Table 2. Overview of the 22 SNPs of *ADAM33* gene on chromosome 20p13 that were genotyped in our case–control association study

SNP No.	Variation*	Location	SNP name in ref. [14]	Forward (f) and reverse (r) primers for PCR†	Annealing temp. (°C)	Genotyping methods
01	−3989 T/C	5′ genomic		5′-CCTCTAAATGCACCAGGCTG-3′ (f) 5′-AGTGTGGATGTCAGGTACAG-3′ (r)	60	Direct sequencing
02	−3982 C/T	5′ genomic		5′-CCTCTAAATGCACCAGGCTG-3′ (f) 5′-AGTGTGGATGTCAGGTACAG-3′ (r)	60	Direct sequencing
03	−3835 C/T	5′ genomic		5′-CCTCTAAATGCACCAGGCTG-3′ (f) 5′-AGTGTGGATGTCAGGTACAG-3′ (r)	60	Direct sequencing
04	−3705 C/A	5′ genomic		5′-CCTCTAAATGCACCAGGCTG-3′ (f) 5′-AGTGTGGATGTCAGGTACAG-3′ (r)	60	Direct sequencing
05	−85 T/C	5′ genomic		5′-CCAGGAGACCACGGAAGATC-3′ (f) 5′-AAGCTGGTTGTGAGAGCGAG-3′ (r)	60	Direct sequencing
06	6716 G/C	Intron 3	D-1	5′-TTTTTCTCCACAACTAAGCCATCACCA*T*G-3′ (f) 5′-TTCCTCCCTAAATGCTAATGG-3′ (r)	58	RFLP, *EcoT*14 I
07	7441 A/G (Thr178Ala)	Exon 6	F1	5′-TTCTGATCTGAGCAAGTCACC-3′ (f) 5′-TTGGAGGGCTATAGATACAGC-3′ (r)	60	Direct sequencing
08	7575 G/A	Intron 6	F+1	5′-GGGGAGCCCTCCAAATCAGAAGAG*CC*-3′ (f) 5′-AGTGGAAGCTGCTGGGCTT-3′ (r)	58	RFLP, *Msp* I
09	7667 A/G	Intron 6	G-1	5′-GCTGTATCTATAGCCCTCCA-3′ (f) 5′-AAGGGCTTGAGCCCTGACCA*TT*AA-3′ (r)	58	RFLP, *PshB* I
10	9073 G/A	Intron 11	L-1	5′-CAAGGGTGCTCGTGTCCTC-3′ (f) 5′-GCTCAGGCAAAGTGGATTCTC-3′ (r)	60	Invader assay
11	10918 G/C (Gly717Gly)	Exon 19	S2	5′-GGAACCGCAGGAGTAGGCTC-3′ (f) 5′-GGAAGAATGCCTTCCAGGAG-3′ (r)	60	Invader assay
12	11188 A/T	Intron 19	S+1	5′-CTTCCTCAGTGGCCTCCCAGTCAA*T*CG-3′ (f) 5′-AGGGTGAGGCAGCCAGCT-3′ (r)	58	RFLP, *Taq* I
13	11434 C/A	Intron 19	ST+4	5′-TCGCCCTGTTTTTCCCTGG-3′ (f) 5′-AAGGCCCAGCATTTGGGAACT*C*CA*T*G-3′ (r)	55	RFLP, *EcoT*14 I
14	12433 T/C (Met764Thr)	Exon 20	T1	5′-TCAAGTTCCTGGAGTGGCTG-3′ (f) 5′-GGTTCTTTGGAAGCTGAGCG-3′ (r)	60	Invader assay
15	12462 C/T (Pro774Ser)	Exon 20	T2	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-3′ (r)	60	Direct sequencing
16	12540 C/T	Intron 20	T+1	5′-TCAAGTTCCTGGAGTGGCTG-3′ (f) 5′-GGTTCTTTGGAAGCTGAGCG-3′ (r)	60	Invader assay
17	12601 T/G	Intron 20	T+2	5′-TGGGTGAGTGAGGCACCA-3′ (f) 5′-TTTACTTGTGAATATGGTCAGCAGG*CG*-3′ (r)	58	RFLP, *Hha* I
18	12946 G/A	Intron 21	V-3	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-3′ (r)	60	Direct sequencing
19	12954 C/T	Intron 21	V-2	5′-TGGACTCTTATCACGTTGCTC-3′ (f) 5′-GGGAAGAAACTTCCAAGCTGC-3′ (r)	60	Direct sequencing
20	13236 T/C	3′ UTR	V3	5′-GCTCCAGAACCTCAGGTGTAGC-3′ (f) 5′-CTGACAGCCACTCCAGGAAC-3′ (r)	60	Invader assay
21	13506 C/G	3′ UTR	V4	5′-GCTCCAGAACCTCAGGTGTAGC-3′ (f) 5′-CTGACAGCCACTCCAGGAAC-3′ (r)	60	Invader assay
22	13527 A/G	3′ UTR	V5	5′-GCTCCAGAACCTCAGGTGTAGC-3′ (f) 5′-CTGACAGCCACTCCAGGAAC-3′ (r)	60	Invader assay

*The position of each variation is given in base pairs according to the GenBank accession numbers AF466287 for *ADAM33* cDNA, and AP002898 for *ADAM33* gene containing clone. Nucleotide numbering starts from the 'A' of the initiation codon. †Italic nucleotide was exchanged to incorporate the polymorphic site, and underlined sequence was added as an elongation of PCR products.
UTR, untranslated region; *ADAM33*, a disintegrin and metalloprotease domain 33; SNPs, single-nucleotide polymorphisms.

Using genomic DNA extracted from whole peripheral blood of study subjects by standard methods, we genotyped all 22 SNPs with one of the following three PCR-based methods: Invader assay [22, 23], restriction fragment length polymorphism (RFLP), or direct sequencing.

Invader assay

To simplify the SNP-typing procedure, a panel of seven SNPs (see Table 2) was genotyped with multiplex PCR-Invader assay, as described in detail elsewhere [23]. In brief, we amplified 5 ng of genomic DNA with PCR primers designed to include one or more SNPs (sequences of the primers are listed in Table 2). Invader assay was then performed through use of multiplex PCR products as template. Third Wave Technologies (Madison, WI, USA) designed probe sets for each locus. Genotyping was performed on an ABI PRISM 7700 Sequence Detection System and attached analysis software SDS 2.0 (Applied Biosystems, Foster City, CA, USA).

PCR-RFLP

PCR-RFLP analysis was used to genotype six SNPs (see Table 2). Briefly, genomic DNA was amplified with PCR primers (for primer sequences see Table 2). PCR fragment generated was then digested overnight with a site-specific restriction endonuclease (see Table 2) according to the manufacturer's recommendation. The digestion products were visualized on a 4% agarose gel stained with ethidium bromide.

Direct sequencing

Remaining nine SNPs, as shown in Table 2, were subsequently determined directly on a capillary-based sequencer, using BigDye-terminator cycle sequencing according to standard procedures. In brief, sequencing reaction was accomplished with PCR products, BigDye Terminator v.2 (Applied Biosystems), and one unlabelled primer either reverse (5′-CGAAGTCTCTGAGAAAGGGT-3′ for four SNPs of −3989T/C, −3982C/T, −3835C/T and −3705C/A, and 5′-ATGCCCTCTGCTTTGCCAGC-3′ for −85T/C) or forward (5′-TGGGATGAGGTGAGCTCT-3′ for 7441A/G, 5′-TGGACTCTTATCACGTTGCTC-3′ for 12462C/T, and 5′-CCTGAGAACTCTCATGAG-3′ for both 12946G/A and 12954C/T). Following refinement of the reaction products by ethanol, the purified products were sequenced on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Genotypes were identified by comparing the peak levels of signals within the expected allele range for all lanes through use of a computer programme SEQUENCHER 3.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistics

Statistical analyses were performed using a SPSS 10.0J for Windows (SPSS Inc., Chicago, IL, USA). The Hardy–Weinberg equilibrium of genotype distribution for each of 22 SNPs in *ADAM33* gene was assessed by χ^2 test. Genotype and allele frequencies in cases and control subjects were compared using Pearson's χ^2 test. If an expected number was less than 5, Fisher's exact test was then used. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated as measures of the association of *ADAM33* polymorphisms with JCPsis. Quantitative traits relating to clinical severity (severe vs. mild-to-moderate) and nEOS counts (positive vs. negative) were also analysed with χ^2 test. When genotypic frequencies were compared between different groups, a 2×2 table was performed. Association between total serum IgE levels and *ADAM33* genotypes was tested with ANOVA, *t*-test and general liner model (GLM). For these analyses, total IgE values were logarithm transformed to normalize the distribution. Non-parametric tests (Kruskal–Wallis test and Mann–Whitney test) were employed for an association analysis of *ADAM33* genotypes with actual values of specific IgE to JCP.

Pairwise linkage disequilibrium (LD) and haplotypes for *ADAM33* polymorphisms were evaluated using the programme SNPAlyze 3.1 (DYNACOM Co. Ltd, Chiba, Japan), by the method described elsewhere [24]. Haplotype frequencies were estimated by the expectation–maximization algorithm. Significance levels were corrected for multiple comparison of the number of correlations by Bonferroni adjustment. Two-tailed *P*-value of less than 0.05 was considered statistically significant.

Results

Association of ADAM33 *polymorphisms with JCPsis*

Overall success rate for the SNP genotyping in the present study was 99.4%. All 22 SNPs investigated were in Hardy–Weinberg equilibrium ($P > 0.05$). Therefore, genotyping errors can most probably be excluded.

Among typed 22 SNPs of *ADAM33* gene, six of them had significant associations with JCPsis (Table 3). The statistical *P*-values were 0.0002–0.022 (OR = 4.81 − 1.66) for absolute allele frequencies. These SNPs were 7575G/A (SNP F+1 in ref. [14]) in intron 6, 9073G/A (SNP L-1) in intron 11, 10918G/C (SNP S2, Gly717Gly) in exon 19, 12433T/C (SNP T1, Met764Thr) and 12462C/T (SNP T2, Pro774Ser) in exon 20 and 12540C/T (SNP T+1) in intron 20. Furthermore, a comparison of allelic frequencies in these SNPs indicated that the frequencies of common alleles (7575G, 9073G, 10918G, 12433T, 12462C and 12540C) were significantly higher for the JCPsis-affected individuals than for the healthy control subjects ($P < 0.05$).

As shown in Table 3, we also evaluated the frequencies of diploid genotypes in cases and controls, and observed that five of the SNPs (9073G/A, 10918G/C, 12433T/C, 12462C/T and 12540C/T) were significantly associated with JCPsis ($P = 0.00004$–0.004, OR = 6.71–2.39).

No significant association was detected ($P > 0.05$) in the analyses for other variants – a coding SNP (7441A/G, Thr178Ala), seven intronic SNPs (6716G/C, 7667A/G, 11188A/T, 11434C/A, 12601T/G, 12946G/A and 12954C/T), five 5′ genomic SNPs (−3989T/C, −3982C/T, −3835C/T, −3705C/A and −85T/C) and three 3′UTR SNPs (13236T/C, 13506C/G and 13527A/G).

In addition, to clarify whether sensitization to HDM would affect the association between *ADAM33* polymorphisms and JCPsis, we analysed genotype and allele frequencies for each of the 22 SNPs in patients with or without positive allergen-specific IgE against HDM, and did not identify any significant correlation ($P > 0.05$). Namely, association of the *ADAM33* gene with JCPsis was independent of allergic sensitization to HDM in our study population.

Intragenic LD and haplotype frequencies in ADAM33 *gene*

To investigate the pattern of LD in the *ADAM33* locus, pairwise LD was measured by $|D'|$ and r^2 among the 22 SNPs genotyped. These 22 SNPs were located in one extended block of LD and some were in tight LD (Fig. 1). However we found that six of the SNPs had significant associations with JCPsis, when pairwise LD was examined between these six SNPs, most of the SNPs were in LD with each other (Table 4). We further analysed the haplotype structure using four SNPs (7575G/A [SNP F+1], 9073G/A [SNP L − 1], 10918G/C [SNP S2] and 12540C/T [SNP T+1]) and identified six common haplotypes covering more than 98% of the population in both case and control groups (Table 5). Estimated frequencies of the four-locus haplotypes were compared between cases and control subjects, and found the most common haplotype was 7575G, 9073G, 10918G and

© 2004 Blackwell Publishing Ltd,

Table 3. Association between *ADAM33* polymorphisms and Japanese cedar pollinosis

SNP*	Location	*n*†	Genotype frequency					Allele frequency		
			Genotype			*P*-value‡	OR (95% CI)¶	Allele	*P*-value§	OR (95% CI)¶
7575 G/A	Intron 6		GG	GA	AA			A		
F+1	Cases	95	0.516	0.432	0.053	0.072	1.70 (0.95–3.05)	0.268	0.022	1.66 (1.07–2.58)
	Controls	91	0.385	0.473	0.143			0.379		
9073 G/A	Intron 11		GG	GA	AA			A		
L-1	Cases	94	0.947	0.043	0.011	0.00004	6.71 (2.45–18.37)	0.032	0.0002	4.81 (1.93–11.98)
	Controls	95	0.726	0.274	0.000			0.137		
10918 G/C	Exon 19		GG	GC	CC			C		
S2	Cases	94	0.691	0.266	0.043	0.004	2.39 (1.32–4.33)	0.176	0.006	1.96 (1.21–3.20)
	Controls	95	0.484	0.442	0.074			0.295		
12433 T/C	Exon 20		TT	TC	CC			C		
T1	Cases	95	0.905	0.084	0.011	0.0003	4.20 (1.86–9.47)	0.053	0.001	3.24 (1.53–6.86)
	Controls	95	0.695	0.305	0.000			0.153		
12462 C/T	Exon 20		CC	CT	TT			T		
T2	Cases	95	0.895	0.095	0.011	0.00007	4.52 (2.08–9.87)	0.058	0.0003	3.55 (1.74–7.24)
	Controls	95	0.653	0.337	0.011			0.179		
12540 C/T	Intron 20		CC	CT	TT			T		
T+1	Cases	95	0.905	0.084	0.011	0.0005	3.99 (1.77–9.03)	0.053	0.002	3.11 (1.47–6.60)
	Controls	95	0.705	0.295	0.000			0.147		

*SNP with *P*-value for genotype or allele frequencies between groups less than 0.05 listed. SNP name in ref. [14] indicated in bold. †Number of valid subjects who were successfully genotyped for each of SNP. ‡Analysis performed by a 2 × 2 table for each SNP using major homozygotes vs. others in cases and controls. §Analysis performed by a 2 × 2 table for the number of each allele in cases and controls. ¶Reference group (controls) designated with an OR of 1.00.
SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33; CI, confidence interval; OR, odds ratio.

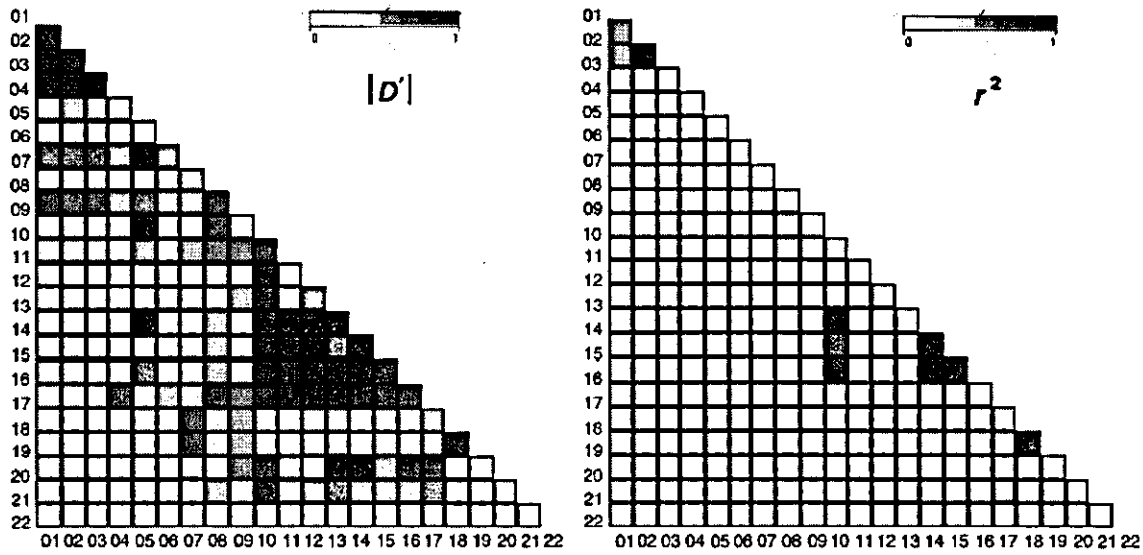

Fig. 1. Pairwise linkage disequilibrium in a disintegrin and metalloprotease domain 33 (*ADAM33*), as measured by *D'*-value (left panel) and r^2 (right panel) between all pairs of single-nucleotide polymorphisms (SNPs) examined. The physical locations of 22 SNPs refer to the SNP number (from 01 to 22) shown in Table 2.

12540C (GGGC haplotype) in both groups. This haplotype was more frequently observed in patients than in healthy controls (68.9% vs. 57.8%, $P = 0.027$, OR = 1.62; see Table 5); however, when Bonferroni's correction was applied to the result we found no significant difference between the groups ($P = 0.188$). In contrast, a tight association with JCPsis was observed with AACT haplotype ($P = 0.0013$, OR = 4.19; see Table 5), and the association was also statistically significant after correcting for multiple testing ($P = 0.009$). In the control subjects, this haplotype had a frequency of 11.8%, whereas the frequency in the JCPsis-affected individuals was substantially lower, 3.1%.

Table 4. Pairwise linkage disequilibrium coefficients between the six SNPs of *ADAM33* gene

SNP*	7575 G/A, F+1	9073 G/A, L-1	10918 G/C, S2	12433 T/C, T1	12462 C/T, T2	12540 C/T, T+1
7575 G/A, F+1	–	0.126	0.348	0.083	0.102	0.078
9073 G/A, L-1	0.812	–	0.300	0.804	0.684	0.828
10918 G/C, S2	0.743	1.000	–	0.345	0.256	0.334
12433 T/C, T1	0.589	1.000	0.961	–	0.851	0.972
12462 C/T, T2	0.602	1.000	0.763	1.000	–	0.827
12540 C/T, T+1	0.579	1.000	0.960	1.000	1.000	–

*The disease-associated polymorphisms in this study are shown. SNP name in ref. [14] indicated in bold.
D'-value (abs) is listed in lower left triangle, and r^2 in upper right.
SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33.

Table 5. *ADAM33* haplotype profiles in our study population

	SNP position†				Haplotype frequency			
Haplotype*	7575, F+1	9073, L-1	10918, S2	12540, T+1	Cases (n = 94)	Controls (n = 91)	P-value‡	OR (95% CI)§
Haplotype 1	G	G	G	C	0.689	0.578	0.027	1.62 (1.06–2.48)
Haplotype 2	A	G	G	C	0.130	0.131	0.985	1.01 (0.55–1.84)
Haplotype 3	A	G	C	C	0.110	0.130	0.554	1.21 (0.64–2.26)
Haplotype 4	A	A	C	T	0.031	0.118	0.0013	4.19 (1.64–10.74)
Haplotype 5	G	G	C	C	0.018	0.013	0.698	1.40 (0.26–7.61)
Haplotype 6	G	G	C	T	0.016	0.011	0.687	1.45 (0.24–8.79)
Others					0.006	0.019	0.272	3.03 (0.38–24.28)

*Haplotype structure using four SNPs analysed. †SNP name in ref. [14] indicated in bold. ‡Analysis using a 2 × 2 table for each haplotype vs. all others combined in cases and controls. § Reference group designated with an OR of 1.00.
SNP, single-nucleotide polymorphism; *ADAM33*, a disintegrin and metalloprotease domain 33; OR, odds ratio; CI, confidence interval.

Association of ADAM33 *polymorphisms with cedar pollinosis-related phenotypes*

Of the 22 SNPs examined, five of them showed statistically significant associations with cedar pollinosis closely related phenotypes including disease severity, nEOS counts and specific IgE to JCP (summarized in Table 6); however, no SNP was associated with two of these three phenotypes.

Ninety-five affected individuals were further stratified into two subgroups: those with mild-to-moderate JCPsis (n = 27) and those with severe JCPsis (n = 68). As shown in Table 6, the difference between two subgroups was significant in the analyses for genotype distribution of two intronic SNPs (P = 0.027, both). These two SNPs were 12946G/A (SNP V-3) and 12954C/T (SNP V-2) in intron 21, which were in complete LD with each other (see Fig. 1). A trend was observed for an association between the allele frequencies and severe JCPsis relative to mild-to-moderate JCPsis, but not at a statistically significant level (47.1% vs. 31.5%, P = 0.050). Analysis of other polymorphisms in *ADAM33* did not identify any significant correlation between clinical severity of JCPsis and genotype distribution, as well as allele frequencies (P > 0.05).

No significant difference was detected in total serum IgE levels (log IU/mL) between the genotypes for each of the 22 SNPs (using codominant, dominant and recessive models), either in patients with JCPsis or in healthy control subjects (P > 0.05). Moreover, we examined the genetic association between *ADAM33* polymorphisms and total IgE levels using a whole study population, while incorporating disease status (case or control) as a covariate in a GLM analysis, and found no evidence of significant association as well (P > 0.05).

However, we found that the genotype distributions for 11188A/T (SNP S+1) in intron 19 and 12601T/G (SNP T+2) in intron 20 were associated with the actual levels of allergen-specific IgE to JCP in sera from patients with JCPsis, but the significance was marginal (P = 0.027 and 0.045, respectively; see Table 6). No significant effects were demonstrated from analysis to determine the associations between any other SNPs of *ADAM33* and specific IgE values for JCP (P > 0.05).

We next investigated the impact of *ADAM33* variants on eosinophil counts in nasal secretion from JCPsis-affected individuals. A 5′ genomic polymorphism in *ADAM33*, −3989T/C, showed a significant difference in either genotype or allele frequencies between nEOS-positive and -negative subjects (see Table 6). Using the TT homozygotes as a reference, we observed a significant association between nEOS counts (positive vs. negative) and genotype frequencies (P = 0.015). Comparison of allele frequencies showed that the C allele frequency was significantly higher in the subjects with positive nEOS than in those with negative nEOS (60.4% vs. 38.5%, P = 0.036). No significant association was obtained in the analyses regarding whether other polymorphisms of *ADAM33* were related to nEOS counts (P > 0.05).

Discussion

ADAM33, which maps to human chromosome 20p13 and consists of 22 exons [25], was recently identified to be a novel asthma susceptibility gene [14]. To our knowledge, this study represents the first evaluation of the *ADAM33* gene as a

© 2004 Blackwell Publishing Ltd,

Table 6. Association between *ADAM33* polymorphisms and cedar pollinosis related phenotypes

SNP*	Location	Clinical severity	Nasal eosinophila	
		Severe vs. mild-to-moderate	nEOS positive vs. negative	Specific IgE levels against Japanese cedar pollen
−3989 T/C	5′ genomic		$P = 0.015$†; $P = 0.036$‡	
11188 A/T, **S+1**	Intron 19			$P = 0.027$§
12601 T/G, **T+2**	Intron 20			$P = 0.045$§; $P = 0.048$¶
12946 G/A, **V-3**	Intron 21	$P = 0.027$†		
12954 C/T, **V-2**	Intron 21	$P = 0.027$†		

*SNP with *P*-value less than 0.05 listed. SNP name in ref. [14] indicated in bold. †Analysis for genotype frequencies by a 2 × 2 table using major homozygotes vs. others. ‡Analysis for allele frequencies by a 2 × 2 table. §Comparison made by a Kruskal–Wallis test using codominant model. ¶Comparison made by a Mann–Whitney test using major homozygotes vs. others.

nEOS positive, eosinophil counts in nasal secretion ranging from 1+ to 3+. nEOS negative, none of eosinophils counted in nasal secretion. *ADAM33*, a disintegrin and metalloprotease domain 33; SNP, single-nucleotide polymorphism.

susceptibility locus for nasal allergy. Using a case–control study, we have typed a total of 22 SNPs in *ADAM33* among a Japanese population living in the same area by using PCR-based molecular methods, and evaluated the genetic variants for evidence of association to JCPsis and related traits. The results showed that three intronic and three coding SNPs at the *ADAM33* locus were significantly associated with JCPsis ($P = 0.0002 - 0.022$, OR = 4.81 − 1.66 for allele frequencies). These SNPs were 7575G/A (SNP F+1) in intron 6, 9073G/A (SNP L − 1) in intron 11, 10918G/C (SNP S2, Gly717Gly) in exon 19 encoding the transmembrane domain, 12433T/C (SNP T1, Met764Thr) and 12462C/T (SNP T2, Pro774Ser) in exon 20 encoding the cytoplasmic domain and 12540C/T (SNP T+1) in intron 20. Unexpectedly, we found significantly increased frequencies of the common alleles (7575G, 9073G, 10918G, 12433T, 12462C and 12540C) in patients with JCPsis compared with healthy control subjects.

Six of these JCPsis-associated SNPs (F+1, L − 1, S2, T1, T2 and T+1) have been previously reported to be associated with asthma and BHR in Caucasian populations from the UK (SNPs F+1 and S2) or the US (SNPs L-1, T1, T2 and T+1), but not significant in both samples combined [14]. Associations with asthma were then replicated in the African American (SNP S2), US white (SNPs T1 and T2) and US Hispanic (SNPs S2 and T2) populations [15], and in a German family-based study sample (SNP F+1) [16]; however, no SNP was consistently associated across ethnically diverse groups. Additionally, we found no association between JCPsis and each of the two SNPs (11434C/A [SNP ST+4] in intron 19 and 13506C/G [SNP V4] in 3′UTR), which was described to be implicated in asthma susceptibility among the UK population and combined UK and US samples [14], the Dutch population (SNP V4 only) [15] and the German families (SNP ST+4 only) [16]. There were pronounced differences in allele frequencies for SNPs S2, ST+4, T1, T2 and V4 (which were tested in ethnically diverse groups) between our population and others [14–16]. The variability of genetic background (including LD pattern) and environmental exposure among the populations may account for the observed differences [26]. There is of course also the possibility that different SNPs of the *ADAM33* gene contribute to asthma or rhinitis susceptibility in specific ethnic populations.

Subsequent assessment of the pattern of LD between all pairs of SNPs showed that most of the disease-associated polymorphisms in this study were in LD with each other. We further analysed the *ADAM33* haplotype structure and frequencies using four SNPs (7575G/A [SNP F+1], 9073G/A [SNP L-1], 10918G/C [SNP S2] and 12540C/T [SNP T+1]), and identified a haplotype (AACT haplotype) that accounted for JCPsis susceptibility risk. Because this haplotype is more frequently present in the healthy controls, it could be interpreted as a protective or resistant allele.

In addition to targeting disease status, as stated above, we observed weak correlations between five of non-coding SNPs in the *ADAM33* gene and JCPsis-related traits that included clinical severity, nasal eosinophila, and specific IgE levels; however, no SNP was associated with two or more phenotypes. These five SNPs were −3989T/C in 5′ genomic region, 11188 A/T (SNP S+1) in intron 19, 12601 T/G (SNP T+2) in intron 20 and 12946 G/A (SNP V-3) and 12954 C/T (SNP V-2) in intron 21. None of the SNPs was overlapped with those found to be associated with susceptibility to JCPsis. Interestingly, five of these SNPs had not been identified as the risk for asthma and BHR in the original report of Van Eerdewegh et al. [14]. It is likely that different SNPs of *ADAM33* may be important in specific phenotypes.

In the present study, we found no evidence for association of SNPs in *ADAM33* with total serum IgE levels. Lack of association of *ADAM33* polymorphisms with total IgE was also reported in the Latino populations [17] but contradicted by the data in others [15, 16], suggesting an existence of race and ethnic difference.

One of the main finding from Van Eerdewegh et al. [14]; original analysis showed that the linkage of *ADAM33* gene to asthma was strongly correlated with BHR. This raises the possibility that asymptomatic BHR might be influencing the results of our study, because there is evidence that a proportion of subjects with pollinosis, even in the absence of asthma, also exhibit non-specific BHR during and out of the pollen seasons [27, 28]. However, some characteristics of aeroallergens, such as inhaled particles' size and allergenic properties, are likely to determine or influence the degree of BHR and the level of bronchial airways inflammation in subjects with AR [28, 29]. One carefully conducted study in Japanese young adult population revealed negative BHR (based on PC_{20} values to methacholine) in non-asthmatic subjects with JCPsis [29]. Furthermore, among these subjects the airway inflammation markers (percentages of eosinophils, and levels of ECP and TNF-α) in induced sputum were below detection limits [29], indicating less inflammatory process in the lower airways in non-asthmatics with JCPsis. It therefore

seems less likely that the finding of our study concerning the association of *ADAM33* with JCPsis is attributable to the impact of BHR, although we did not test it in our subjects. In fact, recent study by Werner et al. [16] has shown that BHR was even absent in many asthmatics, and demonstrated that the *ADAM33* gene was associated with asthma susceptibility in the absence of BHR.

Taken together, despite the relatively small sample size in this study, our findings provided evidence for the *ADAM33* gene as an important determinant of JCPsis susceptibility, and the 3′ portion of the gene that encodes the transmembrane and cytoplasmic domains may be the key region. However, without further functional analysis it is difficult to ascribe primacy to any individual SNP, since the extent of LD exists in this region [14].

ADAM33 is a newly discovered member of the ADAM protein family of zinc-dependent metalloproteinase superfamily and contains multiple domains including signal sequence, pro-domain, followed by catalytic, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains [25]. ADAMs were originally identified as membrane-anchored proteins on the cell surface that mediate adhesion and proteolysis, and play pivotal roles in cell-to-cell interactions, cell signalling and the remodelling of extracellular matrix components [30]. The potential importance of ADAM33 in asthma pathogenesis is supported by the cellular-expression profile of *ADAM33* mRNA [14, 31], as well as protein [32], in human bronchial smooth muscle cells and lung fibroblasts. *ADAM33* polymorphisms may thus accelerate the proliferation of smooth muscle cells, fibroblasts and myofibroblasts and subsequent structural changes leading to airway remodelling in the asthmatic lung [14]. Also, *ADAM33* might affect asthma through playing a role in the alteration of growth factor expression, the regulation of cytokine shedding or a shift toward an immune response mediated by Th2 cells [33], although its function is at present unknown. This raises an intriguing question regarding similar potential mechanisms being implicated in nasal allergy, because the upper and lower airways show similar epithelial features and inflammatory reactions to irritants and allergens [10, 11].

Viewing the airways as a whole, from the nostrils to the alveoli, anatomic differences exist between the two ends of the respiratory tract, plentiful smooth muscle in the lower airways, but venous sinusoids and prominent glands in the nasal airways. Some studies show less tissue remodelling in the upper airways than in the lower airways of allergic individuals [34, 35]. However, allergic nasal mucosa also displays a variety of characteristic histopathological features including thickening and hyperplasia with polypoid degeneration of the epithelium, infiltration with eosinophilic, mononuclear and lymphoid cells; oedema and proliferation of the fibrous tissue with dilatation as well as thickening and compression of blood vessels in the lamina propria [36]. This points towards a potential underlying role for epithelium damage in contributing to nasal airways inflammation and remodelling responses by virtue of interaction of nasal mucosa with inhaled allergen in AR [37–39]. Interestingly, it is quite likely that the nasal mucosa has enhanced mechanisms for epithelial regeneration and repair through EGF receptor (EGFR) and its ligands, such as EGF, heparin-binding EGF-like growth factor (HB-EGF) and TGF-α [40, 41].

Also, it is plausible that the epithelium damage and remodelling processes in AR might link to an imbalance between matrix metalloproteinases (MMPs) and their inhibitors, the specific tissue inhibitors of metalloproteinases (TIMPs) [42]. Several studies have shown that MMPs, especially MMP-9, may contribute to the pathogenesis of asthma via their influence on the function and migration of inflammatory cells, as well as matrix deposition and degradation [43]. In contrast, there are few data available on the role of MMPs in AR [42, 44] and other upper airway diseases characterized by chronic inflammation and tissue remodelling, such as nasal polyposis [45]. These limited studies have, however, shown the presence of MMPs and consequently the potential for epithelial damage and remodelling in the nasal airways. On the other hand, evidence reveals that TIMPs, a group of endogenous inhibitors of MMPs and ADAMs [46], are present in large amounts in the nasal mucosa and that they might suppress MMP-mediated damage in AR [42], suggesting the potential influence of MMP–TIMP interactions on the development of nasal allergy.

A recent study has demonstrated proteolytic activity of human ADAM33 catalytic protein, and found that the activity could be inhibited by TIMPs [47]. Importantly, ADAM33 belongs to a subfamily [25] where members are shown to be catalytically active. For example, ADAM12 sheds HB-EGF [48]; ADAM19 participates in the proteolytic processing of membrane-anchored neuregulin-β1, a member of the EGFR family [49]; ADAM8 and ADAM15 catalyse ectodomain shedding of CD23, the low affinity IgE receptor [50]. It has been speculated that alteration in ADAM33 activity might underlie abnormalities in the regulation function for shedding of growth factors, cytokines and their receptors [33]. This raises the possibility that polymorphic variation in *ADAM33* might contribute to AR through influencing the course of epithelium inflammation, damage, repair and consequent tissue remodelling in the nasal airways.

In summary, we conclude that polymorphisms in the *ADAM33* gene are associated with susceptibility to AR due to JCP, but the functional relationship still needs clarification. Our case–control study has shown that some of the associated SNPs were splice variants located in non-coding sequences of introns, while, there were other disease-related SNPs that cause amino acid changes in the coding sequence; this highlights the potential for alterations in gene function and ADAM33 activity. Thus, further genetic and functional studies are needed to elucidate the role of *ADAM33* polymorphisms in the molecular mechanisms underlying JCPsis and nasal allergy.

Acknowledgements

We wish to thank K. Obara and K. Hasegawa for their contribution to the study, and H. Sekiguchi and M. Kokubo for technical assistance. This work was supported by a research grant from the Japanese Government's Millennium Genome Project; and by the Ministry of Health, Labour and Welfare of Japan, Research on Human Genome, Tissue

Engineering, and Research on Allergic Disease and Immunology. L. Cheng is a fellow of CREST-JST (Core Research for Evolution Science and Technology, Japan Science and Technology Agency).

References

1 Ishizaki T, Koizumi K, Ikemori R, Ishiyama Y, Kushibiki E. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. Ann Allergy 1987; 58:265–70.
2 Okuda M, Shida T. Clinical aspect of Japanese cedar pollinosis. Allergol Int 1998; 47:1–8.
3 Tanihara S, Oki I, Ojima T, Nakamura Y, Yanagawa H. Process and current status of the epidemiologic studies on cedar pollinosis in Japan. J Epidemiol 1999; 9:20–6.
4 Sasazuki T, Nishimura Y, Muto M, Ohta N. HLA-linked genes controlling immune response and disease susceptibility. Immunol Rev 1983; 70:51–75.
5 Matsushita S, Muto M, Suemura M, Saito Y, Sasazuki T. HLA-linked nonresponsiveness to *Cryptomeria japonica* pollen antigen. I. Nonresponsiveness is mediated by antigen-specific suppressor T cell. J Immunol 1987; 138:109–15.
6 Ikagawa S, Matsushita S, Chen YZ, Ishikawa T, Nishimura Y. Single amino acid substitutions on a Japanese cedar pollen allergen (*Cry j* 1)-derived peptide induced alterations in human T cell responses and T cell receptor antagonism. J Allergy Clin Immunol 1996; 97:53–64.
7 Hori T, Kamikawaji N, Kimura A et al. Japanese cedar pollinosis and HLA-DP5. Tissue Antigens 1996; 47:485–91.
8 Nagata H, Mutoh H, Kumahara K et al. Association between nasal allergy and a coding variant of the FcεRIβ gene Glu237Gly in a Japanese population. Hum Genet 2001; 109:262–6.
9 Barnes KC. Evidence for common genetic elements in allergic disease. J Allergy Clin Immunol 2000; 106:S192–200.
10 Grossman J. One airway, one disease. Chest 1997; 111:11–6S.
11 Togias A. Systemic cross-talk between the lung and the nose. Am J Respir Crit Care Med 2001; 164:726–7.
12 Cookson WO, Sharp PA, Faux JA, Hopkin JM. Linkage between immunoglobulin E responses underlying asthma and rhinitis and chromosome 11q. Lancet 1989; I:1292–5.
13 Hoffjan S, Ober C. Present status on the genetic studies of asthma. Curr Opin Immunol 2002; 14:709–17.
14 Van Eerdewegh P, Little RD, Dupuis J et al. Association of the *ADAM33* gene with asthma and bronchial hyperresponsiveness. Nature 2002; 418:426–30.
15 Howard TD, Postma DS, Jongepier H et al. Association of a disintegrin and metalloprotease 33 (*ADAM33*) gene with asthma in ethnically diverse populations. J Allergy Clin Immunol 2003; 112:717–22.
16 Werner M, Herbon N, Gohlke H et al. Asthma is associated with single-nucleotide polymorphisms in *ADAM33*. Clin Exp Allergy 2004; 34:26–31.
17 Lind DL, Choudhry S, Ung N et al. *ADAM33* is not associated with asthma in Puerto Rican or Mexican populations. Am J Respir Crit Care Med 2003; 168:1312–6.
18 Okuda M, Senba O. Effects of beclomethasone dipropionate nasal spray on subjective and objective findings in perennial allergic rhinitis. Clin Otolaryngol 1980; 5:315–21.
19 Okuda M, Sakaguchi K, Ohtsuka H. Intranasal beclomethasone: mode of action in nasal allergy. Ann Allergy 1983; 50:116–20.
20 Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y. JSNP: a database of common gene variations in the Japanese population. Nucleic Acids Res 2002; 30:158–62.
21 Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T. Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. J Hum Genet 2002; 47:605–10.
22 Lyamichev V, Mast AL, Hall JG et al. Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. Nat Biotechnol 1999; 17:292–6.
23 Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001; 46:471–7.
24 Nakajima T, Jorde LB, Ishigami T et al. Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. Am J Hum Genet 2002; 70:108–23.
25 Yoshinaka T, Nishii K, Yamada K et al. Identification and characterization of novel mouse and human ADAM33s with potential metalloprotease activity. Gene 2002; 282:227–36.
26 Postma DS, Howard T. *ADAM33* gene: confirming a gene without linkage. Clin Exp Allergy 2004; 34:1–3.
27 Polosa R, Gotti FL, Mangano G, Mastruzzo C, Pistorio MP, Crimi N. Monitoring of seasonal variability in bronchial hyperresponsiveness and sputum cell counts in non-asthmatic subjects with rhinitis and effect of specific immunotherapy. Clin Exp Allergy 2003; 33:873–81.
28 Di Lorenzo G, Mansueto P, Melluso M et al. Non-specific airway hyperresponsiveness in mono-sensitive Sicilian patients with allergic rhinitis. Its relationship to total serum IgE levels and blood eosinophils during and out of the pollen season. Clin Exp Allergy 1997; 27:1052–9.
29 Obase Y, Shimoda T, Mitsuta K, Matsuo N, Matsuse H, Kohno S. Correlation between airway hyperresponsiveness and airway inflammation in a young adult population: eosinophil, ECP, and cytokine levels in induced sputum. Ann Allergy Asthma Immunol 2001; 86:304–10.
30 Primakoff P, Myles DG. The ADAM gene family: surface proteins with adhesion and protease activity. Trends Genet 2000; 16:83–7.
31 Umland SP, Garlisi CG, Shah H et al. Human *ADAM33* mRNA expression profile and post-transcriptional regulation. Am J Respir Cell Mol Biol 2003; 29:571–82.
32 Garlisi CG, Zou J, Devito KE et al. Human ADAM33: protein maturation and localization. Biochem Biophys Res Commun 2003; 301:35–43.
33 Shapiro SD, Owen CA. *ADAM-33* surfaces as an asthma gene. N Engl J Med 2002; 347:936–8.
34 Chanez P, Vignola AM, Vic P et al. Comparison between nasal and bronchial inflammation in asthmatic and control subjects. Am J Respir Crit Care Med 1999; 159:588–95.
35 Braunstahl GJ, Fokkens WJ, Overbeek SE, KleinJan A, Hoogsteden HC, Prins JB. Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways. Clin Exp Allergy 2003; 33:579–87.
36 Salib RJ, Drake-Lee A, Howarth PH. Allergic rhinitis: past, present and the future. Clin Otolaryngol 2003; 28:291–303.
37 Watanabe K, Kiuna C. Epithelial damage of nasal mucosa in nasal allergy. Ann Otol Rhinol Laryngol 1998; 107:564–70.
38 Nagata H, Motosugi H, Sanai A et al. Enhancement of submicroscopic damage of the nasal epithelium by topical allergen challenge in patients with perennial nasal allergy. Ann Otol Rhinol Laryngol 2001; 110:236–42.
39 Gluck U, Gebbers J. Epithelial changes in seasonal allergic rhinitis throughout the year: evidence of coexistent air pollution and local secretory IgA deficiency? ORL 2000; 62:68–75.
40 Polosa R, Prosperini G, Tomaselli V, Howarth PH, Holgate ST, Davies DE. Expression of c-erbB receptors and ligands in human nasal epithelium. J Allergy Clin Immunol 2000; 106: 1124–31.
41 Matovinovic E, Solberg O, Shusterman D. Epidermal growth factor receptor – but not histamine receptor – is upregulated in seasonal allergic rhinitis. Allergy 2003; 58:472–5.

42 Shaida A, Kenyon G, Devalia J, Davies RJ, MacDonald TT, Pender SL. Matrix metalloproteinases and their inhibitors in the nasal mucosa of patients with perennial allergic rhinitis. J Allergy Clin Immunol 2001; 108:791–6.
43 Shute J. Matrix metalloproteinase-9: marker or mediator of tissue damage in asthma? Clin Exp Allergy 2002; 32:168–71.
44 van Toorenenbergen AW, Gerth van Wijk R, Vermeulen AM. Allergen-induced matrix metalloproteinase-9 in nasal lavage fluid. Allergy 1999; 54:293–4.
45 Lechapt-Zalcman E, Coste A, d'Ortho MP et al. Increased expression of matrix metalloproteinase-9 in nasal polyps. J Pathol 2001; 193:233–41.
46 Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 2002; 115:3719–27.
47 Zou J, Zhu F, Liu J et al. Catalytic activity of human ADAM33. J Biol Chem 2004; 279:9818–30.
48 Asakura M, Kitakaze M, Takashima S et al. Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med 2002; 8:35–40.
49 Shirakabe K, Wakatsuki S, Kurisaki T, Fujisawa-Sehara A. Roles of Meltrin β/ADAM19 in the processing of neuregulin. J Biol Chem 2001; 276:9352–8.
50 Fourie AM, Coles F, Moreno V, Karlsson L. Catalytic activity of ADAM8, ADAM15, and MDC-L (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. J Biol Chem 2003; 278:30469–77.

© 2004 Blackwell Publishing Ltd,

JOURNAL OF ADOLESCENT HEALTH 2004;35:156–158

INTERNATIONAL ARTICLE

Allergic Symptoms and Microflora in Schoolchildren

SANAE FUKUDA, Ph.D., HIROKI ISHIKAWA, Ph.D., YASUHIRO KOGA, M.D., Ph.D., YUJI AIBA, KAZUKO NAKASHIMA, M.M.Sc., LEI CHENG, M.D., AND TARO SHIRAKAWA, M.D., Ph.D.

We studied 867 junior high school children and administered a questionnaire documenting allergic symptoms and environmental variables, and measured Immunoglobulin E serum levels and the immunoglobulin G titers of serum antibody to microflora. A total of 716 subjects were ultimately used for statistics; those with at least two of the following allergic symptoms: asthma, rhinitis, eczema, or food allergy, showed significantly higher IgG titers to *Bactroides vulgatus* than other groups. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora tends to affect the gut issues, but further studies are needed to clarify this. © *Society for Adolescent Medicine, 2004*

KEY WORDS:
Allergy
Schoolchildren
Microflora

The prevalence of allergic diseases has been increasing in developed countries. However, many studies have demonstrated that the prevalence of these diseases is lower in the formerly socialist countries of Central and Eastern Europe than in Western European counties [1]. One factor associated with the rise of allergic diseases may be the decline of many infectious diseases in developed countries as the result of improved living standards and vaccinations [2]. In modern societies, it has been noted that allergies may be "mapped" according to gradients dictated by hygiene and by the individual degree of genetic predisposition to allergy [3]. These concepts are known as the "Hygiene hypothesis" [3–5]. It has been suggested that a reduced microbial stimulation during infancy and early childhood would result in slower postnatal maturation of the immune system and development of an optimal balance between Th1- and Th2-like immunities [6].

To clarify what and when bacterial species of commensal intestinal microbes are recognized as antigens that induce serum antibody responses in allergy patients, we studied the serum antibody responses to microflora in atopic school-aged children.

Methods

From a population of approximately 1000 12–13-year-old schoolchildren attending the 18 junior high schools in the country in 1995, we did a retrospective study of records of tuberculin responses in 867 children. They had been immunized with bacillus Calmette-Guerin (BCG) at 6 and 12 years of age and they showed negative tuberculin responses. Shirakawa et al showed the strong inverse association between delayed hypersensitivity to tuberculin and atopy [7]. The Institutional Review Board of RIKEN approved this study and 787 of all recruited subjects gave informed consent. We administered a questionnaire documenting atopic symptoms; the

From the Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, Japan (S.F., K.N., L.C., T.S.); and Department of Infectious Diseases, School of Medicine, Tokai University, Isehara, Japan (H.I., Y.K., Y.A.).

Address correspondence to: Dr. Sanae Fukuda, Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Yoshida-Konoe, Kyoto 606-8501, Japan. E-mail: fukuda@pbh.med.kyoto-u.ac.jp

Manuscript accepted September 25, 2003.

1054-139X/04/$–see front matter
doi:10.1016/j.jadohealth.2003.09.018

© Society for Adolescent Medicine, 2004
Published by Elsevier Inc., 360 Park Avenue South, New York, NY 10010

Table 1. The Relationship Between Allergy Symptoms and IgG Titers of Serum Antibody Microflora in School Children (Mean ± SE)

Microflora or IgE	Nonallergy (n = 433)	One allergy symptom (n = 200)	2 or more allergy symptoms (n = 83)	F	p
E. coli	3.04 ± 0.01	3.07 ± 0.02	3.04 ± 0.03	0.62	.54
B. vulgatus	2.94 ± 0.01	2.91 ± 0.01	2.97 ± 0.02	3.00	.05
E. faecalis	2.99 ± 0.01	2.99 ± 0.02	3.03 ± 0.03	1.11	.33
B. longum	3.02 ± 0.01	3.01 ± 0.02	3.06 ± 0.02	1.38	.25
IgE	2.19 ± 0.03	2.25 ± 0.04	2.41 ± 0.08	4.23	.02

All were analyzed by one-way analysis of variance. Each gut flora or IgE values were converted to $\log_{10}$ because they didn't show normal distribution. SE = means standard error; *E. coli* = *Escherichia coli*; *B. vulgatus* = *Bacteroides vulgatus*; *E. faecalis* = *Enterococcus faecalis*; *B. longum* = *Bfidobacterium longum*; IgE = Immunoglobulin E.

questions were: "Do you have asthma?," "Do you have rhinitis?," "Do you have eczema?," "Do you have food allergy?" Also included were environmental variables, family allergy history, tuberculin response, etc., and we also measured immunoglobulin E (IgE) serum levels (measured by Mitsubishi BCL, Inc., Kyoto, Japan) and the immunoglobulin G (IgG) titers of serum antibody to *Escherichia coli* (*E. coli*), *Bacteroides vulgatus* (*B. vulgatus*), *Enterococcus faecalis* (*E. faecalis*), and *Bifidobacterium longun* (*B. longun*). This assay was described in detail in a previous article [8]. The titers of IgG class antibodies reacting with bacteria in serum were measured by enzyme-linked immunosorbent assay, and the absorbance was read at 495 nm using a plate reader.

We avoided subjects who did not complete questions about atopic symptoms and whose serum sample could not be measured for technical reasons. Ultimately we used 716 subjects for statistics. The values of the IgG titers of serum antibody to microflora and the level of IgE were converted to $\log_{10}$. Differences in IgG titers to *E. coli*, *B. vulgatus*, *E. faecalis*, and *B. longun* between nonallergic schoolchildren with no allergic symptoms, and symptomatic schoolchildren with more than one allergic symptom among asthma, rhinitis, eczema, and food allergy, were analyzed by two-tailed Student's *t*-test. We also tested the relation in IgG titers by each symptom; asthma, rhinitis, eczema, and food allergy by two-tailed Student's *t*-test. Then we divided subjects into three groups; "nonallergy," who have no allergy symptoms; "one allergy," who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy," who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. We examined the differences in IgG titers to *E. coli*, *B. vulgatus*, *E. faecalis*, and *B. longun* among the three groups by one-way analysis of variance. The statistical analysis for this study was done using SPSS 10.0J for Win.

Results

IgG titers to *E. coli*, *B. vulgatus*, *E. faecalis*, and *B. longun* showed no significant difference between nonallergic schoolchildren (n = 433) with no allergic symptoms, and symptomatic schoolchildren (n = 283) who have more than one allergic symptom among asthma, rhinitis, eczema, and food allergy. We also tested the relation by each symptom; asthma, rhinitis, eczema, and food allergy; there were, however, no differences between nonallergic children and children with one or more allergic symptoms. The subjects were divided into three groups; "nonallergy" (n = 433), who have no allergy symptoms; "one allergy" (n = 200), who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy" (n = 83) who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. In the "two or more allergy" group, IgG titers to *B. vulgatus* were significantly higher than in the other two groups (Table 1) (F = 3.00 df = 2, p = .05). IgG titers to *E. coli*, *B. vulgatus*, and *E. faecalis* showed no significant difference among three groups. Also in the "two or more allergy" group, subjects showed the highest mean IgE levels (2.41 ± 0.08 vs. 2.19 ± 0.03, 2.25 ± 0.04; F = 4.23 df = 2, p = .02) among three groups (Table 1).

Discussion

The children with two or more of the allergic symptoms showed higher IgG titers to *B. vulgatus* than the groups of nonallergic or one-allergic-symptom schoolchildren. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora might be an organism that affects the gut issues and thus, induces inflammation accompanied by an elevation of serum antibodies to this bacterium [8], although the exact mechanism of this remains un-

clear. Some studies reported that there was a difference in the composition of intestinal microflora between allergic and nonallergic infants. They compared 1-year-old infants living in two countries with a low (Estonia) and a high (Sweden) prevalence of atopy, and found that *Lactobacilli* and *Eubacteria* were more frequently found in the intestinal microflora of Estonian infants, whereas *Clostridia* was more prevalent in Swedish infants [9]; 2-year-old allergic children were colonized less often by *Lactobacilli*, and harbored higher counts of aerobic bacteria (coliforms, *Staphylococcus aureus*) than nonallergic children [10]. These subjects were, however, all infants, and the number of samples was small. Kirjavainen et al recently showed that, in 4.2–10.5-year-old children, serum total IgE concentration correlated with bacteroides in heightened risk of subjects with atopic disorders, and those subjects also showed a greater number of lactobacilli/enterococci than those in the low-risk group [11]. Our data is partly consistent with this.

This is the first study to clarify the relationship between atopic symptoms and bacterial species of predominant commensal intestinal microflora in a large group of junior high school children. Further studies are needed to clarify when bacterial species of commensal microbes in the gut are recognized as the antigens that induce serum antibody responses in allergic subjects.

References

1. Bjorksten B, Dumitrascu D, Foucard T, et al. Prevalence of childhood asthma, rhinitis and eczema in Scandinavia and Eastern Europe. Eur Respir J 1998;12:432–7.
2. Doll R. Health and the environment in the 1990s. Am J Public Health 1992;82:933–41.
3. Matricardi PM, Bonini S. High microbial turnover rate preventing atopy: A solution to inconsistencies impinging on the Hygiene hypothesis? Clin Exp Allergy 2000;30:1506–10.
4. Strachan DP. Hay fever, hygiene and household size. BMJ 1989;299:1259–60.
5. Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. Lancet 1999;354(Suppl 2):SII12–5.
6. Bottcher MF, Nordin EK, Sandin A, et al. Microflora-associated characteristics in faeces from allergic and nonallergic infants. Clin Exp Allergy 2000;30:1590–6.
7. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. Science 1997;275:77–9.
8. Saitoh S, Noda S, Aiba Y, et al. Bacteroides ovatus as the predominant commensal intestinal microve causing a systemic antibody response in inflammatory bowel disease. Clin Diagn Lab Immunol 2002;9:54–9.
9. Sepp E, Julge K, Vasar M, et al. Intestinal microflora of Estonian and Swedish infants. Acta Pediatr 1997;86:956–61.
10. Bjorksten B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 1999;29:342–6.
11. Kirjavainen PV, Arvola T, Salminen SJ, Isolauri E. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? Gut 2002;51:51–5.

American Journal of Pathology, Vol. 164, No. 4, April 2004
Copyright © American Society for Investigative Pathology

Pathological Role of Large Intestinal IL-12p40 for the Induction of Th2-Type Allergic Diarrhea

Ayako Hino,*†‡ Mi-Na Kweon,*‡§ Kohtaro Fujihashi,† Jerry R. McGhee,† and Hiroshi Kiyono*†‡

From the Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; the Departments of Oral Biology and Microbiology,† The Immunobiology Vaccine Center, The University of Alabama at Birmingham, Birmingham, Alabama; the Mucosal Immunology Section,§ International Vaccine Institute, Seoul, Korea; and Core Research for Evolution Science and Technology,‡ Japan Science and Technology Corporation, Tokyo, Japan*

IL-12 consists of two disulfide-linked subunits, p40 and p35, that form functionally active heterodimers for the induction of Th1 cells. In contrast to IL-12 heterodimers, p40 monomers and homodimers possess inhibitory effects on Th1 cells leading to the creation of a Th2 environment. Although it has been shown that IL-12p40 acts as antagonist of IL-12p70 *in vitro*, no evidence is currently available whether IL-12p40 is functional *in vivo*. We now report that IL-12p40 plays an important pathological role in an intestinal allergic disease. A high expression of IL-12p40 protein was demonstrated in epithelial cells, dendritic cells, and macrophages in large but not small intestine of allergic diarrhea-induced mice. Interestingly, neutralization with anti-IL-12p40 mAbs reduced the incidence and delayed the onset of disease development. Lower levels of ovalbumin (OVA)-specific IgE Abs in serum were detected in anti-IL-12p40 mAb-treated mice than in control Ab-treated mice. The secretion of Th2 cytokines and eotaxin by the mononuclear cells isolated from the large intestine of anti-IL-12p40 mAb-treated mice was significantly decreased. Finally, the removal of the IL-12p40 gene resulted in complete inhibition of disease development. These results show that over-expression of IL-12p40 is an important contributing factor for the generation of the dominant Th2-type environment in the large intestine of mice with allergic diarrhea. *(Am J Pathol 2004, 164:1327–1335)*

In general, intestinal allergic reactions are provoked by the activation of allergen-specific Th2-type cells, excessive eosinophil and mast cell recruitment, and IgE Ab production.[1,2] We previously reported that systematically primed BALB/c mice developed severe diarrhea after repeated oral challenge with ovalbumin (OVA).[1] Diarrhea-induced mice revealed a Th2-type allergic response characterized by high levels of Ag-specific IgE Abs in serum, increased numbers of IgG1, IgA and IgE Abs in the large intestine, and high numbers of mast cells and eosinophils in the large intestine. Furthermore, large intestinal $CD4^+$ T cells isolated from mice with allergic diarrhea secreted IL-4, IL-5, and IL-13, but not IFN-γ. On the other hand, a murine model of eosinophilic gastrointestinal hypersensitivity induced by challenge with oral allergen, in the form of enteric-coated beads, resulted in marked allergen-induced IL-4 and IL-5 production and eosinophil accumulation in the small intestine. Although several interesting intestinal allergic models were recently reported,[2,3] the exact underlying molecular and cellular mechanisms remain to be elucidated.

In allergic asthma, allergen-specific T cells have been shown to also acquire the Th2 phenotype and to avoid from the Th1-type pathway.[4,5] A recent study has demonstrated that Th1/Th2 imbalance induced allergic disease at the level of transcription factors. Interestingly, a high expression of GATA-3 and/or a lack of T-bet signaling markedly influenced the development of allergic asthma.[6,7] In addition, the expression of not only Th2-type cytokine but also Th1-type cytokine (ie, IFN-γ or IL-12) played a critical role in murine dermatitis and asthma models.[8,9] It has also been demonstrated that natural killer (NK) cells, like Th2 cells, play an important role in the development of allergen-induced asthma.[9]

It has been suggested that antigen-presenting cells (APCs) play a crucial role in the skewing of Th1 and Th2 differentiation.[10,11] IL-12 is a heterodimeric cytokine composed of p40 and p35 which strongly promotes the differentiation of naive $CD4^+$ T cells to the Th1 phenotype and suppresses the synthesis of Th2-type cytokines.[12] IL-12 is produced primarily by APCs and the production is regulated by IL-10 and IFN-γ.[13,14] In addition, biological effects of IL-12 are counter-balanced by IL-12p40 itself, which binds to the receptor complex without induc-

Supported in part by a CREST grant from Japan Science and Technology Corporation (JST) and Grant-in-Aid from the Ministry of Education, Science, Sports and Culture and the Ministry of Health and Welfare of Japan and by US PHS grants DK 44240, AI 18958, AI 43197, and DE 12242.

Accepted for publication December 24, 2003.

Address reprint requests to Dr. Hiroshi Kiyono, Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail: kiyono@ims.u-tokyo.ac.jp.

ing intracellular signals.[15] Thus, murine IL-12p40 inhibits IL-12-mediated responses by means of the competitive binding to IL-12 receptor with an affinity similar to that of IL-12p70.[16,17] Further, IL-12p40 can behave as an IL-12p70 antagonist *in vivo*, delaying the allograft rejection of cardiac myoblast.[18] IL-12p40 transgenic mice also showed increased susceptibility to the malaria infection.[19]

With regard to allergic responses, a potential contribution of IL-12 has been suggested for the development of allergic asthma.[9,20] Mixed Th1- and Th2-associated cytokines, including IFN-γ, IL-2, IL-5, GM-CSF, and IL-12, were secreted by smooth muscle cells located in the sensitized airways of atopic asthma-induced mice.[21] A previous study demonstrated that IL-12p40 mRNA expression was detected in the lung tissue of mice with asthma.[21] However, the role of IL-12 in intestinal allergic disease has not yet been carefully examined. To investigate the potential roles of IL-12 in intestinal hypersensitivity, we have assessed the expression pattern of IL-12p40 or IL-12p35 in the intestinal tract of diarrhea-induced mice and examined the therapeutic effects of modulating IL-12 involvement in allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from Japan Clea Company (Tokyo, Japan). Breeding pairs of IL-12p40-deficient [IL-12p40 knockout (KO)] mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and colonies were established and maintained in the experimental animal facility at the University of Tokyo and the University of Alabama at Birmingham. All mice were 6 to 7 weeks of age at the beginning of individual experiments.

Antibodies

Recombinant murine IL-12 p40 and p70 were obtained from BD PharMingen (San Diego, CA). Biotin-anti-CD11b (M1/70, rat IgG2b) and biotin-anti-CD11c (HL3, hamster IgG) were also purchased from BD PharMingen. Biotin-SP-conjugated, affinity-purified anti-rat IgG (H+L) mouse $F(ab')_2$ and anti-hamster IgG(H+L) goat $F(ab')_2$ were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA[22] and was purified from ascites on a protein G column (Pharmacia Biotech, Uppsala, Sweden). Purified rat IgG was purchased from Sigma Chemical, Inc. (St. Louis, MO).

Induction of Allergic Diarrhea

For the induction of allergic diarrhea, our well-established protocol was used as described previously.[1] Briefly, on the first day of the experiment (day 0), mice were primed by subcutaneous (SC) injection of 1 mg of OVA in Complete Freund Adjuvant (CFA) (Difco Laboratories, Detroit, MI). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral administration (PO) three times per week for several weeks.[1] These mice were sacrificed and analyzed within 1 and 2 hours after a total of 10 times of oral administration with OVA. In a timed kinetics study, mice were sacrificed at indicated intervals, ie, 0, 30 minutes, 1 hour, and 2 hours following the last oral administration of OVA. As controls, mice were repeatedly given oral OVA in phosphate-buffered saline (PBS) without systemic priming or were injected SC with 1 mg OVA in CFA without repeated oral challenge.

Treatment of Mice with Anti-IL-12p40

In vivo Ab treatment was performed as described previously.[23] Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Schering-Plough Research Institute, Dardilly, France.[22] BALB/c mice were intraperitoneally administered with 0.5 mg to 2.0 mg of purified anti-IL-12p40 (C17.8) or control rat IgG (Sigma Chemicals, Inc.) per week for the duration of the experiment. Ab treatment was started 1 week before or at the time systemic priming with OVA in CFA. Among different concentrations tested, the protocol of 1 mg/mouse of per week was the most optimal condition. Further, when the mAb treatment schedule was compared between the start at 1 week before or at the same time as OVA systemic priming, an identical effect was noted in this study. Thus, the mAb anti-IL-12 (1 mg/mouse) was started on 1 week before the OVA systemic priming.

ELISA for OVA-Specific IgE Abs in Serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted.[1] End-point titers of OVA-specific IgE Abs were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of 0.1 higher absorbance than that of sera of non-immune mice as background.

Isolation of Mononuclear Cells and Cytokine-Specific ELISA Assay

To isolate mononuclear cells from small and large intestines, we used a enzymatic dissociation method.[24] Briefly, mononuclear cells were dissociated by collagenase from small and large intestines after the removal of Peyer's patches and were then subjected to a discontinuous Percoll gradient.[25] Mononuclear cells from small and large intestines were then co-cultured in the presence of 1 mg OVA. After 3 days of culture, the supernatant was collected and assayed for cytokines by using an ELISA Kit specific for IL-4 (Endogen, Woburn, MA), IL-5 (Amersham Pharmacia Biotech, Piscataway, NJ), IL-13 (R&D Systems, Minneapolis, MN) and eotaxin (Techne Corporation, Minneapolis, MN).

Table 1. Primers and Probes Used for Qualitative and Quantitative RT-PCR

		Conventional PCR*	
HPRT	primer	F	GTT GGA TAC AGG CCA GAC TTT GTT G
		R	GAG GGT AGG CTG GCC TAT AGG CT
IL-12p40	primer	F	ATG GCC ATG TGG GAG CTG GAG
		R	TTT GGT GCT TCA CAC TTC AGG
IL-12p35	primer	F	ACC CAG TTG GCC AGG GTC
		R	CAA GGC ACA GGG TCA TCA TC
		Real-time PCR†	
HPRT	primer	F	AAC TTT GCT TTC CCT GGT
		R	AGT CAA GGG CAT ATC CAA CA
	probe	FITC	CAG TAC AGC CCC AAA ATG GTT AAG GTTGC
		LCRed640	AGC TTG CTG GTG AAA AGG ACG TCT CG
IL-12p40	primer	F	AGA GGA GGG GTG TAA CCA G
		R	GGG AAC ACA TGC CCA CTT G
	probe	FITC	ACC GAA GTC CAA TGC AAA GGC GG
		LCRed640	AAT GTC TGC GTG CAA GCT CAG G
IL-12p35	primer	F	CCT GTG CCT TGG TAG CAT CT
		R	AGA CTG CAT CAG CTC ATC G
	probe	FITC	ACC AGA CAG AGT TCC AGG CCA TCA
		LCRed640	TGA TGG CCT GGA ACT CTG TCT GGT

*, Primers were designed based on the published sequence.[13]
†, Primers and probes were designed and produced by Nihon Gene Research Laboratories (Sendai, Japan).

Immunoprecipitation and Western Blot Analyses

For the detection of different forms of IL-12, intestinal tissue extracts were prepared as previously described with minor modifications.[26] Small and large intestines were removed, minced in cold PBS with protease inhibitor, homogenized, and incubated to allow cytokine release from the tissue. After centrifugation intestinal tissue extracts were subjected to the measurement of protein concentration and then pre-cleared with protein G Sepharose beads (Pharmacia Biotech, Uppsala, Sweden), subsequently incubated with anti-IL-12p40, mixed with protein G Sepharose beads. The beads were washed, subjected to SDS-PAGE under non-reducing condition. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (PVDF Immobilon; Millipore, Bedford, MA) and the membrane was reacted with biotinylated anti-IL-12 (C17.8) followed by incubation with biotin-streptavidin complex (ABC-AP Kit; Vector Laboratories, Inc.). Visualization of the signal was performed by NBT/BCIP Substrate Kit (BioRad, Hercules, CA).

Analysis for IL-12 mRNA Accumulation

The expression of IL-12p40 or p35 in small and large intestines of mice was examined using conventional RT-PCR as previously described[13] and quantitative real-time PCR method using a Lightcycler (Roche Diagnostics, GmbH Mannheim, Germany), with some modifications.[27,28] The sequences of primers and probes for real-time PCR were designed by Nihon Gene Research Laboratories (Sendai, Japan) (Table 1). Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and 2 μg of extracted RNA was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies).[14] Hypoxanthine phosphoribosyl transferase (HPRT) cDNA was used to standardize the total RNA content. The cDNA from each experimental sample was then subjected to the Lightcycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics). The external standards of HPRT, IL-12p40, and IL-12p35 DNA prepared by PCR between 20 pg to 0.02 fentograms were used for the quantification of specific cDNA in each sample. The ratio of the p40 and p35 increase in experimental mice was calculated and compared with non-treated mice as follows; the ratio = (p40 or p35 mRNA amounts from experimental mice/HPRT mRNA amounts from experimental mice)/(p40 or p35 mRNA amounts from non-treated mice/HPRT mRNA amounts from non-treated mice).

Immunohistochemical Analysis

Following extensive washing, small and large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose-gradient, frozen in OCT-embedding medium as previously described with minor modifications.[29] For IL-12p40 immunostaining, cryosections were subjected to antigen retrieval using 10 mmol/L citric buffer pH 6.0 for 5 minutes at 98°C. Slides were then blocked with normal mouse IgG and incubated with rat anti-IL-12p40 or control rat IgG for 16 hours at 4°C. The section were then treated with biotinylated goat anti-rat IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc.), ABC-AP Kit, and red chromogen (Vector Red; Vector Laboratories, Inc.). For IL-12p35 immunostaining, we used goat anti-IL-12p35 (Santa Cruz, Inc., Santa Cruz, CA) or control goat IgG. The sections were then treated with biotinylated donkey anti-goat IgG $F(ab')_2$ and ABC-AP Kit. In the case of surface marker staining, serial sections were incubated with anti-CD11b (M1/70, BD PharMingen) or anti-CD11c (HL3, BD PharMingen), biotinylated second anti-

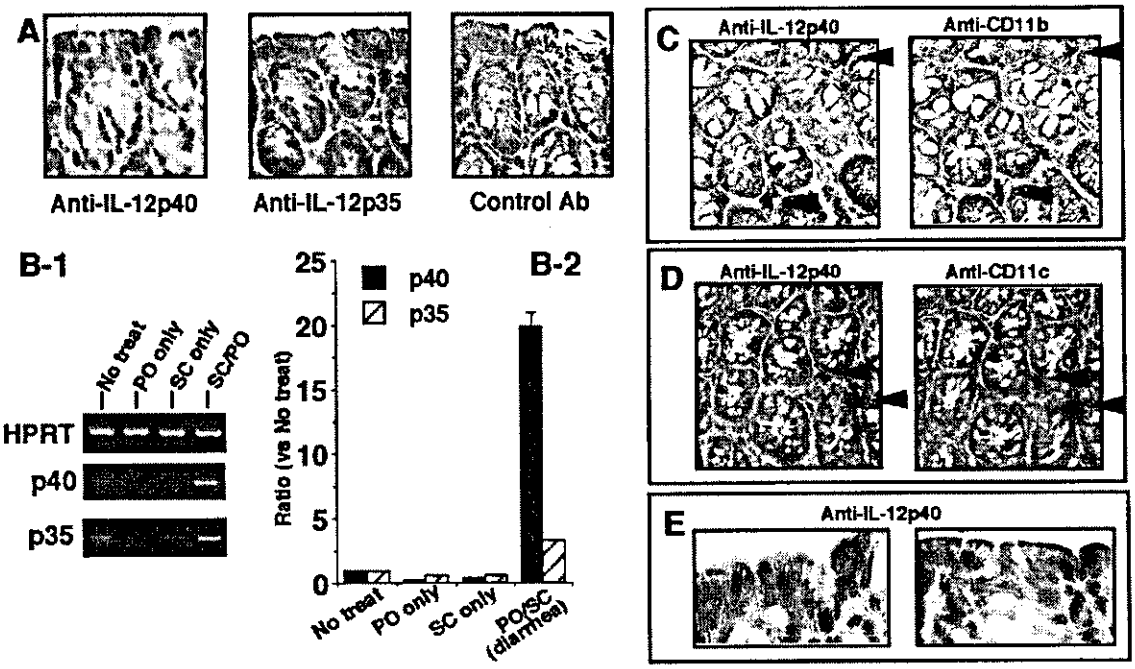

Figure 1. Selective production of IL-12p40 by the large intestine of diarrhea-induced mice. In **A**, large intestinal tissues from diarrhea-induced mice were immunostained with anti-IL-12p40 mAb, anti-IL-12p35 mAb, or control IgG. Control non-disease mice section gave no signal above background (data not shown). In **B-1**, IL-12p40-specific mRNA was expressed selectively in the large intestine of mice with allergic diarrhea. In **B-2**, quantitive real-time PCR analysis of IL-12p40- and p35-specific mRNA expression was performed. The ratio was obtained as the level of IL-12p40 or p35 expression in non-treated mice as a scale of one. The detailed information for the expression of this ratio is described in the Materials and Methods section. In **C-E**, IL-12p40 was detected in MØ and DC and epithelial cells in the large intestine. The serial sections of the large intestine from diarrhea-induced mice were stained with anti-IL-12p40 mAb and anti-CD11b mAb (**C**), with anti-IL-12p40 mAb and anti-CD11c mAb (**D**). The **arrows** point to double-positive cells. Large intestinal epithelial cells were stained with anti-IL-12p40 mAb (**E**).

body and ABC-AP. The color reaction was developed using Vector Red Substrate Kit I.

Statistical Analysis

Statistical analyses were performed by the two sample non-parametric Welch test with a significance level of 0.01 (**) for body weight and Ig levels, respectively. Mouse disease rates were determined using the Wilcoxon rank-sum test with a significance level of 0.01 (**). Values for cytokine-synthesis in the samples between anti-IL-12 p40-treated and control antibody-treated mice were analyzed by using Student's *t*-test at *P* values of <0.01(**).

Results

Detection of IL-12p40 Protein in the Large Intestine of Allergic Diarrhea Mice

To examine whether IL-12p40 was expressed in the large intestine of OVA-induced diarrhea mice, we analyzed IL-12 expression using a variety of available detection methods. First, we performed immunohistochemical analysis to directly demonstrate the enhanced IL-12p40 expression in the large intestine of mice with allergic diarrhea. As shown in Figure 1A, IL-12p40, but not IL-12p35, was expressed in the large intestine of diarrhea-induced mice. To further confirm enhanced expression of IL-12p40 in the large intestine of mice with diarrhea, we next performed IL-12-specific RT-PCR analysis. Interestingly, IL-12p40 mRNA was only detected in the large intestine of diarrhea-induced mice, not in control mice without the disease [eg, SC only or per oral challenge (PO) only; Figure 1B]. In contrast, IL-12p35 mRNA expression was detected in both groups of mice (Figure 1B-1). When IL-12-specific mRNA quantitative real-time PCR analysis was performed, high levels of IL-12p40-specific mRNA were noted in the large intestine of OVA-induced allergic diarrhea mice (Figure 1B-2). In contrast, the level of p35 did not vary among the four different groups including experimental diseased (SC/PO) and control non-diseased mice (non-treated, SC only, and PO only). Taken together, these results clearly indicate that IL-12p40, but not p35, was selectively enhanced at the levels of both mRNA and protein in the large intestine of allergic diarrhea mice.

Inasmuch as the induction of IL-12p40 selectively occurred in the large intestine of OVA-induced allergic diarrhea mice, it was important to determine which cell types produced IL-12p40 in the large intestine. Immunohistochemical analysis demonstrated that IL-12p40-producing cells were co-stained with anti-CD11b mAb [ie, macrophages (MØ)]. Further, $CD11c^+$ cells [ie, dendritic cells (DC)] were also positively stained for IL-12p40 (Figure 1, C and D). Further, some epithelial cells were also positive for IL-12p40 expression (Figure 1E). Taken together, these findings show that large intestinal macrophages, dendritic cells, and epithelial cells are responsi-

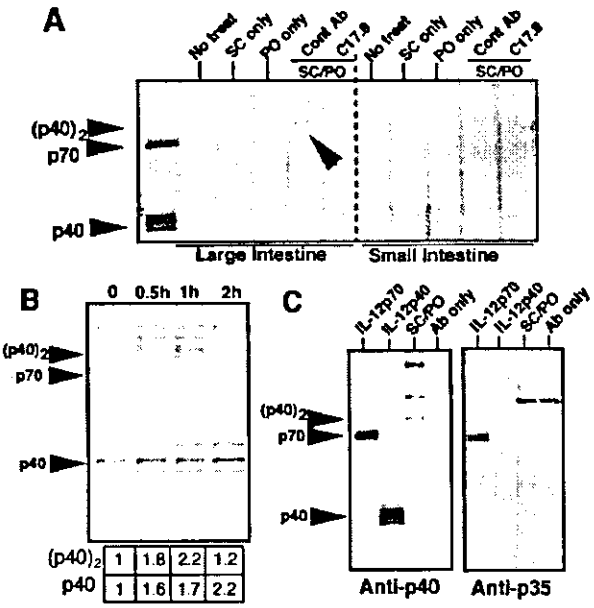

Figure 2. Induction of IL-12p40 homodimer in the large but not small intestine of diarrhea-induced mice. Large and small intestinal tissue extracts were subjected to immunoprecipitation and Western blotting analysis using anti-IL-12p40 (C17.8) mAb under non-reducing conditions (**A**). The captions above the figure indicate the experimental mouse group receiving different *in vivo* treatments. Thus, the samples were obtained from SC/PO mice treated with C17.8 or control antibodies. Further, the samples were isolated from mice treated with PO only, SC only, or non-treated mice. The **arrow** points to IL-12p40 homodimer expression in the large intestine of diarrhea-induced mice. The data represent four independent experiments. In **B**, at the indicated times after oral administration of OVA, large intestinal tissue extracts isolated from diarrhea-induced mice were assayed for IL-12p40 by the same method as in **A**. In **C**, the large intestinal tissue extracts of diarrhea-induced mice were subjected to Western blotting with anti-IL-12p35 Ab as well as anti-IL-12p40. IL-12p70 protein was used as a positive control for the IL-12p35 detection system. As negative control, immunoprecipitation was performed without the tissue specimens (Ab only). The data represent three different experiments.

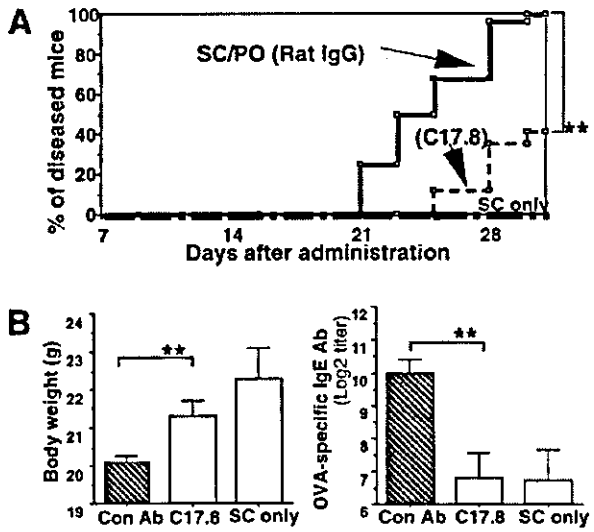

Figure 3. Inhibition of allergic diarrhea disease by the treatment with anti-IL-12p40 mAb. In **A**, anti-IL-12p40 mAb (C17.8) treatment (**thin dashed line**) delayed the development of allergic diarrhea when compared with the rat IgG-treated group (**solid line**). Statistical differences were determined by Wilcoxon rank-sum test and are indicated by **, $P < 0.01$. Mice with SC only were used as controls (**thick dashed line**). In **B**, **left**, body weight was recovered in allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). In **B**, **right**, OVA-specific IgE Abs were reduced in the serum of allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). The data are expressed as the mean of ± SE and are representative of five independent experiments. Statistical differences between anti-IL-12p40 mAb and control rat IgG-treated mice are indicated as **, $P < 0.01$.

ble for the production of IL-12p40 at the disease site of OVA-induced allergic diarrhea.

The Western blotting method was adopted for the examination of IL-12 p40 expression in the small and large intestinal tissue extracts from OVA-induced allergic diarrhea mice within 1 to 2 hours after the last oral challenge. In the large intestine of diarrhea-induced mice, the 80kD form of IL-12 predominated clearly demonstrating the presence of IL-12p40 homodimer but not 70kD IL-12 heterodimer, in contrast to the environment observed in the large intestine of control mice or the small intestine of mice with/without diarrhea (Figure 2A). The multiple bands of p40 and p80 are the result of glycosylation heterogenity.[15] We thus analyzed three bands of p40 and three bands of p80 as specific bands. In the case of spleen, IL-12p40 was detected in control, healthy mice. The levels of IL-12p40 did not change after development of allergic diarrhea (data not shown). To examine the kinetics of the response, we next assessed the time course of IL-12p40 expression in the large intestine of the diarrhea-induced mice. The expression of IL-12p40 or p80 in the large intestine peaked between 1 and 2 hours after the last oral challenge, at the same time that severe symptoms of OVA-induced allergic diarrhea were observed (Figure 2B). These data suggest that there is an intimate relationship between the development of diarrhea and the expression of IL-12p40 in the large intestine.

To further confirm the expression of IL-12p80 or p40 instead of the p70 form, the protein extracts from the large intestine of the diarrhea-induced mice were immunoprecipitated with anti-IL-12p40 mAb and then Western blotting was performed using anti-IL-12p35 mAb. No molecular bands corresponding to IL-12p70 proteins were detected in the large intestine of diarrhea-induced mice, while predominant IL-12p40 protein was detected (Figure 2C). The large molecular weight band above the p70 and p80 bands was non-specific and was caused by the nature of antibody used in the immunoprecipitation, since the large molecular weight band was also seen following immunoprecipitation in the absence of tissue specimens (Ab only in Figure 2C). These results indicate that the secretion of IL-12p40, but not IL-12p70, in the large intestine is critically important in the development of OVA-induced allergic diarrhea.

Anti-IL-12p40 Treatment Reduced the Symptoms of Allergic Diarrhea

Inasmuch as the preferential localization of IL-12p40 was observed in mice with allergic diarrhea, we next performed a neutralization experiment using anti-IL-12p40 mAb (C17.8). We observed a significant delay in the onset of diarrhea and reduced the frequency of diarrhea to 40% by treatment with anti-IL-12p40 mAb (Figure 3A). Obvious body weight loss was seen in control Ig-treated

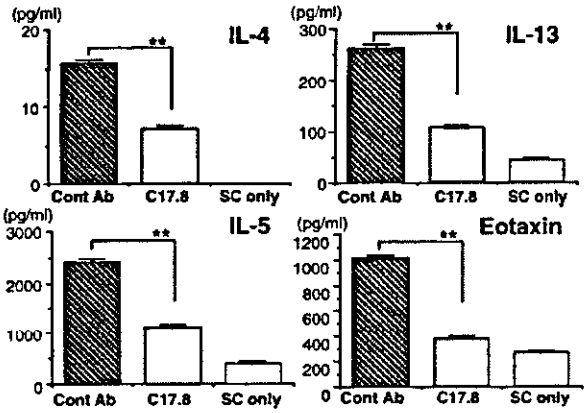

Figure 4. *In vivo* treatment with anti-IL-12p40 (C17.8) reduced the predominant antigen-specific Th2 type responses by large intestinal mononuclear cells isolated from diarrhea-induced mice. The mononuclear cells isolated from the large intestine (1.5×10^5 cells/well) were cultured with OVA (1 mg/ml) for 3 days. Culture supernatants were harvested and then assayed for IL-4, IL-13, IL-5, and eotaxin by ELISA assay. These data are expressed as the mean ± SE and are representative of three independent experiments. The statistical differences between anti-IL-12p40 mAb and control antibody treated mice are indicated as **, $P < 0.01$.

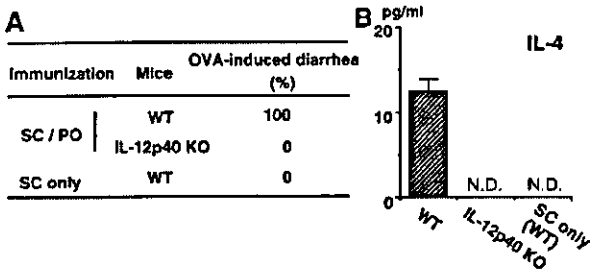

Figure 5. Suppression of allergic diarrhea development in IL-12p40 KO mice. In **A**, the incidence of allergic diarrhea was reduced in the IL-12p40 KO mice when compared with wild-type mice immunized subcutaneously and then given OVA repeatedly by the oral route (SC/PO). In **B**, the large intestinal LP mononuclear cells from IL-12p40 KO mice did not produce IL-4. Mononuclear cells isolated from the large intestine were restimulated with OVA for the assessment of IL-4 synthesis as described in Figure 4A. The data are expressed as the mean ± SE and represent three different experiments.

diarrhea mice, while treatment with anti-IL-12p40 mAb resulted in partial recovery from body weight loss (Figure 3B, left). In addition, high levels of OVA-specific IgE Abs were detected in the serum of diarrhea-induced mice treated with control Ab, whereas the mice treated with anti-IL-12p40 mAb showed low levels of OVA-specific IgE Abs (Figure 3B, right). These results indicate that treatment with anti-IL-12p40 mAb alters the environment from a disease-inducing one to one fastening recovery in OVA-induced allergic diarrhea.

Suppression of Intestinal Th2-Type Cytokine by Anti-IL-12p40 Treatment

To confirm decreased Th2-type responses in the large intestine after anti-IL-12p40 mAb treatment, we next examined antigen-induced cytokine production by the large intestinal mononuclear cells. Interestingly, the anti-IL-12p40 treatment resulted in decreased levels of OVA-induced Th2 cytokine synthesis including those of IL-4, IL-5, and IL-13 (Figure 4). Production levels of the Th2 cytokines were comparable to those of control mice without allergic diarrhea (SC only). In contrast to the alterations observed in OVA-induced Th2 cytokine synthesis, there was no difference in the level of IFN-γ production between the mice treated with anti-IL-12p40 mAb and control IgG (data not shown). We further confirmed that IL-4 producing cells were $CD4^+$ Th2 cells by intracellular staining (data not shown).

Finally, the level of eotaxin, a well-known chemokine for eosinophil recruitment in allergic disease,[30] was also examined, since our previous study demonstrated that the frequency of eosinophils was increased in the large intestine of allergic diarrhea mice.[1] Likewise, the level of eotaxin could also be presumed to be increased in the large intestine of allergic diarrhea mice (Figure 4). Interestingly however, the level of eotaxin synthesis was significantly decreased by the treatment with anti-IL-12p40 mAb (Figure 4). These results indicate that anti-IL-12p40 mAb inhibited the immunopathological Th2 cytokine environment of the large intestine in allergic diarrhea mice. Thus, an interesting scenario could be the presence of high levels of IL-12p40 monomer and/or homodimers instead of IL-12p70 in the disease site of OVA-induced allergic diarrhea mice. Therefore, treatment with anti-IL-12p40 mAb might result in the inhibition of Th2-type responses in the large intestine of allergic disease mice.

IL-12p40-Deficient Mice Do Not Develop Allergic Diarrhea

To directly confirm the pathological role of IL-12p40 in the development of allergic diarrhea, IL-12p40 knockout (KO) mice were used. IL-12p40 KO mice did not develop the allergic diarrhea completely (Figure 5A). OVA-induced IL-4 production by large intestinal LP mononuclear cells was not detected in IL-12p40 mice (Figure 5B). The levels of other Th2-type cytokines (IL-5 and IL-10) were also reduced in IL-12p40 KO mice (data not shown). Taken together, these results clearly show that IL-12p40 plays an important role in the development of this large intestinal allergic disease.

Discussion

Our present findings provide new, strong evidence for an immunopathological role for locally produced IL-12p40 in the development of OVA-induced allergic diarrhea. Here we demonstrate the high expression of IL-12p40, without IL-12p35, in the large intestine but not in the small intestine of mice with allergic diarrhea. These IL-12p40 were locally produced by large intestinal MØ, DC, and epithelial cells. Based on our knowledge, this is the first demonstration of the presence of IL-12p40 in the selected part of the intestinal tract (eg, large intestine) in mice with allergic diarrhea. Although IL-12 is thought to drive the Th1-dominant environment,[31] our present findings provide additional supportive evidence that IL-12p40 contributes to the generation of a Th2-dominant environ-

ment.[18,19] It should be noted that our results directly demonstrate the *in vivo* immunopathological contribution of locally produced mucosal IL-12p40 to the development of OVA-induced diarrhea. Thus, the anti-IL-12p40 treatment reduced the incidence of OVA-induced allergic diarrhea. An attractive explanation would be that large intestinal MØs and DCs as well as epithelial cells contribute to the development of pathological Th2-dominant responses by the production of IL-12p40 in OVA-induced allergic diarrhea. Thus, the administration of anti-IL-12p40 resulted in the inhibition of the locally produced, mucosal IL-12p40-created, pathological Th2 condition, leading to the reduction of disease development.

Our present and previous results clearly show that large intestinal antigen-specific Th cells produce high levels of Th2 cytokine in OVA-induced allergic diarrhea.[1] The presence of monomeric or dimeric forms of IL-12p40, behaving as an antagonist to IL-12p70, is an additional contributing factor for the creation of a dominant pathological Th2 environment. Thus, the severe symptoms of allergic diarrhea were reduced by treatment with anti-IL-12p40 mAb, since the production of Th2 cytokines was significantly decreased in the large intestine. Overall, IL-12p40-supported, Th2-type cytokine synthesis plays a critical and pathological role in the induction of allergic reactions in large intestinal tissues. Although we do not have any specific explanation for the generation of IL-12p40 at the disease site, one possibility could be antigen overload in the intestinal tract. Our previous study demonstrated that oral administration of high doses of OVA induced Th2-mediated allergic diarrhea in systematically pre-sensitized BALB/c mice.[1] In contrast, low doses of oral OVA failed to induce allergic diarrhea. It was also shown that high doses of OVA peptide increased the numbers of naive $CD4^+$ T cells with Th2-like phenotype, which in turn produced dramatically large amounts of IL-4.[32] Therefore, high doses of oral antigen may create an immunological environment favoring Th2 cell development. To support this view, it has also been shown that high doses of oral antigen preferentially inhibit IFN-γ-producing Th1-type cells.[1] Further, the dose of antigen can determine whether Th1- or Th2-type cells are generated by antigen-presenting cells including DC.[33] Taken together, these findings allow us to postulate that an overload of oral antigen may direct mucosal antigen-presenting cells, including DC and MØ, and epithelial cells, to produce monomeric or dimeric forms of IL-12p40 instead of IL-12p70.

IL-12 has been considered as an inhibitory factor for allergic responses induced by preferential Th2-cytokine production. Indeed, endogenous rIL-12 decreased IgE levels and Th2 cytokine production induced by allergic reaction.[34] In contrast, IL-12 has also been shown to be involved in the pathological phase of mucosa-associated allergic diseases of the respiratory tract. In the murine asthma model, IL-12 contributed to the recruitment of eosinophils into the respiratory tract via the induction of VCAM-1 on local vascular epithelial cells.[35] Thus, the deletion of the IL-12 gene (p40) resulted in a substantial reduction in the airway recruitment of eosinophils and in the expression of VCAM-1 when compared with wild-type mice exhibiting an asthma-like reaction induced by systemic sensitization followed by nasal OVA.[36] In addition, selective overexpression of IL-12p40 was noted in airway epithelial cells and bronchoalveolar lavage fluids of patients with asthma.[20] Our present findings also demonstrate that the locally produced p40 form of IL-12 was associated with the development of OVA-induced allergic diarrhea. Thus, IL-12p40 was preferentially expressed only in the large intestine of allergic diarrhea mice. In addition to these results generated through the characterization of an asthma model, our present finding suggests a critical role for IL-12, especially that of p40-associated molecules, for the development of allergic diseases including asthma and food allergy.

The treatment with anti-IL-12p40 mAbs effectively reduced the incidence as well as the severity of allergic diarrhea, an effect most likely due to an alteration in the dominant immunopathological Th2-type response to a Th1-type environment. To support this view, locally overexpressed IL-12p40 may compete with the well-known Th1 promoter IL-12p70 and IL-23 (p40/p19).[37–39] To this end, it has been shown that endogenous IL-12p40 can overcome the Th1-promoting activity of IL-12p70 and/or IL-23.[18] In this regard, our recent and separate study showed that IL-23p19-specific mRNA expression was not detected in either diseased or healthy BALB/c mouse groups (data not shown). The results suggest that IL-23 dose not play an important role in the development of our diarrhea model. Therefore, treatment with anti-IL-12p40 antibody likely eliminated the antagonistic effect of IL-12p40 at the local site, perhaps leading to the creation of an IL-12p70 environment for the initiation of down-regulation of Th2 responses. An alternative explanation would be that anti-IL-12p40 mAb used in this experiment may possess a higher affinity for the monomeric or dimeric form of IL-12 than for the IL-12p70 heterodimer. Although our emphasis has been on the inhibitory effects of anti-IL-12p40 mAb for the prevention of allergic diarrhea, one must accept the fact that complete prevention of disease development was never achieved through use of mAbs. A possible explanation for this finding could be that anti-IL-12p40 mAb inhibited Th1 induction of IL-12p70 in addition to IL-12p40. Thus, this alteration of a Th2 dominant environment and shift to one of a Th1-type may partially occur in the large intestine of mice with allergic diarrhea. To support this possibility, the mAb used in these experiments has been shown to neutralize IL-12p70 in addition to IL-12p40.[13,14] In addition, the experiments using IL-12p40 KO mice suggest that the absence of IL-12p40 results in a complete failure to develop allergic diarrhea. It clearly shows that IL-12p40 play a critical role in the development of this disease. However, one alternative and simple expectation would be that an IL-12p40 deficiency may lead to the creation of Th2 environment due to the lack of Th1 inducing IL-12p70. Thus, it may lead to the more susceptible condition for the development of Th2-mediated diarrhea. Although we do not have any specific data to negate the latter possibility, one possible explanation would be that the deficiency of IL-12p70 formation in IL-12p40 KO mice lead to the lack of ability to active antigen presenting cells. IL-12p70 deficiency may

result in the absence of induction antigen-specific T cell response including the pathological Th2-type cells. It has been shown that IL-12 or IL-12-induced IFN-γ can directly activate antigen presenting cells.[40] To address the issue, a series of interesting experiment would be the adaptive transfer of large intestinal MØ, DC, and epithelial cells into IL-12p40 and/or p35 KO mice. These experiments are, of course, planned for our future study.

Recently, it has been suggested that IL-12 is also one of the key cytokines for the regulation of the intestinal immune response.[41] Mouse IL-12p40 is produced as monomer and homodimer five to ninety times as frequently as IL-12p70 *in vivo* and *in vitro*,[40,42] implying the existence of additional immunological roles for IL-12p40. An interesting possibility would be that excess production of the monomeric and/or the homodimeric form of IL-12p40 could be a key contributing factor to the maintenance of immunological homeostasis at the mucosal compartment. Interestingly, our present findings demonstrate that over-expression of IL-12p40 occurred only in the large but not the small intestine following oral exposure to high doses of protein antigen. At the present time, we cannot offer any specific explanation for this distinct localization of IL-12p40. However, an interesting possibility would be that the expression of negative regulators for IL-12, including sCD40L and IL-10R,[14,43] could differ between the small and large intestine. To support this possibility, epithelial cells have been shown to express CD40 and IL-10R.[34,44] Since the large intestinal tract is continuously exposed to overloaded microflora, the level of co-stimulatory molecule expression such as CD40 by large intestinal epithelial cells could be lower to avoid unnecessary inflammatory responses. Thus, the large intestinal tract may form an immunological environment favoring the generation of IL-12p40. This interesting possibility is currently being tested in our laboratory.

In summary, our results demonstrated that locally produced IL-12p40 contribute to the Th2 cell generation of pathological polarization in the large intestine of OVA-induced allergic diarrhea. This study provides the first evidence for the association of over-expressed IL-12p40 from intestinal epithelial cells, DC and MØ, in the development of allergic diarrhea. Thus, the application of anti-IL-12p40 mAb resulted in the reduction of disease incidence and severity. Further, the disease development was completely eliminated in the deletion of IL-12p40 gene. Taken together, our studies provide an opportunity to consider that anti-IL-12p40 mAbs may be an alternative therapeutical regimen for the control of allergic intestinal disease.

Acknowledgments

We thank members of the Mucosal Immunology Group at Osaka University, The University of Tokyo and The University of Alabama at Birmingham, Immunobiology Vaccine Center for their helpful comments, Dr. Kimberly K. McGhee for editorial help, and Ms. Kelly Stinson and Ms. Sheila Turner for their help in the preparation of this manuscript.

References

1. Kweon MN, Yamamoto M, Kajiki M, Takahashi I, Kiyono H: Systemically derived large intestinal CD4+ Th2 cells play a central role in STAT6-mediated allergic diarrhea. J Clin Invest 2000, 106:199–206
2. Hogan SP, Mishra A, Brandt EB, Royalty MP, Pope SM, Zimmermann N, Foster PS, Rothenberg ME: A pathological function for eotaxin and eosinophils in eosinophilic gastrointestinal inflammation. Nat Immunol 2001, 2:353–360
3. Mishra A, Hogan SP, Brandt EB, Rothenberg ME: Peyer's patch eosinophils: identification, characterization, and regulation by mucosal allergen exposure, interleukin-5, and eotaxin. Blood 2000, 96: 1538–1544
4. Sampath D, Castro M, Look DC, Holtzman MJ: Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma. J Clin Invest 1999, 103:1353–1361
5. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM: The inverse association between tuberculin responses and atopic disorder. Science 1997, 275:77–79
6. Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A: A critical role for NF-κ B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol 2001, 2:45–50
7. Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH: Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. Science 2002, 295:336–338
8. Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS: Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. J Clin Invest 1999, 103:1103–1111
9. Korsgren M, Persson CG, Sundler F, Bjerke T, Hansson T, Chambers BJ, Hong S, Van Kaer L, Ljunggren HG, Korsgren O: Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. J Exp Med 1999, 189:553–562
10. Rodriguez-Sosa M, Satoskar AR, Calderon R, Gomez-Garcia L, Saavedra R, Bojalil R, Terrazas LI: Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low interleukin-12 production and Th2-biasing ability. Infect Immun 2002, 70:3656–3664
11. Jankovic D, Liu Z, Gause WC: Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. Trends Immunol 2001, 22:450–457
12. Yoshimoto T, Kojima K, Funakoshi T, Endo Y, Fujita T, Nariuchi H: Molecular cloning and characterization of murine IL-12 genes. J Immunol 1996, 156:1082–1088
13. Hino A, Nariuchi H: Negative feedback mechanism suppresses interleukin-12 production by antigen-presenting cells interacting with T helper 2 cells. Eur J Immunol 1996, 26:623–628
14. Hino A, Igarashi O, Tagawa YI, Iwakura Y, Nariuchi H: Interferon-γ priming is not critical for IL-12 production of murine spleen cells. Cytokine 2000, 12:12–20
15. Heinzel FP, Hujer AM, Ahmed FN, Rerko RM: In vivo production and function of IL-12 p40 homodimers. J Immunol 1997, 158:4381–4388
16. Wang X, Wilkinson VL, Podlaski FJ, Wu C, Stern AS, Presky DH, Magram J: Characterization of mouse interleukin-12 p40 homodimer binding to the interleukin-12 receptor subunits. Eur J Immunol 1999, 29:2007–2013
17. Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, Su C, Pan YC, Hakimi J: Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. J Immunol 1995, 154:116–127
18. Piccotti JR, Chan SY, Li K, Eichwald EJ, Bishop DK: Differential effects of IL-12 receptor blockade with IL-12 p40 homodimer on the induction of CD4+ and CD8+ IFN-γ-producing cells. J Immunol 1997, 158:643–648
19. Yoshimoto T, Wang CR, Yoneto T, Waki S, Sunaga S, Komagata Y, Mitsuyama M, Miyazaki J, Nariuchi H: Reduced T helper 1 responses in IL-12 p40 transgenic mice. J Immunol 1998, 160:588–594
20. Walter MJ, Kajiwara N, Karanja P, Castro M, Holtzman MJ: Interleukin 12 p40 production by barrier epithelial cells during airway inflammation. J Exp Med 2001, 193:339–351
21. Hakonarson H, Maskeri N, Carter C, Grunstein MM: Regulation of TH1- and TH2-type cytokine expression and action in atopic asth-

matic sensitized airway smooth muscle. J Clin Invest 1999, 103:1077–1087
22. Wysocka M, Kubin M, Vieira LQ, Ozmen L, Garotta G, Scott P, Trinchieri G: Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. Eur J Immunol 1995, 25:672–676
23. Fujihira K, Nagata M, Moriyama H, Yasuda H, Arisawa K, Nakayama M, Maeda S, Kasuga M, Okumura K, Yagita H, Yokono K: Suppression and acceleration of autoimmune diabetes by neutralization of endogenous interleukin-12 in NOD mice. Diabetes 2000, 49:1998–2006
24. Fujihashi K, McGhee JR, Kweon MN, Cooper MD, Tonegawa S, Takahashi I, Hiroi T, Mestecky J, Kiyono H: γ/δ T cell-deficient mice have impaired mucosal immunoglobulin A responses. J Exp Med 1996, 183:1929–1935
25. Kweon MN, Fujihashi K, VanCott JL, Higuchi K, Yamamoto M, McGhee JR, Kiyono H: Lack of orally induced systemic unresponsiveness in IFN-γ knockout mice. J Immunol 1998, 160:1687–1693
26. Li L, Sad S, Kagi D, Mosmann TR: CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. J Immunol 1997, 158:4152–4161
27. Stordeur P, Zhou L, Byl B, Brohet F, Burny W, de Groote D, van der Poll T, Goldman M: Immune monitoring in whole blood using real-time PCR. J Immunol Methods 2003, 276:69–77
28. Kinoshita N, Hiroi T, Ohta N, Fukuyama S, Park EJ, Kiyono H: Autocrine IL-15 mediates intestinal epithelial cell death via the activation of neighboring intraepithelial NK cells. J Immunol 2002, 169:6187–6192
29. Romijn HJ, van Uum JF, Breedijk I, Emmering J, Radu I, Pool CW: Double immunolabeling of neuropeptides in the human hypothalamus as analyzed by confocal laser scanning fluorescence microscopy. J Histochem Cytochem 1999, 47:229–236
30. Cuvelier SL, Patel KD: Shear-dependent eosinophil transmigration on interleukin 4-stimulated endothelial cells: a role for endothelium-associated eotaxin-3. J Exp Med 2001, 194:1699–1709
31. Trinchieri G: Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994, 84:4008–4027
32. Hosken NA, Shibuya K, Heath AW, Murphy KM, O'Garra A: The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-α β-transgenic model. J Exp Med 1995, 182:1579–1584
33. Ruedl C, Bachmman FB, Kopf M: The antigen dose determined T helper subset development by regulation of CD40 ligand. Eur J Immunol 2000, 30:2056–2064
34. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M: Interleukin 12 inhibits antigen-induced airway hyper-responsiveness, inflammation, and Th2 cytokine expression in mice. J Exp Med 1995, 182:1527–1536
35. Kaminuma O, Fujimura H, Fushimi K, Nakata A, Sakai A, Chishima S, Ogawa K, Kikuchi M, Kikkawa H, Akiyama K, Mori A: Dynamics of antigen-specific helper T cells at the initiation of airway eosinophilic inflammation. Eur J Immunol 2001, 31:2669–2679
36. Wang S, Fan Y, Han X, Yang J, Bilenki L, Yang X: IL-12-dependent vascular cell adhesion molecule-1 expression contributes to airway eosinophilic inflammation in a mouse model of asthma-like reaction. J Immunol 2001, 166:2741–2749
37. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA: Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 2000, 13:715–725
38. Camoglio L, Juffermans NP, Peppelenbosch M, te Velde AA, ten Kate FJ, van Deventer SJ, Kopf M: Contrasting roles of IL-12p40 and IL-12p35 in the development of hapten-induced colitis. Eur J Immunol 2002, 32:261–269
39. Belladonna ML, Renauld JC, Bianchi R, Vacca C, Fallarino F, Orabona C, Fioretti MC, Grohmann U, Puccetti P: IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. J Immunol 2002, 168:5448–5454
40. Trinchieri G: Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995, 13:251–276
41. MacDonald TT, Monteleone G: IL-12 and Th1 immune responses in human Peyer's patches. Trends Immunol 2001, 22:244–247
42. D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf SF, Trinchieri G: Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med 1992, 176:1387–1398
43. Wittmann M, Kienlin P, Mommert S, Kapp A, Werfel T: Suppression of IL-12 production by soluble CD40 ligand: evidence for involvement of the p44/42 mitogen-activated protein kinase pathway. J Immunol 2002, 168:3793–3800
44. Young LS, Eliopoulos AG, Gallagher NJ, Dawson CW: CD40 and epithelial cells: across the great divide. Immunol Today 1998, 19:502–506