AJRCMB Articles in Press. Published on July 23, 2010 as doi:10.1165/rcmb.2009-0418OC
Media embargo until 2 weeks after above posting date; see thoracic.org/go/embargo

TSLP Promoter Polymorphisms are Associated with Susceptibility to Bronchial Asthma

Michishige Harada[1], Tomomitsu Hirota[1], Aya I. Jodo[1], Yuki Hitomi[1], Masafumi Sakashita[1], Tatsuhiko Tsunoda[2], Takehiko Miyagawa[3], Satoru Doi[4], Makoto Kameda[4], Kimie Fujita[5], Akihiko Miyatake[6], Tadao Enomoto[7], Emiko Noguchi[8], Hironori Masuko[9], Tohru Sakamoto[9], Nobuyuki Hizawa[9], Yoichi Suzuki[10], Shigemi Yoshihara[11], Mitsuru Adachi[12], Motohiro Ebisawa[13], Hirohisa Saito[14], Kenji Matsumoto[14], Toshiharu Nakajima[14], Rasika A. Mathias[15], Nicholas Rafaels[15], Kathleen C. Barnes[15], Blanca E. Himes[16], Qing Ling Duan[16], Kelan G. Tantisira[16], Scott T. Weiss[16], Yusuke Nakamura[17], Steven F. Ziegler[18], and Mayumi Tamari[1]

[1]Laboratory for Respiratory Diseases, [2]Laboratory for Medical Informatics, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Kanagawa, Japan
[3]Miyagawa Clinic, Gifu, Japan
[4]Department of Pediatric Allergy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino, Osaka, Japan
[5]School of Human Nursing, The University of Shiga Prefecture, Shiga, Japan
[6]Miyatake Asthma Clinic, Osaka, Japan
[7]NPO Japan Health Promotion Supporting Network, Wakayama, Japan
[8]Department of Medical Genetics, Majors of Medical Sciences, Graduate School of Comprehensive Human Sciences, [9]Division of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan
[10]Department of Public Health, Graduate School of Medicine, Chiba University, Chiba, Japan
[11]Department of Pediatrics, Dokkyo University School of Medicine, Tochigi, Japan
[12]The Division of Respiratory and Allergy, Department of Internal Medicine, Showa University School of Medicine, Tokyo, Japan
[13]Department of Pediatric Allergy, National Sagamihara Hospital, Clinical Research Center for Allergy and Rheumatology, Kanagawa, Japan
[14]Department of Allergy and Immunology, National Research Institute for Child Health & Development, Tokyo, Japan
[15]The Johns Hopkins Asthma & Allergy Center, Johns Hopkins University, Baltimore, MD, USA
[16]Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA
[17]Laboratory of Molecular Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[18]Immunology Program, Benaroya Research Institute, Seattle, WA, USA

*Correspondence author: Mayumi Tamari, MD, PhD; Laboratory for Respiratory Diseases, Center for Genomic Medicine, Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan; Phone: +81-45-503-9616, Fax: +81- 45-503-9615, E-mail: tamari@src.riken.jp

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and from the Ministry of Health, Labor, and Welfare, Japan.

Running title: An association study of *TSLP* in bronchial asthma

This article has an online data supplement that is accessible from this issue's table of contents online at www.atsjournals.org.

AT A GLANCE COMMENTARY
TSLP initiates Th-2-mediated immune responses and plays crucial roles in allergic inflammation. The influence of genetic changes in this crucial cytokine on the etiology of asthma is unclear.

What This Study Adds to the Field
Our findings suggest that functionally *TSLP* polymorphisms contribute to the disease susceptibility of asthma. The susceptible functional allele might contribute to the Th2-polarized immunity in asthma during viral respiratory infections. We also found synergistic suppression of poly(I:C)-induced TSLP production by dexamethasone and salmeterol.

ABSTRACT
Rationale: Thymic stromal lymphopoietin (TSLP) triggers dendritic cell-mediated T helper (Th) 2 inflammatory responses. A single nucleotide polymorphism (SNP), rs3806933, in the promoter region of the *TSLP* gene creates a binding site for the transcription factor activating protein (AP)-1. The variant enhances AP-1 binding to the regulatory element and increases promoter-reporter activity of *TSLP* in response to poly(I:C) stimulation in normal human bronchial epithelium (NHBE).
Objectives: We investigated whether polymorphisms including the SNP rs3806933 could affect the susceptibility to and clinical phenotypes of bronchial asthma.
Methods: We selected three Tag SNPs and conducted association studies of the *TSLP* gene using two independent populations (639 childhood atopic asthma patients and 838 controls, and 641 adult asthma patients and 376 controls, respectively). We further examined effects of corticosteroids and a long-acting β_2-agonist (LABA) (salmeterol) on expression levels of the *TSLP* gene in response to poly(I:C) in NHBE.
Measurements and Main Results: We found promoter polymorphisms, rs3806933 and rs2289276, significantly associated with disease susceptibility in both childhood atopic and adult asthma. The functional SNP rs3806933 was associated with asthma (meta-analysis, P = 0.000056; odds ratio, 1.29; 95% confidence interval, 1.14-1.47). A genotype of rs2289278 was correlated with pulmonary function. We also found that the induction of *TSLP* mRNA and protein expression induced by poly(I:C) in NHBE was synergistically impaired by a corticosteroid and salmeterol.
Conclusions: *TSLP* variants are significantly associated with bronchial asthma and pulmonary function. Thus, TSLP might be a therapeutic target molecule for combination therapy.

Abstract: 244 words
The body of the manuscript: 3100 words
Key Words: asthma; TSLP; bronchial epithelial cells; combination therapy; genetic polymorphisms

Abbreviations: confidence interval, CI; linkage disequilibrium, LD; odds ratio, OR; single nucleotide polymorphism, SNP; T helper type, Th; untranslated region, UTR.

INTRODUCTION
Thymic stromal lymphoprotein (TSLP) is an epithelial cell-derived cytokine that triggers dendritic cell-mediated T helper (Th) 2 inflammatory responses and plays an important role in the initiation and maintenance of the allergic immune response (1-6). A recent study has shown that TSLP is

released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells (7). In humans, TSLP is highly expressed by airway epithelial cells during allergic inflammation (2), and the *TSLP* expression in asthmatic airways is correlated with both the expression of Th2-attracting chemokines and disease severity (3).

Large numbers of association studies on asthma and asthma-related phenotypes using genetic polymorphisms have been conducted in different populations (8). Recent studies have shown roles of human genetic polymorphisms of the *TSLP* gene. A variant in *TSLP* was associated with reductions in IgE in response to cockroaches and total IgE in a sex-stratified analysis (9). A functional SNP, rs3806933, has been identified in the regulatory element of *TSLP* (10). The variant creates a binding site for AP-1, and affects the transcriptional efficiency of the long form of *TSLP* induced by stimulation with poly(I:C) in bronchial epithelial cells (10).

The majority of exacerbations of asthma coincide with respiratory viral infections, most commonly by rhinoviruses (RVs) (11). DsRNA, a TLR3 ligand, is a potent stimulus for innate antiviral immune responses, and poly(I:C) is thought to mimic the effects of dsRNA (12). Inflammatory mediators IL-1β and TNF-α regulate human *TSLP* gene expression, and human *TSLP* mRNA levels increase after exposure to Toll-like receptor (TLR) 2, TLR3, TLR8, and TLR9 ligands in airway bronchial epithelial cells (13, 14). A suppressive effect of glucocorticoid on the expression of TSLP induced in airway epithelial cells by stimulation with the TLR3 ligand and Th2 cytokines has been reported (14). In addition, several clinical studies have shown that the combination of an inhaled corticosteroid and long-acting β-adrenergic agonist (LABA) is more efficacious in asthma than either alone and reduces exacerbations (15-18). We investigated the effects of dexamethasone (DEX) and salmeterol (SAL) on the induction of TSLP by poly(I:C).

In this study, we found that the promoter polymorphisms rs3806933 and rs2289276 were significantly associated with susceptibility to both childhood atopic and adult asthma using a case-control association study. We also found a significant correlation between lung function and the genotype of rs2289278. Functional analyses of the related variant rs2289276 were conducted. We further found that corticosteroids and LABA (salmeterol) synergistically suppressed the expression levels of *TSLP* mRNA and TSLP protein production induced by dsRNA in bronchial epithelial cells.

Some of the results of these studies have been previously reported in the form of an abstract (19).

METHODS

Additional details on methods are provided in the online supplement.

Study Subjects and Genotyping

All subjects with asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute) as described (20-22). Data for forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and FEV_1:FVC were available for subjects with adult asthma. The clinical parameters of the participants are summarized in Table 1. Genomic DNA was prepared in accordance with standard protocols. Genotyping was performed by the TaqMan allele-specific amplification

(TaqMan-ASA) method (Applied Biosystems, Foster City, CA). All individuals were recruited with written informed consent to participate in the study in accord with the rules of the process committee at the Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN).

Statistical Analysis

A total of 23 polymorphisms in the *TSLP* gene have been identified in the Japanese population (10). Pairwise linkage disequilibrium (LD) was calculated as D'/LOD and r^2 and three Tag SNPs, rs3806933, rs2289276 and rs2289278, were selected among seven SNPs with a frequency of greater than 10% by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/) (10) (Figure 1A). The functional promoter SNP rs3806933 had an allele-specific effect on expression through altering affinity for AP-1, and the SNP was in strong LD with rs2289276 (r^2 = 0.82) (Figure 1B). To test the association between *TSLP* variants and bronchial asthma, we conducted contingency chi-square test. We applied Bonferroni corrections, the multiplication of *P* values by three, the number of Tag SNPs. In the association study, corrected *P* values of less than 0.05 were judged to be significant. The Mantel-Haenszel method was used to combine allele frequency data sets. ORs with 95%CIs were also calculated. Haplotype frequencies for three loci were estimated, and haplotype association tests were performed using Haploview 4.1. We further investigated associations between asthma-related phenotypes. We examined associations between asthma-related phenotypes (eosinophil count, serum total IgE, lung functions and disease severity) and variants within patients with asthma as described (22, 23). Comparisons in mRNA expression analysis and protein expression analysis were performed with Student's t-test. Statistically significant differences in the luciferase assay were assessed with the Bonferroni-Dunn test with two-factor factorial analysis of variances (ANOVA). Statistical significance was defined at the standard 5% level.

Computational Analysis of Transcription Factor-Binding Sites and Biotinylated Oligonucleotide Precipitation Assay

TRANSFAC® Professional 10.3 (http://www.biobase.de/pages/) was used to predict putative transcription factor-binding sites under the minimizing condition of the sum of false positives and false negatives.

Biotinylated oligonucleotide precipitation assay was conducted as described (10). The oligonucleotides for the precipitation assay are listed below: -82C oligo, 5'-TGGCCCCTAAGGCAGGCCTTACAG-3'; -82T oligo, 5'-TGGCCTCTAAGGCAGGCCTTACAG -3'. AP-2α was detected by immunoblotting with an anti-AP-2α antibody (C-18; Santa Cruz Biotechnology, Inc.).

Quantitative Real-Time RT-PCR, Enzyme-Linked Immunosorbent Assay (ELISA) and Luciferase Assay

The expression of *TSLP* was determined by real-time quantitative RT-PCR using SYBR Premix Ex Taq (Takara, Shiga, Japan). TSLP in culture supernatants was measured using ELISA kits (R&D Systems Inc., Minneapolis, MN), and the reporter luciferase assays were conducted as described (10).

RESULTS

Identification of *TSLP* Polymorphisms and Haplotypes Associated with Asthma Susceptibility

All genotype and allele frequencies are shown in Table 2, and those for the control and asthma groups were in Hardy-Weinberg equilibrium. We found that the functional SNP rs3806933 was associated with childhood atopic asthma (P = 0.0063; odds ratio, 1.25; 95% confidence interval, 1.07-1.47) and adult asthma (P = 0.0023; odds ratio, 1.37; 95% confidence interval, 1.12-1.67). Another SNP, rs2289276, was also significantly associated with childhood atopic asthma (P = 0.00066; odds ratio, 1.33; 95% confidence interval, 1.13-1.57) under allelic model (Table 2). The directions of associations of the two SNPs were similar in both of the populations. We combined the results using Mantel-Haenszel meta-analysis, and observed the most significant association at rs3806933 (meta-analysis, P = 0.000056; odds ratio, 1.29; 95% confidence interval, 1.14-1.47) (Table 2). We also found a significant association under dominant model at rs3806933 (meta-analysis, P = 0.00013; odds ratio, 1.37; 95% confidence interval, 1.16-1.62) and at rs2289276 (meta-analysis, P = 0.00019; odds ratio, 1.36; 95% confidence interval, 1.16-1.61) (Table 2). We further conducted an association study using the adult asthma cases that were diagnosed in childhood (see Table E1 in the online data supplement). We confirmed a significant association between rs3806933 and adult asthma diagnosed in childhood with a similar direction of association. A recent study has shown a female-specific association between a variant of the *TSLP* gene and total serum IgE and IgE to cockroach (9). We further conducted a sex-stratified analysis, however we could not determine the female-specific effect on the associations. Although we surveyed associations between the three SNPs and asthmatic patients who had high eosinophil counts, high serum IgE levels and disease severity, we could not find any association. However, the rs2289278 C allele was significantly correlated with decreased FEV_1:FVC in adult asthma (P = 0.00021 by the Jonckheere-Terpstra test) (Figure 2). We next constructed the haplotypes of the three SNPs and estimated the frequency of each haplotype in controls and those with bronchial asthma (Table E2). We identified three common haplotypes in the population. Haplotype T-T-C of *TSLP* was significantly associated with childhood atopic asthma and adult asthma. We obtained P values of 0.00070 and 0.032, respectively, by using the Haploview 4.1 program.

To test the generalizability of our findings in other ethnic populations, we compared our results with two recent genome-wide association studies (GWAS) of asthma (24, 25). Markers rs3806932, rs2289276, and rs11466741 were included in the Illumina panels used in the studies. Marker rs3806933 was in complete LD with rs3806932 (D' = 1.00 and r^2 = 1.00), and rs2289276 was in complete LD with rs11466741 (D' = 1.00 and r^2 = 1.00) in a Japanese population (10). However, we could not replicate the association between these *TSLP* variants and asthma in the African- and European-ancestry samples (Table E3).

Transcription factor binding to the rs2289276 SNP

We predicted a potential allelic difference in cis-acting regulatory function in transcription via a bioinformatics approach, and rs2289276

(-82C/T) was found to possibly alter the affinity of a transcription factor, AP-2α, between two alleles (Figure 3A). The sequence containing the -82C SNP on the protective allele corresponded to the putative binding site to AP-2α, a possible transcription suppression factor (26). We next examined the binding of AP-2α protein to the sequences containing the -82C/T SNP, and binding was clearly detectable in both oligonucleotides without stimulation (Figure 3B). The binding ability of -82T was diminished compared with that of -82C, regardless of poly(I:C) stimulation, suggesting that the higher AP-2α binding to -82C (on the protective allele) might have reduced its transcriptional activity through repressive effects on the transcription.

The Induction of *TSLP* mRNA and Protein Expression Induced by Poly(I:C) in NHBE is Synergistically Impaired by a Corticosteroid and Salmeterol

We investigated the effects of dexamethasone (DEX) and salmeterol (SAL) on the induction of *TSLP* by poly(I:C). We first confirmed, by ELISA, the capability of DEX to suppress poly(I:C)-induced TSLP protein production in normal human bronchial epithelial (NHBE) cells. The addition of DEX 0.5 h before or simultaneously with poly(I:C) stimulation dramatically reduced TSLP production in a dose-dependent manner (Figure E1). Even if DEX was added to the NHBE medium at 1 h after poly(I:C) stimulation, TSLP production was also significantly impaired (more than 50% suppression) (Figure E1). Next we investigated effects of SAL on expression of the *TSLP* gene and TSLP protein production. NHBE cells were stimulated with or without poly(I:C) for 4 h. DEX (1 μM) and/or SAL (1 μM) was added to medium 0.5 h before the stimulation with poly(I:C). Relative expression of *TSLP* in NHBE was normalized with *GAPDH* expression. The *TSLP* mRNA expression was decreased by DEX and/or SAL. The greatest suppressive effect was observed by concurrent exposure of the cells to DEX and SAL (Figure 4A). Expression data are representative of three independent experiments, and similar results were obtained using NHBE cells from three individuals.

We also measured the protein levels of TSLP under the same conditions. NHBE were stimulated with or without poly(I:C), and the concentrations of TSLP in supernatants 24 h after stimulation were measured by ELISA. DEX and SAL were added to the medium 0.5 h before the stimulation with poly(I:C) at the indicated doses. Synergistic suppression of TSLP protein production was also observed by concurrent exposure to DEX and SAL, and DEX and/or SAL caused a dose-dependent decrease in TSLP protein production (Figure 4B). Data represent mean ± SD. Data are representative of two independent experiments performed using NHBE cells from two individuals, and similar results were obtained. These results implied that DEX and SAL might synergistically suppress TSLP production in human airway epithelial cells during viral respiratory infections.

Effects of Dexamethasone and Salmeterol on Luciferase Transcription

Rs3806932 (-1914T/G) was in complete LD with rs3806933 (-847C/T) (D' = 1.00 and r^2 = 1.00) (10). The promoter activity of reporter constructs containing the -1914G-847T-82T (minor) haplotype showed significantly greater activity than the other haplotype,

-1914A-847C-82C (major) in response to poly(I:C) as described (10). We here examined the effects of DEX and SAL on the promoter activities of these reporter constructs (Figure 5A). DEX and SAL suppressed reporter activities and their synergistic suppression of luciferase activities was also observed by concurrent exposure to DEX and SAL in both of the haplotypes (Figure 5B). However, there was no difference in the luciferase activities between the major and minor haplotypes under concurrent exposure to DEX and SAL (P = 0.096 by the Bonferroni-Dunn test with two-factor factorial ANOVA).

DISCUSSION

We have reported that a functional promoter SNP (rs3806933) of *TSLP* that creates a binding site for the transcription factor AP-1 enhances AP-1 binding to the regulatory element (10). The functional variant also increases promoter-reporter activity of long-form *TSLP* in response to poly(I:C) stimulation in NHBE(10). In this study, we found that the *TSLP* functional polymorphism was associated with both childhood atopic and adult asthma in a Japanese population. There is a synergistic relationship between viral infection and allergen sensitization and exposure in provoking exacerbations and the major trigger for exacerbations in both children and adults is viral infection (27-29). Clinical studies suggest that asthmatics are more susceptible to rhinovirus infections than normal individuals and have longer duration of lower respiratory tract symptoms when infected with rhinoviruses (30-32). TSLP appears to be involved in the development of bronchial asthma through functional genetic polymorphisms that might contribute to Th2-polarized immunity through higher TSLP production by bronchial epithelial cells in response to viral respiratory infections.

A recent study has shown that *TSLP* expression is increased in asthmatic airways and correlates with lung function (3). The numbers of both epithelial and submucosal cells expressing *TSLP* mRNA correlated inversely with FEV_1 (3). We found a significant correlation between the rs2289278 genotype and lung function (FEV_1:FVC). The rs2289278 variant is located in intron 2 of long-form *TSLP* and the 5' untranslated region of short-form *TSLP*. However, the genetic influences of the polymorphism on the function of the *TSLP* gene in asthma are unclear, and further investigation of the functional role of the variant needs to be conducted.

A recent study has shown that a SNP in *TSLP* is associated with cockroach-allergy IgE in Costa Rican girls and with total IgE in girls in two populations (9). The study has shown significant evidence of linkage to IgE produced in response to cockroach allergy on chromosome 5q23 in female subjects (9). *TSLP* is located near the linkage peak and has female-specific effects on lung disease in mice (33). It has also been reported that rs2289276 is associated with reductions in IgE in the response to the cockroach and total IgE (9). In our Japanese population, we could not find an association between total IgE and the rs2289276 SNP among asthmatic subjects in this study and female-specific effect on the associations. Although inconsistent associations may reflect sample size, phenotype heterogeneity, or gene-environment interactions in the population, further replication studies of these findings are needed.

We could not replicate our findings in the populations that were examined in two recent GWAS (24, 25).

There were differences of minor allele frequencies of the variants among the control populations. The MAFs of the three variants, rs3806932, rs2289276, and rs11466741, in the Japanese population were 0.29, 0.26 and 0.26, respectively (10). To test for replication at the level of the gene rather than the SNP, further fine mapping around the *TSLP* gene and association studies seem to be needed.

We could not replicate our findings in European and African-ancestry populations for which recent GWAS's were performed (24, 25). There were differences of minor allele frequencies of the variants among the control populations. The MAFs of the three variants, rs3806932, rs2289276, and rs11466741, in the Japanese population were 0.29, 0.26 and 0.26, respectively (10), compared to 0.43, 0.29, and 0.29 in the CAMP population, and 0.63, 0.18, and 0.31 in the African American population, and 0.66, 0.17, and 0.28 in the African Caribbean population. Failure to observe a SNP-for SNP replication in ethnically diverse populations is not uncommon, and can result from variation in allele frequencies, population admixture, heterogeneity of the phenotype, and environmental factors. Our failure to replicate at the SNP level is therefore not surprising, and it is likely that variants other than those tested in this study are the causal variants. Elsewhere it has been argued that a gene-based approach, rather than a SNP-for SNP, may provide evidence for genetic analysis at the functional level (25). Our findings suggest that replication at the level of the gene rather than the SNP, and further fine mapping around the TSLP gene are warranted.

We previously have shown that a functional rs3806933 SNP in the promoter region of long-form *TSLP* (-847T) creates a binding site for AP-1 and enhances AP-1 binding to the regulatory element (10). In this study, we found the binding of AP-2α protein, a possible transcription suppression factor, to the sequences containing the rs2289276 (-82C/T) SNP and higher binding to -82C (on the protective allele). Both of the SNPs, rs3806933 (-847C/T) and rs2289276 (-82C/T), were significantly associated with asthma susceptibility. We also identified a common disease-susceptible haplotype, T(-847)-T(-82)-C(1560), in both childhood atopic and adult asthma. The two different transcription factors on the two promoter SNPs might lead to preferential transcription from the susceptible haplotype T-T-C through their cooperative effects in bronchial epithelial cells. In this study, we did not examine the functional effects of polymorphisms rs3806932, rs2289277, rs10073816, and rs11466741, which were in strong LD with the related variants, rs3806933 and rs2289276. The functions of these linked polymorphisms remain to be elucidated.

Combination therapy with LABA and inhaled corticosteroids reduces exacerbation frequency in asthma, and it is also efficacious as intervention therapy in asthma exacerbations (16-18). In this study, SAL was able to suppress TSLP production in NHBE cells and, furthermore, we demonstrated synergistic suppression of poly(I:C)-induced TSLP production by DEX and SAL in NHBE cells. Respiratory viral infections are associated with the majority of exacerbations of bronchial asthma, and a recent study has shown that combined corticosteroid/β_2 agonists synergistically suppress rhinovirus-induced neutrophil (CXCL8) and lymphocyte (CCL5, CXCL10)

chemokine production in airway epithelial cells (34). Glucocorticosteroids inhibit both NF-κB and AP-1 through glucocorticoid-induced leucine zipper protein (35, 36), and an NF-κB binding site has been identified 3.7kb upstream from the start of *TSLP* transcription (13). Although further investigation of the molecular mechanisms mediating suppressive effects of DEX and/or SAL on TSLP and chemokine production is needed, combination therapy might exert an anti-Th2 polarized inflammatory effect during respiratory viral infections through suppressive effects on TSLP and production of other chemokines in airway epithelial cells.

In summary, we identified *TSLP* as a susceptibility gene for childhood atopic and adult asthma by means of a case-control study with SNPs, and demonstrated that glucocorticoid/LABA treatment synergistically suppressed poly(I:C)-induced TSLP in NHBE. Our data strongly support the important role of TSLP in bronchial asthma and the clinical benefits of combination therapy in asthma management.

Acknowledgments: We thank all the participants in the study. We are grateful to the members of The Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan for supporting our study. We also thank Makiko T. Shimizu, Hiroshi Sekiguchi and Nami Kawaraichi for technical assistance, Chinatsu Fukushima for providing patients' data, and Dr. Takashi Naito for correcting samples. The authors thank investigators from the Genomic Research on Asthma in the African Diaspora (GRAAD) consortium, including Terri H. Beaty, Ingo Ruczinski, Audrey V. Grant, Candelaria Vergara, Tanda Murray, Cassandra Foster, Peisong Gao, Alkis Togias, Nadia Hansel, Gregory Diette, Patrick Breysse, N. Franklin Adkinson, Mark Liu, Mezbah Faruque, Georgia Dunston and Harold Watson. K.C.B. was supported in part by the Mary Beryl Patch Turnbull Scholar Program. GRAAD was supported by NIH HL087699.

References

1. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu Mr, Travis M, Zurawski SM, Johnston J, Liu YJ, Spits H, de Waal Malefyt R, Kastelein RA, Bazan JF. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. J Immunol 2001;167:336-343.
2. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt Rd R, Bazan F, Kastelein RA, Liu YJ. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673-680.
3. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, Corrigan C. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. J Immunol 2005;174:8183-8190.
4. Ziegler SF, Liu YJ. Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. Nat Immunol 2006;7:709-714.
5. Liu YJ. Thymic stromal lymphopoietin: Master switch for allergic inflammation. J Exp Med 2006;203:269-273.
6. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, Omori M, Zhou B, Ziegler SF. TSLP: An epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 2007;25:193-219.
7. Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, Paquette N,

Ziegler SF, Sarfati M, Delespesse G. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 2007;204:253-258.
8. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol 2008;8:169-182.
9. Hunninghake GM, Lasky-Su J, Soto-Quiros ME, Avila L, Liang C, Lake SL, Hudson TJ, Spesny M, Fournier E, Sylvia JS, Freimer NB, Klanderman BJ, Raby BA, Celedón JC. Sex-stratified linkage analysis identifies a female-specific locus for IgE to cockroach in Costa Ricans. Am J Respir Crit Care Med 2008;177:830-836.
10. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, Miyatake A, Enomoto T, Noguchi E, Yoshihara S, Ebisawa M, Saito H, Matsumoto K, Nakamura Y, Ziegler SF, Tamari M. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. Am J Respir Cell Mol Biol. 2009;40:368-374.
11. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. BMJ 1995;310:1225-1229.
12. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3. Nature 2001;413:732-738.
13. Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF-kB. Proc Natl Acad Sci U S A 2007;104:914-919.
14. Kato A, Favoreto S, Jr., Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. J Immunol 2007;179:1080-1087.
15. Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. N Engl J Med 1997;337:1405-1411.
16. Calverley P, Pauwels R, Vestbo J, Jones P, Pride N, Gulsvik A, Anderson J, Maden C; TRial of Inhaled STeroids ANd long-acting beta2 agonists study group. Combined salmeterol and fluticasone in the treatment of chronic obstructive pulmonary disease: A randomised controlled trial. Lancet 2003;361:449-456.
17. Kankaanranta H, Lahdensuo A, Moilanen E, Barnes PJ. Add-on therapy options in asthma not adequately controlled by inhaled corticosteroids: A comprehensive review. Respir Res 2004;5:17.
18. O'Byrne PM, Bisgaard H, Godard PP, Pistolesi M, Palmqvist M, Zhu Y, Ekström T, Bateman ED. Budesonide/formoterol combination therapy as both maintenance and reliever medication in asthma. Am J Respir Crit Care Med 2005;171:129-136.
19. Harada M, Hirota T, Jodo AI, Ziegler SF, Tamari M. Functional SNP in promoter of TSLP gene is associated with susceptibility to childhood atopic asthma. Am J Respir Crit Care Med 2008;177:A774.
20. Hirota T, Harada M, Sakashita M, Doi S, Miyatake A, Fujita K, Enomoto T, Ebisawa M, Yoshihara S, Noguchi E, Saito H, Nakamura Y, Tamari M. Genetic polymorphism regulating ORM1-like 3 (Saccharomyces cerevisiae) expression is associated with childhood atopic asthma in a Japanese population. J Allergy Clin Immunol 2008;121:769-770.
21. Hirota T, Suzuki Y, Hasegawa K, Obara K, Matsuda A, Akahoshi M, Nakashima K, Cheng L, Takahashi N, Shimizu M, Doi S, Fujita K, Enomoto T, Ebisawa M, Yoshihara S, Nakamura Y, Kishi F, Shirakawa T, Tamari M. Functional haplotypes of IL-12B are associated with childhood atopic asthma. J Allergy Clin Immunol 2005;116:789-795.
22. Harada M, Nakashima K, Hirota T, Shimizu M, Doi S, Fujita K, Shirakawa T, Enomoto T, Yoshikawa M, Moriyama H, Matsumoto K, Saito H, Suzuki Y, Nakamura Y, Tamari M. Functional polymorphism in the suppressor of cytokine signaling 1 gene associated with adult asthma. Am J Respir Cell Mol Biol 2007;36:491-496.

23. Harada M, Obara K, Hirota T, Yoshimoto T, Hitomi Y, Sakashita M, Doi S, Miyatake A, Fujita K, Enomoto T, Taniguchi M, Higashi N, Fukutomi Y, Nakanishi K, Nakamura Y, Tamari M. A functional polymorphism in IL-18 is associated with severity of bronchial asthma. Am J Respir Crit Care Med. 2009;180:1048-55.
24. Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, Wilk JB, Willis-Owen SA, Klanderman B, Lasky-Su J, Lazarus R, Murphy AJ, Soto-Quiros ME, Avila L, Beaty T, Mathias RA, Ruczinski I, Barnes KC, Celedón JC, Cookson WO, Gauderman WJ, Gilliland FD, Hakonarson H, Lange C, Moffatt MF, O'Connor GT, Raby BA, Silverman EK, Weiss ST. Genome-wide association analysis identifies PDE4D as an asthma-susceptibility gene. Am J Hum Genet 2009;84:581-593.
25. Mathias RA, Grant AV, Rafaels N, Hand T, Gao L, Vergara C, Tsai YJ, Yang M, Campbell M, Foster C, Gao P, Togias A, Hansel NN, Diette G, Adkinson NF, Liu MC, Faruque M, Dunston GM, Watson HR, Bracken MB, Hoh J, Maul P, Maul T, Jedlicka AE, Murray T, Hetmanski JB, Ashworth R, Ongaco CM, Hetrick KN, Doheny KF, Pugh EW, Rotimi CN, Ford J, Eng C, Burchard EG, Sleiman PM, Hakonarson H, Forno E, Raby BA, Weiss ST, Scott AF, Kabesch M, Liang L, Abecasis G, Moffatt MF, Cookson WO, Ruczinski I, Beaty TH, Barnes KC. A genome-wide association study on African-ancestry populations for asthma. J Allergy Clin Immunol 2010;125:336-346.
26. Wang X, Bolotin D, Chu DH, Polak L, Williams T, Fuchs E. AP-2alpha: a regulator of EGF receptor signaling and proliferation in skin epidermis. J Cell Biol 2006;172:409-421.
27. Murray CS, Simpson A, Custovic A. Allergens, viruses, and asthma exacerbations. Proc Am Thorac Soc 2004;1:99-104.
28. Contoli M, Caramori G, Mallia P, Johnston S, Papi A. Mechanisms of respiratory virus-induced asthma exacerbations. Clin Exp Allergy 2005;35:137-145.
29. Sears MR. Epidemiology of asthma exacerbations. J Allergy Clin Immunol 2008;122:662-668.
30. Gern JE, Calhoun W, Swenson C, Shen G, Busse WW. Rhinovirus infection preferentially increases lower airway responsiveness in allergic subjects. Am J Respir Crit Care Med 1997;155:1872-1876.
31. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective type 1 response to rhinovirus in atopic asthma. Thorax 2002;57:328-332.
32. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. J Exp Med 2005;201:937-947.
33. Taneda S, Segerer S, Hudkins KL, Cui Y, Wen M, Segerer M, Wener MH, Khairallah CG, Farr AG, Alpers CE. Cryoglobulinemic glomerulonephritis in thymic stromal lymphopoietin transgenic mice. Am J Pathol 2001;159:2355-2369.
34. Edwards MR, Johnson MW, Johnston SL. Combination therapy: Synergistic suppression of virus-induced chemokines in airway epithelial cells. Am J Respir Cell Mol Biol. 2006;34:616-624.
35. Barnes PJ. Corticosteroid effects on cell signalling. Eur Respir J 2006;27:413-426.
36. Mittelstadt PR, Ashwell JD. Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. J Biol Chem 2001;276:29603-29610.
37. Yamaguchi-Kabata Y, Nakazono K, Takahashi A, Saito S, Hosono N, Kubo M, Nakamura Y, Kamatani N. Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am J Hum Genet. 2008;83:445-456.

TABLE 1. BASELINE CHARACTERISTICS OF THE PARTICIPANTS

Characteristic	Childhood asthma	Control 1	Adult asthma	Control 2
Total no.	639	838	641	376
Male - %	59.4	73.4	42.8	46.8
Age - year				
Mean	9.3	49.8	51.8	50.7
Range	4-15	20-75	20-75	29-72
Atopy - no./no. tested (%)	639/639		531/596	
Serum IgE - IU	1101.5±94.9		621.9±62.6	93.9±6.9
Eosinophil count - no./μl	518.1±15.0		392.4±16.0	
FVC- % of predicted value			84.0±0.84	
FEV1.0 - % of predicted value			71.1±0.88	
FEV1.0/FVC - %			66.8±0.78	

Plus-minus values are means ± standard error.

TABLE 2. GENOTYPE COUNTS, FREQUENCIES AND CASE-CONTROL ASSOCIATION TEST RESULTS

db SNP ID	Case				Control				MAF		*P* values, ORs (95% c.i.)		
Allele1/2	1/1	1/2	2/2	Sum	1/1	1/2	2/2	Sum	Case	Control	Allelic	Dominant	Recessive
Childhood atopic asthma					Control 1								
rs3806933	294	269	68	631	446	311	72	829	0.32	0.27	0.0063	0.0064	0.18
-847C/T	0.47	0.43	0.11		0.54	0.38	0.09				1.25 (1.07-1.47)	1.34 (1.09-1.64)	1.27 (0.90-1.80)
rs2289276	322	256	60	638	493	288	56	837	0.29	0.24	0.00066	0.0013	0.055
-82C/T	0.5	0.4	0.09		0.59	0.34	0.07				1.33(1.13-1.57)	1.41(1.14-1.73)	1.45(0.99-2.12)
rs2289278	418	195	25	638	537	261	38	836	0.19	0.20	0.52	0.61	0.56
1560C/G	0.66	0.31	0.04		0.64	0.31	0.05				0.94 (0.78-1.13)	0.95 (0.76-1.17)	0.86 (0.51-1.43)
Adult asthma					Control 2								
rs3806933	289	274	71	634	204	143	27	374	0.33	0.26	0.0023	0.0060	0.039
-847C/T	0.46	0.43	0.11		0.55	0.38	0.07				1.37 (1.12-1.67)	1.43 (1.11-1.85)	1.62 (1.02-2.58)
rs2289276	322	264	53	639	213	138	23	374	0.29	0.25	0.034	0.044	0.21
-82C/T	0.5	0.41	0.08		0.57	0.37	0.06				1.25 (1.02-1.53)	1.30 (1.01-1.68)	1.38 (0.83-2.29)
rs2289278	415	187	29	631	232	127	17	376	0.19	0.21	0.28	0.19	0.96
1560C/G	0.66	0.30	0.05		0.62	0.34	0.05				0.88 (0.71-1.11)	0.84(0.64-1.09)	1.02(0.55-1.88)
Combined (Mantel-Haenszel)													
rs3806933	583	543	139	1265	650	454	99	1203	0.32	0.27	0.000056	0.00013	0.022
-847C/T	0.46	0.43	0.11		0.54	0.38	0.08				1.29 (1.14-1.47)	1.37 (1.16-1.62)	1.39 (1.05-1.85)
rs2289276	644	520	113	1277	706	426	79	1211	0.29	0.24	0.000076	0.00019	0.026
-82C/T	0.50	0.41	0.09		0.58	0.35	0.07				1.3 (1.14-1.48)	1.36 (1.16-1.61)	1.42 (1.04-1.96)
rs2289278	833	382	54	1269	769	388	55	1212	0.19	0.21	0.25	0.23	0.69
1560C/G	0.66	0.30	0.04		0.63	0.32	0.05				0.92 (0.79-1.06)	0.90 (0.76-1.07)	0.92 (0.61-1.39)

P values of the two populations represent the chi square test for case-control comparisons under each

model.

Figure Legends

Figure 1. SNPs and pairwise LD map of the *TSLP* gene. (*A*) A graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *TSLP* gene. Three polymorphisms were genotyped in this study. The ATG, UTR, and the ORF are shown by closed triangles, white boxes, and black boxes, respectively. (*B*) Pairwise D'/LOD (left) and r^2 (right) for all combinations of SNP pairs are shown.

Figure 2. Relationship of *TSLP* rs3806933, rs2289276 and rs2289278 genotype with lung function in adult asthma patients. The percentage of forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) is plotted, and horizontal bars represent the mean for each genotype group.

Figure 3. (*A*) The DNA sequences of transcription factor-binding motifs around rs2289276 SNP. The positions of potential AP-2α binding sites are shown in the open box and the asterisks (*) represent SNPs. (*B*) Binding affinity of transcription factors to oligonucleotides in vitro. NHBE were stimulated with or without 10μg/ml poly(I:C) for 1h. Proteins interacting with the double-stranded oligonucleotides were precipitated and analyzed by immunoblotting with the indicated antibodies. Three independent experiments were performed with similar results.

Figure 4. Suppression of TSLP production by SAL and DEX in NHBE. (*A*) Quantitative RT-PCR assay of the *TSLP*. $P < .001$, by Student's t-test. (*B*) Effects of DEX and SAL on TSLP protein production in NHBE stimulated by poly(I:C). The concentrations of TSLP in supernatants were measured by ELISA. *, not detectable.

Figure 5. (*A*) Luciferase constructs. The gray box indicates the NF-κB regulatory region. (*B*) Effects of dexamethasone and salmeterol on luciferase transcriptional activities of haplotypes of the long form of *TSLP* in NHBE. Data represent mean ± SD and are from three experiments in triplicate. $*P < 0.0001$ and $\dagger P = 0.0021$ by the Bonferroni-Dunn test with two-factor factorial ANOVA.

Figure 1

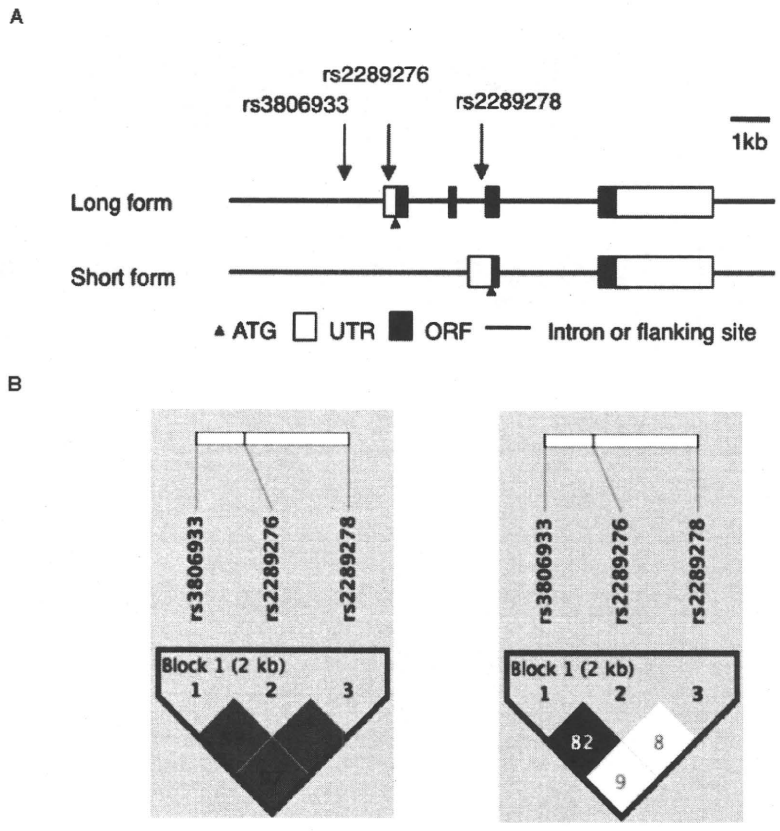

A
rs2289276
rs3806933
rs2289278
1kb
Long form
Short form
ATG
UTR
ORF
Intron or flanking site
B
rs3806933
rs2289276
rs2289278
Block 1 (2 kb)
1
2
3
82
8
9

Figure 2

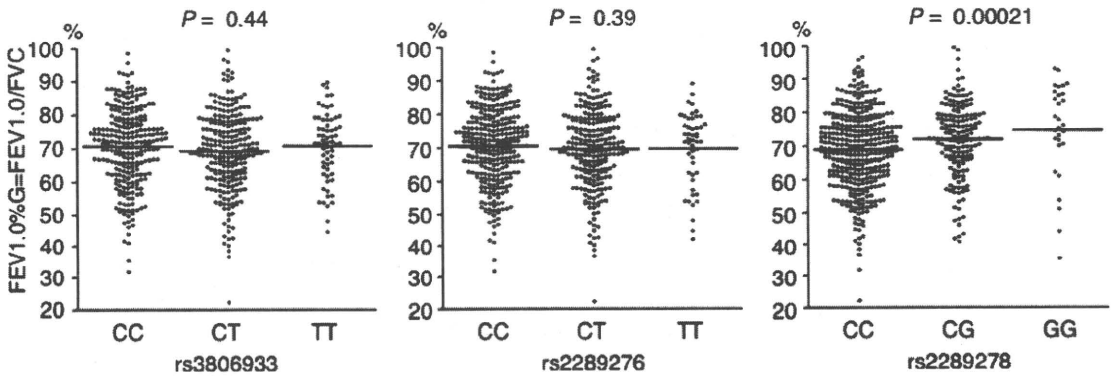

FEV1.0%G=FEV1.0/FVC
%
100
90
80
70
60
50
40
30
20
P = 0.44
CC
CT
TT
rs3806933
P = 0.39
CC
CT
TT
rs2289276
P = 0.00021
CC
CG
GG
rs2289278

Figure 3

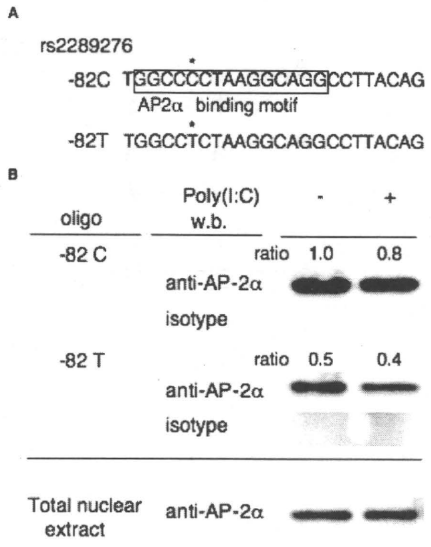

A
rs2289276
-82C TGGCCCCTAAGGCAGGCCTTACAG
AP2α binding motif
-82T TGGCCTCTAAGGCAGGCCTTACAG
B
oligo
Poly(I:C) w.b.
-
+
-82 C
ratio 1.0 0.8
anti-AP-2α
isotype
-82 T
ratio 0.5 0.4
anti-AP-2α
isotype
Total nuclear extract
anti-AP-2α

Figure 4

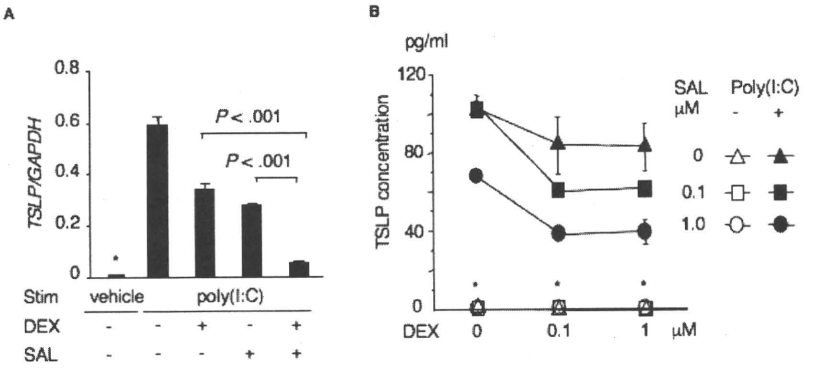
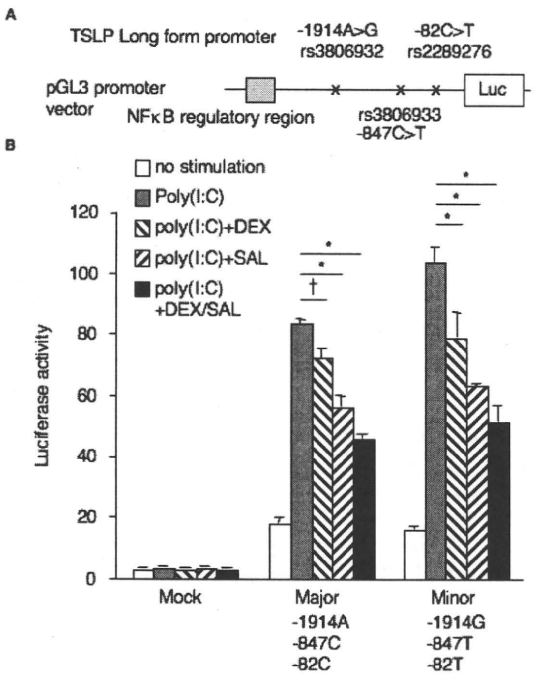

A
TSLP/GAPDH
0.8
0.6
0.4
0.2
0
P < .001
P < .001
Stim
vehicle
poly(I:C)
DEX
SAL
B
pg/ml
TSLP concentration
120
80
40
0
SAL
μM
Poly(I:C)
0
0.1
1.0
DEX
0
0.1
1
μM

Figure 5


A
TSLP Long form promoter
-1914A>G
rs3806932
-82C>T
rs2289276
pGL3 promoter
vector
NFκB regulatory region
rs3806933
-847C>T
Luc
B
no stimulation
Poly(I:C)
poly(I:C)+DEX
poly(I:C)+SAL
poly(I:C)
+DEX/SAL
Luciferase activity
120
100
80
60
40
20
0
Mock
Major
-1914A
-847C
-82C
Minor
-1914G
-847T
-82T

Online Data Supplement

TSLP Promoter Polymorphisms are Associated with Susceptibility to Bronchial Asthma

Michishige Harada, Tomomitsu Hirota, Aya I. Jodo, Yuki Hitomi, Masafumi Sakashita, Tatsuhiko Tsunoda, Takehiko Miyagawa, Satoru Doi, Makoto Kameda, Kimie Fujita, Akihiko Miyatake, Tadao Enomoto, Emiko Noguchi, Hironori Masuko, Tohru Sakamoto, Nobuyuki Hizawa, Yoichi Suzuki, Shigemi Yoshihara, Mitsuru Adachi, Motohiro Ebisawa, Hirohisa Saito, Kenji Matsumoto, Toshiharu Nakajima, Rasika A. Mathias, Nicholas Rafaels, Kathleen C. Barnes, Blanca E. Himes, Qing Ling Duan, Kelan G. Tantisira, Scott T. Weiss, Yusuke Nakamura, Steven F. Ziegler, and Mayumi Tamari

Study Subjects and Genotyping

All subjects with bronchial asthma were diagnosed according to the criteria of the National Institutes of Health (National Heart, Lung, and Blood Institute, National Institutes of Health, 1991) by doctors who were specialists for asthma (20-22). We recruited 639 subjects with childhood atopic asthma from the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Dokkyo University School of Medicine, National Research Institute for Child Health & Development, and National Sagamihara Hospital. A total of 641 subjects with adult asthma were recruited from the Miyatake Asthma Clinic and Showa University School of Medicine. After exclusion of individuals who had been diagnosed with asthma, atopic dermatitis or nasal allergies by physicians' interviews, a total of 838 healthy individuals were recruited as described (20-22). There was an age difference between the childhood asthma and control groups. We performed linear regression analysis between age and the genotypes of the three SNPs (Table E4), and found no evidence of association between age and genotype. A total of 376 healthy individuals who had never been diagnosed with asthma, atopic dermatitis or nasal allergies were recruited during their annual health checkup as a second control group in the University of Tsukuba. The serum IgE level was $\log_{10}$-transformed before analysis. A recent study of Japanese population structure has shown that most Japanese individuals fall into two main clusters, Hondo and Ryukyu; the Hondo cluster includes most of the individuals from the main islands in Japan (37). All subjects in this study were Japanese and were recruited from Hondo area. We confirmed no obvious population stratification in subjects with adult asthma and healthy individuals (22). Genotyping completion rates were ≥99% for all three SNPs. All SNPs were in Hardy Weinberg equilibrium in the two control groups.

Statistical Analysis

We resequenced the *TSLP* gene using 24 subjects with asthma and 12 control volunteers (10). We selected three Tag SNPs for association study by using the LD map shown in a recent study (10). The three Tag SNPs captured all seven of the SNPs with a mean r^2 of 1.00 among the 36 Japanese subjects (10). Rs3806933 was in complete LD with rs3806932, rs2289277, and rs10073816 (D' = 1.00 and r^2 = 1.00). Rs2289276 was in complete LD with rs 11466741 (D' = 1.00 and r^2 = 1.00) (10) (Figure

1B). Although we searched the HapMap release 27 database to find whether any additional information was available, the database did not show any novel SNP in the *TSLP* gene.

To test the association between *TSLP* variants and bronchial asthma, we compared differences in the allele frequency and genotype distribution of each polymorphism between case and control subjects by using a contingency chi-square test. We further investigated associations between asthma-related phenotypes (eosinophil count, serum total IgE, lung functions and disease severity) and variants within patients with asthma (22). Serum total IgE, eosinophil counts and lung functions (% of predicted FEV_1, % of predicted FVC and FEV_1:FVC) were analyzed as quantitative levels by the Kruskal-Wallis test. The correlations between the lung functions and the alleles of SNPs were evaluated with the Jonckheere-Terpstra test. We surveyed associations between the SNPs and disease severity as described (23). The clinical severity of adult asthma was classified according to the criteria of the National Institutes of Health/Global Initiative for Asthma 2002. The distribution of subjects was as follows: step 1, mild intermittent 2.2% (14 individuals); step 2, mild persistent 51.4% (327 individuals); step 3, moderate persistent 28.8% (183 individuals); and step 4, severe persistent 17.6% (112 individuals). We divided the subjects with asthma into two groups, steps 1 and 2 versus steps 3 and 4 by sample size (53.6 vs. 46.4%).

Biotinylated Oligonucleotide Precipitation Assay

NHBE cells were lysed on ice for 15 min and insoluble material was removed by centrifugation as described (10). The supernatant was diluted 1:3 with buffer, and the lysate was preabsorbed using ImmunoPure streptavidin-agarose beads (Pierce, Rockford, IL) for 1 h. The sample was then incubated with 3 μg of biotinylated double-stranded oligonucleotides, together with 3 μg of poly(dI-dC) for 1 h. Biotinylated DNA-protein complexes were recovered using streptavidin-agarose beads for 1 h, and separated on SDS-polyacrylamide gels. AP-2α was detected by immunoblotting with an anti-AP-2α antibody. The band intensity was determined by densitometry, and the intensity ratio of each band to that of -82C without poly(I:C) were shown (Figure 3B).

Quantitative Real-Time RT-PCR

NHBE cells were purchased and maintained using medium kits (BulletKit) (Cambrex, East Rutherford, NJ). Cells were stimulated with 10 μg/ml poly(I:C) (InvivoGen, La Jolla, CA). Poly(I:C) stimulated NHBE cells were cultured with dexamethasone (ICN Biomedicals, Costa Mesa, CA) and/or salmeterol (TOCRIS Inc., Ellisville, MO). The expression of *TSLP* was determined by real-time quantitative RT-PCR, and the amounts of cDNA were standardized by quantification of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) in all experiments as described (10).

Luciferase Assay

The reporter plasmids for TSLP were generated using the reporter gene pGL3-promoter vector (Promega, Madison, WI) as described (10). NHBE (5×10^4/well) were transfected with these reporter constructs (500ng) and pRL-TK Renilla luciferase vector (10ng) as a normalization control using FuGENE 6 transfection reagent (Roche