium bromide.

Statistical Analysis

Pairwise linkage disequilibrium (LD) was calculated as |D′| and r^2 by using the Haploview 3.2 program (http://www.broad.mit.edu/mpg/haploview/). We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. To test the association between *SOCS1* variants and adult asthma, we compared differences in allele frequency and genotype distribution of each polymorphism between case and control subjects by using a

TABLE 2. POLYMORPHISMS IN THE *SOCS1* GENE

SNP*	Location	Nucleotide	Position†	Amino Acid	Minor allele frequency (%)	Primers‡	JSNP ID IMS-JST	NCBI dbSNP
SNP 1	5′ genome	C > T	−5635		27	F1R1		rs243324
SNP 2	5′ genome	G > A	−5485		27	F1R1		rs1559392
SNP 3*	5′ genome	C > T	−5388		38	F1R1		
SNP 4	5′ genome	G > A	−5162		28	F1R1		rs243325
SNP 5*	5′ genome	C > T	−3969		27	F2R2		rs243327
SNP 6	5′ genome	G > A	−3741		4	F2R2		
SNP 7	5′ genome	T > C	−3223		4	F3R3	113573	rs3760113
SNP 8	5′ genome	A > G	−1656		30	F4R4		rs243330
SNP 9*	5′ genome	CA > del	−1478		10	F4R4		
SNP 10	5′ genome	G > A	−892		2	F5R5		
SNP 11	5′ genome	G > T	−820		27	F5R5		
SNP 12	exon 2	G > C	678	'UTR	2	F8R8		
SNP 13	3′ genome	G > C	1125		27	F8R8		

Definition of abbreviation: SNP, single-nucleotide polymorphism.
Position 1 is the A of the initiation codon.
* SNPs were genotyped in this study.
† Numbering according to the genomic sequence of SOCS1 (AC009121.8).
‡ Primer set listed in Table 1.

TABLE 3. PAIRWISE LINKAGE DISEQUILIBRIUM FOR ALL POSSIBLE TWO-WAY COMPARISONS AMONG NINE SNPS IN *SOCS1*

		D′								
		SNP 1	SNP 2	SNP 3*	SNP 4	SNP 5*	SNP 8	SNP 9*	SNP 11	SNP 13
r^2	SNP 1		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	SNP 2	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00
	SNP 3	0.62	0.62		1.00	1.00	0.87	1.00	1.00	1.00
	SNP 4	1.00	1.00	0.61		1.00	1.00	1.00	1.00	1.00
	SNP 5	1.00	1.00	0.62	1.00		1.00	1.00	1.00	1.00
	SNP 8	0.90	0.90	0.57	0.90	0.90		1.00	1.00	1.00
	SNP 9	0.04	0.04	0.19	0.05	0.04	0.04		1.00	1.00
	SNP 11	1.00	1.00	0.62	1.00	1.00	0.90	0.04		1.00
	SNP 13	1.00	1.00	0.62	1.00	1.00	0.90	0.04	1.00	

For definition of abbreviation, *see* Table 2.
* SNPs were genotyped in this study.

contingency χ^2 test. Odds ratios (ORs) with 95% confidence intervals (95% CI) were also calculated. Haplotype frequencies for multiple loci were estimated, and haplotype association tests were performed using Haploview 3.2. We also performed permutation tests for our association results, and one thousand permutated data were generated for the three single markers and haplotypes in blocks using the Haploview 3.2 program. We investigated associations between asthma-related phenotypes (eosinophil count, serum total IgE, disease severity, and atopic asthma) and variants within patients with asthma (case-only association study). Serum total IgE levels and eosinophil counts were examined as a dichotomized phenotype using the 75th percentile as the cut point. In addition, serum total IgE and eosinophil counts were analyzed as quantitative levels by the Mann-Whitney *U*-test or the Kruskal-Wallis test. Comparisons in reporter assays, mRNA expression analysis, and protein expression analysis were performed with Student's *t* test. Statistical significance was defined at the standard 5% level.

Luciferase Assay

The human lung alveolar epithelial cell line A-549 (lung adenocarcinoma, ATCC CCL-185) was purchased from American Type Culture Collection (Manassas, VA). Three concatenated copies of the 29-bp DNA fragments were cloned into pGL3-basic vector (Promega, Madison, WI) in the 5′-3′ orientation. The DNA fragments were: for −1478CA, 5′-CTAGCCCTCCCCAGCCTCAGTTTCTTCCGCAT-3′; and for −1478 del, 5′-CTAGCCCTCCCCAGCCTGTTTCTTCCG CAT-3′. We then transfected subconfluent A549 cells (5×10^4) cultured in 24-well plates with 0.125 μg of each construct and 0.0025 μg of pRL-TK Renilla luciferase vector (Promega), an internal control for transfection efficiency, using 0.75 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 h, we lysed the cells and measured firefly and Renilla luciferase activities in a luminometer using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity of the mock and *SOCS1* reporter constructs is represented as the ratio of the firefly luciferase activity to that of Renilla. Each experiment was repeated three times, and each sample was studied in triplicate as described (22). The mock-transfected average is represented as 1. Comparison in reporter assays was performed with Student's *t* test. A P value of < 0.05 was considered statistically significant.

Protein Expression of SOCS1 and Phospho-STAT1 in Nasal Fibroblasts

Nasal fibroblasts were established from nasal polyp biopsy specimens and maintained in DMEM/F-12 HAM medium containing 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL), and 3 μg/ml amphotericin B (Sigma, St. Louis, MO) as described (25). Polyinosinic acid:polycytidylic acid (poly (I:C)) and IFN-β were purchased from InvivoGen (San Diego, CA) and PeproTeck Inc. (Rocky Hill, NJ), respectively. Human NPF cells, homozygous for CA ($n = 7$) and heterozygous for CA/del ($n = 7$), were examined. We treated cultured NPF cells with the poly (I:C) (100 μg/ml) for 0, 1.5, 3, 6, and 12 h, and with IFN-β (50 ng/ml) for 1 h. Cells were washed with ice-cold PBS and made soluble in 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8/2% SDS/10% glycerol/50 mM DTT/0.01 wt/vol bromophenol). Cell lysates were collected, boiled for 5 min, and run on SDS-PAGE. Proteins were transferred to a PVDF membrane and then immunoblotting was performed with antibodies against SOCS1 (IMGENEX, San Diego, CA), β-actin (Sigma), phospho-STAT1 (Cell Signaling Technology, Santa Cruz, CA) or STAT1 (Cell Signaling Technology). The intensity of each band was quantified using the by a Luminescent Image Analyzer LAS-3000 (Fujifilm, Tokyo, Japan). The relative levels of protein expression of SOCS1 and phospho-STAT1 were calculated by comparison with β-actin protein and STAT1 protein, respectively.

RESULTS

Association of *SOCS1* Polymorphisms with Asthma Susceptibility

We performed screening of polymorphisms with genomic DNA from 24 randomly selected individuals with asthma. After extensive examination of *SOCS1* by direct sequencing, we identified 13 polymorphisms (11 SNPs in the promoter region and one SNP within the transcript) (Table 2). Six polymorphisms were contained in the two available public databases: NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and IMS-JST JSNP DATABASE (http://snp.ims.u-tokyo.ac.jp/). To examine the linkage disequilibrium (LD) between identified SNPs, pairwise LD coefficients D′ and r^2 were calculated using the Haploview program. Since four of the SNPs were quite rare, pairwise LD was measured by D′ and r^2 among the nine SNPs with a frequency of greater than 5% (Table 3). –3969C > T was in complete LD (D′ = 1.00 and r^2 = 1.00) with –5635C > T, –5485G > A, –5162G > A, –820G > T, and 1125G > C, and in strong LD (D′ = 1.00 and r^2 = 0.90) with –1656A > G. However, –5388C > T was in marginal LD with other SNPs (maximum r^2 = 0.62), and it was the SNP with the highest minor allele frequency. –1478CA > del was not in strong LD with other SNPs (maximum r^2 = 0.19). We finally selected three polymorphisms, –5388C > T, –3969C > T, and –1478CA > del, for association studies (Figure 1).

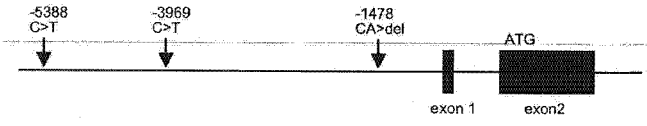

Figure 1. A graphical overview of genotyped polymorphisms identified in relation to the exon/intron structure of the human *SOCS1* gene. Two exons are shown by *black boxes*, and positions for polymorphisms are relative to the translation start site (+1).

TABLE 4. GENOTYPE FREQUENCIES FOR *SOCS1* SNPS AND ASTHMA SUSCEPTIBILITY

	Genotype	Case (%) (n = 462)	Control Subjects (%) (n = 639)	Allele	Case (%) (n = 462)	Control Subjects (%) (n = 639)	Genotype P Value	Dominant P Value	Recessive P Value	Allelic P Value
SNP3	CC	206 (46)	305 (49)	C	615 (68)	878 (70)	0.513	0.288	0.457	0.257
	CT	203 (45)	268 (43)	T	287 (32)	368 (30)				
	TT	42 (9)	50 (8)							
SNP5	CC	279 (61)	380 (60)	C	716 (78)	987 (78)	0.915	0.711	0.925	0.782
	CT	158 (35)	227 (36)	T	198 (22)	281 (22)				
	TT	20 (4)	27 (4)							
SNP9	CACA	365 (80)	534 (86)	CA	817 (89)	1150 (93)	0.023	0.0063	0.424	0.0063
	CA del	87 (19)	82 (13)	del	97 (11)	90 (7)				
	del del	5 (1)	4 (1)							

For definition of abbreviation, *see* Table 2.

All genotype frequencies in the present investigation for the control group were in Hardy-Weinberg equilibrium. The results for genotype frequencies in the asthma group and the control group are shown in Table 4. We found a significant association between the −1478CA > del genotype and adult asthmatics (CA:del ratio, 817:97 [0.89:0.11] in the asthma group; CA:del ratio, 1150:90 [0.93:0.07] in the control group) (allele frequency: OR, 1.52; 95%CI, 1.12–2.05; χ^2 = 7.47, P = 0.0063; dominant model: OR, 1.57; 95%CI, 1.13–2.16; χ^2 = 7.47, P = 0.0063). We also obtained a significant P value (0.0140) estimated by permutation tests. None of the other SNPs tested in this study showed a significant association with adult asthma.

In addition, we surveyed associations between the three SNPs and patients with asthma who had a high eosinophil count, high serum IgE level, disease severity, and atopic asthma. In the case-control study, an association of −1478CA > del on *SOCS1* with adult atopic asthma was observed in an allelic test (OR, 1.64; 95%CI, 1.20–2.24; χ^2 = 9.81, P = 0.0017) (Table 5). In the case-only study, there was a positive association in the genotype frequency of −3969C > T of the *SOCS1* gene and the most severe cases of adult asthma in an allelic test (OR, 1.72; 95%CI, 1.18–2.51; χ^2 = 8.13, P = 0.0044) (Table 5). Although total serum IgE levels and eosinophil counts were analyzed as dichotomized and quantitative phenotypes, both in case-control and in case-only studies, we could not find any association between the three SNPs and eosinophil counts or total serum IgE levels.

Haplotype Frequencies in *SOCS1* Gene

We next constructed the haplotypes of the three SNPs and estimated the frequency of each haplotype in the control and adult asthma groups (Table 6). We identified three common haplotypes covering more than 99% of the population in both case and control groups, found a positive association with a haplotype of *SOCS1* in adult asthma (χ^2 = 6.69, P = 0.0097) (haplotype T-C-del [−5388T, −3969C, and −1478 del] versus others) and obtained a significant P value of 0.024 with 1,000 permutations using the Haploview program.

Reporter Gene Analysis in *SOCS1* Gene

We examined transient expression of the −1478CAdel luciferase reporter constructs. Polymorphism −1478CAdel, in the promoter of *SOCS1*, affected relative luciferase activity (P < 0.001 for comparison between allele −1478CA and −1478 del by Student's t test, 3.25 [SD 0.49] versus 13.95 [SD 1.97], respectively) (Figure 2). The −1478 del construct had 4.29-fold higher luciferase reporter activity than the −1478CA construct.

Relationship of SOCS1 −1478 Promoter Genotype with Protein Expression of SOCS1 and Phosphorylation of STAT1 in Nasal Fibroblasts

A recent study has shown that poly(I:C) induces SOCS1, which is a negative feedback regulator of STAT1 signaling (13). To investigate whether the −1478 promoter genotype affected the protein levels of SOCS1 and phosphorylated STAT1, we measured relative protein expression and compared it in subjects with different genotypes, homozygous for CA and heterozygous for CA/del. Primary nasal fibroblasts with the CA/del allele exhibited significantly higher expression of SOCS1 protein under no stimulation and in response to poly (I:C) for 12 h (no stimulation, P = 0.036; after 12 h, P = 0.033) (Figure 3A). There was a tendency toward lower phosphorylation of STAT1 in fibroblasts with the CA/del genotype (after 3 h, P = 0.160) (Figure 3A). Higher expression of SOCS1 in cells with the CA/del allele was also observed with stimulation by 50 ng/ml IFN-β for 1 h (IFN-β, P = 0.014) (Figure 3B). The CA/del allele showed a tendency toward a lower phosphorylated STAT1 protein expression level (IFN-β stimulation, P = 0.178) (Figure 3B).

DISCUSSION

We identified polymorphisms in *SOCS1*, and performed case-control and case-only association studies as well as haplotype

TABLE 5. ASSOCIATIONS BETWEEN SNPS AND ASTHMA-RELATED PHENOTYPES

Name	Number (%)				OR (95% CI) χ^2 (df = 1)	P Value
SNP5 (−3969C > T)		C/C	C/T	T/T		
Severity = 1, 2, 3	370	234 (63)	124 (33)	12 (3)		
Severity = 4	83	41 (49)	34 (41)	8 (10)	1.72 (1.18–2.51) 8.13	0.0044*
SNP9 (−1478CA > del)		CA/CA	CA/del	del/del		
Control subjects	620	534 (86)	82 (13)	4 (1)		
Atopic asthma	369	290 (76)	74 (20)	5 (1)	1.64 (1.20–2.24) 9.81	0.0017†

Definition of abbreviations: 95% CI, 95% confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

* C allele versus T allele.

† CA allele versus deletion (del) allele.

TABLE 6. HAPLOTYPE FREQUENCIES OF POLYMORPHISMS OF THE *SOCS1* GENE

Haplotype	SNP 3	SNP 5	SNP9	Adult Asthma (*n* = 462)	Control Subjects (*n* = 639)
1	C	C	CA	0.68	0.70
2	T	T	CA	0.21	0.22
3	T	C	del	0.10	0.07
Others				0.01	0.01

analyses using patients with clinically characterized asthma. We showed here a significant association between adult asthma susceptibility and *SOCS1* promoter polymorphism −1478CA > del, which affects higher levels of transcription in the human lung epithelial cell line.

Viral infections can influence both the development and the severity of asthma (26). An epidemiologic study showed that ~ 50% of adult asthma attacks are associated with viral upper respiratory infections (27). IFNs play a central role in host defense against invasive viruses (28). A recent study has shown that SOCS1 is crucial in attenuating type I interferon signaling *in vivo* and in limiting the host response to viral infection (10). SOCS1 associates with and regulates IFNAR1-specific signals, abrogating tyrosine phosphorylation of transcription factor STAT1 and reducing the duration of antiviral gene 2′-5′-OAS expression (10). Another report showed that SOCS1 proteins inhibit IFN-α–induced activation of the JAK-STAT pathway and expression of antiviral proteins, MxA and 2′-5′-OAS (12). MxA and 2′-5′-OAS play an important role in mediating at least some of the antiviral activities induced by type I interferons (12). *In vivo*, SOCS1 cardiac myocyte-specific transgenic expression during the early stages of viral infections decreases the antiviral defense by inhibiting IFNs and promotes tissue injury in the heart (11). Transgenic expression of SOCS1 inhibits viral-induced JAK signaling and STAT, with accompanying increases in viral replication, cardiomyopathy, and mortality in virus-infected mice (11). It has been previously reported that asthmatic bronchial epithelial cells have abnormal innate immune responses to viral infection characterized by impaired type I interferon production and impaired virus-induced apoptosis, resulting in increased viral replication (29). A recent study has shown that poly(I:C) induces SOCS1 and IFN-α/β expression, and SOCS1 inhibits STAT1-dependent TLR3, IRF-7, and MIP-1α production by blocking the JAK-STAT1 pathway (13). In this study, we found that the −1478 promoter genotype affected the protein levels of SOCS1

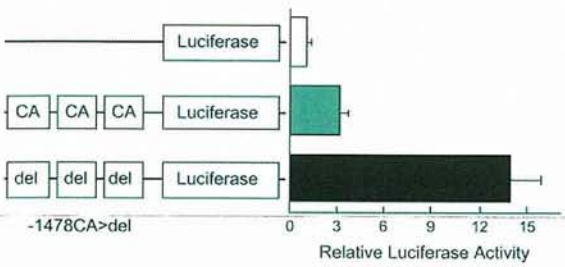

Figure 2. Effect of −1478CA > deletion polymorphism on the transcriptional activity of the human *SOCS1* promoter in a human lung epithelial cell line. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for three independent experiments ($P < 0.001$ by Student's *t* test).

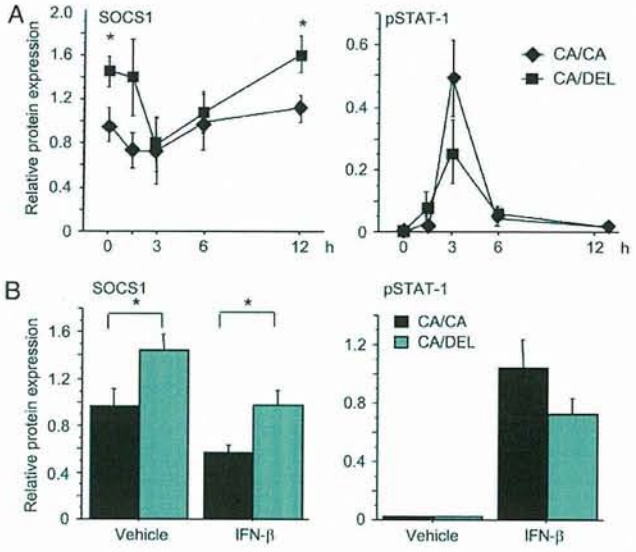

Figure 3. The relative protein expression levels of SOCS1 and phosphorylated STAT1 according to the promoter genotype were measured in primary nasal fibroblasts. Data are presented as mean values ± SEM normalized to 1.0 for the β-actin control (*$P < 0.05$). (*A*) Cells were stimulated with 100 μg/ml poly (I:C) for the indicated number of hours. Nasal fibroblasts with the CA/del allele exhibited significantly higher expression of SOCS1 protein under no stimulation and in response to poly (I:C) for 12 h. (*B*) Higher expression of SOCS1 in cells with the CA/del allele was observed with or without stimulation with 50 ng/ml IFN-β for 1 h.

and phosphorylated STAT1 under no stimulation and in response to poly (I:C) or IFN-β. Dysfunction of SOCS1 molecules could contribute to the etiology of asthma by affecting the antiviral defense in the respiratory tract. The susceptibility allele might affect the increased sensitivity to viral infection through higher SOCS1 production.

A growing body of evidence suggests that two subsets of T helper (Th) cells, Th1 and Th2, may play an important role in allergic and autoimmune disorders (30). In the case-control study, an association of −1478CA > del on *SOCS1* with adult atopic asthma was observed. The hallmark of allergic disease is infiltration by Th2 cells (1, 2). T cells from atopic subjects respond to allergens *in vitro* by inducing cytokines produced by Th2 cells, rather than cytokines produced by Th1 cells (31). A recent study has shown that the normal type 1–like antiviral response is defective in atopic asthma, both at the inducing (IL-12) and effector (IFN-γ) levels (32). IFN-γ is induced by IL-12, a cytokine regulated by SOCS1 (33). Exposure of PBMC to rhinoviruses resulted in time- and dose-dependent up-regulation of IFN-γ and IL-12 in both normal subjects and in subjects with atopic asthma. Deficient production of the type 1 cytokines IFN-γ and IL-12 in the atopic asthma group compared with the normal subjects was observed (32). Given the role of SOCS1 in attenuating Th1 cytokine signaling, the functional genetic variant in SOCS1 might contribute to the atopic phenotype in adult asthma.

In the case-only study, there was a positive association between −3969C > T of the *SOCS1* gene and the most severe cases of adult asthma. Recent studies reported reduced production of IFN-γ by PBMC of patients with asthma, which correlates with disease severity (34). In another study, patients with severe ongoing asthma had significantly reduced house dust mite–induced

IFN-γ production compared with control subjects and patients with resolved asthma (35). Furthermore, a cytokine imbalance with a deficient Th1 response, decreased generation of IFN-γ, to rhinovirus is associated with asthma severity (36). The *SOCS1* variant might be involved in severe phenotypes of adult asthma through alteration of IFN-γ signaling. In this study, we did not analyze the functional effects of the −5635C > T, −5485G > A, −5162G > A, −1656A > G, −820G > T, and 1125G > C, which were in linkage disequilibrium with promoter variant −3969C > T. To validate the involvement of the *SOCS1* gene in the severity of asthma, however, it will be necessary to elucidate the functional effects of the polymorphisms.

Our data strongly suggest the important role of SOCS1 in asthma, and further investigations of the connection between genotypes and the functional role of *SOCS1* will be helpful to clarify the etiology of asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Expression of Syk is associated with nasal polyp in patients with allergic rhinitis

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Abstract

Objective: Numerous signalings are involved in allergic inflammation. The non-receptor protein tyrosine kinase, Syk, is widely expressed in immune-potentiated cells and plays critical roles in initiating signal transduction in response to the activation of cytokine, chemokine and other types of receptors. It has been hypothesized that Syk expression in allergic nasal mucosa and polyps with allergy is different from non-allergic mucosa, and that changes in Syk expression contribute to the activation of allergic reactions.
Methods: We examined whether the expression of Syk is found in allergic nasal mucosa and polyps. We investigated the expression of Syk in 46 nasal mucosa and polyps (14 samples from patients with allergic rhinitis and 32 samples with non-allergic chronic sinusitis) using an immunohistochemical technique.
Results: Allergic polyps had more Syk positive cells than non-allergic polyps. Syk positive cells were determined to mainly be eosinophils. There was no difference in Syk expression in the lamina propria and nasal gland between allergic mucosa and non-allergic mucosa.
Conclusion: Eosinophils in allergic polyps receive an intracellular signal, although the signal is not able to determine the function in the present state. Syk appears to be a promising target molecule for anti-allergic inflammation in allergic rhinitis.

Keywords: Nasal polyp; Syk; Eosinophil; Mast cell; Nasal allergy

1. Introduction

Nasal polyps are recurrent protrusions of the nasal sinus mucosa that prolapses into the nasal cavity. The origin of nasal polyps can be divided into two categories: allergic rhinitis (AR) and non-allergic chronic sinusitis (NACS). In AR, exposure to the allergen promotes the cross-linking of IgE molecule on mast cells and mast cells release histamine and other proinflammatory mediators within minutes. The released chemical mediators induce many signals in the cells of the nose and the sum of signals causes allergic inflammation. Chemoattractants play a dual role by triggering integrin activation and directing leukocyte migration. Some chemokines and cytokines (RANTES, Eotaxin, IL-5, etc.) have been shown to attact and activate eosinophils *in vitro* and to recruit eosinophils into the inflammatory region in the nasal mucosa. The addition of several factors (ex. edema, a change in architecture of the epithelium, a large influx of water, an alteration of the structure of gland) to the AR contributes to the development of nasal polyp [1].

NACS may originate from or be perpetuated by local or systemic factors that predispose one to sinus ostial obstruction and infection [2]. Japanese NACS is different from Western NACS, and has a characteristic that neutrophils are the predominant infiltrating cells in nasal mucosa [3]. A high level of IL-8 concentration in the nasal lavage from patients with nasal polyps is typical in Japanese NACS [3–5]. Persistent inflammation due to bacterial

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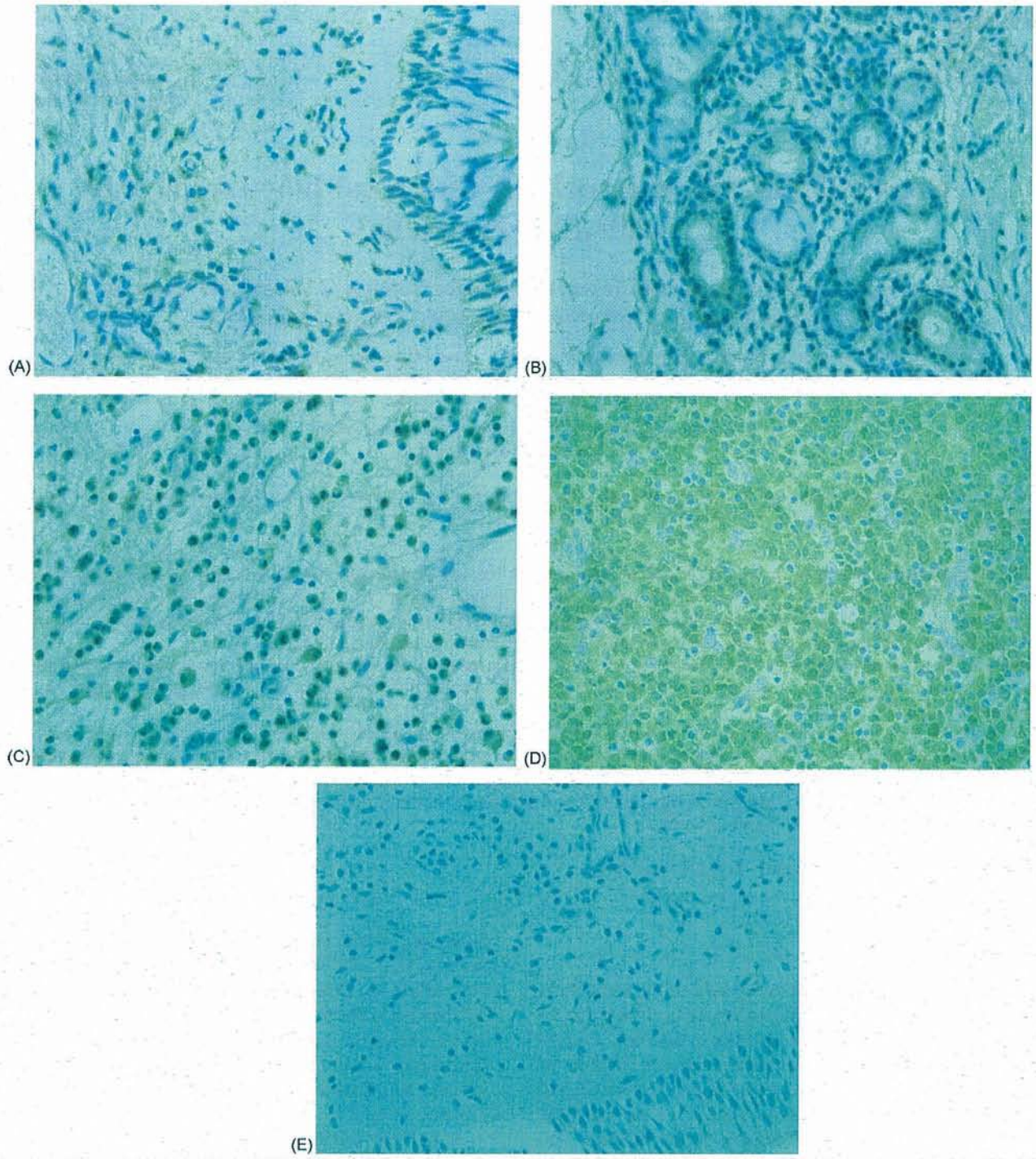

Fig. 1. Immunohistchemical staining of Syk in the nasal mucosae, intestinum of lamina propria (a: Syk score = 29.5), the nasal grand (b: Syk score = 46.5), nasal polyp (c: Syk score = 57.5), the tonsil was for the positive staining of Syk (d: Syk score = 48.8) nasal polyp (e: negative control) (magnification 200×).

infection and sequential cytokine secretion (IL-1β, TNFα, IL-8, TGFα and TGFβ) induced many signals in inflammatory cells, fibroblasts and epithelial cells [6,7]. The activation of epidermal growth factor receptor kinase and platelet-derived growth factor receptor kinase leads to hyperplasia of epithelial cells and nasal glands for the pathogenesis of nasal polyps in NACS [8,9].

The complicated activation in cells through cytokine and chemokine receptors is associated with protein tyrosine kinase. Protein tyrosine kinases can be divided into two

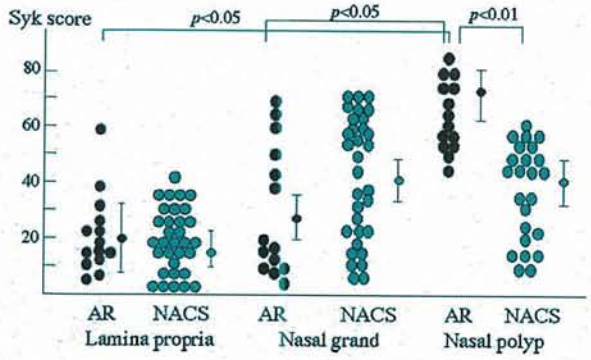

Fig. 2. The number of Syk positive cells. The Syk score determined the number of Syk positive cells in all cells. The Syk score in the nasal polyp with allergic rhinitis (AR) was higher than those with non-allergic chronic sinusitis (NACS).

families: receptor tyrosine kinases and non-receptor tyrosine kinases. Syk has non-receptor protein tyrosine kinases function as critical components in signaling cascades from membrane receptors lacking intrinsic tyrosine kinase activity, and is widely expressed on hematopoietic cells: B cells [10,11], mast cells [12], eosinophils [13], T cells [14], neutrophils [15] and other cells. Recently, it has been shown that Syk is also expressed in non-hematopoietic cells; human nasal fibroblasts [16,17], breast epithelium [18] and human hepatocytes [19]. The level of Syk expression in nasal polyp-derived fibroblasts was correlated with RANTES production by LPS [16].

In this study, we investigated the Syk expression on nasal polyps between AR and NACS using an immunohistochemical technique, since there are few reports about the expression of signal molecules in nasal polyps.

2. Materials and methods

2.1. Patients and sample collections

We studied the inferior turbinate mucosa and nasal polyps of 14 patients (10 males and 4 females) with perennial AR caused by *Dermatophagoides Pteronyssinus* (DP), and 32 control subjects (20 males and 12 females) with NACS. All of the patients underwent endoscopic sinus surgery, because all conservative treatments had no effect on their nasal congestion. We obtained informed consent from all patients. Both the inferior turbinate mucosa and the nasal polyps were excised during surgery. All of the patients with AR had a high titer of anti-DP-specific IgE without Japanese Cider and Ragweed in the serum. All patients with NACS had negative evidence for these allergies. No patients in either group had histories of aspirin-induced asthma. There were no significant differences in the background of the patients in AR and NACS groups except for nasal allergy. The operation was performed under local anesthesia by injection with 0.5% lidocaine with 1:100,000 adrenalin and 10% cocaine.

2.2. Immunohistochemical staining

Specimens were immediately fixed in 4% paraformaldehyde for over 48 h. After fixation, samples were embedded in paraffin and sectioned at 2–4 μm thickness. They were deparaffinated and treated with ethanol, then rinsed with pH7.4 phosphate-buffered saline (PBS). Immunohistchemical staining was performed using the avidin–biotin-complex technique [20].

Specimens were washed in distilled water, and rinsed with pH 7.4 PBS, and incubated in 0.3% H_2O_2 solution dissolved in absolute methanol at room temperature for 15 min to inhibit endogenous peroxidase activity. After washing, specimens that would be stained for Syk were treated by microwave irradiation for 10 min in distilled water. They were rinsed with PBS and incubated with rabbit anti-human Syk polyclonal antibodies diluted at 1:200 at 4 °C for 24 h. After rinsing with PBS, all specimens were treated with polymerized peroxidase anti rabbit-IgG (DAKO, Glostrup, Denmark) for 1 h at room temperature. After rinsing with PBS, peroxidase color visualization was carried out with 15 mg of 3–3′-diaminobenzidine tetrahydrochroride (WAKO, Tokyo, Japan) dissolved in 100 ml PBS with 8 μl of 30% H_2O_2 for 10 min. Nuclear counter staining was carried out with Mayer's haematoxylin for 2 min before mounting. For positive controls, we used the tissue of the tonsil, which was already known to be positive for Syk. For the negative control, we used rabbit anti-human IgG for the first antibody.

2.3. Double immunofluorescence techniques

The standard double immunofluorescence technique was employed. Mouse anti-human EG2 monoclonal antibody (mAb) as a marker of eosinophils, anti-CD30 mAb for T cells, anti-CD14 mAb for macrophages, anti-elastase mAb for neutrophils, anti-tryptase for mast cells, and anti-CD20 mAb for B cells were used. After being incubated with anti-human Syk antibody, rinsing with tris buffered saline (TBS), we applied swine FITC conjugated anti rabbit immunoglobulins for 2 h at room temperature. After rinsing with TBS, we applied the second antibody (for example: CD20) at 4 °C for 24 h. After rinsing with TBS, we treated rabbit RPE conjugated anti-mouse immunoglobulin for 2 h at room temperature. After washing with TBS, nuclear counter staining was carried out with Mayer's haematoxylin for 2 min before mounting. Then, we counted the positive cells with fluorescence microscopy. In this experiment, 7 NACS samples were randomly selected.

2.4. Evaluation of Syk expression

For microscopic analysis, we randomly selected five images of strongly stained sections of Syk in each specimen. The mean number of Syk positive cells per field, that had infiltrated the intestinum of the lamina propria, goblet cell,

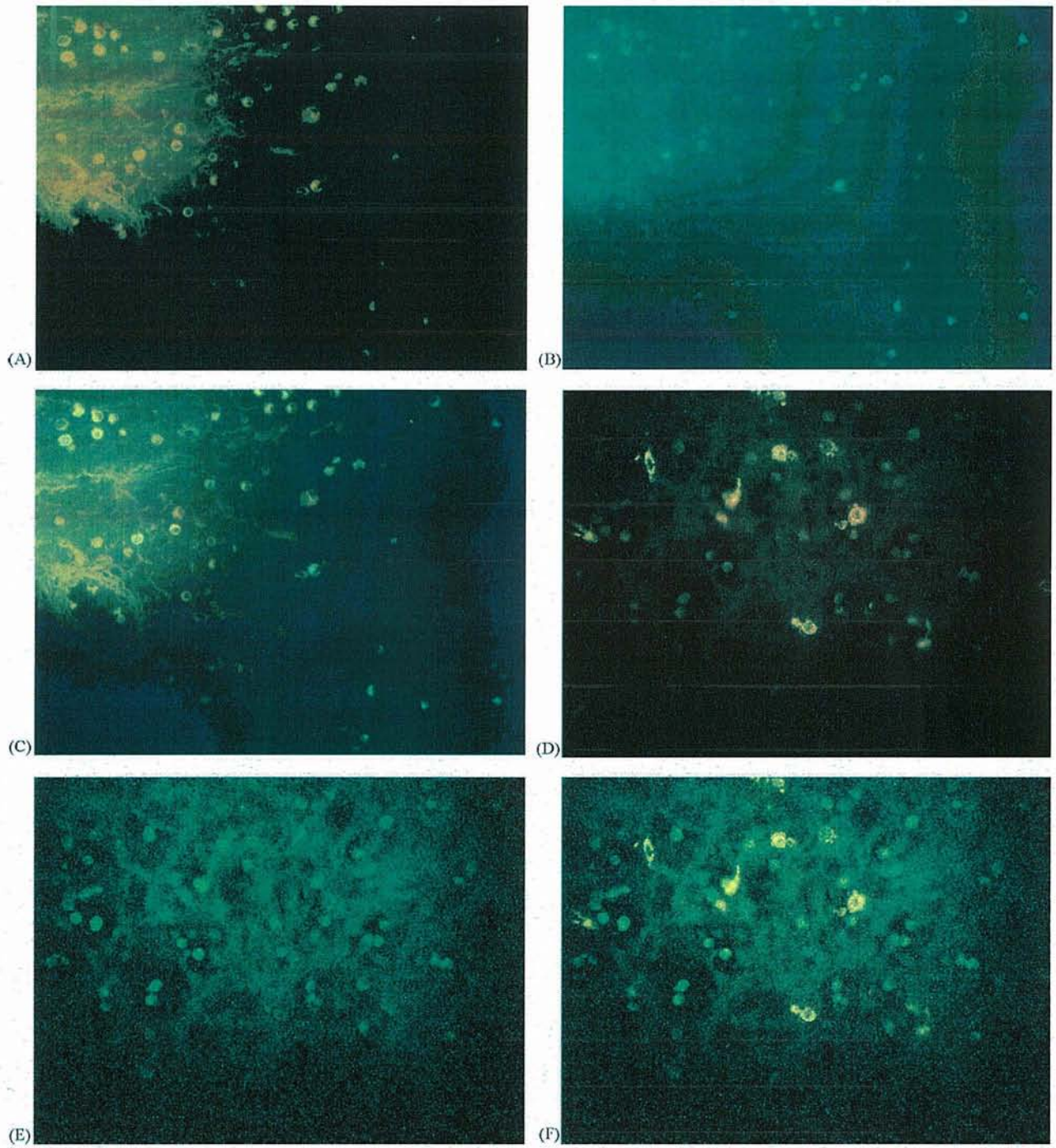

Fig. 3. Double immunohistchemical staining. (A) EG2 positive cells (red); (B) Syk positive cells (green); (C) merged view Syk score is 0.44 in this section (magnification 200×); (D) mast cell positive cells (red); (E) Syk positive cells (green); (F) merged view Syk score is 0.16 in this section (magnification 200×) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

epithelial layer were counted. We counted at least 1000 cells, including Syk positive cells, and estimated the positive cells as the Syk score [20].

For fluorescence analysis, we used a microscope (BX51-33-FL-3, Olympus, Tokyo, Japan). We randomly selected three images of strongly stained sections of Syk. The mean number of Syk and other per field that had infiltrated the nasal polyp were counted. These analyse were performed at a magnification of 400× (Syk positive cells). Macintosh computers (Stat view software; Abacus Concepts Inc., Berkeley, CA) were used for all statistical analysis.

3. Results

3.1. Syk expression in the nasal mucosa and polyp

Syk positive cells were observed in the lamina propria, nasal gland and nasal polyp. Syk staining was positive in the cells that had infiltrated the lamina propria and the nasal gland (Fig. 1A and B). The usual pattern of positive staining for Syk involved the cytoplasm. The mean Syk positive cells per field (Syk score) in the intestinum of lamina propria from all 46 patients was 15.9 ± 11.4 (mean ± S.D.), that in the nasal gland was 32.2 ± 22.4, and that in nasal polyp was 38.0 ± 19.0. In the AR group, the mean Syk score in the intestinum of lamina propria was 18.0 ± 11.7, that in the nasal gland was 25.8 ± 21.1, that in the nasal polyp was 50.9 ± 17.9. Syk score in the nasal polyp was higher than that in the lamina propria and nasal gland ($p < 0.05$, $p < 0.05$). In the NACS group, the mean of the Syk score in the intestinum of the lamina propria was 14.8 ± 11.2, that in the nasal grand was 35.3 ± 22.6, that in the nasal polyp was 30.2 ± 15.2 (no difference among the NACS group). In the AR group, the Syk score in the nasal polyp was significantly higher than that in the NACS group (50.9 ± 17.9 versus 30.2 ± 15.2, $p < 0.01$, Fig. 2). Few Syk positive cells were detected in the epithelial layer (data not shown).

3.2. Double staining

To clarify which cells expressed Syk in the nasal polyp from patients with AR, double immunostaining was performed. EG2 and Syk double positive cells were mainly observed in the allergic polyp (Fig. 3). In the AR group, the mean percentage of double positive staining for Syk and EG2 was higher than the NACS group (29.4 ± 21.4% versus 11.5 ± 8.7%, $p < 0.05$, Fig. 4). Since the Syk score of the nasal polyp in AR was higher than that in the NACS group, the absolute number of double positive nasal polyp cells in AR was also higher than that in NACS. Double positive cells

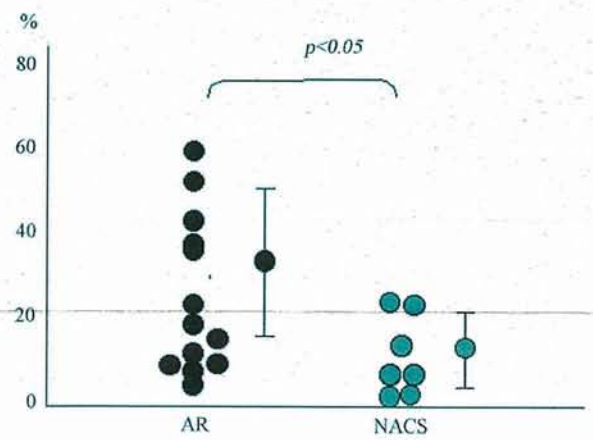

Fig. 4. The mean percentages of EG2 and Syk double positive cells. The double positive cells in AR weres higher than those in NACS ($p < 0.05$).

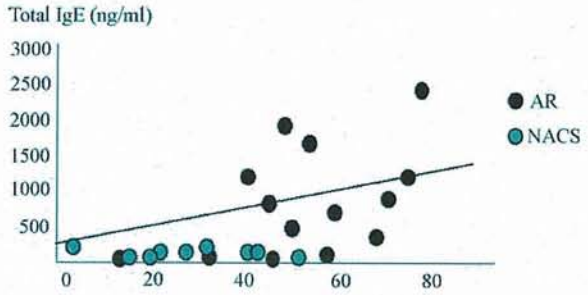

Fig. 5. The correlation with serum IgE titer and Syk score in AR. Total IgE in the serum was correlated with Syk score of nasal polyp from AR patients.

for Syk with each CD30 or elastase or CD14 were only minimally detected in both the AR and NACS group (data not shown). Although CD20 positive cells were stained in AR, the mean percentage of double positive cells in the CD20 cells was 1.1 ± 0.9%. And the mean percentage of double positive cells in the tryptase positive cells was 10.92 ± 16.85%.

3.3. Correlation of Syk score and IgE in the serum

Serum total IgE values in AR range from 16 to 2400, while those values in NACS range from 8 to 130. Total IgE in the sera is significantly associated with the Syk score in the nasal polyp ($r = 0.640$, $p < 0.01$, Fig. 5). There is no difference between the Syk score in the lamina propria and serum IgE (data not shown). Nine NACS samples which has IgE data were included.

4. Discussion

In this study, we demonstrated that Syk expression in nasal polyps from AR patients was significantly higher than that those from NACS patients. There was no difference in the Syk expression between nasal mucosa from AR and that from NACS. The Syk-positive cells are mainly eosinophils in the nasal polyp. Syk was stained in the cytoplasm of eosinophils. The mean percent of Syk positive cells in nasal polyps were associated with serum total IgE.

Eosinophils are well known to be induced and activated by several cytokines and chemokines. In allergic disease, the up-regulation of Interleukin-5 (IL-5), granulocyte/macrophage colony-stimulating factor (GM-CSF), eotaxin and RANTES cause blood and tissue eosinophilia. Patients with allergic nasal polyposis had significantly higher tissue densities of IL-4, IL-5, GM-CSF and IL-3 compared with those with non-allergic nasal polyposis [21,22]. Fan et al. also reported that EG2 and IL-5 positive cells were abundant in the submucosa of patients with allergic sinusitis, especially in the superficial layer. About half of the IL-5 producing cells were eosinophils and apoptotic eosinophils were less numerous in the superficial layer [23]. Human eosinophils have IL-3/IL-5/GM-CSF receptor on

the surface. IL-5 receptor activation in eosinophils has been shown to stimulate JAK2, STAT1, Lyn and Syk [24]. IL-5 and GM-CSF inhibited the apoptosis of eosinophils *in vitro* and *in vivo*. Both Syk and Lyn are essential signal molecules for the activation of the anti-apoptotic pathway(s) induced by the IL-3/IL-5/GM-CSF receptor subunit in human eosinophils [25]. Thus, one possibility is that Syk expression in the nasal polyps of AR may indicate the activation of Syk by IL-5 receptor to lead to the elongation of eosinophil-survival.

Conversely, the analysis of Syk-knock out mice ($Syk^{-/-}$) demonstrated that the anti-apoptotic effect of IL-5 in cells does not require Syk despite the activation of this tyrosine kinase upon IL-5 receptor ligation [26]. However, Syk is important in activation events (oxidative burst or phagocytosis) induced by Fcγreceptor (FcγR) stimulation [26]. FcγR is found on the surface of eosinophils and plays a critical role in eosinophil activation in cooperation with Syk phosphorylation. Among several FcγRs, allergen-specific IgG1 and IgG3 induces degranulation of eosinophils as inflammatory reaction through FcγRII (CD32) [27]. Also, FcγRII may pivotally regulate both the survival and death of eosinophils, depending on the manner of receptor ligation and β2 integrin involvement [28]. The integrin family of cell adhesion receptors mediates both cell–cell and cell–matrix interaction and plays critical roles in development, inflammation, angiogenesis, migration, metastasis and other important biological processes [29]. The binding of β2 integrin receptors to their ligands (ICAM-1) is critical for firm attachment, spreading and the transendothelial migration of eosinophils [30]. Syk is essential to activate signal transduction cascades initiated by the binding of β2 integrin receptors to their ligands [31]. Thus, Syk expression might suggest that signal transduction from β2 integrin receptors in eosinophils was working to migrate into the nasal polyp. As Syk is regulated by multiple classes of integrins, Syk is deeply associated with the integrin family [32]. Additionally, signaling by integrin and ICAM-1 prolong eosinophil survival [33].

Aggregation of the high affinity IgE receptor (FcεRI) by IgE binding results in the sequential activation of Syk and Lyn on mast cells [34]. Local IgE class switchings and local IgE syntheses were demonstrated in human allergic nasal mucosa [35,36]. IgE itself up-regulates FcεRI, which prevents protease digestion at the cell surface [37]. Recently, nasal polyps have been characterized by a high concentration of IgE in the nasal polyp associated with presence of *Staphylococcus aureus* enterotoxin-specific IgE [38]. These data led us to speculate that Syk-dependent FcεRI signaling is working well in nasal polyps of AR. However, the positive expression of Syk in mast cells was less than eosinophils in this study (Fig. 3D–F). Although eosinophils express FcεRI, most of the protein is confined to the cytoplasm [39]. Our data showed that IgE in the serum was correlated with the Syk score in nasal polyps in AR patients. However, there is little evidence for IgE-dependent function in eosinophils. IgG appears to be more important for eosinophil activation in allergic disease than IgE.

Recently, it was reported that enhanced IFN-α signaling and proinflammatory function were dependent on the tyrosine kinase Syk and on adaptor proteins that activate Syk through immunoreceptor tyrosine activation motifs [40]. IFN-α inhibited IL-5 and GM-CSF generation of cord blood. IFN-α receptor was found on eosinophils collected from patients with various eosinophilic disorders and inhibited the release of eosinophil granule proteins, such as eosinophil cationic protein, neurotoxin, or IL-5 [41]. The oromucosal administration of IFN-α reduced allergen-specific IgE production and allergen-induced eosinophil recruitment in the absence of detectable toxicity for the treatment of allergic disease [42]. However, our previous study showed that IFN-α was not detected in the nasal lavage from patients with AR and nasal polyps [5] and in the supernatant of nasal polyp-derived fibroblasts (data not shown). There might be the possibility that Syk activation in eosinophils of allergic rhinitis polyp induces suppressive signaling for allergic disease, but this possibility was low.

The delivery of Syk antisense oligodeoxynucleotides (ASO) by aerosol to rat lungs *in vivo* has the potential to reduce Syk expression in infiltrated immune cells and to suppress Ag-pulmonary inflammation [43]. Additionally, the treatment of Syk ASO greatly inhibited the number of eosinophils in the lung parenchyma [44]. They suggested that Syk ASO may be a useful anti-inflammatory agent. Intranasal application of Syk inhibitor R112 improved allergic symptoms of seasonal allergy in a park setting [45]. They suggested that intranasal application of Syk inhibitor become a new treatment of a seasonal allergy.

The degree of Syk expression is not equal to the activity of Syk in cells, while no expression of Syk does not means any activities of Syk in cells. The autophosphorylation and activation of Syk (phosphorylation of adaptor molecule) produce the signal pathway in eosinophils. Constitutive phosphorylated Syk was detected in nasal polyps with a high Syk expression from patients with AR by Western blotting in this study (data not shown). Although further study is necessary to investigate how Syk works in nasal polyps with AR, Syk may be a target molecule for the treatment of nasal polyps with allergy.

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ARTICLE

Early-Life Risk Factors for Occurrence of Atopic Dermatitis During the First Year

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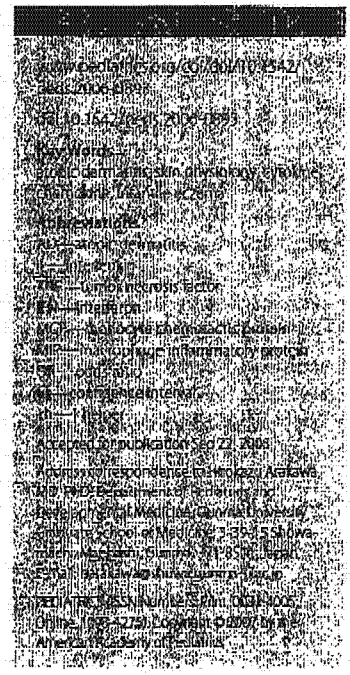
ABSTRACT

OBJECTIVE. In a prospective birth cohort study, we sought to identify perinatal predictors of the occurrence of atopic dermatitis in the first year of life.

METHODS. Associations of family history, infection during pregnancy, cord blood cytokine concentrations, and skin function parameters with atopic dermatitis were analyzed. Stratum corneum hydration was measured with an impedance meter until 5 days after delivery and again at 1 month.

RESULTS. Complete data were obtained for 213 infants, including 27 diagnosed by a physician as having atopic dermatitis during their first year and 26 diagnosed as having infantile eczema during their first month. The risk of atopic dermatitis during the first year of life was related to maternal atopic dermatitis, lower concentrations of macrophage inflammatory protein-1β in cord blood, and greater skin moisture in the surface and stratum corneum of the forehead and cheek at 1 month of age but not to viral or bacterial infection during pregnancy or breastfeeding. Paternal hay fever was associated negatively with the development of atopic dermatitis. High concentrations of interleukin-5, interleukin-17, and macrophage chemotactic protein-1 and only surface moisture in the cheek were associated with greater risk of infantile eczema in the first month.

CONCLUSIONS. The association of atopic dermatitis in infancy with reduced neonatal macrophage inflammatory protein-1β levels suggests a link with immature immune responses at birth. Stratum corneum barrier disruption in atopic dermatitis may involve impairment of cutaneous adaptation to extrauterine life. The majority of risk factors had different effects on infant eczema and atopic dermatitis, indicating different causes.



ATOPIC DERMATITIS (AD), a chronic inflammatory skin disease that usually occurs in the first few years of life,[1] has increased dramatically in prevalence in developed countries over the past several decades. AD and other allergic diseases seem to have multifactorial origins, arising from complex interactions between genes and the environment; however, the relative importance of genetic and prenatal environmental factors is not yet clear. AD is one of the earliest manifestations of allergic/atopic diseases in children. Furthermore, AD is considered a significant risk factor for aeroallergen sensitization at 5 years and a predictor of the subsequent development of asthma.[2] Asthma is a worldwide problem, and the disease's social burden and costs to public and private health care systems are substantial. Therefore, establishing early risk factors for AD may help provide intervention strategies for the primary prevention of asthma.[3]

Cytokines are considered important regulators of functional maturation in the developing fetal immune system. However, factors that determine the degree of immune competence at birth and during early infancy are not fully understood. Several studies have linked cytokine profiles at birth to subsequent development of allergic disorders.[4–7] Tang et al[4] found that infants who either exhibited symptoms of atopic disease or had a positive skin test at 1 year of age produced significantly less interferon (IFN)-γ at birth than did infants without atopy. Macaubas et al[5] found negative relationships of interleukin (IL)-4, IFN-γ, and tumor necrosis factor (TNF)-α concentrations in cord blood to the risk of asthma, atopy, or both by 6 years of age. These findings suggested that some immune functions, including the capacity to secrete both T helper (Th) type 1 and 2 cytokines, are attenuated at birth in children who develop atopy subsequently. Most previous studies focused on the relationship between Th1/Th2 cytokines at birth and future development of atopic diseases. However, a few reports examined specifically links between levels of serial cytokines, which play roles in inflammation, maturation of T cells, and production and maintenance of the Th1/Th2 balance, at birth and future development of atopic diseases.

The pathogenesis of AD involves both allergic predisposition and nonallergic environmental factors. Skin barrier disruption has attracted attention as a nonallergic etiologic factor for AD, characterized by disorders of water retention and skin barrier function.[8] One study reported that levels of ceramide, a lipid contributing to skin function, were significantly lower in lesional and nonlesional skin of subjects with AD, compared with control subjects.[9] However, we know of no reported data concerning when in life dry skin and subsequent skin barrier disruption first become apparent in children who develop AD.

The purpose of the present prospective birth cohort study was to assess the risk of prenatal maternal factors and immunologic profiles at birth and skin functional parameters just after delivery and at 1 month of age in development of AD during the first year of life. To do this, we measured levels of 17 kinds of cytokines, including proinflammatory cytokines, Th1/Th2 distinguishing cytokines, nonspecifically acting cytokines, and chemokines, in serum from cord blood and measured stratum corneum hydration with an impedance meter until 5 days after delivery and again at 1 month.

METHODS

Subjects

This was a prospective cohort study examining multiple prenatal and perinatal factors in relation to child health outcomes. Participants were recruited at Ozawa Obstetric Clinic in Gunma Prefecture, where we explained the study to pregnant women and obtained informed consent at the routine clinic visit corresponding to 35 to 37 weeks of gestation. We enrolled 279 pregnant women (93.9% of eligible subjects) between June 1, 2002, and May 31, 2003. Only children born at term without significant neonatal respiratory difficulties or pathologic jaundice were included in the study, to avoid influences on infant skin physiologic features from humidification in an infant incubator or dehydration during phototherapy. These criteria excluded only 10 children.

Cord blood was collected from participants at birth. Blood was centrifuged at 3000 × *g* for 15 minutes, and serum was separated and stored at −30°C until cytokine measurement. Mothers were provided a self-administered questionnaire and interviewed briefly. Pregnancy and perinatal data were collected from perinatal records. The main factors taken into account were parental history of atopic disease (asthma, eczema, or hay fever), maternal age, viral infection (upper airway or gastrointestinal infection) or bacterial infection (urinary tract or vaginal infection attributable to *Escherichia coli*, *Chlamydia* spp, Gram-positive group B streptococcus, or other bacteria) during the prenatal period, gestational age, infant birth weight, and method of delivery. This study was approved by the committee of ethics at the Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine.

Follow-up Examinations

In physical examinations at the 1-month checkup visit in our clinic, one of the authors noted the presence or absence of eczema on the face. At that time, physiologic skin measurements also were performed. Children were diagnosed as having infantile eczema when facial eczema was present at 1 month of age. All children were monitored for at least 1 year, for assessment of the development of atopic diseases such as AD, asthma-like illness, and food allergies. Parents were asked directly,