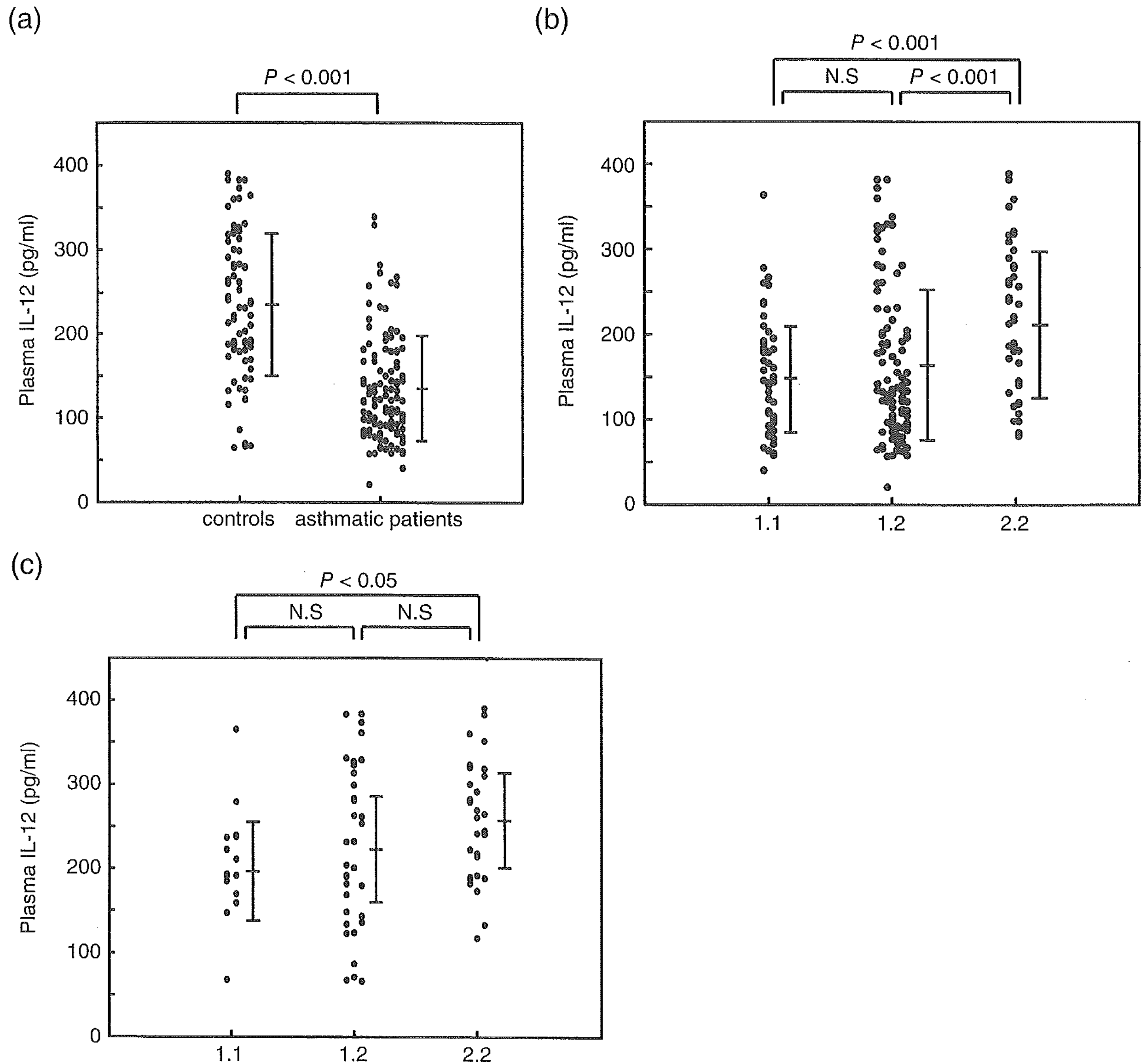

Fig. 2 (**a**) Plasma IL-12 concentrations in asthmatic patients and controls. The plasma IL-12 concentrations in the asthmatic patients were significantly lower than those in the controls (P<0.001). (**b**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in both the controls and asthmatic patients (n=189). The plasma IL-12 concentrations in the subjects with a 1.1 genotype were significantly lower than those in the subjects with a 2.2 genotype (P<0.001). (**c**) Relationship between *IL-12B* promoter polymorphism and plasma IL-12 concentrations in the controls (n=78). The plasma IL-12 concentrations in the controls with a 1.1 genotype were significantly lower than those in the controls with a 2.2 genotype (P<0.05).

IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS IN THE CONTROLS AND ASTHMATIC PATIENTS

Since most asthmatic patients had positive CAP-RAST scores for D farinae (Derf1), their PBMCs were cultured with the specific antigen Derf1 for 24 hours. IL-12 production by Derf1-stimulated PBMCs in the asthmatic patients (378.0 ± 271.4 pg/mL) was significantly lower than that in the controls (663.0 ± 364.2 pg/mL) (P < 0.001 ; Fig. 3a).

ASSOCIATION BETWEEN *IL-12B* PROMOTER POLYMORPHISM AND IL-12 PRODUCTION BY DERF1-STIMULATED PBMCS

We examined IL-12 production by Derf1-stimulated PBMCs in each genotype in both the controls and asthmatic patients. The subjects with a 1.1 genotype (347.2 ± 229.2 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (690.2 ± 331.1 pg/mL) (P < 0.001 ; Fig. 3b). In the controls, the subjects with a 1.1 genotype

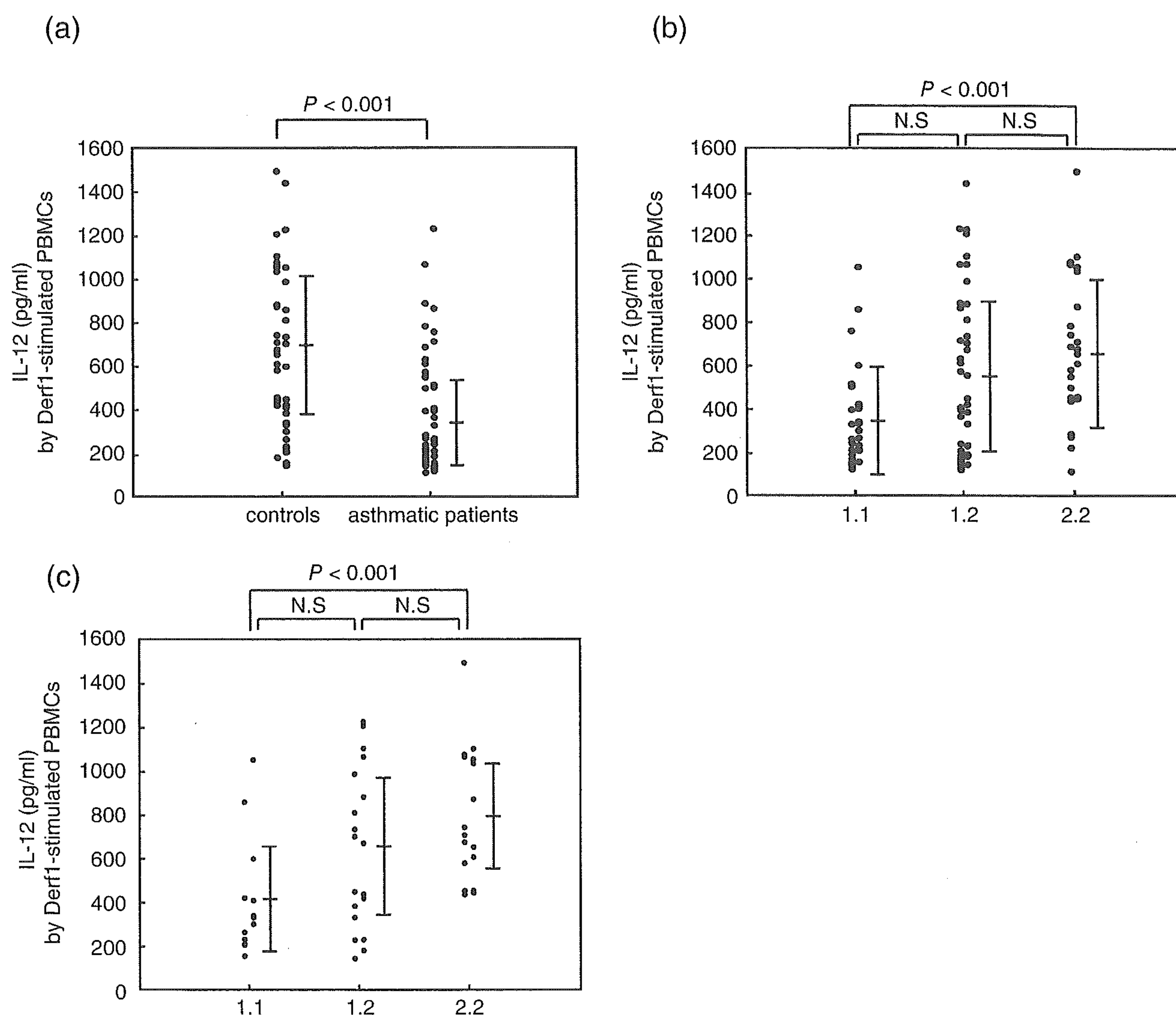

Fig. 3 (**a**) IL-12 production by Derf1-stimulated PBMCs in asthmatic patients and controls. The IL-12 production in the asthmatic patients was significantly lower than that in the controls ($P < 0.001$). (**b**) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in both the controls and asthmatic patients (n = 189). The IL-12 production in the subjects with a 1.1 genotype was significantly lower than that in the subjects with a 2.2 genotype ($P < 0.001$). (**c**) Relationship between *IL-12B* promoter polymorphism and IL-12 production by Derf1-stimulated PBMCs in the controls (n = 78). The IL-12 production in the controls with a 1.1 genotype was significantly lower than that in the controls with a 2.2 genotype ($P < 0.001$).

(412.2 ± 315.0 pg/mL) had a lower IL-12 production by Derf1-stimulated PBMCs than those with a 2.2 genotype (807.2 ± 292.2 pg/mL) ($P < 0.001$; Fig. 3c). IL-12 production by Derf1-stimulated PBMCs positively correlated with IFN-γ production by IL-12-stimulated PBMCs (data not shown). Therefore, the subjects with a 1.1 genotype showed lower IL-12 and IFN-γ productions than those with a 2.2 genotype.

EFFECTS OF PROMOTER POLYMORPHISM ON PROMOTER ACTIVITY

To examine the functional activity of promoter polymorphism, we cloned the −2808/−2303 region of *IL-12B* into the PGV-P2 firefly luciferase reporter plasmid. The activity of the *IL-12B* promoter-reporter constructs was assessed in a transient transfection assay using RAW264 cells. As shown in Figure 4, the luciferase activity of the PGV-P2 plasmid with the *IL-12B* promoter-reporter constructs was significantly higher than that of the PGV-P2 plasmid only. A significantly lower luciferase activity was observed for haplotype 1 construct than for haplotype 2 construct (32.8% decrease ; P = 0.0083). Furthermore, we constructed mutant vectors with homozygotes for mut-1 (the CTCTAA/C allele) and mut-2 (the GC/T allele). The luciferase activity of the construct with mut-2 was

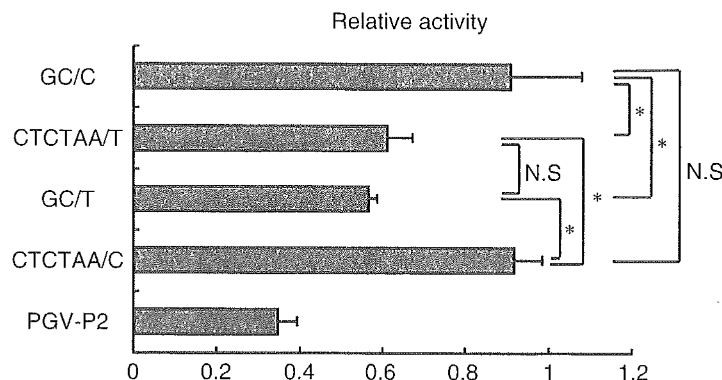

Fig. 4 Effects of *IL-12B* promoter polymorphism on promoter activity. RAW 264 cells were transfected by lipofectamine with reporter-constructs and stimulated with IFN-γ and LPS. To examine whether NP1 or NP2 affect the *IL-12B* transcriptional activity, mutated vectors (mut-1 and mut-2) were constructed. Luciferase activity was measured using the Dual-Luciferase reporter assay system. The presented results are mean ± SD from three independent experiments. * $P < 0.05$.

significantly lower than that of the construct with mut-1 and haplotype 2. This result indicates that *IL-12B* promoter polymorphism, that is, not the CTCTAA/GC allele but the T/C allele, has a major influence on the basal transcription rate of *IL-12B*.

DISCUSSION

IL-12 is a key mediator of immune responses. IL-12 is a heterodimeric molecule composed of two disulfide-linked subunits, a 35-kd subunit encoded by *IL-12A* on chromosome 3p12-q13.2 and a 40-kd subunit encoded by *IL-12B* on chromosome 5q31-q33.[18] A previous study has reported the association between *IL-12B* and asthma as determined by a genome-wide search.[7] We sequenced exons 1-8 and region 3 kb upstream from the transcriptional start site of *IL-12B*, and found three novel polymorphisms. In nineteen polymorphisms, NP1 + 2 that existed in the promoter region was shown to have a significant difference between the asthmatic patients and the controls in the genome frequency analysis. The frequencies of *IL-12B* polymorphisms in the coding region (NP3 and NP19) were not significantly different between the controls and asthmatic patients (data not shown). This result is similar to the result of Noguchi *et al.*[12]

Furthermore, the subjects with a 1.1 genotype had significantly lower plasma IL-12 concentrations and the lower IL-12 production by Derf 1-stimulated PBMCs than those with a 2.2 genotype. IL-12 plays an important role in the inhibition of Th2 cytokine production and the promotion of IFN-γ production by binding to plasma membrane receptors on activated T cells or NK cells.[2] IFN-γ inhibits IgE synthesis by human PBMCs *in vitro*.[19-22] Reduced IL-12 production and IL-12-dependent IFN-γ concentrations have been reported in asthmatic patients.[23-25] Therefore, IL-12 was shown to be associated with atopic dermatitis and asthma.[26-28] According to our result, *IL-12B* promoter polymorphism is likely to be associated with asthma prevalence by reducing IL-12 production.

Since the IL-12 levels measured during an exacerbation of asthma or bacterial/viral infection are analyzed in relation to the polymorphism, the functional consequences of the polymorphism cannot be fully explored. Hence, we investigated the functional activity of *IL-12B* promoter polymorphism. The transcriptional activity of the construct with a 1.1 genotype was lower than that of the construct with a 2.2 genotype. The transcriptional activity with mut-2 was significantly lower than that with mut-1 and haplotype 2. These results indicate that *IL-12B* promoter polymorphism, not NP1 but NP2, reduces the *IL-12B* transcriptional activity and IL-12 production. Since the NF-IL6 binding site in the *IL-12B* promoter region is shown to be in the −2405 to −2397 area of the 5'-upstream region, the T/C allele at this point may affect *IL-12B* transcriptional activity.[29] The transcriptional activity of the 296-bp construct from the transcriptional start site was reduced to half compared with that of the 3.3-kb construct.[17] This result shows that there are significant binding sites influencing the transcriptional activity from −296 to −3.3 kb of the transcriptional start site. The sequence near the T/C allele may be one of the binding sites affecting *IL-12B* transcriptional activity.

Morahan *et al.*[13] reported the association between asthma severity and a 4-bp microinsertion, which exists at a region 3 kb upstream from the transcriptional

start site of *IL-12B*. Since we identified only NP1 around the region 3 kb upstream from the transcriptional start site, the 4-bp microinsertion is likely to be NP1. According to their report, heterozygosity for *IL-12 B* promoter polymorphism is associated with asthma severity, reduced *IL-12B* transcription level and decreased IL-12 secretion. Inconsistent results may have occurred due to differences in methods and population. In another study, Morahan *et al.* also reported that a 1.1 genotype is associated with mortality from cerebral malaria and with reduced production of nitric oxide in Tanzanian children, and that the *IL-12B* mRNA expression in the subjects with a 1.1 genotype is lower than that in the subjects with a 2.2 genotype.[30] These results are consistent with our results.

Khoo *et al.*[14] have recently shown that *IL-12B* promoter polymorphism is not associated with asthma prevalence, but that there is an association was between 1.1 genotype and elevated serum IgE levels in male subjects, and reduced pulmonary function in female subjects in childhood. In our data, the subjects with a 1.1 genotype had high IgE levels, however, there was no correlation with sex (data not shown). The subjects with a 1.1 genotype had reduced IL-12 production and *IL-12B* transcriptional activity compared with those with a 2.2 genotype. Therefore, homozygosity for haplotype 1 may elevate serum IgE by reducing IL-12 production.

In conclusion, the frequency of homozygosity for haplotype 1 in asthmatic patients was significantly higher than that in controls. The subjects with a 1.1 genotype had reduced plasma IL-12 concentrations and IL-12 production by Derf1-stimulated PBMCs compared with those with a 2.2 genotype. The *IL-12B* transcriptional activity was reduced by the $^{-2403}$T allele, not by the $^{-2703}$CTCTAA allele. Hence, *IL-12B* promoter polymorphism ($^{-2403}$T/C) can be a risk factor for the development of asthma.

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A novel single-nucleotide substitution, Glu 4 Lys, in the leukotriene C4 synthase gene associated with allergic diseases

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Abstract. Cysteinyl leukotrienes (cysLTs) play important roles in bronchial asthma, and can mediate bronchial smooth muscle constriction and increase mucous secretion, vascular permeability and cellular infiltration. We identified a novel heterozygous single-nucleotide substitution 10G>A (Glu 4 Lys) in the first exon of the leukotriene C4 synthase gene (*LTC4S*). This substitution was detected in 5 of 141 allergic patients, but not in 110 nonallergic subjects. There was a difference in the Glu 4 Lys frequency between the allergic patients and nonallergic subjects (Fisher's exact test, p=0.0460). The five patients with Glu 4 Lys had allergic diseases such as bronchial asthma and/or allergic dermatitis. Furthermore, a familial analysis of Glu 4 Lys revealed a link with allergic diseases. Thus, our results suggest that Glu 4 Lys in the *LTC4S* might be associated with allergic diseases.

Introduction

Susceptibility to allergic diseases, such as bronchial asthma (BA), atopic dermatitis (AD) and allergic rhinitis (AR), is determined by the interaction between an unknown number of genetic and environmental factors (1). Single-nucleotide substitutions in several genes have been reported to be associated with allergic diseases (2-5).

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Abbreviations: BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; *LTC4S*, leukotriene C4 synthase gene; LTE4, leukotriene E4; 11DTXB2, 11-dehydro-thromboxane B2

Key words: allergic disease, bronchial asthma, cysteinyl leukotriene, leukotriene C4 synthase gene, single-nucleotide substitution, morbidity

The locus of *LTC4S* is on chromosome 5q35(6) and has been associated with allergic diseases on the basis of a genome-wide search (7). Cysteinyl leukotrienes (cysLTs) play important roles in BA and can mediate bronchial smooth muscle constriction and increase mucous secretion, vascular permeability and cellular infiltration (8,9). LTC4 synthase converts LTA4 to LTC4 by conjugation to reduced glutathione. A single-nucleotide promoter polymorphis (A-444C) in *LTC4S* has been associated with aspirin-sensitive asthma in Polish patients (3), although recent studies found no association between this promoter polymorphis and aspirin-sensitive asthma (4). In a small study of 23 severe asthmatics taking oral zafirlukast for 2 weeks, there was a trend (p=0.1) for those who had 1 or 2 copies of the -444C allele to show improvements in lung function (5). We hypothesized that *LTC4S* is one of the candidate genes for BA. Here, we report that a novel single-nucleotide substitution 10G>A (Glu 4 Lys) in *LTC4S* is associated with BA.

Materials and methods

Patients and control subjects. One hundred and forty-one allergic patients (89 males and 52 females; mean age ± 1 SD, 8.5±7.9 years old; 130 BA, 3 AD, 1 AR, 3 BA and AD, and 4 BA and AR) and 110 nonallergic subjects (60 males and 50 females; mean age ± 1 SD, 23.4±11.1 years old) were studied. The allergic patients were selected on the basis of having major allergic diseases such as BA, AD and/or AR. BA was diagnosed according to the criteria of the American Thoracic Society, AD according to the criteria of Hanifin (10) and AR according to the criteria of Skoner (11,12). The nonallergic subjects were healthy and did not have a history of allergic diseases. All of the subjects were randomly selected from patients attending our hospitals. Informed consent was obtained from all subjects or their parents.

Detection of single-nucleotide substitution in the LTC4S. Neutrophils obtained from heparinized blood were stored at -30°C. Genomic DNA was extracted from the neutrophils with a Sepagene kit (Sanko Junyaku, Tokyo, Japan).

For 15 allergic patients, the five coding regions of *LTC4S* (GenBank accession no. U50136) DNA were amplified and

Table I. Sequence of oligonucleotides for PCR.

Primer			Sequence	Position	Size of the amplified fragment (bp)	Annealing temp. (°C)
LTC4S	1	Forward	5'-AGGCTGCTCTTCCTCTCCTG-3'	-96-58	154	62
		Reverse	5'-CTTCCTAGATAGGAACCAGC-3'			
	2	Forward	5'-CCTTAGGGAGAGGACACG-3'	1504-1603	100	62
		Reverse	5'-CTCACTGGGCTCGGTAGACG-3'			
	3	Forward	5'-CACCGCAGGGTGAACTGCAG-3'	1706-1776	71	50
		Reverse	5'-ACCTTCATGAAAGAAGATGCCG-3'			
	4	Forward	5'-TGCGTACCTCTCGCAGGCGC-3'	1861-1942	82	56
		Reverse	5'-CTCACCTGAGCTGCGCGGAGCG-3'			
	5	Forward	5'-GCAGGCTGGCACCGCTGTAC-3'	2173-2429	257	53
		Reverse	5'-GGTCTCGGTCACTAGAAC-3'			

Table II. Distribution of genotype of Glu 4 Lys in *LTC4S*.

Genotype	Nonallergic subjects (n=110)	Allergic patients (n=141)	Total (n=251)	p-value
GAG (Glu)/GAG (Glu)	110 (100%)	136 (96.5%)	246 (98%)	p=0.046[a]
GAG (Glu)/AAG (Lys)	0 (0%)	5 (3.5%)	5 (2.0%)	
AAG (Lys)/AAG (Lys)	0 (0%)	0 (0%)	0 (0%)	

[a]Statistical analyses were performed using Fisher's exact test.

Table III. The clinical features of allergic patients with Glu 4 Lys in *LTC4S*.

Patient	Sex	Age	Allergic diseases	Serum IgE levels (IU/ml)	LTE4 (pg/mg creatinine)	11DTXB2 (pg/mg creatinine)
1	F	15	BA	889.8	ND	ND
2	F	8	BA, AD	755.5	411.0	2049.9
3	F	7	BA	1300.0	259.0	1837.0
4	M	5	BA	171.7	256.0	1532.3
5	F	9	BA	1900.0	215.0	1226.0
Nonallergic subjects[a]						
Mean				19.5	233.0	252.0
Range				6.8-56.2	103.0-389.0	41.0-716.0

[a]They were quoted from ref. 13.

sequenced using the PCR technique and an ABI 3100 DNA sequencer (Applied Biosystems, CA, USA). The conditions for PCR and the primers used are shown in Table I. To detect the Glu 4 Lys substitution, PCR products of the 126 allergic patients and 110 controls were electrophoresed on a 12% polyacrylamide gel following *Mnl 1* (New England BioLabs, MA, USA) digestion at 37°C for 1 h.

Measurement of LTE4 and 11DTXB2. Urine samples were collected from patients with the Glu 4 Lys substitution, 50 randomly selected allergic patients and 7 randomly selected controls. They were stored at -80°C and analyzed within a month of collection.

Urinary leukotriene E4 (LTE4) was measured using the leukotriene C4/D4/E4 enzyme-immunoassay system (Amersham, Buckinghamshire, UK) after purification by high-performance liquid chromatography (HPLC; 486 Tunable Absorbance Detector, Waters, MA, USA) using a C18 reverse-phase column (Cappcell PAC UG 120; Shiseido, Tokyo, Japan). Urinary LTE4 level was expressed as pg/mg creatinine.

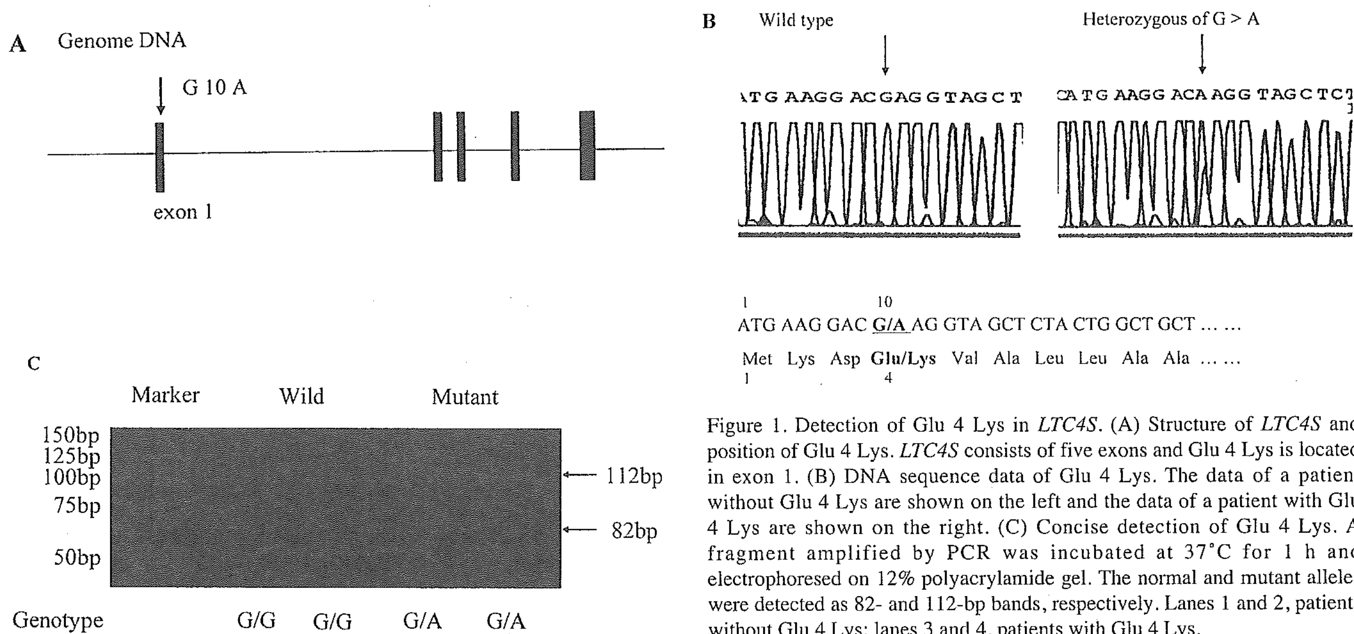

Figure 1. Detection of Glu 4 Lys in *LTC4S*. (A) Structure of *LTC4S* and position of Glu 4 Lys. *LTC4S* consists of five exons and Glu 4 Lys is located in exon 1. (B) DNA sequence data of Glu 4 Lys. The data of a patient without Glu 4 Lys are shown on the left and the data of a patient with Glu 4 Lys are shown on the right. (C) Concise detection of Glu 4 Lys. A fragment amplified by PCR was incubated at 37˚C for 1 h and electrophoresed on 12% polyacrylamide gel. The normal and mutant alleles were detected as 82- and 112-bp bands, respectively. Lanes 1 and 2, patients without Glu 4 Lys; lanes 3 and 4, patients with Glu 4 Lys.

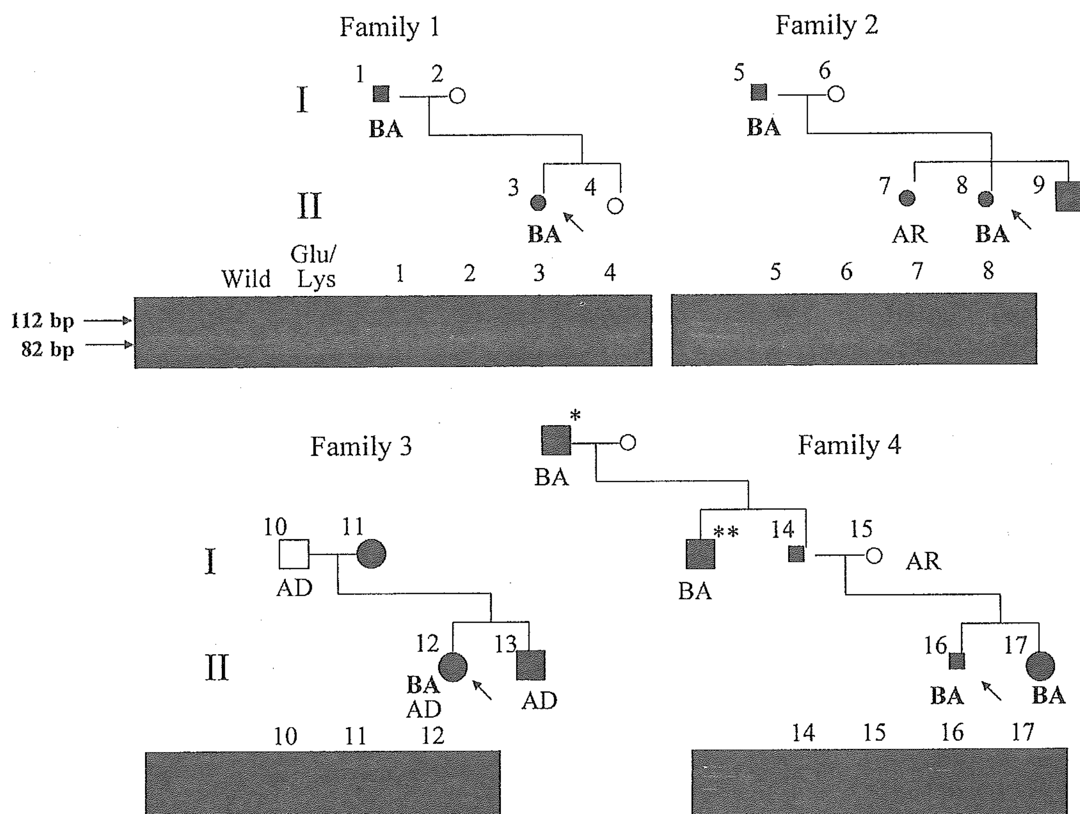

Figure 2. Genetic analysis for Glu 4 Lys in four atopic families. (Family 1) II-3 was the proband with the heterozygous Glu 4 Lys and BA. The father (I-1) had the heterozygous Glu 4 Lys and BA. (Family 2) II-8 was the proband with the heterozygous Glu 4 Lys and BA. The father (I-5) had the heterozygous Glu 4 Lys and BA. The elder sister (II-7) also had the heterozygous Glu 4 Lys and was suffering from AR. We could not get informed consent from the brother (II-9). (Family 3) II-12 was the proband with Glu 4 Lys and BA and AD. The mother (I-11) had the heterozygous Glu 4 Lys without any allergic history. We could not get informed consent from her brother (II-13). (Family 4) II-16 was the proband with Glu 4 Lys and BA. The father (I-14) had the heterozygous Glu 4 Lys without any allergic symptoms. His grandfather (*) and uncle (**) had a history of BA. We could not get informed consent from his sister (II-17).

Urinary 11-dehydrothromboxane B2 (11DTXB2) was measured using an 11-dehydrothromboxane B2 [^{125}I] RIA kit, (Perking-Elmer Life and Analytical Sciences, Boston, MA, USA) after extraction using octadecylsilyl silica (ODS) powder and purification using an open silica mini column (Bond Elute SI; Varian, Palo Alto, CA, USA). The urinary 11DTXB2 level was also expressed as pg/mg creatinine and the urinary creatinine level was determined

using a creatinine test kit (Pure Auto® S CRE-L, Daiichi-kagaku, Tokyo, Japan).

Statistical analyses. The distribution of the genotype of Glu 4 Lys in *LTC4S* was analyzed using Fisher's exact test. The Mann-Whitney unpaired *U* test was performed for allergic patients with Glu 4 Lys and controls, and allergic patients with and without Glu 4 Lys. p-values <0.05 were considered statistically significant.

Results

Glu 4 Lys in LTC4S. We identified a novel heterozygous single-nucleotide substitution 10G>A (Glu 4 Lys) in the genomic *LTC4S* DNA by direct sequencing in some of the allergic patients (Fig. 1A and B). We determined the prevalence of Glu 4 Lys in the *LTC4S* in allergic patients and non-allergic subjects using the restriction enzyme assay (Fig. 1C). The normal and mutant alleles were detected as 82- and 112-bp bands, respectively (Fig. 1C).

Glu 4 Lys was found in 5 (3.5%) of the 141 allergic patients. However, Glu 4 Lys was not detected in any of the 110 nonallergic subjects. There was a difference in Glu 4 Lys frequency between the allergic patients and nonallergic subjects (Fisher's exact test, p=0.0460) (Table II). The clinical features of the allergic patients with Glu 4 Lys are shown in Table III. All of the patients with Glu 4 Lys suffered from BA. The urinary LTE4 levels of patients with Glu 4 Lys (mean, 285.3 pg/mg creatinine; range, 215.0-411.0 pg/mg creatinine) did not show a significant difference from those of allergic patients with wild-type *LTC4S* (mean, 364.0 pg/mg creatinine; range, 86.9-1840.0 pg/mg creatinine) or control subjects (mean, 204.0 pg/mg creatinine; range, 103.0-347.0 pg/mg creatinine). The urinary 11DTXB2 levels of patients with Glu 4 Lys (mean, 1661.2 pg/mg creatinine; range, 1226.0-2050.0 pg/mg creatinine) did not show a significant difference from those of allergic patients with wild-type *LTC4S* (mean, 1294.8 pg/mg creatinine; range, 40.0-6070.0 pg/mg creatinine); however, they were significantly different from those of the control subjects (mean, 596.5 pg/mg creatinine; range, 239.0-1376.0 pg/mg creatinine; p=0.0140) (data not shown).

Genetic analysis of Glu 4 Lys in atopic families. The link between Glu 4 Lys and allergic diseases was investigated in four families (Fig. 2). In family 1, II-3 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-1) who had BA. A younger sister (II-4) without any allergic symptoms did not have this substitution.

In family 2, II-8 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-5) who had BA. An elder sister (II-7) also had the heterozygous Glu 4 Lys and suffered from AR. We could not obtain informed consent from the brother (II-9).

In family 3, II-12 was the proband with Glu 4 Lys and suffering from BA and AD. Glu 4 Lys was inherited from the mother (I-11) who had no allergic history. We could not get informed consent from the brother (II-13).

In family 4, II-16 was the proband with Glu 4 Lys and suffering from BA. Glu 4 Lys was inherited from the father (I-10) who did not have any allergic symptoms. The sister (II-17) also had the heterozygous Glu 4 Lys and suffered from BA.

In families 1 and 2, the probands with Glu 4 Lys (II-3 and -8) all suffered from BA. Glu 4 Lys was inherited from their fathers (I-1 and -5) who had a history of BA in childhood. In families 3 and 4, the proband with Glu 4 Lys (II-12 and -16) also suffered from BA. However, Glu 4 Lys was inherited from the mother (I-11) or father (I-14) who did not have allergic symptoms. Interestingly, the grandfather (*) and uncle (**) in family 4 had a history of BA. In all four families, the subjects with a history of BA also had the Glu 4 Lys substitution.

Discussion

Through linkage and association studies, a number of putative atopy genes have been identified. Studies of the chromosome 5q region, in which the *LTC4S* is located (6), revealed the linkage to atopy and bronchial hyper-responsiveness. Other candidate genes for asthma are located in the 5p region, including the IL-4 cytokine cluster (IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF) and the β_2-adrenergic receptor.

In this study, we identified a novel single-nucleotide substitution 10G>A (Glu 4 Lys) in the first exon of the *LTC4S* gene. All patients who had a Glu 4 Lys substitution suffered from BA or both BA and AD. Furthermore, the familial analysis of Glu 4 Lys revealed a link between allergic diseases and Glu 4 Lys. All patients with BA in the four families we studied had Glu 4 Lys in *LTC4S*. These results showed that Glu 4 Lys in *LTC4S* may be associated with the phenotype of allergic diseases such as BA.

We have reported that the levels of urinary LTE4 and 11DTXB2 are significantly higher in asthmatic children than in control subjects (13). In our present study, the levels of urinary 11DTXB2 were significantly higher in patients with Glu 4 Lys than in control subjects (p<0.05). However, there was no significant difference in the level of urinary LTE4 between the allergic patients with Glu 4 Lys and control subjects. This mutation in the *LTC4S,* which changes the charge from negative to positive, may affect the stability of the *LTC4S* protein. Therefore, this mutation is induced to inhibit the synthesis of cysLTs. Unfortunately, in this study, the urinary prostanoids were measured only in the four patients with Glu 4 Lys. Further studies may be necessary to define the mechanism underlying the activity of *LTC4S* with a Glu 4 Lys substitution.

In conclusion, our study suggested that Glu 4 Lys in *LTC4S* might be associated with BA. Further large-scale clinical studies are required to confirm the effects of the Glu 4 Lys substitution in the *LTC4S* in patients with allergic diseases, together with studies of clustering SNPs on the 5q chromosome affecting BA.

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Original Article

Characterization of T-cell clones specific to Ovomucoid from patients with egg-white allergy

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Summary. *Background:* Allergic reactions to foods are specific problems for infants and young children. Ovomucoid (OM) is one of the major allergens found in egg-white. We previously established several T-cell clones (TCCs) specific to OM in non-polarizing conditions from 4 patients (TM and YN are immediate-type, IH and YT are non-immediate-type) with egg-white allergy. We characterized their reactive epitopes, antigen-presenting molecules (HLA class II), and usage of TCR alpha and beta genes and the CDR3 loop sequence.
Objective: The objective of this study was to characterize these seven clones (TM1.3,TM1.4,YN1.1,YN1.5,IH3.1,IH3.3 and YT6.1) for cytokine production patterns and cell-surface-marker phenotypes.
Methods: We measured the production of cytokines, namely interleukin (IL)-4, IL-5 and interferon-γ (IFN-γ) by stimulation with ovomucoid peptides and stained intracellular IL-4 and IFN-γ, and determined cell-surface markers using anti-interleukin-12 receptor (IL-12R) ß1, anti-IL-12Rß2 and anti-interleukin-18 receptor α (IL-18Rα).
Results: Most TCCs secreted both IL-4 and IFN-γ in response to the OM peptide mixture, but the secretion patterns were variable; an IFN-γ dominant pattern was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3 and TM1.4, an IL-4> IFN-γ pattern in YN1.5. In intracellular IFN-γ and IL-4 staining, IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3. All TCCs were IL-12Rß1-positive, and TM1.3, IH3.1, IH3.3 and YT6.1 were both IL-12Rß2- and IL-18Rα-positive. TM1.4 and YN1.1 were both IL-12Rß2- and IL-18Rα-negative. Based on these results, TM1.3 and TM1.4, IH3.1 and YT6.1 had a predominantly Th1 character and YN1.1, YN1.5, and IH3.3 possessed a predominantly Th0 character.
Conclusions: The phenotypes of TCCs were not in accordance with their clinical manifestations. TCCs established from patients with immediate-type hypersensitivity had either the Th1 or Th0 phenotype as well as those with non-immediate-type hypersensitivity.

Key Words: AD (atopic dermatitis), IFN-γ (interferon gamma), IL-12Rß2 (interleukin-12 receptor beta 2), IL-18Rα (interleukin-18 receptor alpha), OM (ovomucoid), TCC (T cell clone), Th (helper T cell).

Abbreviations used:

AD: atopic dermatitis
FITC: Fluorescein isothiocyanate
HLA: Human leukocyte antigen
IFN-γ : Interferon gamma
IL: Interleukin
IL-12R: IL-12 receptor
IL-18R: IL-18 receptor
OM: Ovomucoid
PBMC: Peripheral blood mononuclear cell
PE: phycoerythrin
RAST: Radioallergosorbent test
rIL: recombinant Interleukin
TCC: T cell clone
TCL : T cell line
TCR: T cell receptor
Th: helper T cell

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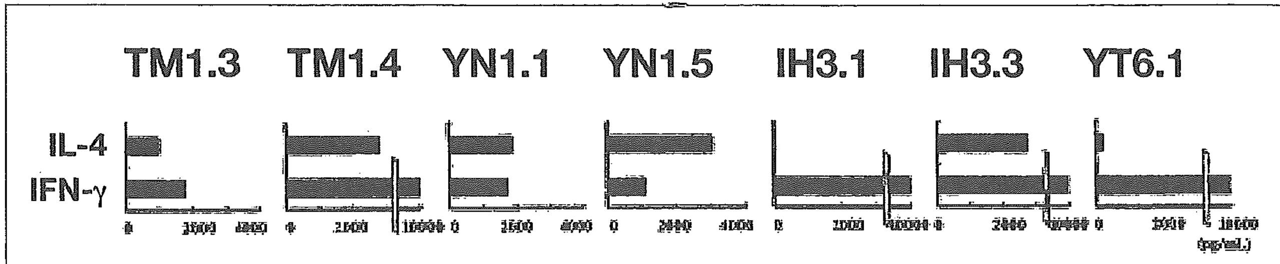

Figure 1. Cytokine production patterns of our TCCs. T-cells were cultured in the presence of an OM peptide mixture (1mM each). After incubation for 56 hours, culture supernatants were collected immediately, and the cytokines were measured by ELISA. Net cytokine concentration in culture supernatants is expressed as the mean value of duplicate cultures.

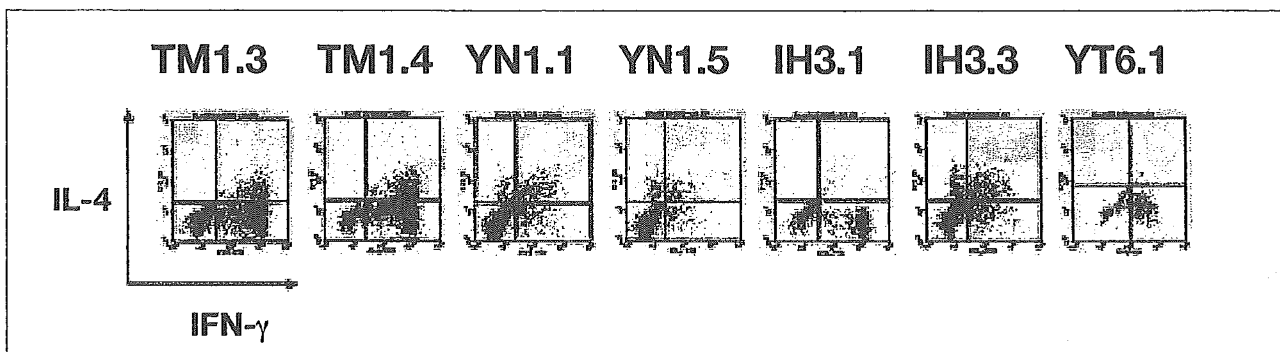

Figure 2. Intracellular IFN-γ and IL-4 staining. TCCs specific to OM were cultured at a density of 2 x 10^6 cells per mL in an RPMI 1640 medium with PMA and ionomycin in the presence of Brefeldin-A for 4 hr at 37°C. After Pma stimulation, the cells were directly stained with a FITC-conjugated anti-CD4 monoclonal antibody. After permeabilization, the cells were stained with IFN-γ FITC/IL-4 PE. Flow cytometric analysis was performed using a FACS Calibur.

Cell-surface-marker phenotypes of TCCs

A FITC-labeled monoclonal anti-IL-12Rß1 antibody (PharMingen, San Diego, CA), a phycoerythrin (PE)-labeled monoclonal anti-IL-12Rß2 antibody (kindly donated by Dr. F Sinigaglia, Italy), anti-IL-18Rα, and anti-human CCR-3 (R&D Systems, McKinley Place, MN) were used to analyze the phenotype of our TCCs. FITC-labeled anti-Leu4/CD3, anti-Leu3a/CD4 (Becton Dickinson), anti-TCR-α/ß-1-WT31 (Becton Dickinson), anti-TCR-γ/δ (Endogen, Woburn, MA), and PE-labeled anti-Leu2a/CD8 (Becton Dickinson) were also used to analyze the phenotypes of our TCCs by double-color staining. The stained cells were analyzed using a FACScan instrument (Becton Dickinson). The forward scatter threshold was set to exclude debris only from the preparation.

Results

Production of IL-4 and IFN-γ in supernatants of TCCs

Cytokine secretion was investigated in the TCCs specific to OM. Since these clones were maintained in the presence of IL-2 and IL-4, they were washed with the culture medium and then cultured with the OM peptide mixture (1 μM) and irradiated autologous PBMC, but in the absence of IL-2 and IL-4, for 56 hr. Cell proliferation was similar among these clones when examined using a 3H-thymidine uptake (data not shown). Most TCCs secreted both IL-4 and IFN-γ in response to the peptide mixture, as well as OM crude protein (data not shown), but the secretion patterns were variable; an IFN-γ dominant pattern (IFN-γ >> IL-4) was seen in IH3.1 and YT6.1, an IFN-γ >IL-4 pattern in TM1.3,TM1.4 and IH3.3, IL-4> IFN-γ pattern in YN1.5, as shown in Figure 1.

Intracellular IL-4 and IFN-γ staining

Since the patterns of IL-4 and IFN-γ secretion were variable among the TCCs, intracellular IL-4 and IFN-γ staining was analyzed. As shown in Figure 2, intracellular IFN-γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN-γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3.

Together with cytokine secretion data, TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype.

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Introduction

Allergic reactions to foods are specific problems for infants and young children and present a wide spectrum of clinical reactions, including cutaneous, gastrointestinal and respiratory symptoms as well as systemic anaphylactic symptoms. It is also known that food allergies are more prevalent in children due to an immature gastrointestinal epithelial membrane barrier that allows more proteins to cross the barrier and get into circulation [1]. Among various food antigens, the hen's egg, particularly its egg-white, is one of the most common causes of food allergy in young children. One of the major components of egg-white, comprising approximately 10% of the total egg-white proteins, is ovomucoid (OM) [2], and it has been reported to play a more important role in the pathogenesis of allergic reactions to egg-white than other egg-white proteins [3,4].

Interferon-γ (IFN-γ) production characterizes the Th1 subset while interleukin (IL)- 4 production characterizes the Th2 subset [5]. The differentiation into either Th1 or Th2 cells is a crucial step that determines the direction of subsequent adaptive immune responses. Interleukin-12 receptor (IL-12R) ß2 mRNA synthesis is restricted to Th1 cells [6,7], and the mRNA expression of Interleukin-18 receptor α (IL-18Rα) was observed only in the Th1 clones [8]. We recently established OM-specific TCCs from four Japanese atopic dermatitis (AD) patients with egg-white allergy [9]. In that study, all the TCCs specific to OM exhibited the $CD4^+$ phenotype. These clones were established in non-polarizing conditions, and it was of interest to see whether they had Th1 or Th2 characters in relation to clinical phenotypes. We determined the cytokine production patterns and cell-surface-marker phenotypes of TCCs specific to OM.

Materials and Methods

Subjects

Clinical information on the four patients whose TCCs have been established was reported previously [9]. The diagnosis of allergy to hen's egg-white was based on clinical symptoms, hen's egg-white challenge test results and CAP-RAST [10] against hen's egg-white and OM. All the patients had AD symptoms. Patient TM and YN presented immediate-type hypersensitivity symptoms, such as systemic urticaria and severe coughing, which occurred within thirty minutes after the antigen challenge, and their levels of total IgE and CAP-RAST for egg-white and OM were high. Patient IH and YT had presented non-immediate symptoms, such as systemic eczema, which occurred more than two hours after antigen challenge, and their levels of total IgE and CAP-RAST against egg-white and OM were low [11].

TCC culture

OM-specific TCC establishment was reported previously [9]. These TCCs were cultured in an RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 10% pooled A.B, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). The plates were incubated at 37°C in 5% CO_2 humid air. After 7-9 days, irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5/well) pulsed with OM peptide mixture (1 mM each for 5 hr), human recombinant interleukin (rIL)-2 (50 U/ml) (Genzyme, Cambridge, MA), and human rIL-4 (10 U/ml) (Biosource International, Camario, CA) were added to the culture wells. Thus, the TCCs were maintained for another 7 days.

Production of IL-4 and IFN-γ in supernatants of TCCs

The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble OM peptide mixture (1μM) and irradiated autologous PBMC (1.5×10^5/well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80°C until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay (ELISA) kits for detecting human IL-4 (Biosource Int'l), and IFN-γ (Ohtsuka, Tokyo, Japan) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Intracellular IFN-γ and IL-4 staining

TCCs specific to OM were cultured at a density of 2×10^6 cells /mL in an RPMI 1640 medium for 4 hr at 37°C. During the 4-hr incubation, the cells were stimulated with a combination of 25 ng/mL of phorbol 12-myristate 13-acetate (PMA) (SIGMA) and 2μg/mL of ionomycin (SIGMA) in the presence of 10 ng/mL of Brefeldin-A (SIGMA). Then the cells were directly stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a FACS Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were preincubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE IFN-γ FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. The cells were then washed twice and resuspended in a phosphate-buffered saline. Flow cytometric analysis was performed using a FACS Calibur. The results were expressed as the percentage of each cytokine-producing cell population in a total population of CD4 cells.

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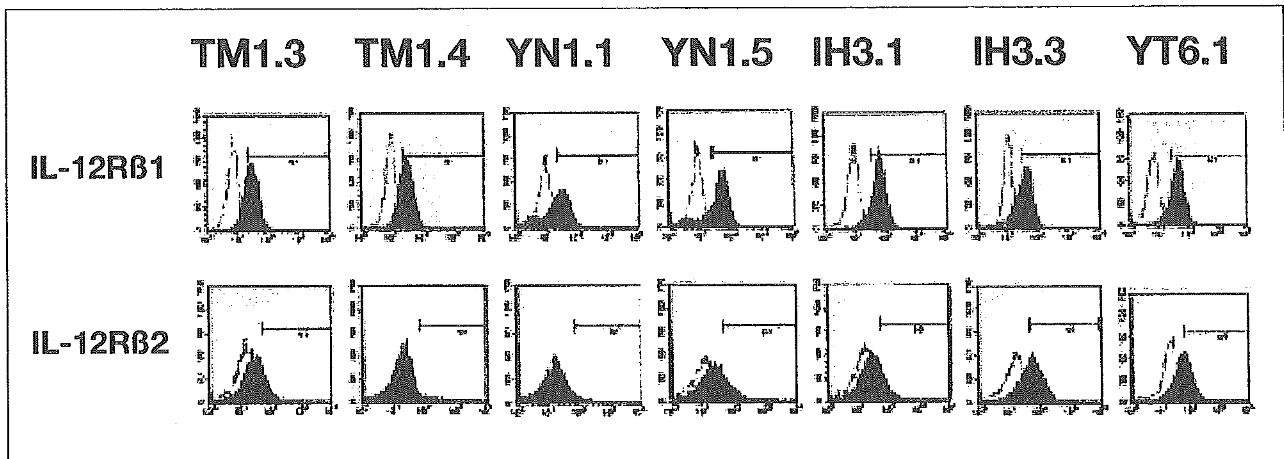

Figure 3. Flow cytometric analysis of our TCCs. FITC-labeled monoclonal antibody, anti-IL-12R ß1, and a PE-labeled monoclonal antibody, anti-IL-12R ß2, were used. Stained cells were analyzed using a FACScan instrument.

Cell-surface-marker phenotypes of TCCs.

All TCCs specific to OM exhibited $CD3^+$, $CD4^+$, $CD8^-$, $\alpha\beta TCR^+$ and $\gamma\delta TCR^-$ phenotypes (data not shown). Moreover, all TCCs were IL-12Rß1-positive (Fig.3). TM1.3, YN1.5, IH3.1, IH3.3 and YT6.1 were IL-12Rß2-positive and TM1.4 and YN1.1 were IL-12Rß2-negative (Fig.3). IL18Rα was co-expressed with IL-12Rß2 in these clones (data not shown).

Discussion

Murine helper T-cells are divided into two subsets, Th1 and Th2 cells. The former produce IL-2 and IFN-γ and the latter produce IL-4 and IL-5 [12]. Such dichotomy is also evident in human cells, albeit to a less polarized extent than in murine T-cells [13]. Th1 cells cause non-immediate-type hypersensitivity reactions whereas Th2 cells promote IgE production leading to immediate-type hypersensitivity.

In our previous study, it was shown that the proliferative responses of PBMCs to Ovalbumin (OVA) in children with AD who are sensitive to hen's eggs were significantly higher than those of healthy children and hen-egg-sensitive children with immediate symptoms. However, in patients with AD there were no significant correlations between the proliferative response to PBMCs and the RAST values [14]. We also reported on the high sensitivity and specificity of proliferative responses of lymphocytes to OVA for the detection of hen-egg allergy in patients with AD [15]. These studies indicate that the molecular basis of non-immediate-type hypersensitivity, as seen in AD patients who are sensitive to hen's eggs is different from that of immediate-type hypersensitivity to hen's eggs.

Chicken OM has been reported to be the most important allergenic protein in egg-white [2,3] and consists of three tandem homologous domains [16]. Hence, we previously established several OM-specific TCCs in non-polarizing conditions from 4 patients with egg-white allergy to investigate the molecular basis of hen-egg allergy [9]. In this paper, we characterized these TCCs with regard to helper T-cell phenotypes. Patients TM and YN presented immediate-type hypersensitivity symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were high. On the other hand, patients IH and YT presented non-immediate symptoms, and their levels of total IgE and CAP-RAST for egg-white and OM were low. These clinical manifestations suggested that Th1 and Th2 cells mainly contribute to the pathogenesis of hen-egg allergy in the former two patients and the latter two patients, respectively. All of the twenty-four Der pI-specific TCCs from a patient with severe atopic disease were reported to have the Th2-type character [17]. This may indicate that imbalance between Th1 and Th2 in a patient's condition influences the subtypes of these TCCs. Hence we expected that Th1 clones would be established from IH and YT and that Th2 clones would be from TM and YN.

In the present study, we analyzed IFN-γ and IL-4 production patterns by stimulation with OM, intracellular IFN-γ and IL-4 staining in 7 T-cells clones.

The results were further confirmed by the cell-surface marker of Th1 cells. TM1.3, TM1.4, IH3.1, and YT6.1 had a Th1-dominant phenotype and YN1.1, YN1.5, and IH3.3 had a Th0-dominant phenotype. IH3.1 and YT6.1 had a Th1-dominant phenotype in accordance with the fact that they were established from patients who presented non-immediate-type hypersensitivity. However, TM1.3 and TM1.4 were established from patient TM who presented immediate-type hypersensitivity symptoms, but both had a Th1-dominant phenotype. YN1.1 and YN1.5 were from patient YN who

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also presented immediate-type hypersensitivity symptoms but both had a Th0 phenotype. Our results indicate that the cell phenotype of TCCs does not always reflect clinical manifestations corresponding to immediate or non-immediate hypersensitivity. The phenotypes of TCCs could not be simply predicted, so that establishment of further TCCs and their characterizations are now under way.

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The cell cycle associated protein, HTm4, is expressed in differentiating cells of the hematopoietic and central nervous system in mice

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Summary

HTm4 is a member of a newly defined family of human and murine proteins, the MS4 (membrane-spanning four) protein group, which has a distinctive four-transmembrane structure. MS4 protein functions include roles as cell surface signaling receptors and intracellular adapter proteins. We have previously demonstrated that HTm4 regulates the function of the KAP phosphatase, a key regulator of cell cycle progression. In humans, the expression of HTm4 is largely restricted to cells of the hematopoietic lineage, possibly reflecting a causal role for this molecule in differentiation/proliferation of hematopoietic lineage cells. In this study, we show that, like the human homologue, murine HTm4 is also predominantly a hematopoietic protein with distinctive expression patterns in developing murine embryos and in adult animals. In addition, we observed that murine HTm4 is highly expressed in the developing and adult murine nervous system, suggesting a previously unrecognized role in central and peripheral nervous system development.

Introduction

HTm4 is a member of a newly defined, extensive family of human and murine proteins. Each member of the MS4 (membrane-spanning four) protein group has a distinctive four-transmembrane structure. To date, few members of this family are well understood, but a diverse functionality is beginning to emerge. These functions include roles as cell surface signaling receptors and intracellular adapter proteins (Hulett *et al.* 2001, Ishibashi *et al.* 2001, Liang *et al.* 2001, Liang & Tedder, 2001, Barnhill *et al.* 2004). We demonstrated that HTm4 is an adapter molecule that regulates the function of the KAP phosphatase, a key regulator of cell cycle progression (Donato *et al.* 2002). HTm4, in humans, is a 214 amino acid protein whose expression is largely restricted to both mature and precursor cells of the hematopoietic lineage, which may reflect a causal role in driving the differentiation/proliferation of hematopoietic lineage-specific cells (Adra *et al.* 1994, 1999, Donato *et al.* 2002, Nakajima *et al.* 2004).

A second important feature of the biology of human *HTm4* is its genetic localization. Human *HTm4* maps to chromosome 11q13.1 (Adra *et al.* 1994), in close

**JLK and XY contributed equally to this work.

proximity to the FcεRI antigen receptor beta chain gene (Ra *et al.* 1989, Ravetch & Kinet 1991) and the gene for CD20 (Tedder *et al.* 1988), both of which are MS4 family members. The 11q13.1 region is linked significantly to human atopic phenotypes (Collee *et al.* 1993, Shirakawa *et al.* 1994, van Herwerden *et al.* 1995, Folster-Holst *et al.* 1998, Adra *et al.* 1999). Specifically, TaqI restriction fragment length polymorphism (RFLP) in the 3rd intron of the human *HTm4* showed a strong association with atopic asthma. This variant showed similar odds ratios to that of intron 2 of the FcεRIβ gene in severe asthma, as well as in severe atopy phenotypes. Interestingly, we found that HTm4 is highly expressed in basophils at both the mRNA and protein levels (Nakajima *et al.* 2004); hence, human *HTm4* is considered an atopy gene candidate on locus 11q13.1 (Adra *et al.* 1999).

In this report, we present data on the genetic sequence, genomic organization, protein structure prediction, biochemistry and tissue expression pattern of the murine HTm4 homologue. Our data show that, like the human homologue, murine HTm4 is also predominantly a hematopoietic protein with distinctive expression patterns in developing murine embryos and adults. We also report the unexpected finding that murine HTm4 is expressed in the developing murine nervous system. Moreover, we demonstrate that the functional interaction with the KAP phosphatase, previously demonstrated by our group in human cells, is also present in the murine system. Our work suggests a conservation of structure and function for HTm4 between the mouse and human systems.

Materials and methods

Cloning of murine HTm4 cDNA

Rapid Amplification of cDNA Ends (RACE)-PCR was used for the cloning of murine *HTm4* cDNA. Marathon-Ready BALB/c mouse spleen cDNA library (Clontech Laboratories Inc, Palo Alto, CA) was used as the template. Two gene specific primers were derived from a single EST sequence that was highly homologous to human *HTm4*. A gene specific antisense primer, 5′-CAT TCG TGT GGG GTT TCT CCT GC-3′, was paired with the 5′ primer supplied by the manufacturer to generate PCR product that would cover the 5′ end region of cDNA of interest, murine *HTm4*. In the same manner, a gene specific sense primer, 5′-GTT TAC AAC ACG TGT CCC ACC ACT TCA G-3′, was used to generate PCR product that would cover the 3′ end region of murine *HTm4* cDNA, when paired with the 3′ primer from the manufacturer. PCR products from both experiments were sequenced and compared. The longest contiguous murine *HTm4* cDNA sequence was generated through the merging of largest 5′ and 3′ PCR fragments at the overlapping region that was flanked by the aforementioned gene specific primers. Based on this sequence, a pair of murine *HTm4* specific primers was generated for the procurement of a full-length cDNA. The sequences for this pair of primers were 5′-CAA AGA AGA GAT TCG TTA AGC CTG AGG-3′, the sense primer, and 5′-CGC CTA AGG AAA TAT ATT TAT TCA TCC AGA TC-3′, the antisense primer. The final full-length murine *HTm4* cDNA was cloned into the pGEM-T vector (Promega, Madison, WI) and analyzed by DNA sequencing.

Cloning and sequencing of the murine HTm4 genomic sequence

A BAC genomic library of murine 129/SvJ DNA (Genome Systems Inc., St. Louis, MO) was screened by PCR using primers designed for the 5′ and 3′ ends of the gene. The positive clone was isolated, and its DNA purified, restricted and sub-cloned in the pBluescript vector (Stratagene, CA). The mouse genomic DNA insert (accession number: AY258288) was sequenced by direct BAC sequencing and also by sequencing the sub-clones using the BigDye Terminator Cycle Sequencing Kit and ABI prism-377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Murine HTm4 protein modeling

Murine HTm4 protein modeling was predicted and compared to human HTm4 by program 'SOSUI' (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) which was made for prediction of existence of transmembrane helices and determination of transmembrane helical regions in the proteins based on physicochemical properties of amino acid sequences such as hydrophobicity and charges. A 'primary' transmembrane helix is composed of the highly hydrophobic and successive region of the AA sequence by a hydropathy profile based on the Kyte–Doolittle hydropathy index (Kyte & Doolittle 1982), while a 'secondary' transmembrane helix is composed of less hydrophobic and some polar AA sequences of which parameters are calculated with amphiphilicity index of polar amino acids (Mitaku *et al.* 1995).

Generation of a polyclonal antiserum directed against murine HTm4

A peptide derived from the amino-terminus of murine HTm4 (amino acids 4–19: EETGGSVYQPLDESRH) was coupled to keyhole limpet hemocyanin and used to generate a rabbit polyclonal antiserum. We produced amino- and carboxyl-terminal fusion proteins (aa 4–19 and aa 197–213) of murine HTm4 with the GST protein using pGEX6P1 vector (Amersham

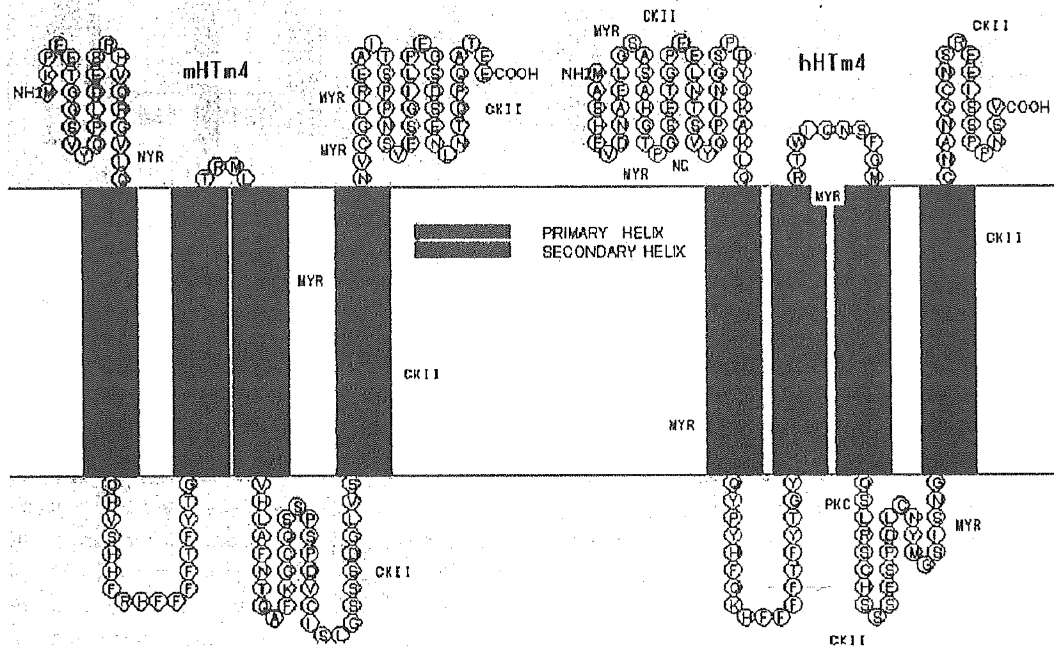

Figure 1. Comparison of the predicted model of murine HTm4 and human HTm4 proteins. The predicted murine (m)HTm4 (left panel) and human (h)HTm4 (right panel) protein structures were compared. Each figure shows topology with AA sequence from outer to inner side through membrane portion. Two outside loops are predicted for each protein as 'primary' transmembrane helixes (dark green loops), while two inside loops are predicated as 'secondary' transmembrane helixes (light green loops).

Pharmacia Biotech, Piscataway, NJ) in order to screen the reactivity of these antibodies. Affinity purification was performed by standard methods using the immunizing peptide.

Immunoprecipitation and western blotting

Samples consisted of either 5 μg murine HTm4 GST fusion proteins, or 20 μg proteins derived from BALB/c mouse spleen cells lysed in a standard SDS buffer. Protein samples were boiled for 8 min before loading. Cells were pelleted in a microcentrifuge (2000*g*, 2 min) and washed once in 1 ml ice cold phosphate buffered saline to remove serum proteins. 10^7 cells were lysed on ice for 30 min in 350 μl of the following buffer: 50 mM HEPES pH 7.4, 75 mM NaCl, 20 mM NaF, 10 mM iodoacetamide, 0.5% (w/v) Triton X-100, 1 mM phenlymethylsulfonyl fluoride (PMSF), 500 mg/ml Aprotinin, 1.0 mg/ml Leupeptin and 2.0 mg/ml Chymostatin. Lysates were clarified by microcentrifugation (10,000*g*, 5 min). Supernatants were transferred to clean tubes and either mixed with 1.4 volumes acetone and placed at −20 °C for 1 h (to pellet total protein) or tumbled at 4 °C for 2 h with the indicated antibody. Acetone precipitates were harvested by centrifugation at 10,000*g* for 5 min. Immunocomplexes were captured using 15 μl/tube Protein G-sepharose beads and washed using two rounds of microcentrifugation and re-suspension in 1 ml lysis buffer. Beads and protein pellets were dried by aspiration and then incubated at 95 °C for 8 min in a reducing SDS polyacrylamide gel electrophoresis (PAGE) loading buffer. After 10% SDS–PAGE, the resolved proteins were transferred to PVDF membrane by electroblotting and the membrane was blocked for 1 h using 5% BSA bovine serum albumin (Figure 3B) or 5% non-fat milk (Figure 5) in PBS pH 7.0. The membrane was then probed for either 2 h at RT (Figure 3B) using 1:1000 dilution of murine HTm4 antiserum, or 16 h at 4°C (Figure 5) using 1:2000 dilution monoclonal anti-KAP (BD Pharmingen, San Diego, CA.). After extensive washing (4 × 5 min in PBS/0.5% Tween-20) a developing antibody was applied (1 h at RT, 1:10,000 dilution of donkey anti-rabbit (Amersham, Piscataway, NJ) or sheep anti-mouse (Amersham, Piscataway, NJ) IgG coupled to HRP). After further washing bands were visualized using ECL according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Northern blotting procedures

Northern blot filters of Poly (A)+ RNA from normal mouse tissues were purchased from BD Biosciences, CLONTECH, Palo Alto, CA. The filters were probed with 32p-labelled full-length murine cDNA according to the published protocols (Adra *et al.* 1994, 1997). The blots have been normalized to beta-actin by the manufacturer.

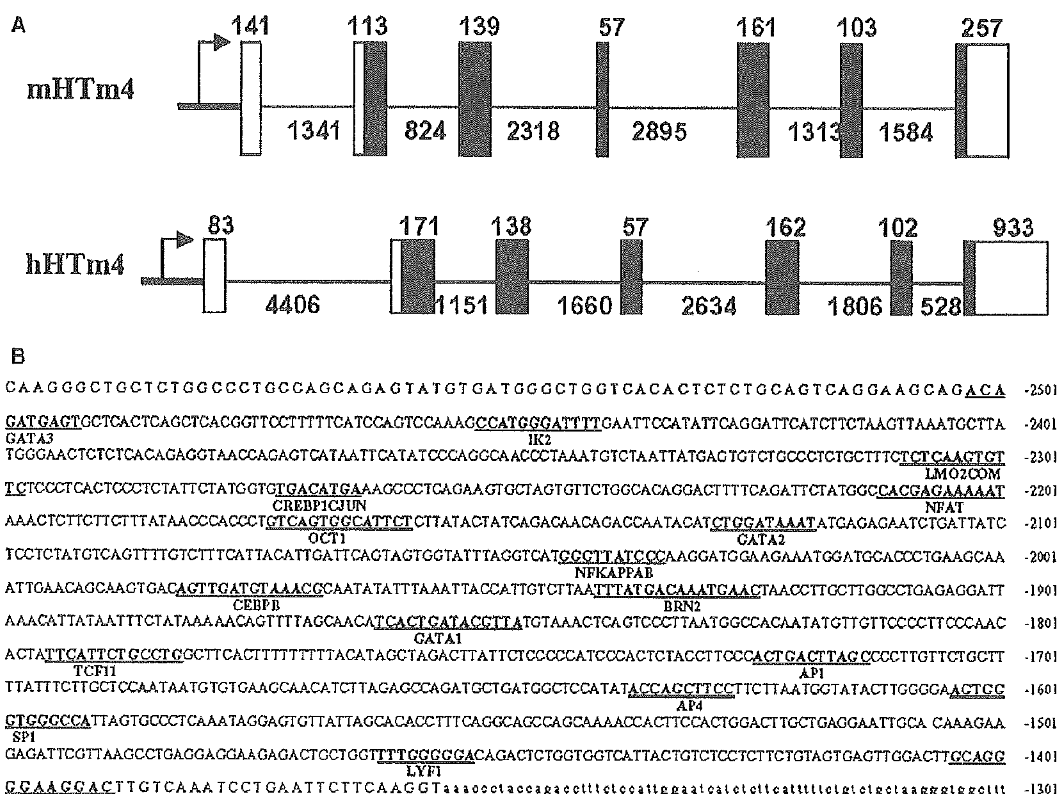

Figure 2. Structure and 5′ nucleotide sequence of the murine HTm4 gene. (A) Comparison of the complete gene structure of mouse HTm4 and human HTm4. Exons are shown as boxes and introns are connecting lines. The coding region is shaded. Exon and intron lengths are marked. Arrows are used to indicate the direction of transcription. (B) 5′nucleotide sequence of the murine HTm4 gene.
Accession number is AY258288. Sequence analysis started up to 1 kb upstream from the ATG (+1). The potential transcription factor binding sites were searched using MatInspector V2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl) (Quandt *et al.* 1995) and are shown in boldface and underlined.

Immunohistochemical studies for murine HTm4

All staining was performed by standard immunoperoxidase methods. Briefly, either embryonic murine tissue slides from day 8-day 16 or adult murine BALB/c tissue (Novagen, Madison, WI) were deparaffinized and pre-treated in 10 mM sodium citrate, pH 6.0 using a pressure cooker system (Decloaking chamber, Biocare Medical, Walnut Creek, CA as per manufacturer's instructions). All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (DAKO, Carpinteria, CA) for 5 min to quench endogenous peroxidase activity, and a 1:5 dilution of goat serum in 50 mM Tris-Cl, pH 7.4, for 20 min to block non-specific binding sites. Affinity purified rabbit anti-murine HTm4 antibody (1:100 dilution in 50 mM Tris–Cl, pH 7.4 with 3% goat serum) was applied at room temperature for 1 h. After washing in 50 mM Tris–Cl, pH 7.4, secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Envision detection kit, DAKO, Carpinteria, CA) was applied for 30 min. After further washing, immunoperoxidase staining was developed using a DAB chromogen kit (DAKO, Carpinteria, CA), per manufacturer's instruction, and counterstained with hematoxylin. To assure specificity, murine HTm4 antibody was pre-incubated with the immunizing peptide in most experiments and applied to identical tissues, revealing complete blocking of staining.

Bone marrow cell preparation for flow cytometry analysis

C57/BL6 mice were used. Either femurs or tibias were harvested from mice without exsanguinations following cervical dislocation. The bone marrow contents were flushed with 4 °C PBS using a 26G hypodermic needle, thoroughly resuspended by gentle pipetting several times, and filtered through 30-μm-nylon mesh to remove remaining particulate material. Bone marrow cells were then washed twice with PBS buffer, pelleted by centrifugation at 1000 rpm for 10 min at 4 °C, resuspended, and counted. Cells were permeabilized with the Fix & Cell Permeabilization Kit, as described

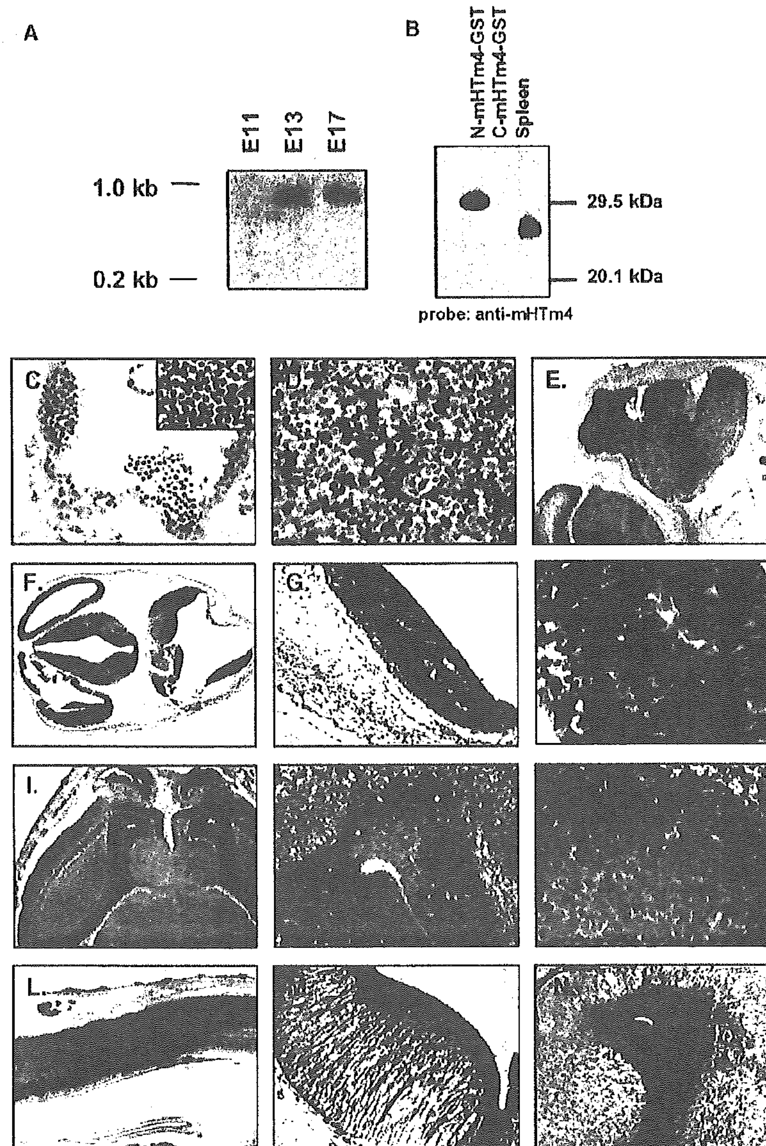

Figure 3. Murine HTm4 expression patterns in the embryonic mouse. (A) Northern blot analysis of HTm4 transcription during mouse embryogenesis. Poly A^+ RNA (approximately 2 μg/lane) was prepared from mouse embryos at the indicated stage of development (The Northern membrane was probed as described in Methods to detect the 1.0 kb HTm4 transcripts, which are detectable by this technique after day E11. This commercial blot has been normalized to beta-actin by the manufacturer (BD Biosciences, CLONTECH, Palo Alto, CA). (B) Validation of anti-murine HTm4 antibody by specific western blot. Polyclonal anti-murine HTm4 was generated against N-terminal amino acids 4-19 in rabbit as described in Methods. Protein samples corresponding to 5 μg/lane GST fusion proteins of either the amino- or carboxyl-terminal cytoplasmic tails of murine HTm4 (N-murine HTm4-GST, C-murine HTm4-GST), or total lysate from $1x10^6$ murine spleen cells, were resolved by 10% SDS–PAGE. After electrotransfer to PVDF membrane, western analysis was performed with 1 μg/ml anti-murine HTm4. Note the presence of approximately 25 kDa band in murine spleen and immunoreactivity for only the N-murine HTm4-GST fusion protein, which contains the immunogenic peptide. (C–N) Immunohistochemisty for murine HTm4 in embryonic murine hematopoietic and neural tissues. Representative sections of formalin-fixed paraffin embedded embryonic (E) murine tissues were stained with anti-murine HTm4 antibody. Positive staining (DAB chromogen with hematoxylin counterstain) was restricted to either developing hematopoietic cells within the blood islands (C; E8; 400× original magnification; inset 1000×) and liver (D; E14; 400× original magnification), or neural derived cells within the developing brain (E for E15, sagittal section, 40× original magnification; F–H for E12, cross-section, 20×, 100×, or 400× original magnifications, respectively; and I–K for E16, cross section, 20×, 100×, or 400× original magnifications, respectively). Strong expression of murine HTm4 was also detected within the spinal cord and dorsal and ventral ganglia starting at E14 (L; representative E15 embryo, 40× original magnification). Nestin staining is shown in panel M (E12, 40× original magnification) and panel N (E16 original magnification, 100×). Note the distinct patterns of murine HTm4 and nestin expression (panel G versus M for E12 and panel H versus N for E16) indicating diminished murine HTm4 protein expression in the more primitive periventricular cells of the developing brain compared to nestin.