interleukin-4 gene promoter −589C/T single-nucleotide polymorphism (SNP) and an allele of interferon regulatory factor 1 (IRF-1) gene GT repeat polymorphism, and the T allele of −88 G/T SNP of *MxA* gene encoding an antivirally active protein, MxA, were more frequent in SSPE patients than in controls (Inoue *et al*, 2002; Torisu *et al*, 2004).

Innate immunity is responsible for the detection of and the initial protective response to viral infections (Kawai and Akira, 2006). Specific receptors of the innate immune system play a key role in detecting the presence of viruses. In MV infection, viral double-stranded (ds) RNA is formed during the replication cycle and recognized by toll-like receptor 3 (TLR3) (Tanabe *et al*, 2003). Other cytosolic receptors for dsRNA including retinoic-acid-inducible protein I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are also involved in antiviral response to MV infection (Berghall *et al*, 2006; Plumet *et al*, 2007). Recognition of dsRNA derived from MV triggers the production of type I interferon (IFN), which plays an important role in MV clearance.

It has been reported that common mutations of genes encoding molecules related to innate immunity influences susceptibility to viral infections. *TLR4* mutations were associated with an increased risk of severe respiratory syncytial virus bronchiolitis (Tal *et al*, 2004). Rapid progression of HIV-1 infection was associated with *TLR9* polymorphisms (Bochud *et al*, 2007). To determine the role of *TLR3*, *RIG1*, and *MDA5* genes as a host genetic factor for the development of SSPE, we have performed an association study on SNPs of these genes in the Japanese SSPE patients and controls.

Results

The frequencies of each allele of tag SNPs of *TLR3* L412F (rs3775291), *RIG1* rs277729, and *MDA5* rs4664463, and of three additional SNPs of *TLR3* (−7C/A; rs3775296, IVS3+71C/A; and c.1377C/T; rs3775290) for haplotype analysis in SSPE patients and controls are shown in Table 1. The distribution of the genotypes in the controls was in Hardy-Weinberg equilibrium. The frequency of *TLR3* 412Phe allele (rs3775291) in SSPE patients was significantly higher than that in controls ($P=.03$). There were no significant differences in allele frequencies of SNPs of *RIG1* and *MDA5* genes between SSPE patients and controls.

Table 2 shows linkage disequilibrium (LD) analysis of the four SNPs spanning 6.5 kb of *TLR3*. A significant linkage disequilibrium was observed among them. To investigate if a particular haplotype constituted by the four SNPs was associated with SSPE, haplotype frequencies were estimated and association analysis was done (Table 3). We observed only one haplotype containing 412Phe allele. The frequency of −7C/IVS3+71C/Phe412/c.1377C haplotype, the sole haplotype containing 412Phe allele, was significantly higher in SSPE patients than in controls ($P=.006$, odds ratio: 2.2, confidence interval: 1.3–3.9).

Discussion

In the current study, we first demonstrated an association between a SNP of *TLR3* and SSPE in Japanese population. These data suggest that 412Phe allele of *TLR3* and the haplotype containing this allele may be host genetic factors that confer a predisposition to SSPE. These results imply that TLR3 may participate in the pathogenesis of SSPE.

TLR3 is expressed in conventional dendritic cells (DCs), a variety of epithelial cells including airway, genital tract, biliary and intestinal epithelial cells, and in the brain (Rock *et al*, 1998). TLR3 is localized primarily in endosomal membrane and recognizes virus-derived dsRNA. TLR3 signaling activates the transcription factor-κB (NF-κB) and IRF-3. IRF-3

Table 1 Allele frequencies of each polymorphism

Gene	Polymorphism	Allele	Control (%)	SSPE (%)	*P* value
RIG1	rs277729	G	120 (71.4)	50 (62.5)	.16
		T	48 (28.6)	30 (37.5)	
	rs9695310	C	118 (70.2)	54 (67.5)	.66
		G	50 (29.8)	26 (32.5)	
MDA5	rs4664463	T	137 (81.5)	70 (87.5)	.24
		C	31 (18.5)	10 (12.5)	
TLR3	−7C/A	C	130 (77.4)	60 (75.0)	.68
		A	38 (22.6)	20 (25.0)	
	IVS3+71C/A	C	132 (78.6)	61 (76.3)	.68
		A	36 (21.4)	19 (23.8)	
	c.1243C/T (L412F)	C (L)	122 (72.6)	47 (58.8)	.03
		T (F)	46 (27.4)	33 (41.3)	
	c.1377C/T	C	107 (63.7)	53 (66.3)	.69
		T	61 (36.3)	27 (33.8)	

Note. *P* values were calculated using 2×2 chi-square test.

Table 2 Linkage disequilibrium between all pairs of loci (D') in *TLR3* gene

	IVS3+71C/A	Leu412Phe	c.1377C/T
−7C/A	0.83	0.77	0.84
IVS3+71C/A		0.88	0.90
Leu412Phe			1

induces expression of type I interferons (IFNs), which contribute to antiviral effect via activation of other genes, including 2′-5′-oligoadenylate synthetase and M × A genes (Matsumoto *et al*, 2004). In the brain, both neurons and glial cells have been shown to express TLR3 and initiate inflammatory and antiviral responses upon being triggered with dsRNA (Lafon *et al*, 2006; Town *et al*, 2006), whereas neither RIG-I nor MDA5 is expressed in the brain (Daffis *et al*, 2007; DiSepio *et al*, 1998; Kang *et al*, 2004).

Studies using TLR3-deficient mice showed that TLR3 played contradictory roles in viral infection and general outcome depended on several factors, such as the type of virus, the cell type that is infected, and the stage of infection (Vercammen *et al*, 2008). TLR3-deficient mice had resistance to influenza A virus and West Nile virus (WNV) infections due to the absence of TLR3-mediated inflammatory signaling (Le Goffic *et al*, 2006; Wang *et al*, 2004), whereas they showed normal resistance to lymphocytic choriomeningitis virus, vesicular stomatitis virus, and reovirus (Edelmann *et al*, 2004), and were susceptible to encephalomyocarditis virus and mouse cytomegalovirus (Hardarson *et al*, 2007; Tabeta *et al*, 2004). The association between genetic variation of *TLR3* and human diseases has been suggested. Polymorphisms in the *TLR3* gene may be associated with type 1 diabetes, Stevens-Johnson syndrome, and toxic epidermal necrolysis (Pirie *et al*, 2005; Ueta *et al*, 2007). Heterozygous variant for Leu412Phe of the *TLR3* gene were associated with low antibody and lymphoproliferative responses to measles vaccination, suggesting that the SNP influenced modulation of the immune response to measles vaccine (Dhiman *et al*, 2008). Recently, Zhang *et al* identified a dominant-negative *TLR3* allele in patients with herpes simplex virus 1 (HSV-1) encephalitis (Zhang *et al*, 2007). A heterozygous *TLR3* mutation (Phe554Ser) was associated with impaired TLR3-dependent induction of IFNs in response to HSV-1. 412Leu is located near the concave surface of the TLR3 ectodomain, which binds directly to short dsRNA (Bell *et al*, 2006). Based on the analysis of the crystal structure of TLR3 ectodomain, it was predicted that 412Phe would destabilize the solenoid structure and might disrupt potential glycosylation of neighboring residue Asn413, which was observed to have *N*-acetylglucosamines attached (Bell *et al*, 2005; Ranjith-Kumar *et al*, 2007). By a reporter assay of TLR3-dependent activation, 412Phe allele of *TLR3* gene showed reduced IFN production in response to a synthetic dsRNA (Ranjith-Kumar *et al*, 2007). Accordingly, it is likely that 412Phe allele is associated with reduced ability to control viral infection as Phe554Ser mutation in HSV-1 encephalitis patients.

MV seems to gain access to the CNS at the time of primary infection, as the MV genome was detected on reverse transcription polymerase chain reaction (PCR) in cerebrospinal fluid (CSF) samples not only from patients with measles encephalitis and SSPE but also from those with acute measles (Nakayama *et al*, 1995). In SSPE brains, neurons, oligodendrocytes, astrocytes, and microvascular endothelial cells have been found to be infected (Allen *et al*, 1996). Microglia and astrocytes are endogenous cells of the CNS that are key players in the immune responses that occur within this compartment (Becher *et al*, 2000). MV infection induces type I IFN via TLR3 and type I IFN dramatically up-regulates TLR3 expression in a positive-feedback fashion in virus-infected cells (Tanabe *et al*, 2003). Previous studies have shown that type I IFN acts as a barrier to efficient MV replication in mice (Mrkic *et al*, 1998). Although the role of TLR3 signaling in MV infection in the brain has not been evaluated in TLR3-deficient mice, it is likely that 412Phe allele of *TLR3* gene with reduced TLR3-dependent IFN production or haplotype including this allele may be associated with high levels of viral invasion and replication in the brain in primary MV infection. Initial high MV titer in the brain would contribute to the establishment of persistent CNS infection, an essential step in the pathogenesis of SSPE. In our previous study, we reported an association of *M × A* promoter −88T allele with a high MxA-producing capability and SSPE. It is suggested that, once

Table 3 Estimated haplotype frequencies in healthy controls and SSPE patients

Haplotype	Controls	SSPE	*P* value	OR (95%CI)
−7C/IVS3+71C/Leu412/c.1377C	0.341	0.25	.15	0.6 (0.4 ~ 1.2)
−7C/IVS3+71C/Phe412/c.1377C	0.241	0.412	.006*	2.2 (1.3 ~ 3.9)
−7A/IVS3+71A/Leu412/c.1377T	0.162	0.237	.16	1.6 (0.8 ~ 3.2)
−7C/IVS3+71C/Leu412/c.1377T	0.147	0.067	.19	0.6 (0.2 ~ 1.3)

Note. OR: odds ratio, CI: confidence interval.
The frequencies were estimated using the EH software program.
Haplotype with frequency > 0.05 were shown.
*Corrected *P* value was .024.

persistent CNS infection has established, MV might benefit from IFN signaling through attenuation of viral gene expression and resultant escape from immunologic clearance. We could not find any synergism of Leu412Phe of *TLR3* gene and −88G/T of *MxA* gene (data not shown).

In summary, among SNPs of the genes encoding molecules related to dsRNA recognition, frequencies of 412Phe allele of *TLR3* gene and haplotype including it were significantly higher in SSPE patients. These findings suggested that *TLR3* might confer host genetic susceptibility to SSPE in Japanese population.

Materials and methods

Subjects

The study population comprised 40 unrelated SSPE patients (27 males and 13 females) and 84 unrelated normal school children. All the SSPE patients fulfilled the diagnostic criteria, that is, clinical features, increased MV antibody titer in the CSF, and typical electroencephalograph (EEG) showing periodic slow wave complexes early in the disease. The age at onset of SSPE ranged between 2 and 15 years (mean ± SD, 8.0 ± 3.1 years). Thirty-five patients had contracted natural measles occurring between ages 0.4 and 4 years (1.3 ± 0.9 years). The history of natural measles was unknown in the other five patients, including one with a history of live attenuated measles vaccination. The places where SSPE patients lived and had contracted measles were distributed all over Japan. The age of SSPE patients, when studied, ranged between 5 and 26 years (median 11 years, mean ± SD 12.4 ± 5.1 years). The control subjects were randomly selected from among normal schoolchildren around 10 years of age and were not matched for age or histories of measles and measles vaccination. Informed consent was obtained from their parents. The current study was approved by the Ethics Committee of Kyushu University, Japan.

DNA extraction

Genomic DNA was extracted from peripheral blood using a QIAmp DNA Blood Kit (Qiagen, Tokyo, Japan).

Selection of SNPs

We used data on common SNPs from HapMap (Japanese samples, October 2005 release) to identify tag SNPs. We selected one tag SNPs with the highest minor allele frequency within one linkage disequilibrium block: *TLR3* L412F (rs3775291), *RIG1* rs277729 and rs9695310, and *MDA5* rs4664463. We selected two tag SNPs of *RIG1* gene, because this gene consists of two LD blocks. For haplotype analysis, we selected additional three SNPs of *TLR3* gene based on the previous study; −7C/A (rs3775296), IVS3+71C/A, and c.1377C/T (rs3775290) (Noguchi *et al*, 2004).

Genotype analysis of SNPs in the TLR3, RIG1, and MDA5 genes

SNPs and genotyping methods are described in Table 4. Genotyping of each subject was performed by either TaqMan method or PCR restriction fragment length polymorphism (RFLP). TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) was performed following the manufacturer's instructions. PCR was carried out with mixes consisting of 8 ng of genomic DNA, 5 μl of Taqman master mix, 0.5 μl of 20 × assay mix, and double distilled H_2O up to 10 μl of final volume. The following amplification protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s and annealing and extension at 60°C for 1 min. After PCR, the genotype of each sample was attributed automatically by measuring the allelic specific fluorescence on the ABI PRISM 7700 Sequence Detection Systems using the SDS 2.2.2 software for allelic discrimination (Applied Biosystems). Additional three SNPs of *TLR3* gene were analyzed by PCR-RFLP as previously described (Noguchi *et al*, 2004). PCR was performed with a listed primer pair under the following conditions: 94°C for 5 min; then 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1min. The PCR products were digested with each restriction enzyme, followed by separation in 3%

Table 4 Genotyping methods of each polymorphism

Gene	Polymorphism	Method	Primer	Enzyme
TLR3	−7C/A (rs3775296)	RFLP	5'-gcatttgaaagccatctgct 5'-aagttggcggctggtaatct	*MboII*
	IVS3+71C/A	RFLP	5'-gctggaaaatctccaagagc 5'-gaggctagagagcattacattcat	*NlaIII*
	L412F (rs3775291)	TaqMan		
	c.1377 C/T (rs3775290)	RFLP	5'-ccaggcataaaaagcaatatg 5'-ggaccaaggcaaaggagttc	*TaqI*
RIG1	rs277729	TaqMan		
	rs9695310	TaqMan		
MDA5	rs4664463	TaqMan		

agarose gels and visualization by ethidium bromide staining.

Haplotype analysis of SNPs in the TLR3 gene
We performed haplotype analysis using four SNPs in the *TLR3* gene. Haplotype frequencies were estimated using the Estimating Haplotype Frequencies (EH) software program (ftp://linkage.rockefeller.edu/software/rh). LD coefficients D' values, odd's ratio, and 95% were calculated using data in the EH software program.

Statistical analyses
The Hardy-Weinberg equilibrium of alleles in controls was assessed by means of 2×2 χ^2 statistics. Differences in the allele or genotype frequencies between two groups were evaluated by means of the χ^2 analysis with a 2×2 or 2×3 contingency table. A P value of .05 was considered to be significant except for haplotype analysis, in which a P value of .05/4 (= .0125) was considered to be significant, taking Bonferroni's multiple adjustments into consideration.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Association study between *B- and T-lymphocyte attenuator* gene and type 1 diabetes mellitus or systemic lupus erythematosus in the Japanese population

M. Inuo,* K. Ihara,* T. Matsuo,* H. Kohnot & T. Hara*

Summary

This study is to elucidate whether the *B-* and *T-lymphocyte attenuator* (*BTLA*) gene is a new susceptibility gene for the development of type 1 diabetes (T1D) and systemic lupus erythematosus (SLE). As a result, this study did not find any genetic contribution of the *BTLA* gene to the development of T1D and SLE in Japanese population.

Introduction

The efficient T-cell activation requires two signals; an antigen-specific signal mediated via the TCR, and a non-cognate co-stimulatory signal. The CD28–B7 interaction was initially recognized as the co-stimulatory pathway and then CD28-homologous receptor, CTLA-4, which is an inducible and inhibitory receptor, was identified as a negative regulator of T-cell activation (Sharpe & Freeman, 2002). Recently, PD-1 and B- and T-lymphocyte attenuator (BTLA) have been described as new inhibitory receptors of the CD28 superfamily (Watanabe *et al.*, 2003). They have various expression patterns among both lymphoid and non-lymphoid (parenchymal) tissues (Greenwald *et al.*, 2005). Since the negative or inhibitory co-stimulatory pathways regulate T-cell activation and play a role in peripheral tolerance, the lack of the *CTLA-4*, *PD-1* or *BTLA* gene in mice demonstrated increased severity or duration of experimental inflammation as well as accelerated development of autoimmunity (Tivol *et al.*, 1995; Nishimura *et al.*, 2001; Carreno & Collins, 2003). In human, the *CTLA-4* and *PD-1* genes significantly contributed to the development of various autoimmune diseases in different genetic backgrounds (Nielsen *et al.*, 2003; Ferreiros-Vidal *et al.*, 2004; Prokunina *et al.*, 2004; Gough *et al.*, 2005; Johansson *et al.*, 2005; Kong *et al.*, 2005). Type 1 diabetes (T1D) is an organ-specific autoimmune disease that is characterized by the selective destruction of insulin-secreting β cells in the islets of Langerhans (Casares & Brumeanu, 2001), while systemic lupus erythematosus (SLE) is a chronic and systemic autoimmune disease (Croker & Kimberly, 2005). Both *CTLA-4* and *PD-1* genes played individual roles for the development of these different types of autoimmune diseases (Ihara *et al.*, 2001; Ni *et al.*, 2007), whereas there is no study report on the genetic contribution of *BTLA* gene to the development of any autoimmune diseases.

The purpose of our study is to elucidate whether the *BTLA* gene is another susceptibility gene in the CD28 family for the development of T1D and/or SLE in the Japanese children.

Materials and methods

Study subjects

The study population consisted of 182 unrelated T1D patients, 77 unrelated SLE patients and 228 normal children (Sasaki *et al.*, 2004). T1D was diagnosed by endocrinologists according to the criteria of the National Diabetes Data Group (National Diabetes Data Group, 1979). Diabetes can be diagnosed by the presence of classical signs and symptoms of diabetes (abrupt clinical onset, insulinopenia, proneness to ketosis even in the basal state, and dependence on exogenous insulin to sustain life), the presence of autoantibodies against component of beta cells, and unequivocally elevated blood glucose levels; by a fasting plasma glucose greater than or equal to 140 mg dL^{-1}; or by an abnormal oral glucose tolerance test, with a venous plasma glucose value greater than or equal to 200 mg dL^{-1} at 2 h after 75 g oral glucose. Fulminant T1D patients were not included in the study population. All patients were under the treatment of daily insulin injection. We accepted the diagnosis of SLE only when four of the 11 diagnostic criteria (serositis, oral ulcers, arthritis, photosensitivity, blood changes, renal involvement, antinuclear antibody test, immunological

* Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, and † Department of Endocrinology and Metabolism, Fukuoka Children's Hospital Center for Infectious Diseases, Fukuoka, Japan

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Correspondence: Mika Inuo, MD, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashiku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5421; Fax: +81 92 642 5435; E-mail: mikam@pediatr.med.kyushu-u.ac.jp

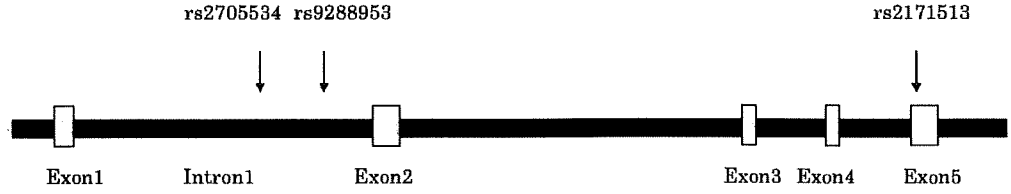

Figure 1. Schematic structures and polymorphisms of the *BTLA* gene.
The exons are depicted as white boxes and the introns as horizontal lines. Exon numbers are shown below the exon boxes. Polymorphisms are indicated by arrows with rs numbers (NCBI dbSNP database).

changes, neurological signs, malar rash, discoid rash) from the revised criteria for SLE of the American Rheumatism Association were fulfilled (Tan *et al.*, 1982). Informed consent was obtained from the patients and/or their parents. They were comprised of only Japanese people, excluding minor distinct ethnic groups (Ainu in Hokkaido and Ryukyu in Okinawa), and immigrants from Korea and China. The use of all materials in this study was approved by the Ethics Committee of Kyushu University.

Genomic DNA extraction

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA extraction kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

SNPs selection

We selected three single nucleotide polymorphisms (SNPs) by using SNP browser 3.5 (Applied Biosystems, Tokyo, Japan). The +10372 T/G SNP (rs2705534 in the NCBI SNP database), +15505 T/C SNP (rs9288953), those located in the intron 1 of the human *BTLA* gene, and +33830 C/T SNP (rs2171513) in the exon 5 were selected for the assay (Fig. 1). The surrounding sequences of these SNPs are as follows: TTGCAACCATTCAAA [T/G] AAATGAATGACTG for +10372 T/G, TTCAAATGCTAACA [T/C] ATTGAGCGAATTTG for +15505 T/C, and TCCAACAGGGACCA [C/T] TGAATGATCAGCAT for +33830 C/T, respectively.

Genotyping of polymorphisms in the BTLA genes

Genotyping by the TaqMan method was performed on an ABI PRISM 7700 Sequence Detection System, using TaqMan® SNP Genotyping Assays.

Estimating haplotype

Using genotype data collected from the study populations, the putative haplotype frequencies of the polymorphic sites in the *BTLA* gene were estimated individually in the patients and control groups. Pairs of alleles were estimated using the estimating haplotype frequencies (EH) software program (http://linkage.rockefeller.edu/ott/eh.htm).

Statistical analysis

Differences in allele, genotype and haplotype frequencies between respective groups were evaluated by chi-square analysis using 2×2 contingency tables with StatMate III (ATMS, Tokyo, Japan). For the multiple comparisons of the allele, genotype, and estimated haplotype frequencies in the *BTLA* gene, Bonferroni's multiple adjustments were made to the level of significance, which were set at a P-value of < 0.016 (0.05/3) for alleles and genotypes, and < 0.00625 (0.05/8) for estimated haplotypes, respectively.

Results

Polymorphisms in the *BTLA* gene

The locations of polymorphisms we analysed are shown in the Fig. 1. The minor allele frequencies of these polymorphisms were between 0.19 and 0.47 (Table 1). All of the genotype frequencies of the analysed SNPs in the control group were in Hardy–Weinberg equilibrium. As a result, none of the allele or genotype frequencies of the *BTLA* gene polymorphisms showed significant differences between T1D or SLE and controls.

Haplotype analysis

Four major haplotypes with frequencies more than 0.1 were estimated to exist in the *BTLA* gene. These haplotypes accounted for more than 89% of all haplotypes in T1D or SLE patients and controls. The frequencies of these estimated haplotypes were not significantly different between T1D or SLE patients and controls (Table 2).

Discussion

Engagement of co-stimulatory and co-inhibitory molecules plays key roles in the activation and termination of the immune response, and these molecules were intensively studied as candidate genes for autoimmune diseases (Sun *et al.*, 2003; Peggs & Allison, 2005). There were many reports suggesting the involvement of CTLA-4 and PD-1 to the development of T1D, SLE or other autoimmune diseases. A new member of the co-inhibitory molecules, BTLA, is expressed on T and B lymphocytes, natural killer cells, dendritic cells, myeloid cells and on various somatic

Table 1. Allele and genotype frequencies of three polymorphisms in the *BTLA* gene

Position (NCBI SNP no.)	Genotype/Allele	Control $n = 228$	T1D $n = 182$	SLE $n = 77$	Control versus T1D			Control versus SLE		
					P-value	OR	95%CI	*P*-value	OR	95%CI
+10372 (rs2705534)	TT	120 (52.6)	107 (58.8)	51 (66.2)	0.24			0.11		
	TG	97 (42.6)	63 (34.8)	23 (29.9)						
	GG	11 (4.8)	12 (6.4)	3 (3.9)						
	Allele T	340 (73.9)	277 (76.2)	125 (81.2)	0.47	1.12	0.82–1.55	0.069	1.52	0.97–2.40
	Allele G	120 (26.1)	87 (23.8)	29 (18.8)						
+15505 (rs9288953)	TT	77 (33.8)	62 (34.0)	21 (27.0)	0.032			0.14		
	TC	89 (39.0)	89 (48.9)	40 (51.9)						
	CC	62 (27.2)	31 (17.0)	16 (20.8)						
	Allele T	243 (53.3)	213 (58.5)	82 (53.2)	0.13	1.24	0.94–1.63	0.99	1.00	0.69–1.44
	Allele C	213 (46.7)	151 (41.5)	72 (46.8)						
+33830 (rs2171513)	CC	146 (63.9)	118 (64.8)	58 (75.3)	0.54			0.18		
	CT	76 (33.5)	62 (34.2)	17 (22.1)						
	TT	6 (2.6)	2 (1.0)	2 (2.6)						
	Allele C	368 (80.7)	298 (81.9)	133 (86.4)	0.67	1.08	0.76–1.54	0.11	1.51	0.9–2.54
	Allele T	88 (19.3)	66 (18.1)	21 (13.6)						

P-value for each allele distribution was calculated by the chi-square test with 2 × 2 contingency table. After Bonferroni's correction of multiple comparison, a *P*-value < 0.05/3 (= 0.0017) for each allele frequencies and genotypes was considered to be statistically significant.CI, confidence interval; OR, odds ratio; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

Table 2. Summary of the estimated haplotype frequencies of the *BTLA* gene

No.	Haplotype	Control $n = 228$	T1D $n = 182$	SLE $n = 77$	Control versus T1D *P*-value	Control versus SLE *P*-value
1	T-T-C	0.505	0.526	0.5325	0.54	0.79
2	T-C-C	0.194	0.180	0.259	0.61	0.086
3	G-C-C	0.102	0.082	0.072	0.31	0.32
4	G-C-T	0.152	0.105	0.116	0.04	0.46
5	Others*	0.047	0.107	0.020	—	—

After Bonferroni's correction of multiple comparison, a *P*-value < 0.05/8 (= 0.00625) for each haplotype was considered to be statistically significant.
* Other haplotypes include minor haplotypes with their frequencies less than 0.05 in the control population.
SLE, systemic lupus erythematosus; T1D, type 1 diabetes.

tissues. It was weakly expressed on naïve T cells, but was induced during activation and remained expression on T helper type 1 (Th1) but not on Th2 cells (Murphy *et al.*, 2006). BTLA also interacts with herpes virus-entry mediator (HVEM) which belongs to a tumour necrosis factor receptor (TNFR) superfamily, suggesting that there exits a unique crosstalk between these superfamilies (Sedy *et al.*, 2005; Murphy *et al.*, 2006).

In this study, we did not find any significant differences in alleles, genotypes and haplotypes of the *BTLA* gene between T1D or SLE patients and controls therefore the contribution of the *BTLA* gene to the development of T1D and SLE was not apparent. Since two SNPs we analysed located in the intron 1 and one in the 3′ UTR of exon 5, the functional significance of these SNPs would be, if any, small. Otherwise actually functional polymorphisms outside the linkage disequilibrium block consisted of these SNPs or haplotypes might affect the expression level. Further intensive study would be needed to conclude the results about the contribution of *BTLA* gene to the development of T1D or SLE.

There is still possibility that the *BTLA* gene works as a predisposing gene for other autoimmune diseases. Binding of BTLA and its ligand HVEM activated the inhibitory signals of regulatory T cells for preventing intestinal chronic inflammation (Steinberg *et al.*, 2008), accordingly, genetic contribution of *BTLA* gene to the development of chronic inflammatory diseases such as ulcerative colitis or Crohn's disease, would be of interest. Further evaluation in other ethnic groups with a larger study population for other autoimmune diseases may show some different results, and disclose the genetic roles of the *BTLA* gene.

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Expansion of FOXP3-positive CD4$^+$CD25$^+$ T cells associated with disease activity in atopic dermatitis

Yasunori Ito, MD, PhD*; Yuichi Adachi, MD, PhD*; Teruhiko Makino, MD, PhD†; Hiroyuki Higashiyama, MD*; Tatsuya Fuchizawa, MD, PhD*; Tadamichi Shimizu, MD, PhD†; and Toshio Miyawaki, MD, PhD*

Background: FOXP3-positive CD4$^+$CD25$^+$ T cells are known to have an immunoregulatory function by means of preventing T-cell reactivity to both self- and non–self-antigens. However, the role of these cells in the pathogenesis of allergic diseases is not clear.

Objective: To evaluate the quantity and quality of circulating FOXP3-positive T cells in patients with atopic dermatitis (AD).

Methods: Peripheral blood mononuclear cells were isolated from 35 AD patients (mean [SD] age, 27.1 [7.5] years) and 36 controls (mean [SD] age, 27.5 [10.0] years). Cellular FOXP3 expression was analyzed using flow cytometry. Characteristics of FOXP3-positive T cells were evaluated with respect to cytokine production capability and suppressive function.

Results: Frequencies of circulating FOXP3$^+$CD25$^+$ cells in the CD4$^+$ T-cell population of AD patients were significantly higher than those in controls (mean [SD], 7.4% [4.6%] vs 4.5% [1.3%]; $P = .002$) and correlated with their Scoring Atopic Dermatitis (SCORAD) scores ($r = 0.74$, $P = .008$) and peripheral blood eosinophil counts ($r = 0.72$, $P < .001$). In the patients whose samples were analyzed at intervals of 1 to 2 months, frequencies of FOXP3-positive T cells were decreased as their skin lesions improved, regardless of medicines used. FOXP3-positive CD4$^+$ T cells from patients, as well as those from controls, showed little capability to synthesize interferon γ and interleukin 4. No differences were found in suppression abilities of CD4$^+$CD25$^+$ T cells between AD patients and controls.

Conclusions: Our data suggest that dynamic fluctuation in numbers of circulating FOXP3-positive regulatory T cells might contribute to the pathogenesis of AD.

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INTRODUCTION

Immune regulation and tolerance are essential functions of the immune system to prevent and limit harmful immune responses to self- and non–self-antigens. CD4$^+$CD25$^+$ regulatory T (Treg) cells represent a unique lineage of immunoregulatory cells in both humans and animals and play a central role in the maintenance of immunologic self-tolerance. Most recently, FOXP3 has emerged as a master control transcription factor for the generation and function of Treg cells, as illustrated by the demonstration that naturally occurring defects in FOXP3 cause characteristic autoimmune syndrome in mice and humans.[1] Human FOXP3-expressing Treg cells, which are mostly found in the CD4$^+$CD25$^+$ T-cell population, are generated in the thymus and enter the circulation as naive thymus-derived Treg cells, and they convert into memory Treg cells on exposure to specific antigens.[2] FOXP3 expression is also induced in CD4$^+$CD25$^-$ T cells after activation.[3,4] However, activation-induced FOXP3 expression is transient and the cells fail to develop suppressor function, suggesting that a sustained high level of FOXP3 expression is required for Treg phenotype and function.[5] Another source for memory Treg cells, which are generally termed *adaptive Treg cells*, is the generation of FOXP3-expressing Treg cells from FOXP3-negative CD4$^+$ T cells after T-cell receptor stimulation in the periphery.[6,7] These cells control immune responses not only to self-antigens but also to various non–self-antigens, such as alloantigen, exogenous antigens, and tumor antigens. Although these peripherally induced adaptive Treg cells resemble naturally occurring thymus-derived Treg cells in phenotype and aspects of their function, interaction between these 2 populations has not been clarified yet.

Atopic dermatitis (AD) is a common pruritic and chronically relapsing allergic inflammatory skin disease, which is caused by impaired balance of T_H1/T_H2 immune responses to environmental factors in genetically susceptible individuals. Lack of FOXP3$^+$CD4$^+$CD25$^+$ T cells leads to immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome,[8] and affected patients often have AD-like skin lesions, increased IgE levels, and enhanced T_H2 responses.[9] However, conflicting results regarding the numbers and functions of Treg cells in AD have been reported. Verhagen and

Affiliations: * Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; † Department of Dermatology, Graduate School of Medicine, University of Toyama, Toyama, Japan.

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colleagues[10] demonstrated the absence of FOXP3-positive T cells in skin, which suggested a dysregulated control of inflammation. In contrast, Ou and colleagues[11] showed the elevated number of circulating Treg cells with a normal suppressive function in patients with AD, using the expression of CD25 molecule as a marker for Treg cells. In this study, we evaluated the quantity and quality of circulating FOXP3-positive T cells in patients with AD compared with healthy individuals. Using flow cytometric analysis and an anti-FOXP3 monoclonal antibody, we explored whether frequency of FOXP3-positive cells correlated with disease activity and clinical course of AD.

METHODS

Study Participants

Peripheral blood samples were obtained from 35 AD patients (mean age, 27 years; age range, 14–41 years) and from 36 healthy volunteers (mean age, 27 years; age range, 15–49 years) with no history of allergic diseases and autoimmune diseases. Profiles of the AD patients are given in Table 1. The diagnosis of AD was based on Hanifin and Rajka's criteria.[12] No patients had received systemic glucocorticoids or immunosuppressive drugs. Disease activity was evaluated by the Scoring Atopic Dermatitis (SCORAD) index.[13] The study was conducted according to the ethical standards of the University of Toyama, which requires informed consent from each study participant.

Flow Cytometric Analysis

Mononuclear cells (MNCs) were isolated from heparinized blood by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St Louis, Missouri). FOXP3 expression was analyzed by flow cytometry according to the protocol described previously.[4,8] Briefly, fresh MNCs were washed in phosphate-buffered saline, fixed in 1 mL of phosphate-buffered saline with 1% paraformaldehyde and 0.05% Tween 20, and kept overnight at 4°C. Cells were treated twice with 0.5 mL of DNAse (100 Kunitz units/mL) according to the manufacturer's instructions (Sigma-Aldrich). Cells were incubated with a murine anti–human FOXP3 monoclonal antibody (Abcam, Cambridge, England) for 1 hour at room temperature and washed with FACS buffer (phosphate-buffered saline, 3.0% fetal calf serum, 0.5% Tween 20, and 0.05% azide). FOXP3 staining was detected using Alexa Fluor-488 goat anti–mouse IgG antibody (Invitrogen, Eugene, Oregon) and washed as described herein. Cell surface staining was then performed using PC5–conjugated anti–human CD4 (Immunotech, Marseille, France) and phycoerythrin-conjugated anti–human CD25 (Miltenyi Biotec, Bergish Gladbach, Germany) monoclonal antibodies for 20 minutes at room temperature. Cells were analyzed using a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan). Intracellular synthesis of cytokines was evaluated at the single-cell level by a flow cytometric analysis with a modification of the previously described method.[14,15] Briefly, 1×10^6 per well of fresh MNCs were suspended in RMPI 1640 (Nipro K.K., Tokyo, Japan) supplemented with 2 mL of L-glutamine, 10% fetal bovine serum (Equitch-Bio, Ingram, Texas), 2×10^{-5}M 2-mercaptoethanol, 10 U/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, and 10 μg/mL of gentamycin (GIBCO BRL, Grand Island, New York) and then stimulated with 20 ng/mL of phorbol myristate acetate (Sigma-Aldrich) and 1 μg/mL of ionomycin (Sigma-Aldrich) in the presence of 10 μg/mL of brefeldin A (Sigma-Aldrich) for 6 hours at 37°C in 5% carbon dioxide and 95% air. After washing, the stimulated cells were fixed and permeabilized, then stained with anti-FOXP3 monoclonal antibodies as mentioned herein. Finally, intracellular cytokines were stained with phycoerythrin-conjugated anti–interleukin 4 and anti–interferon γ monoclonal antibodies (Immunotech).

Table 1. Characteristics of Study Participants[a]

Characteristic	Patients (n = 35)
Age, mean (SD), y	27.1 (7.5)
M/F	22/13
Allergic disease	
Bronchial asthma	8 (22.8)
Rhinitis	5 (14.3)
Conjunctivitis	1 (2.8)
Treatment	
Oral	
Corticosteroids	0 (0)
Antihistamines	22 (62.8)
Topical	
Corticosteroids	25 (71.4)
Calcineurin inhibitors	6 (17.1)
PUVA therapy	2 (5.7)

Abbreviation: PUVA, psoralen-UV-A.

[a] Data are given as number (percentage) except where indicated otherwise. Of the 36 control subjects, 24 were men and 12 were women (mean [SD] age, 27.5 [10.0] years).

Suppression Assay

In vitro suppression assays were performed as previously reported.[11] Briefly, $CD4^+$ T cells purified by means of negative selection with immunomagnetic beads (by depletion of $CD8^+$, $CD11b^+$, $CD16^+$, $CD19^+$, $CD36^+$, and $CD56^+$ cells) were separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ fractions by positive selection with immunomagnetic beads with a $CD4^+CD25^+$ regulatory T-cell isolation kit (Miltenyi Biotec). The purity of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells was always more than 90%. All steps were performed according to the manufacturer's instructions. To analyze the suppressive capacity of $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells (6×10^4) were stimulated with soluble 1 μg/mL of anti-CD3 (Nichirei, Tokyo, Japan) in the presence of irradiated (30 Gy) autologous MNCs as antigen-presenting cells (6×10^4 per well) for 72 hours. Variable numbers of $CD4^+CD25^+$ T cells were added, giving final $CD25^+$ cell to $CD25^-$ cell ratios of 1:1, 0.5:1, 0.2:1, 0.1:1, or 0:1. Proliferation was determined using a bromodeoxyuridine proliferation enzyme-linked im-

munosorbent assay kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Briefly, bromodeoxyuridine was added into the incubated cells 2 hours before the termination of the culture. After transferring into 96-well flat bottom plates, the cells were treated with FixDenat solution for 30 minutes and were subsequently incubated with anti–bromodeoxyuridine-peroxidase antibodies for 90 minutes at room temperature. Substrate solution was added, and the absorbance of samples was measured with an enzyme-linked immunosorbent assay reader at 450 nm (reference wavelength, 690 nm). Suppression was expressed as percentage proliferation compared with $CD4^+CD25^-$ stimulated T cells.

Statistical Analysis

Comparison between unpaired groups was performed using the Mann-Whitney *U* test. Linear regression analysis was performed to determine whether a relationship existed among frequencies of FOXP3-positive $CD4^+CD25^+$ T cells, serum IgE levels, peripheral eosinophil counts, and SCORAD scores. Differences were considered statistically significant at $P < .05$.

RESULTS

FOXP3-Positive $CD4^+CD25^+$ T Cells in the Peripheral Blood of Patients With AD

Flow cytometric analysis of peripheral blood mononuclear cells revealed that FOXP3 expression was detectable mainly within the $CD4^+CD25^+$ T-cell population and only rarely in $CD4^+CD25^-$ T cells (Fig 1-A). It was marked that percentages of FOXP3-positive $CD25^+$ cells within the circulating $CD4^+$ T cells in patients with AD were significantly higher than those in control subjects (mean [SD], 7.4% [4.6%] vs 4.5% [1.3%]; $P = .002$) (Fig 1B). Because our clinical observation suggested that an increase of FOXP3-positive $CD4^+CD25^+$ T cells might be related to the severity of AD, we evaluated the relationship between the percentage of FOXP3-positive cells and disease activity. Frequencies of FOXP3-positive $CD4^+CD25^+$ T cells significantly correlated with SCORAD scores ($r = .74$, $P = .008$) and peripheral blood eosinophil counts ($r = .72$, $P < .001$) but not with serum IgE levels ($r = .22$, $P = .37$) (Fig 2). We next examined whether numbers of circulating FOXP3-positive $CD4^+CD25^+$ T cells in AD patients were changing during their clinical course (Table 2). Of 35 patients, 6 had been chosen because of their poor adherence to treatment. After thorough education, adherence improved except for one, and these samples were reanalyzed 1 to 2 months later. Regardless of medicines used for the treatment, frequencies of FOXP3-positive $CD4^+CD25^+$ T cells clearly decreased in 5 patients whose skin lesions were improved as their SCORAD scores decreased but increased in 1 patient who had refused treatment and experienced deterioration of the skin symptoms (patient 1).

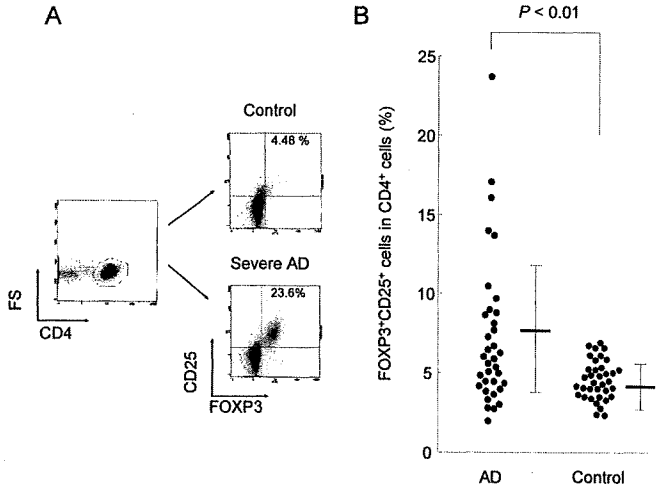

Figure 1. Flow cytometric analysis of FOXP3 expression in circulating $CD4^+$ T cells. A, Representative data of a healthy individual and a patient with severe atopic dermatitis (AD). The numbers in the upper right quadrant indicate percentages of FOXP3-positive $CD25^+$ T cells in a $CD4^+$ T-cell population. B, Percentages of FOXP3-positive $CD25^+$ T cells in a $CD4^+$ T-cell population of patients with AD (n = 35) and control subjects (n = 36). Thick horizontal bars indicate the mean for each group, and error bars indicate the SD.

Phenotypes of FOXP3-Positive $CD4^+CD25^+$ T Cells in Patients With AD

We next sought to determine whether expanded FOXP3-positive $CD4^+CD25^+$ T cells of AD patients elicited the characteristics compatible with Treg cells. It has been shown that FOXP3-positive Treg cells lack an ability to synthesize T_H1 and T_H2 cytokines by themselves[16]; thus, we evaluated cytokine production patterns of circulating T cells on stimulation with phorbol myristate acetate and ionomycin. Extent of FOXP3 expression was not affected by these stimuli. Although patients with AD had a higher ratio of interleukin 4– and interferon γ–producing $CD4^+$ T-cell numbers, reflecting their T_H2-skewed conditions, FOXP3-positive $CD4^+$ T cells from patients, as well as those from control subjects, showed little capability to synthesize both cytokines (Fig 3A

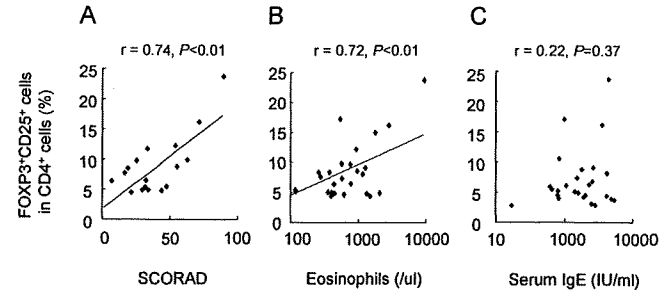

Figure 2. Correlations between the frequencies of FOXP3-positive $CD25^+$ T cells in a $CD4^+$ T-cell population of patients with atopic dermatitic and their Scoring Atopic Dermatitis (SCORAD) scores (A; n = 17), peripheral blood eosinophil counts (B; n = 29), and serum IgE levels (C; n = 27).

Table 2. Profiles of 6 Patients Whose Samples Were Analyzed Twice

Patient No./ sex/age, y	SCORAD score		FOXP3+CD25+ cells, %		Treatment			Interval, mo
	First	Second	First	Second	TCS	TCI	PUVA	
1/M/31	53.5	79.0	10.8	12.9	–	–	–	2
2/M/24	17.0	12.5	8.7	6.7	+	+	–	1
3/M/34	64.5	54.4	8.5	7.2	–	+	–	1
4/F/14	66.6	37.1	7.2	6.9	+	–	–	1
5/M/33	82.8	41.2	14.9	6.9	+	–	–	1
6/F/29	89.0	21.2	23.6	7.3	+	–	–	2

Abbreviations: SCORAD, Scoring Atopic Dermatitis; TCS, topical corticosteroids; TCI, topical calcineurin inhibitor; PUVA, psoralen-UV-A.
Symbols: Minus sign, negative; plus sign, positive.

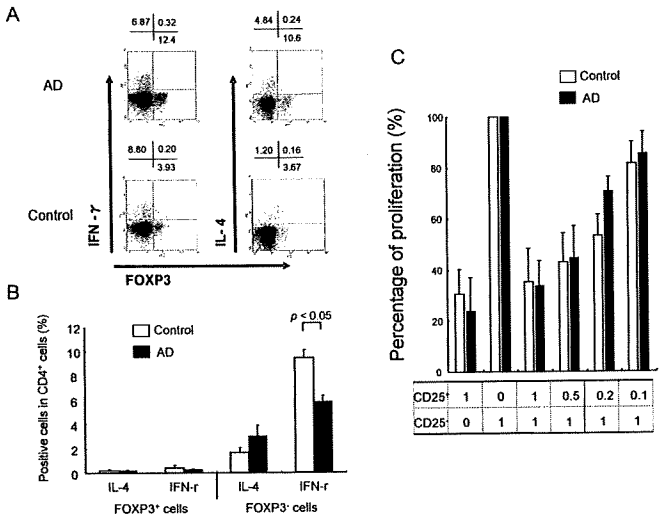

Figure 3. Phenotypes of FOXP3-positive CD4+ T cells in patients with atopic dermatitis (AD). Flow cytometric analysis of intracellular interferon γ and interleukin 4 production of CD4+ T cells. A, Representative data of 3 patients with AD and 3 control subjects. The numbers in each quadrant indicate percentages of the respective subpopulation in gated CD4+ T cells. B, Combined data with SEM within each cell population. C, Suppressive capabilities using CD4+CD25+ T cells as a surrogate for FOXP3-expressing T cells. Variable numbers of CD4+CD25+ T cells were added, giving the indicated final CD25+ to CD25− cell ratios. Suppression was expressed as percentage proliferation compared with CD4+CD25− stimulated T cells. Results (mean ± SEM) are shown for patients with AD (n = 3) and control subjects (n = 3).

and B). It also has been known that FOXP3-positive Treg cells have an ability to suppress the activation and proliferation of other T cells in a contact-dependent manner.[17] In vitro suppression assay was performed using CD4+CD25+ T cells as a surrogate for FOXP3-expressing Treg cells. Proliferation of CD4+CD25− T cells in response to soluble anti-CD3 monoclonal antibody in the presence of accessory cells was suppressed as the numbers of cocultured CD4+CD25+ T cells increased. No differences were found in the suppression abilities of CD4+CD25+ T cells between AD patients and control subjects (Fig 3C).

DISCUSSION

There is accumulating evidence that Treg cells participate in allergic diseases.[18] In this study, we demonstrated that there was an increase in the numbers of FOXP3-positive CD4+CD25+ T cells in the peripheral blood of patients with AD using FOXP3 monoclonal antibody. Although Ou and colleagues[11] showed similar results, the reported frequencies of Treg cells from patients with allergic diseases are inconsistent. Children with asthma had decreased numbers of CD4+CD25+ T cells compared with healthy individuals without allergy.[19] In adults, the rate of FOXP3+ cells in CD4+ cells was lower in symptomatic patients with AD and asthma compared with that in healthy volunteers who had similar levels of IgE and eosinophils.[20] In contrast, other studies showed that numbers of circulating CD4+CD25+ T cells in patients with allergic rhinitis[21] and AD[22] were comparable to those in nonatopic individuals. The differences in these observed frequencies might be related to the disease status. Pediatric patients with more severe asthma had more CD4+CD25+ T cells in their peripheral blood than did patients with less severe disease, and there was a positive correlation between numbers of CD4+CD25+ T cells and serum IgE levels.[19] These findings are consistent with ours in that the frequencies of FOXP3-positive CD4+CD25+ T cells in patients with AD were correlated with their SCORAD scores and peripheral eosinophil counts, which reflect disease severity. Also, frequencies of FOXP3-positive CD4+CD25+ T cells in AD patients of this study normalized after short periods as their skin lesions were improved. These rapid changes in numbers of FOXP3-positive CD4+CD25+ T cells might be explained by rapid peripheral turnover of Treg cells, which was reported by Vukmanovic-Stejic and colleagues.[23] They explored the origin of circulating FOXP3-expressing Treg cells and demonstrated that these cells are continuously recruited from the memory CD4+ T-cell pool and are susceptible to apoptosis. It also has been reported that FOXP3 is transiently induced by stimulation of anti-CD3 and CD28 in FOXP3-negative CD4+CD25− T cells, but this transient expression is not able to lead to the suppression of cytokine production in vitro.[24,25] However, expanded FOXP3-positive CD4+CD25+ T cells in AD patients of this study were hyporesponsive for proliferation and cytokine production, and

their suppressive capacity was equal to healthy volunteers, suggesting that these cells could be functional as Treg cells.

Treatments for AD, such as glucocorticoid steroids and immune suppressants, might affect dynamics of Treg cell expansion. It had been reported that adult asthmatic patients receiving inhaled or systemic glucocorticoid had higher FOXP3 messenger RNA expression in circulating $CD4^+$ T cells compared with that seen in untreated asthmatic patients or healthy volunteers[26] and that 4 weeks of treatment with inhaled glucocorticoids increased percentages of $CD4^+CD25^+$ T cells and FOXP3 messenger RNA expression levels in peripheral blood and bronchoalveolar lavage fluid.[27] In contrast, Caproni and colleagues[28] performed immunohistochemical evaluation of eczematous skin lesions before and after topical treatment with calcineurin inhibitor or corticosteroid and demonstrated that both treatments did not modify the number of FOXP3-positive cells in the skin. In this study, increased numbers of FOXP3-positive cells in AD patients clearly decreased as their skin lesions improved even when they were treated with topical glucocorticoids or calcineurin inhibitor. These results suggest that FOXP3-positive Treg cells could be expanded in response to systemic and local allergic inflammation and might play a role in the negative feedback loop of T-cell activation.

It is not clear whether our results obtained using peripheral blood T cells directly reflect the topical inflammatory response in the skin. Verhagen and colleagues reported the absence of FOXP3-positive T cells in AD skin specimens.[10] However, Treg cells express a wide variety of trafficking receptors, including CLA, CCR4, and CCR6, which are well known as skin homing receptors.[29,30] Recent literature shows that FOXP3-positive cells are present in the skin of inflammatory dermatoses, including AD and psoriasis.[28,31–33] Interestingly, Chen and colleagues[34] demonstrated that frequencies of Treg cells in the peripheral blood and skin concordantly changed with the clinical features of psoriasis. Consistent with our findings, Reefer and colleagues[35] recently reported that $CD25^{high}$ $CD4^+$ T cells in the peripheral blood of AD patients were increased compared with control subjects and that the frequency of $CD25^{high}$ $CD4^+$ T cells decreased after allergen avoidance in parallel with improvement of skin condition. Furthermore, they demonstrated that among these increased cells CCR6-negative cells had capability to promote T_H2 responses, speculating that activated $CD25^{high}$ T cells in the skin might lose expression of CCR6 and leave the skin to join the circulation. Analysis of T cells at the skin level in the context of skin homing receptors, including CCR6, may provide insights into the role of FOXP3-positive T-cell subtypes in immune homeostasis at this site. Collectively, dynamic migration of FOXP3-positive Treg cells may contribute to the pathogenesis of AD. Further studies are required to improve our understanding of the role of Treg cells in allergic inflammation.

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Requests for reprints should be addressed to:
Yuichi Adachi, MD, PhD
Department of Pediatrics
Graduate School of Medicine
University of Toyama
2630 Sugitani
Toyama 930-0194, Japan
E-mail: yadachi@med.u-toyama.ac.jp

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A novel immunoregulatory protein in human colostrum, syntenin-1, for promoting the development of IgA-producing cells from cord blood B cells

Mostafa M. Sira[1], Taketoshi Yoshida[1], Makoto Takeuchi[2], Yoshinori Kashiwayama[3], Takeshi Futatani[1], Hirokazu Kanegane[1], Akiko Sasahara[1], Yasunori Ito[1], Mineyuki Mizuguchi[2], Tsuneo Imanaka[3] and **Toshio Miyawaki[1]**

[1]Department of Pediatrics, [2]Department of Structural Biology and [3]Department of Biological Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan

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Abstract

Human colostrum contains many bioactive factors that must promote the development of intestinal mucosal immunity in infants. Especially, the presence of certain cytokines such as transforming growth factor (TGF)-β or IL-10 has been of great interest for IgA production as a function of mucosal immune response. In the present study, we attempted to investigate whether unidentified factors inducing generation of IgA-producing cells from naive B cells might exist in colostrum. For this purpose, colostrum samples were directly added to a culture consisting of naive B cells and dendritic cells from cord blood and CD40 ligand-transfected L cells, comparing with recombinant IL-10 (rIL-10) and/or rTGF-β. It was noted that most colostrum samples alone were able to induce IgA-secreting cells at higher levels than rIL-10 and/or rTGF-β. IgA-inducing activity of colostrum was abolished by neither anti-neutralizing mAbs against IL-10 nor TGF-β, though partially by anti-IL-6 mAb. We prepared partially purified fractions from both pooled colostrums with and without IgA-inducing activity and comparatively performed quantitative proteomic analysis by two-dimensional difference gel electrophoresis followed by liquid chromatography-mass spectrometry. As a result, syntenin-1 was identified as a candidate for IgA-inducing protein in colostrum. Western blot analysis indicated that levels of syntenin-1 in colostrum samples were correlated with their IgA-inducing activities. Moreover, we demonstrated that recombinant syntenin-1 could induce preferentially IgA production from naive B cells. These results suggest that syntenin-1 serves as one of IgA-inducing factors for B cells.

Introduction

There is ample evidence that, in addition to the essential nutrients, human breast milk contains a variety of bioactive components such as soluble proteins like IgA or lactoferrin, anti-microbial peptides, cytokines, growth factors, nucleotides and immune competent cells, seemingly compensating physiological delay of the immune responses in growing infants (1, 2). This has been supported by many epidemiological and clinical studies that breastfeeding attenuates the susceptibility to infectious illnesses or certain diseases and the development of allergic disorders in infants (3–5). Colostrum is the first milk produced after birth and is particularly rich in non-nutritional compositions (2). Thus, colostrum is often used to search for some milk components, which must be important for facilitating optimal maturation of the immune responses in the early period of human life (1).

IgA is the predominant Ig isotype in mucosal secretions and plays a crucial role in the first defense against pathogens at the mucosal surfaces. How naive B cells in the mucosal tissues differentiate into IgA-producing cells through Ig class-switch process has been steadily understood (6). The IgA class switch appears to be mediated by T-cell-dependent and T-cell-independent pathways. It has been

Correspondence to: T. Yoshida; E-mail: ytake@med.u-toyama.ac.jp

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shown that the ligation of CD40–CD40 ligand (CD40L) between B cells and activated T_h cells triggers the Ig class switch (7, 8) and also that certain cytokines appear to be requisite for its completion (9). Among them, transforming growth factor (TGF)-β is considered to be an essential IgA class-switch factor (10, 11). Other cytokines such as IL-2, IL-5, IL-6 or IL-10 may be important for terminal differentiation of naive B cells into IgA-producing cells as well (12–16). These cytokines have been well known to be present in human breast milk (17), implying the involvement for IgA production by infants as a function of mucosal immune response. Regarding the T-cell-independent pathway leading to IgA class switch and production, recent studies have shown that naive B cells can be triggered through production of a proliferation-inducing ligand and a B-cell-activating factor of the tumor necrosis factor (TNF) family by dendritic cells (DCs), macrophages and intestinal epithelial cells (18–20). In the present study, we have identified a novel factor in human colostrum, syntenin-1, as a candidate of IgA-inducing factor for naive B cells. We also showed that recombinant syntenin-1 could induce preferentially IgA production from naive B cells under culture conditions without other known cytokines. The results suggest that syntenin-1 serves as one of IgA-inducing factors for B cells.

Methods

Colostrum samples

Thirty-five mothers with their full-term newborn infants were recruited from Okada Obstetrics Hospital (Toyama, Japan). After the written informed consent was taken, colostrum samples (~10 ml) were collected from the mother within 7 days postpartum and immediately stored at −70°C. For culture use, each sample was thawed, and the fatty layer and cellular elements were removed by centrifugation at 3000 r.p.m. for 30 min at 4°C. Samples were then dialyzed with PBS for 1 day and next RPMI 1640 for 3 days using Spectra/Pro® Membrane with molecular weight cut off 1000 Da (Spectrum Laboratories, Rancho Dominguez, CA, USA) divided into small aliquots and stored at −70°C until the time of use. This study was approved by the Research Ethic Committee of University of Toyama.

Flow cytometry

Anti-CD19-PE, anti-CD34-FITC and anti-CD1a-FITC mAbs were obtained from DAKO A/S (Carpentaria, CA, USA), and goat anti-human IgA-FITC and IgD-FITC Abs from Southern Biotechnology Associates (Birmingham, AL, USA). Flow cytometric analysis was performed using Coulter EPICS® XL-MCL. System II™ software (Version 3) was used for data processing.

Preparation of naive B cells and DC from cord blood

Cord blood samples were obtained from the umbilical cord of full-term newborns after uneventful delivery. Mononuclear cells (MNC) were isolated from cord blood by the Ficoll-Paque density gradient centrifugation. B cells were purified from cord blood MNC by T-cell depletion with E-rosetting followed by magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Auburn, CA, USA). IgD was expressed on >99% of the B cell population as assessed by a flow cytometer, indicating naive B cells. DCs were generated by the cultures of $CD34^+$ cells isolated from cord blood MNC by MACS in the presence of 100 ng ml^{-1} recombinant granulocyte macrophage colony-stimulating factor, 2.5 ng ml^{-1} rTNF-α and 25 ng ml^{-1} recombinant stem cell factor (all from R&D, Minneapolis, MN, USA) as described previously (21). CD1a was expressed on >86% of the DC population after 12–14 days assessed by a flow cytometer.

CD40L culture system

Induction of Ig-secreting cells (SC) from cord blood B cells was carried out in the CD40L culture system as described (21). The cultures were performed in Iscove's modified Dulbecco's medium supplemented with 50 μg ml^{-1} human transferrin, 5 μg ml^{-1} bovine insulin (all from Sigma, St Louis, MO, USA), 5% FCS (Flow Laboratories) and 10 μg ml^{-1} gentamicin (GIBCO, Carlsbad, CA, USA). A combination of 1×10^5 ml^{-1} B cells and 1×10^5 ml^{-1} DC (irradiated at 3400 rad) were cultured in the presence of 3.75×10^4 ml^{-1} irradiated (7500 rad) CD40L-transfected L cells (a gift of Yong-Jun Liu, DNAX, Palo Alto, CA, USA) in a final volume of 200 μl in 96-well flat microtiter plates (Becton Dickinson, Franklin Lakes, NJ, USA). Colostrum (25% vol/vol) alone, recombinant IL-10 (rIL-10) (200 ng ml^{-1}) and/or rTGF-β1 (0.3 ng ml^{-1}) or rIL-6 (0.3 ng ml^{-1}) (all obtained from R&D Systems) were added to the culture. In some experiments, neutralizing mAbs against human cytokines such as IL-10, latency-associated protein TGF-β1, IL-1β or IL-6 (all obtained from R&D systems) were added to the cultures with colostrum. The cultured cells were harvested on the 6th day for evaluation of IgG, IgA and IgM SC as below.

Enzyme-linked immunosorbent spot

IgG, IgA and IgM SC were evaluated by enzyme-linked immunosorbent spot (ELISPOT). Briefly, cultured cells were added to the ELISPOT plate coated with goat $F(ab')_2$ anti-human Ig (H+L) antibody (Southern Biotechnology Associates). After discarding cells, the plates were washed and biotinylated goat $F(ab')_2$ anti-human IgG, anti-human IgA and anti-human IgM antibodies (all from Southern Biotechnology Associates) were used for secondary antibody. The plates were incubated with avidin–biotin–peroxidase complex in PBS (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), and 3-amino-9-ethylcarbazole substrate (Sigma) was used for development. Spots were counted using computer-aided ELISPOT manual counting system, (ELIPHOTO, Minerva Tech, Tokyo, Japan).

Partial purification of the colostrum factor that induces IgA SC

Pooled colostrum with IgA-inducing activity, termed active samples, was first applied to a Superdex 200, 10/300 GL column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM ammonium hydrogen carbonate. The column was eluted with the same buffer at a flow rate of 0.4 ml min^{-1} and the concentration of eluted proteins was monitored by UV detector. Twenty-four fractions (1 ml each) were collected and aliquot of each fraction was tested in the B cell culture and ELISPOT assay for IgA-inducing activity as described above. Fractions with IgA-inducing activity were

pooled and applied to a UNO Q1 anion exchange column (Bio-Rad, Hercules, CA, USA) equilibrated with 20 mM ammonium hydrogen carbonate. Then, absorbed proteins were eluted with a linear concentration gradient of 0–1 M NaCl and 45 fractions were collected. Aliquot of each fraction was dialyzed against 20 mM ammonium hydrogen carbonate and was freeze-dried. The fractions were tested for IgA-inducing activity and active fractions were pooled and applied to a second Superdex 200, 10/300 GL column equilibrated with 20 mM ammonium hydrogen carbonate. Eluted factions were tested for activity. The active fractions were pooled and stored at −70°C. Another pooled colostrum without IgA-inducing activity, termed non-active samples, was partially purified by the same procedures. The fractions of the non-active samples in the second gel filtration chromatography (GFC) corresponding to the fractions with IgA-inducing activity of the active sample were also pooled. Eluted fractions (of both the active and non-active samples) were applied to SDS–PAGE and the gels were stained with silver.

Two-dimensional difference gel electrophoresis and protein identification

The procedure has been described in details elsewhere (22). Partially purified proteins in active and non-active samples were labeled with Cy5 and Cy3 in a ratio 100 μg of proteins to 200 pmol of dye, respectively. A pool of the two fractions was labeled with Cy2 as internal standard on each gel. The labeled samples were mixed with equal volume of 2× sample buffer [8M urea, 4% (wt/vol) CHAPS, 20 mg ml^{-1} dithiothreitol (DTT) and 2% (vol/vol) immobilized pH gradient (IPG) buffer] (GE Healthcare). Labeled samples were combined (50 μg each labeled with Cy5, Cy3 and Cy2 per gel) and subjected to iso-electric focusing (IEF) on a MultiPhor II electrophoresis unit (GE Healthcare) using IPG strips (24 cm, pI 3–10, GE Healthcare). IEF was performed for 40KV hr at 20°C in dark condition. The strips were equilibrated for 10 min in 50 mM Tris–HCl pH 8.8, 6 M urea, 30% (vol/vol) glycerol and 2% SDS containing 65 mM DTT and then for 10 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 24 × 20 cm, 12% polyacrylamide gels made between low fluorescence glass plates. Strips were overlaid with 0.5% (wt/vol) low melting point agarose in 25 mM Tris-base, 0.1% SDS and 192 mM glycine containing 0.1% bromophenol blue. Gels were run in Ettan DALTTM II system (GE Healthcare) with 3W per gel at 15°C, until the dye front had run off the bottom of the gels. The 2-D gels were scanned directly with a 2920 2D-Master Imager (GE healthcare). Normalization among three Cy dyes was accomplished by adjusting the maximum pixel values to 55 000 counts with changing the exposure time. The image generated was exported as tagged image format (.tif) files for further protein profile analysis by Decyder software (GE Healthcare). The difference in-gel analysis of DeCyder was used to merge the Cy2, Cy3 and Cy5 images for each gel and detect spot boundaries for calculation of normalized spot volumes/protein abundance. A spot matching between gels and statistical analysis was done by the Decyder biological variation analysis (BVA) software (GE Healthcare). For in-gel digestion, spots of interesting proteins were excised from 2-D gels using an automated spot picker (GE Healthcare). The spots were collected in 200 μl of water in 96-well plates. The recovered gel pieces are washed with aqueous 500 mM ammonium bicarbonate and acetonitrile and then incubated with 12.5 ng μl^{-1} trypsin at 30°C for 15 h. The generated peptides were concentrated and mass spectrometry (MS) analysis was carried out by liquid chromatography-mass spectrometry (LC-MS/MS) (22). HPLC (CapLC, Waters, Milford, MA, USA) was coupled with the quadrupole-time of flight micro mass spectrometer (Micromass, Milford, MA, USA). Instrument operation, data acquisition and analysis were performed using MassLynx 3.2 software (Micromass).

Western blot

Proteins were separated on 10–20% SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with rabbit antibodies against human syntenin-1 (H-48) and lactadherin (H-60) obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bound antibody was detected with HRP-conjugated secondary goat anti-rabbit antibody (Biosource International, Carlsbad, CA, USA) and an enhanced chemiluminescence detections system (GE Healthcare).

Preparation of recombinant proteins

To prepare glutathione *S*-transferase (GST)-syntenin-1 and maltose-binding protein (MBP)-lactadherin fusion proteins, entire coding regions of human syntenin-1 (GeneBank accession number; BC013254) and human lactadherin (GeneBank accession number; U58516) were amplified by PCR using complementary DNA (cDNA) library from PBMC. The PCR-generated fragment of syntenin with *Bam*HI and *Not*I restriction sites was subcloned in frame into pGEX-5X-2 vector (GE Healthcare). The fragment of lactadherin with *Xba*I and *Hin*dIII restriction sites was subcloned into pMAL-C2X vector (New England Biolabs, Ipswich, MA, USA). The oligonucleotide primers used for construction of plasmid were as follows: syntenin-1 forward primer, 5′-CGCGGATCCCCTCTCTCTATCCATCTCTCGAAGAC-3′; syntenin-1 reverse primer, 5′-ATAAGAATGCGGCCGCTTAAACCTCAGGAATGGTGTGGTC-3′; lactadherin forward primer, 5′-CTAGTCTAGAATGCCGCGCCCCCGCCTGCTGGCCGCGCTG-3′ and lactadherin reverse primer, 5′-CAGGCAAGCTTCTAACAGCCCAGCAGCTCCAGGCGCAGGGC-3′. *Pfu* polymerase (Stratagene, La Jolla, CA, USA) was used for all the PCR amplification. The identity of subclones was confirmed by semiautomated sequencing on an ABI 310 DNA sequencer (Perkin Elmer Life Science, Waltham, MA, USA). The plasmids pGEX-5X-2/syntenin-1 and pMAL-C2X/lactadherin were transfected to competent BL21 (DE3) *Escherichia coli* cells (Stratagene) according to standard procedures. The *E. coli* cells harboring pGEX-5X-2/syntenin-1 and pMAL-C2X/lactadherin were grown at 37°C in LB media containing 0.1 μg ml^{-1} ampicillin. At a cell density of 0.5 (OD600), protein expression was induced with isopropyl β-D-thiogalactoside (IPTG) for 3 h. The cells expressing GST-syntenin-1 were harvested by centrifugation at 4000 × *g* for 20 min, resuspended in the washing buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, supplemented with the proteinase inhibitors: 3 mM aprotinine, 5 mM benzamidine, 20 mM leupeptine, 3 mM pepstatin, 5 mM 6-aminohexanoic acid and 200 mM phenylmethylsulfonyl fluoride). The cells

were disrupted 20 times for 20 s in an ice bath by Astrason XL-2020 ultrasonic processor (Misonix, Farmingdale, NY, USA). The lysate was centrifuged at 20 000 × *g* for 30 min and GST-syntenin-1 fusion protein in the supernatant was immediately applied to glutathione-Sepharose 4B column (GE Healthcare) equilibrated with the washing buffer. After extensive washing, the GST-syntenin-1 was eluted with the washing buffer containing 10 mM glutathione. In the case of MBP-lactadherin, the cell lysate in the column buffer (20 mM Tris–HCl pH 7.4, 200 mM NaCl and 1 mM EDTA containing above proteinase inhibitors) was applied to amylose resin (New England BioLabs) equilibrated with the column buffer. After extensive washing, MBP-lactadherin was eluted with the column buffer containing 10 mM maltose. The purity of the fusion proteins was confirmed by SDS–PAGE and Coomassie Brilliant Blue staining and western blot using specific antibodies against syntenin-1 and lactadherin.

Semiquantitative reverse transcription–PCR

The messenger RNA (mRNA) was isolated from naive B cells in the CD40L culture system treated with IL-10 (200 ng ml^{-1}) or GST-syntenin-1 (5 μg ml^{-1}) for 6 days using QIAamp RNA Blood Mini (QIAGEN). cDNA was synthesized from total RNA using SuperScript™ III First-Strand Synthesis System (Invitrogen). Semiquantitative reverse transcription (RT)–PCR was performed to evaluate activating-induced cytidine deaminase (AID), which is crucial for class-switch recombination. PCR conditions were: 5 min denaturation at 95°C, amplification of cDNA for 30 cycles, each cycle programmed for denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min 10 s and followed by a final extension phase of 15 min at 72°C. The primers for AID were: forward primer 5′-GAGGCAAGAAGACACTCTGG-3′ and reverse primer 5′-GTGACATTCCTGGAAGTTGC-3′. The PCR products were separated on 2% agarose gel electrophoresis.

Statistical analysis

Each experiment was performed three to four times, and data were presented as mean ± SEM. The significance between different culture conditions, we used non-parametric test (Mann–Whitney *U* test), and correlation was tested by Pearson's χ^2 test. Results were considered significant if $P \leq 0.05$ ($^{**}P \leq 0.01$ and $^{*}P \leq 0.05$). Statistical analysis was done using SPSS software.

Results

Colostrum alone can induce the generation of IgA SC from cord blood naive B cells in the CD40L culture system

We employed the CD40L culture system as described (21) to evaluate the generation of IgA SC from cord blood naive B cells. For this, cord blood B cells were cultured in the presence of DC and CD40L-transfected L cells. Colostrum samples alone were added to the B cell culture, and the added effect of each sample was compared with that of rIL-10, which is a well-known potent IgA-inducing factor (21). As expected, the addition of rIL-10 to the culture resulted in the appreciable generation of IgA as well as IgG and IgM SC. We found that most colostrum samples alone were able to induce the generation of IgA SC at levels comparable to and higher than rIL-10 (Fig. 1). In contrast, nearly all colostrum samples induced lower IgG and IgM SC compared with that induced by rIL-10. Next, we prepared the pooled colostrum sample from five donors showing strong IgA-inducing activity, which we called the active sample. The added effect of this pooled active colostrum sample on the generation of IgA, IgM and IgG SC in the same culture was compared with that of rIL-10 and/or rTGF-β1 or rIL-6 (Fig. 2). Although rIL-10 induced IgG, IgA and IgM SC similarly, it was noted that the pooled active colostrum alone preferentially induced the generation of IgA SC. Human colostrum has been observed to contain approximately equal levels of IgA1 and IgA2 (23), which was confirmed for the pooled active colostrum used here. Nevertheless, regarding subclasses of IgA SC, we found that IgA1 SC were mainly induced by the addition of the pooled active colostrum to the cultures. The similar results were obtained in the cultures with rIL-10 (data not shown). Neither rTGF-β1 nor rhIL-6 induced IgA SC, and a combination of IL-10 and TGF-β1 did not show the enhanced generation of IgA SC as well. To examine the possible roles of IL-10, TGF-β, IL-6 or IL-1β for IgA-inducing activity seen in colostrum, we added neutralizing mAbs against cytokines such as IL-10, TGF-β1, IL-6 or IL-1β to the B cell cultures with the pooled active colostrum sample. As shown in Fig. 3, there was no effect of antibodies against IL-10, TGF-β1 or IL-1β on IgA induction by colostrum. However, it was observed that anti-IL-6 mAb partially inhibited IgA induction by colostrum, suggesting that IL-6 might contribute partly to IgA-inducing activity in colostrum. Based on these observations, we suspected the existence of other IgA-inducing factors in colostrum.

Partial purification of a factor that induces IgA SC from colostrum

In preliminary experiments, we found that IgA-inducing factors in colostrum might be proteinaceous, since IgA-inducing activity in colostrum was abolished by

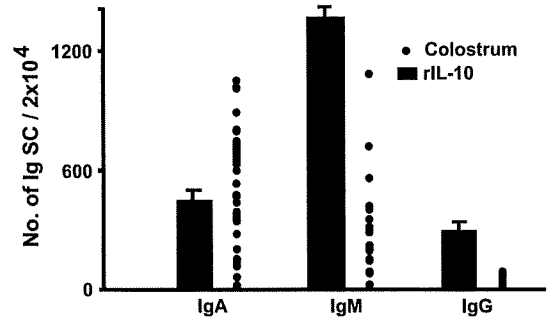

Fig. 1. The influence of colostrum samples on generation of IgA, IgM and IgG SC from cord blood B cells in the CD40L culture system. Thirty-five different colostrum samples (25% vol/vol) or rIL-10 (200 ng ml^{-1}) were added to the B cell culture and each Ig SC was examined on the 6th day by ELISPOT. It was notable that the addition of most colostrum samples to the culture resulted in the predominant generation of IgA SC. In contrast, rIL-10 induced IgA, IgM and IgG similarly. Values in the cultures with rIL-10 are means ± SEMs (n = 3). Results are representative of three separate experiments.

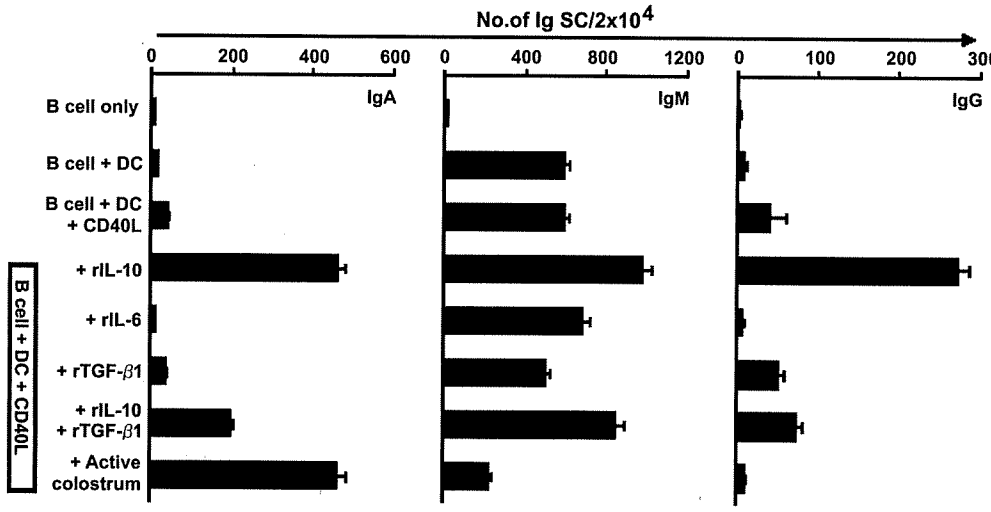

Fig. 2. Preferential IgA induction by the active pooled colostrum. Generation of Ig SC in the CD40L culture system was evaluated as described in the legend of Fig. 1. Cord blood B cells were cultured in different conditions with the active pooled colostrum, rIL-10 and/or rTGF-β1 or rIL-6. Whereas the presence of rIL-10 in culture induced the generation of IgA, IgM and IgG SC similarly, the cultures with the pooled active colostrum sample resulted in the preferential generation of IgA. On the other hand, rIL-6 and rTGF-β1 did not promote IgA production. Values are means ± SEMs (n = 3). Results are representative of three separate experiments.

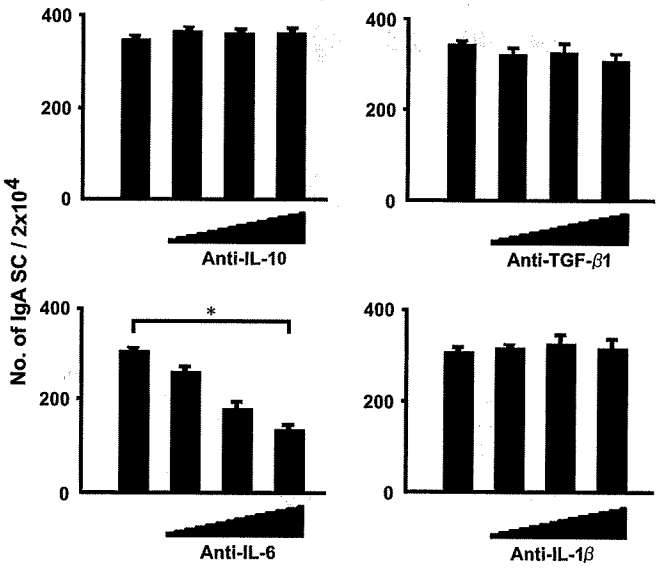

Fig. 3. The added effect of neutralizing mAbs against IL-10, IL-6, TGF-β1 or IL-1β on IgA-inducing activity of colostrum. All cultures were performed in the CD40L culture system with the pooled active colostrum as described in the legend of Fig. 1. The first bar indicates the cultures without neutralizing mAbs, and the other bars represent the added effect of different concentrations of neutralizing antibodies. It was found that anti-IL-10 mAb (0.1, 0.5 and 2.5 μg ml^{-1}), anti-LAP TGF-β1 mAb (0.2, 10 and 50 μg ml^{-1}) or anti-IL-1β mAb (0.2, 0.4 and 0.8 μg ml^{-1}) showed no effect on IgA-inducing activity of colostrum. On the other hand, anti-human IL-6 mAb inhibited partially IgA induction of colostrum in increasing doses (0.2, 0.4 and 0.8 μg ml^{-1}), $*P < 0.05$. Values are means ± SEMs (n = 3). Results are representative of three separate experiments.

pre-treatment with trypsin and chymotrypsin. In addition, the factor was stable through freeze-drying. The pooled active colostrum sample was applied to a GFC. The IgA-inducing activity was eluted as a single peak after a slight delay from the void volume. The molecular mass of the activity was estimated to be ≥440 kDa, suggesting that it might be associated with macromolecules. The activity recovered from GFC was applied to a UNO Q1 anion exchange column. The activity was absorbed to the resin and eluted with 0.4 M NaCl as a single peak. The activity was applied to a second GFC (Fig. 4A). IgA-inducing activity was eluted as a single peak with molecular mass of ≥440 kDa (Fig. 4C). The activity was purified 13.6 times and recovery of the activity was 60%. As we found that some colostrums did not contain IgA-inducing activity, we also prepared non-active fractions by the same procedure (Fig. 4B). IgA-inducing activity fraction and the corresponding non-active one were applied to SDS–PAGE and stained with Cy5. The protein pattern of both partially purified fractions was nearly identical (short time exposure to Cy5) and major band of proteins were estimated to be secretory component or lactoferrin (upper band), IgA heavy chain (middle band) and casein or IgA light chain (lower band), respectively, according to their molecular mass and abundance in colostrum (Fig. 4D). The activity seemed to be associated with minor proteins and further purification was required.

Identification of a factor by two-dimensional difference gel electrophoresis followed LC-MS/MS analysis

Partially purified proteins from active and non-active colostrum samples were labeled with different fluorescent dyes and then mixed together and electrophoretically separated in the same 2-D gel. The Cy5 (Fig. 5A) and Cy3 (Fig. 5B) images are corresponding to active and non-active fractions, respectively. Differences in spot population and intensity were visible between two images. Image analysis, i.e. spot detection, within gels and as well as between gels matching with subsequent determination of significant alterations in protein abundances based on normalization by the internal standard was performed by DeCyder BVA. We identified 37