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Clinical and Genetic Features of Familial Mediterranean Fever in Japan

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ABSTRACT. ***Objective.*** Familial Mediterranean fever (FMF) is thought to be a rare disorder in Japan, and the clinical features of Japanese patients with FMF remain unclear. Our aim was to elucidate the clinical characteristics of FMF in Japanese patients.

Methods. We analyzed clinical and genetic data of 80 patients based on the results of a nationwide questionnaire survey and review of the literature.

Results. From clinical findings of 80 Japanese patients, high-grade fever was observed in 98.8%, chest attacks (pleuritis symptoms) in 61.2%, abdominal attacks (peritonitis symptoms) in 55.0%, and arthritis in 27.5%. Twenty-four percent of patients experienced their first attacks before 10 years of age, 40% in their teens, and 36% after age 20 years. Colchicine was effective in many patients at a relatively low dose (< 1.0 mg/day). AA amyloidosis was seen in only 1 patient. Common *MEFV* mutation patterns were E148Q/M694I (25.0%), M694I alone (17.5%), and L110P/E148Q/M694I (17.5%), and no patient carried the M694V mutation, the most common mutation in Mediterranean patients with FMF.

Conclusion. A larger than expected number of patients with FMF exist in Japan, and the clinical presentation of Japanese FMF patients seems to be relatively milder than those of Mediterranean FMF patients. AA amyloidosis rarely occurs in Japanese patients, probably due to difference in patterns of the *MEFV* genotype between Japanese and Mediterranean patients. (First Release June 15 2009; J Rheumatol 2009;36:1671–6; doi:10.3899/jrheum.081278)

Key Indexing Terms:
FAMILIAL MEDITERRANEAN FEVER | *MEFV* GENE | JAPANESE PATIENTS
NATIONWIDE QUESTIONNAIRE | AA AMYLOIDOSIS

Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrence of fever, polyserositis, and erysipelas-like skin lesions[1]. This disorder is the most common form of hereditary periodic fevers and there are over 100,000 patients around the world[2], but it predominately affects populations from the Mediterranean basin including non-Ashkenazi Jews, Arabs, Armenians, and Turks[1,3]. FMF is caused by mutations in the Mediterranean fever gene (*MEFV*) on chromosome 16p13.3, encoding a 781-amino acid protein denoted pyrin/marenostrin[4,5]. Over 170 sequence variants have been recorded in the dedicated database of the Registry of Familial Mediterranean Fever and Hereditary Auto-inflammatory Disorders Mutations, infevers (http://fmf.igh.cnrs.fr/ISSAID/infevers/). The variants V726A, M694V, M694I, M680I, and E148Q are the most frequent, accounting for 74% of all sequence variants[6]. Development of reactive AA amyloidosis is the most devastating complication of the disease[1,7]. The mainstay of therapy is daily colchicine, which prevents the attacks and the development of reactive AA amyloidosis[7].

In Japan, several patients with recurrent fever were clinically diagnosed as having FMF after 1976[8]. In 2002, the *MEFV* gene mutation was confirmed in a few Japanese patients with periodic fever[9,10], and since then a number of FMF patients diagnosed by DNA analysis have also been described. However, FMF is still recognized as quite rare in Japan, and it remains unclear whether the clinical features of Japanese patients are the same as those of Mediterranean patients or not. To elucidate the clinical features of Japanese patients with FMF, we studied clinical findings from 80 patients.

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MATERIALS AND METHODS

Patients. Clinical records of 80 Japanese FMF patients with *MEFV* gene mutations were studied. Clinical diagnosis of FMF was performed according to the Tel-Hashomer criteria[11]. Thirty-nine patients were diagnosed at Shinshu University Hospital between 2002 and 2007, including some previously reported[9,12-15]. Clinical data of the remaining 41 patients were obtained from a nationwide questionnaire survey (described below) and/or by review of the literature.

Nationwide questionnaire. To determine clinical features of patients, we carried out a nationwide questionnaire survey on FMF in 2006. The questionnaire was mailed to 1850 departments of internal medicine and pediatrics in Japan, asking about the number of FMF patients clinically diagnosed on the basis of the Tel-Hashomer criteria and/or the number of patients confirmed genetically, and the number of FMF patients with reactive systemic AA amyloidosis, between 1996 and 2006. Departments that answered that they had patients with FMF were sent another questionnaire asking for more detailed clinical information including the type of *MEFV* gene mutations. The protocol of these surveys was approved by the ethical committee of Shinshu University.

DNA testing of MEFV gene. DNA analysis of the *MEFV* gene was performed in patients with suspected FMF. Exon 2 and exon 10 with their flanking intronic sequences of the *MEFV* gene were amplified by polymerase chain reaction (PCR) using primers shown in Table 1. Exon 2 was amplified in 2 overlapping PCR fragments, exon 2a and exon 2b. Amplified PCR products were analyzed by direct sequencing (DNA Analyzer 3730xl; Applied Biosystems, Foster City, CA, USA). In patients without mutations in either exon 2 or 10 of the *MEFV* gene, other exons were also analyzed by direct sequencing after amplification of each exon[16]. An L110P mutation in exon 2 was analyzed by restriction fragment-length polymorphism (RFLP) analysis with *Sma* I restriction enzyme in addition to the DNA sequence analysis. An E148Q in exon 2 was also detected by RFLP with *BstN* I restriction enzyme after amplification using Exon2E148QF and Exon2E148QR as primers (Table 1).

Allele frequency analysis. Allele frequencies of L110P, E148Q, and M694I were analyzed in 51 healthy individuals and were compared to those in 39 patients with genetically diagnosed FMF at our institution. L110P and E148Q were analyzed by RFLP and M694I by the amplification refractory mutation system[17]. Differences in allele frequencies between the 51 healthy controls and the 39 FMF patients were compared statistically by the chi-square test.

Prior to the study, detailed informed consent was obtained from all patients following a clear explanation of the purpose of the study. Our genetic study protocol was approved by the local ethics committee.

RESULTS

The results of the questionnaire survey are shown in Table 2. Total response rate was 37.9%. The total number of patients who met the diagnostic criteria[11] was 131. Of the 131, 86 patients carried *MEFV* gene mutations (Table 2). Among these, detailed clinical data including the type of *MEFV* mutation were obtained from 58 patients (Figure 1); 39 of these patients were diagnosed at Shinshu University. The clinical data of the remaining 19 patients were obtained by the second survey; 13 of these patients had also been reported previously[10,18-22]. Unfortunately, further information such as the genotype in the other 28 of the 86 patients could not be obtained in the second survey.

In the nationwide survey, reactive AA amyloidosis associated with FMF was noted in 5 patients (3.8%; Table 2). One of them had already been described[10], but detailed clinical information on the other 4 patients could not be obtained from the second survey.

Clinical data. The results for the 58 patients whose clinical data were obtained by the nationwide survey (Table 2) and also those of 22 patients who had been described elsewhere[12,22-27] were studied (total 80 patients; Figure 1), as summarized in Table 3. Forty-nine patients (61.3%) did not have a family history suggestive of FMF (data not shown in the table). The male to female ratio was 33:47. The mean age at onset was 17.3 ± 10.7 years (data not shown); 19 patients (23.8%) experienced their first attacks before 10 years of age, 32 patients (40.0%) in their teens, 20 patients (25.0%) in their twenties, and 9 patients (11.3%) after age 30 years. Surprisingly, the age of onset was 53 years in one patient[26]. The mean age at diagnosis was 29.5 ± 13.7 years and the mean period from disease onset to diagnosis was 13.2 ± 11 years (data not shown).

High-grade fever (febrile attack) was the symptom seen most frequently (98.8%). Chest attack (pleuritis symptoms) was observed in 61.2% of patients and abdominal attack (peritonitis symptoms) in 55.0%. The frequency of arthritis was 27.5% and erysipelas-like erythema was seen in 10% of patients.

Colchicine was orally administered to 47 patients, and a favorable therapeutic effect was seen in at least 40 (85.1%). Information on efficacy was not obtained in the questionnaire survey in 5 patients. The daily dose of colchicine in 28 patients is shown Table 4, and 26 of these were treated with a relatively low dose (< 1.0 mg/day), among whom were 3 patients under 15 years of age. No patient required over 2.0

Table 1. Primers and polymerase chain reaction conditions.

	Primer	Annealing Temperature, °C
Exon2aF	5'-GCA TCT GGT TGT CCT TCC AGA ATA TTC C-3'	62
Exon2aR	5'-CTT TCC CGA GGG CAG GTA CA-3'	
Exon2bF	5'-CAG GCC GAG GTC CGG CTG CG-3'	62
Exon2bR	5'-CTT TCT CTG CAG CCG ATA TAA AGT AGG-3'	
Exon10F	5'-CCG CAA AGA TTT GAC AGC TG-3'	60
Exon10R	5'-TGT TGG GCA TTC AGT CAG GC-3'	
Exon2E148QF	5'-GCC TGA AGA CTC CAG ACC ACC CCG-3'	55
Exon2E148QR	5'-AGG CCC TCC GAG GCC TTC TCT CTG-3'	

Table 2. Results of the nationwide questionnaire survey.

Feature	Internal Medicine	Pediatrics	Total
Departments surveyed	1338	512	1850
Response rate (%)	437 (32.7)	264 (51.6)	701 (37.9)
Total no. of FMF patients	86	45	131
No. of FMF patients determined by gene analysis	49	37	86*
FMF patients with AA amyloidosis	4	1	5

* Clinical data of 58 out of 86 patients were available in this study.

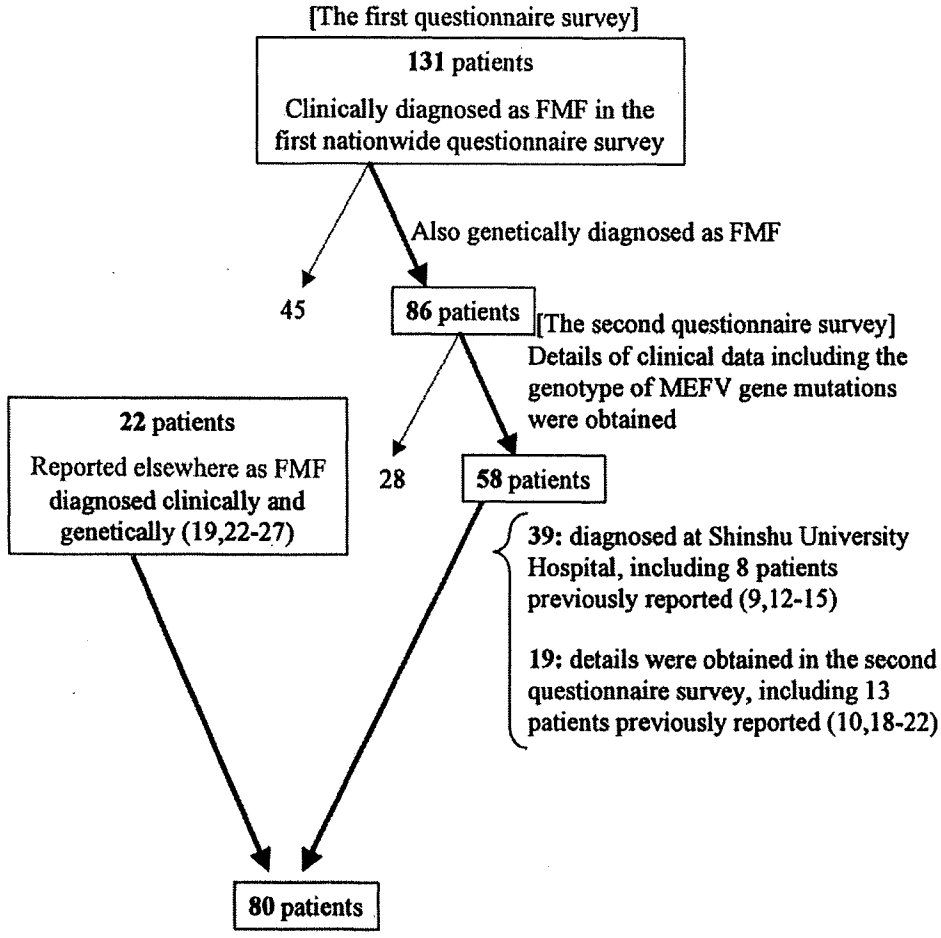

Figure 1. The process of patient selection in this study.

mg/day colchicine to prevent attacks. No effect was observed in 2 patients receiving 1.0 mg/day colchicine (Table 4), but the daily dose could not be increased due to severe diarrhea and bone marrow suppression[10,12,13]. At least 21 patients had not been treated with colchicine. As alternative treatments to colchicine, azelastine was used in one patient, with mild effectiveness, and a combined therapy with infliximab and low-dose methotrexate was effective in one patient[12,13]. In one patient interferon-α was also effective[25], and the herbal medicine "Sho-Saiko-To (TJ-9)" (Tsumura, Tokyo, Japan) was reported to be effective in another patient[20].

Five patients (6.3%) had also been diagnosed as having Behçet's disease (data not shown) before the *MEFV* mutation was identified. Of the 80 patients, only one (1.3%), who was homozygous for the M694I mutation, had reactive systemic AA amyloidosis[10].

MEFV gene mutations. The genotypes of the *MEFV* gene in the 80 patients are shown in Table 5. Common *MEFV* mutation patterns were E148Q/M694I (20 patients, 25.0%),

Table 3. Clinical presentations of 80 patients with FMF.

Age at Onset, yrs, n (%)						Sex, Male,	Clinical Manifestations, n (%)					
< 10	10–19	20–29	30–39	40–49	> 50	n (%)	Fever	Pleuritis	Peritonitis	Arthritis	Erysipelas-like Erythema	Amyloidosis
19 (23.8)	32 (40.0)	20 (25.0)	6 (7.5)	2 (2.5)	1 (1.3)	33 (41.3)	79 (98.8)	49 (61.2)	44 (55.0)	22 (27.5)	8 (10.0)	1 (1.3)

Table 4. Dose of colchicine in 28 patients. Number of patients in whom colchicine was effective is given in parentheses.

Dose mg/day	No. Patients
≤ 0.5	13 (11)*
1	13 (10)**†
1.5	1 (1)
2	1 (1)
> 2.0	0

* The efficacy was unclear in 2 patients. ** Efficacy was unclear in one patient in our survey. † No efficacy was observed in 2 patients.

Table 5. Genotypes of *MEFV* gene of the 80 cases.

MEFV mutation	No. Patients (%)
E148Q/M6941	20 (25.0)
M6941/normal	14 (17.5)
L110P/E148Q/M6941	14 (17.5)
L110P/E148Q	9 (11.3)
M6941/M6941	5 (6.3)
L110P-E148Q/E148Q	4 (5.0)
L110P/M6941	2 (2.5)
E148Q/P369S/R408Q/S503C	2 (2.5)
L110P-E148Q/L110P-E148Q	2 (2.5)
R202Q/M6941	1 (1.3)
E148Q/E148Q-R761H	1 (1.3)
L110P/E148Q/P369S/R408Q	1 (1.3)
E148Q/P369S/R408Q	1 (1.3)
P369S/R408Q	1 (1.3)
E148Q/R202Q	1 (1.3)
E148Q/E148Q	1 (1.3)
E84K/normal	1 (1.3)

M694I alone (14 patients, 17.5%), and L110P/E148Q/M694I (14 patients, 17.5%). Nine patients (11.3%) had L110P/E148Q and 5 (6.3%) were homozygous for the M694I mutation. The majority of patients carried E148Q (56 patients) or M694I (56 patients) at least on an allele, but L110P was also identified in 32 patients. As minor mutations, E84K[19], R202Q, P369S, R408Q, S503C[18], and R761H were found in some patients (Table 5), but most of those mutations were detected with L110P, E148Q, or M694I. Only 2 patients, who had P369S/R408Q or E84K alone[19], did not carry L110P, E148Q, or M694I. The other mutations, including M694V, M680I, and V726A in exon 10, which were common in Mediterranean patients with FMF[28], were not found in these 80 patients.

Allele frequencies of L110P, E148Q, and M694I in 51 healthy individuals (102 alleles) were 0.039, 0.26, and 0, respectively. On the other hand, allele frequencies of these 3 mutations were examined in 39 FMF patients and the results were 0.31 (L110P), 0.44 (E148Q), and 0.35 (M694I). The differences in allele frequencies between healthy populations and those with FMF were statistically significant ($p < 0.001$ for L110P and M694I; $p < 0.02$ for E148Q).

DISCUSSION

Clinical features of Japanese patients with FMF. Our study shows that the clinical pictures of Japanese patients with FMF seem to be different from those of Mediterranean patients. The frequencies of cardinal clinical symptoms during attacks in Japanese and Mediterranean FMF patients[2] are shown in Table 6. Mediterranean patients almost always have abdominal symptoms due to peritonitis[2]. However, the frequency of abdominal symptoms in Japanese patients was relatively low (55.0%). Because the frequencies of chest symptoms due to pleuritis, arthropathy, and erysipelas-like erythema are quite variable even among Mediterranean FMF patients, no clear differences were seen in such symptoms between Mediterranean and Japanese patients. In the literature, the relation between severity of the disease and the diet low in animal fat is discussed, and in particular, it was reported that butter ingestion appeared to provoke peritonitis attacks[29]. Although the mechanism of the low frequency of abdominal symptoms in Japanese patients remains unclear, the difference in diet between Japanese and Mediterranean FMF patients may have effects on the difference of phenotype.

Because of atypical symptoms like high fever or abdominal pain, 5 patients with Behçet's disease underwent the *MEFV* gene analysis. All of them clinically met the Tel-Hashomer criteria and were therefore diagnosed as having both FMF and Behçet's disease. However, there was no significant difference between the patients with con-

Table 6. Frequency of symptoms during attack (%) in different races.

	Japanese	Mediterranean populations[2]			
	(80 cases)	Turks	Jews	Arabs	Armenians
Fever	98.8	93	100	100	100
Peritonitis	55.0	94	95	82	96
Pleuritis	61.3	31	40	43	87
Arthritis	27.5	47	77	37	37
Erysipelas-like erythema	10.0	21	46	3	8

comitant Behçet's disease and the patients with FMF alone in terms of the clinical severity of FMF symptoms.

With regard to age at disease onset, 90% of FMF patients experience their first attacks before the age of 20 years and the percentage of patients with onset at age over 30 is less than 5% in the Mediterranean area[2,3]. In Japanese patients, 63.8% of patients experienced their first attack before age 20 and 11.3% of patients after age 30, indicating that FMF onset in Japanese patients was much later than in Mediterranean patients. The Turkish FMF Study Group reported that the mean period from disease onset to diagnosis of FMF in Turkey was 6.9 ± 7.65 years[28], and there may also be a delay in the diagnosis of FMF in Japanese patients, probably due to the low recognition of this disorder in Japan.

Administration of colchicine is known to be the most effective therapy for FMF to reduce the frequency, duration, and severity of attacks in most patients, and it has commonly been used in doses of 1.0–2.0 mg/day[2]. Moreover, Pras, *et al* noted that 30% of North African Jewish patients needed 2 mg or more of colchicine to control their symptoms[30]. In our study a small dose of colchicine, not over 1.0 mg/day, showed a favorable therapeutic effect in the majority of Japanese patients, so a relatively lower dose of colchicine may control the attacks of FMF symptoms in Japanese as described[21].

Prevalence of reactive systemic AA amyloidosis in Japanese patients with FMF. Although the incidence of reactive systemic AA amyloidosis in Mediterranean FMF patients varies in different ethnic groups, AA amyloidosis occurs very frequently in North African Jews (12.4%–26%), Iraqi Jews (9.5%–15%), Ashkenazi Jews (11%), Arabs (12%), Armenians (24%), and Turks (12.9%)[28,30-32]. On the other hand, the prevalence of AA amyloidosis in our study was quite low. Of 80 patients, only one male patient[10] had AA amyloidosis, which had been detected 3 years before the *MEFV* gene mutation (M694I) was identified. At the time he was diagnosed as having amyloidosis, he did not receive treatment with colchicine. However, in 21 out of 80 FMF patients who had not been treated with colchicine, to date no patient has had AA amyloidosis. Thus, the prevalence of AA amyloidosis associated with FMF in Japanese would appear to be lower than in Mediterranean patients, regardless of treatment with colchicine.

Genotype of MEFV gene in Japanese patients with FMF. The characteristics of the genotype of the *MEFV* mutations in Japanese patients were that almost all patients were homozygous, heterozygous, compound heterozygous, and/or complex allele for L110P, E148Q, and/or M694I. The correlation between the *MEFV* genotype and phenotype (severity of the disease) in FMF has been well discussed. The C-terminal B30.2 domain of pyrin encoded by exon 10 is known to play an important role in its function, interacting directly with caspase-1 to modulate interleukin 1ß production[33]. In addition, the methionine residue in codon 694 makes a crucial contribution to the function of pyrin[34]. Thus, the mutations in codon 694 are considered to produce severe symptoms with early onset and high frequency of attacks and the necessity of a high dose of colchicine to prevent attacks[2]. In particular, the M694V mutation is regarded as a significant risk factor for secondarily developing amyloidosis[3,7,35]. However, in our study none of the 80 patients carried this mutation. While the M694I mutation was the one most frequently found in Japanese patients, the majority of the patients were compound heterozygous or complex allele for M694I and other mutations producing a relatively milder phenotype such as E148Q .and/or L110P, or heterozygous for M694I alone. In addition, numbers of Japanese patients were compound heterozygous or complex allele for E148Q and L110P, so the characteristics of Japanese patients such as late onset and low prevalence of AA amyloidosis would be associated with differences of *MEFV* genotype compared with Mediterranean patients.

It remains controversial whether the E148Q mutation is a disease-causing mutation or a simple polymorphism because of high allele frequency in healthy controls[36-39]. However, it has been reported that most homozygote or compound heterozygote patients associated with other *MEFV* mutations are symptomatic[40,41], and it has also been noted that the allele frequency of E148Q is significantly higher among patients with AA amyloidosis and chronic fever of unknown origin[41]. Moreover, the E148Q mutation was described as producing a milder FMF phenotype with low penetrance[2,6]. While in our study 5 healthy controls were proved to be homozygous for E148Q, the allele frequency of E148Q in patients with FMF was significantly higher than in healthy individuals. Hence we also consider that this mutation can cause FMF, especially when patients are compound heterozygous for E148Q and other *MEFV* mutations or homozygous for E148Q[14].

The L110P mutation was first reported in 2000[42], and to date, several patients have been reported to be compound heterozygote with other mutations even in Japan[19,21]. In our study, 30 out of the 80 patients carried L110P as heterozygote with other mutations, and among these, 28 were compound heterozygous or complex allele for L110P and E148Q. Moreover, there was a significant difference in the frequency between FMF and healthy populations. Therefore, it is considered that L110P can also be associated with the onset of FMF.

Although it is true that *MEFV* gene analysis is needed to establish a definite diagnosis in suspected cases of FMF, *MEFV* mutations are not always found on both alleles even in typical FMF patients[7]. Therefore, diagnosis based on the clinical diagnostic criteria, family history, and the patient's response to colchicine treatment is of great importance in this disorder.

Our study indicates that the clinical presentations and the

MEFV genotype of Japanese patients with FMF seem to be different from those of Mediterranean patients, and our survey suggests that there will be a large number of FMF patients even in Japan.

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tis also may experience long-lasting remission after a single canakinumab injection. This would make IL-1 inhibition even more appealing as a potential treatment of recurrent idiopathic pericarditis.

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Interleukin-1β suppression in Blau syndrome: comment on the article by Martin et al

To the Editor:

We read with great interest the recent article by Martin et al on the role of interleukin-1β (IL-1β) secretion in Blau syndrome (1). To evaluate synergistic effects, the authors measured IL-1β secretion levels in peripheral blood mononuclear cells (PBMCs) isolated from 5 patients with Blau syndrome, compared with levels in the PBMCs of 5 controls, after cells were stimulated with muramyldipeptide, Pam_3Cys (a Toll-like receptor-2 [TLR-2] agonist), lipopolysaccharide (LPS), or with combinations of muramyldipeptide and either Pam_3Cys or LPS. In addition, Martin and colleagues presented 2 case reports in which recombinant human IL-1 receptor antagonist (anakinra) was not effective in treating Blau syndrome. Finally, the authors stated that Blau syndrome is not mediated by excess IL-1 activity.

We would like to present evidence that is consistent with the findings of Martin et al and to offer our own hypotheses. We also have data on IL-1β secretion levels in the PBMCs of 2 patients with Blau syndrome with an arginine-to-tryptophan mutation at position 334 of NOD-2 (R334W) (Figure 1A). These patients (whose cases have been reported previously [2]) had both been receiving prednisolone (15 mg/day [0.3 mg/kg]). The methods of isolation of PBMCs and the analysis of cytokine concentrations in culture supernatants were described by us in a previous report (3), and the analysis of patient materials was approved by the Human Research Ethical Committee of Shinshu University. We evaluated secretion levels of IL-1β, tumor necrosis factor α (TNFα), IL-6, and IL-8 in the culture supernatants of PBMCs that were left untreated for 8 hours or incubated with muramyldipeptide (10 ng/ml or 1 μg/ml) (a NOD-2 stimulatory ligand), LPS (0.1 ng/ml or 10 ng/ml) (a TLR-4 stimulatory ligand), or muramyldipeptide (10 ng/ml or 1 μg/ml) combined with a low amount of LPS (0.1 ng/ml). The data showed that IL-1β secretion from PBMCs isolated from patients with Blau syndrome remained

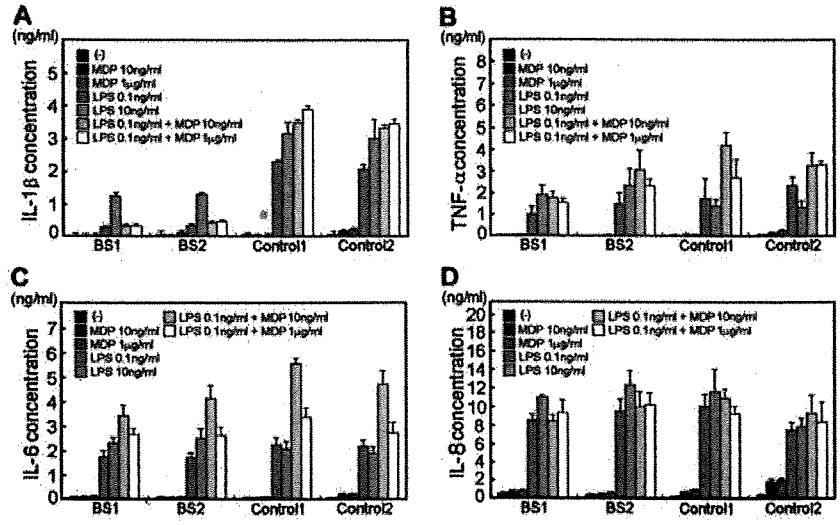

Figure 1. Secretion levels of interleukin-1β (IL-1β) (**A**), tumor necrosis factor α (TNFα) (**B**), IL-6 (**C**), and IL-8 (**D**) in peripheral blood mononuclear cells (PBMCs) isolated from the 2 patients with Blau syndrome (BS1 and BS2) and from 2 healthy controls. PBMCs were incubated with muramyldipeptide (MDP), lipopolysaccharide (LPS), or MDP combined with LPS, or were left untreated (−) for 8 hours. Secretion levels in the supernatants were measured by enzyme-linked immunosorbent assay. Values are the mean and SD from triplicate cultures.

undetectable without stimulation and was low after treatment with muramyldipeptide alone. These findings were the same as in healthy controls. PBMCs isolated from patients exhibited a lower response to LPS stimulation than cells from healthy controls. Notably, the synergistic stimulatory effect of the combination of muramyldipeptide and LPS on IL-1β secretion, which was observed in healthy controls, was not observed in patients with Blau syndrome, while the secretion levels of TNFα, IL-6, and IL-8 in the PBMCs of patients exhibited normal responses compared with healthy volunteers (Figures 1B–D). These observations are consistent with those reported by Martin et al (1).

IL-1β synthesis is known to be regulated by a 2-step process: transcriptional and translational regulation, and post-translational regulation. In the first step, activation of the transcription factor NF-κB leads to transcription of the proIL-1β gene, which can be translated to proIL-1β. The next step is maturation of proIL-1β by inflammasome, which is known to be an IL-1β–processing platform composed of Nod-like receptors, ASC, and caspase 1 (4). The secretion of IL-1β in PBMCs is known to be synergistically induced by muramyldipeptide and LPS. It has also been reported that this synergistic effect of NOD-2 and TLR on IL-1β maturation is caspase 1 dependent and that the activation of caspase 1 and the release of mature IL-1β by muramyldipeptide is NOD-2 dependent (5).

Mutated *NOD2* in patients with Blau syndrome is thought to be a constitutive active form of NOD-2, which has been found to lead to constitutive NF-κB activation in studies performed in vitro (6,7). The results of those studies do not contradict the data presented by Martin et al (1) or our own data (Figure 1), because the in vitro observation in HEK 293T cells transfected with the mutated form of *NOD2* (R334W) reflects primary initiated high-level NF-κB activation. Activation of NF-κB by constitutive activated mutated NOD-2 occurs via induction of a signaling complex, including RICK/RIP2 and IKK complex. Constitutive NF-κB activation then induces a negative feedback regulator, such as A20, a downstream regulator of RICK/RIP2 (8); therefore, we hypothesize that such a negative feedback regulator may affect inflammasome for IL-1β secretion either directly or indirectly. IL-1β secretion due to a synergistic effect of muramyldipeptide and LPS was not observed in PBMCs isolated from patients with Blau syndrome. Therefore, it is possible that the pathogenesis of Blau syndrome may be related to suppression of IL-1β synthesis.

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Using "real clinic" definitions to predict the course of juvenile dermatomyositis: comment on the article by Stringer et al

To the Editor:

We read with interest the recent article by Stringer et al, in which the authors analyze a cohort of patients from their center in order to determine whether the course of juvenile dermatomyositis (DM) can be predicted (Stringer E, Singh-Grewal D, Feldman BM. Predicting the course of juvenile dermatomyositis. Arthritis Rheum 2008;58:3585–92).

At present, there are no validated criteria for clinical remission and inactive disease in juvenile DM. In the study by Stringer et al, disease remission was defined as a clinical state in which rash is absent, there is no evidence of active myositis or arthritis, and the patient has not received immunosuppressive medications for a minimum of 6 months, and remission of skin disease was defined as the absence of heliotrope rash, Gottron's papules, and skin ulcers for at least 3 successive visits. We believe that using these definitions results in a significant number of patients who are classified as having "active" disease, when the standard clinical impression would be that the disease is in fact "inactive." In particular, classifying patients in whom there is clinical disease remission (no evidence of skin, muscle, or joint inflammation), but whose medications continue to be tapered slowly, as having active disease is problematic. The clinical treatment protocol used at the authors' center would require a minimum of 30 months of treatment, with methotrexate tapered over time. When taken together, the remission definition and the methotrexate tapering protocol exaggerated the median time to remission and caused the majority of patients in the study to be classified as having a chronic disease course.

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Antibodies to myelin oligodendrocyte glycoprotein are not involved in the severity of chronic non-remitting experimental autoimmune encephalomyelitis

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ABSTRACT

To elucidate the role of antibodies in development of chronic non-remitting experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice, which is a well-established Th1-mediated autoimmune disease, and the involvement of activation-induced cytidine deaminase (AID) in Th1-mediated function, we have investigated the myelin oligodendrocyte glycoprotein (MOG)-induced EAE in mice deficient of AID, which is absolutely required for class switching and somatic hypermutation. Following immunization with MOG, $AID^{-/-}$ had completely same levels of clinical and pathological severity of EAE when compared with $AID^{+/-}$ and $AID^{+/+}$, although $AID^{-/-}$ did not produce IgG and anti-MOG IgG. Similar levels of T cell proliferation and a modest increase of anti-MOG IgM synthesis were found in spleen cells of $AID^{-/-}$ stimulated with MOG. These results indicate that antibodies are not involved in development of EAE in C57BL/6 mice.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an immunologically mediated disease of central nervous system (CNS) that provides an animal model for acute or chronic human demyelinating disorders: multiple sclerosis (MS) and acute disseminated encephalomyelitis [1]. EAE is induced in a variety of animals by sensitization of myelin basic protein (MBP), proteolipid protein, myelin oligodendrocyte glycoprotein (MOG) or their derived peptide [2]. Among these, the injection of MOG or its encephalitogenic peptide MOG35–55 in C57BL/6 mice produces a paralytic neurological disease with extensive plaque-like demyelination. The mice develop a chronic non-remitting neurological disease 12–16 days postimmunization lasting entire 45 days of observation and the antibody reactivity to MOG35–55 is detected in mice 4 weeks postimmunization [3]. A number of studies have reported that anti-MOG antibodies are able to induce demyelination in vivo or in vitro [4–7]. However, the role of anti-MOG35–55 antibodies in acute/chronic EAE induced by sensitization of MOG35–55 C57BL/6 mice remains to be elucidated. EAE is considered a Th1-mediated autoimmune disease. Recent study has shown that in B6 mice MOG-reactive $CD8^+$ cells are pathogenic [8]. It is believed that the presence of pro-inflammatory cytokines such as INF-γ and TNF-α lead to damage of myelin [9]. Furthermore, the idea that Th17 cells have an important role in EAE has emerged in light of the following observations. Deficiency in either p40 or p19, which form IL-23, results in a decreased number of Th17 cells and protection from EAE, although deficiency in the IL-12p35 subunit, which is specific for IL-12, does not alter the progression of EAE [10,11]. In addition, transfer of myelin-reactive IL-17-producing T cells expanded with IL-23 in vitro induced severe EAE. Although IL-17-deficient mice, nevertheless, develop attenuated EAE, Th17 cells are considered potent inducers of autoimmunity [12].

The role of B cells and myelin-specific Abs in mediating myelin loss is controversial. While the pathology of multiple sclerosis implicates a role for B cells and antibodies in the disease process [13], results from animal models have yielded conflicting results. B cell-deficient mice do not develop EAE when immunized with MOG [14] or developed disease with a reduced disease severity [15]. Conversely, no difference is observed in the onset or severity of disease in the absence of mature B cells by deletion of their mu chain transmembrane region, suggesting that B cells and Abs are not necessary for primary demyelination in MOG-induced EAE

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Distinct response in maintenance of human naive and memory B cells via IL-21 receptor and TCL1/Akt pathways ☆

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ABSTRACT

The molecular mechanisms involving in B-cell survival/proliferation are poorly understood. Here we investigated the molecules affecting the survival of human naïve and memory B cells. Without stimulation, naïve B cells survived longer than memory B cells. Moreover, the viability of memory B cells decreased more rapidly than that of naïve B cells following with *Staphylococcus aureus* Cowan strain (SAC), anti-immunoglobulin (Ig), or anti-CD40 stimulation, but displayed the same levels of survival following CpG DNA stimulation. We analyzed the transcriptional differences between B-cell subsets by gene expression profiling, and identified 15 genes significantly correlated to survival/proliferation. Among them, IL-21 receptor (IL-21R) and T-cell leukemia 1 (*TCL1*) proto-oncogene were highly expressed in naïve B cells. IL-21 induced the proliferation of both naïve and memory B cells. Marked phosphorylation of Akt was found in naïve B cells compared with memory B cells. This study suggests that naive and memory B cells are regulated by several distinct molecules, and the IL-21R and TCL1/Akt pathways might play crucial roles in naïve B cells for their maintenance.

1. Introduction

As the production of antibodies generally continues throughout our lifetime, prolonged B-cell lifespan is considered important in maintaining humoral immunity. Various kinds of signals, in addition to B-cell receptor (BCR) engagement, promote human B-cell survival, activation, proliferation, differentiation, and immunoglobulin (Ig) secretion [1,2]. Human circulating B lymphocytes can be broadly divided into two distinct populations: naive B lymphocytes and memory B lymphocytes [3], but the molecules involved in their survival remain poorly understood. Prolonged CD40 stimulation promotes naïve B-lymphocyte proliferation, expansion, and differentiation into IgM- or IgG-secreting cells, while leading memory B lymphocytes to cell death [4]. On the other hand, memory B cells, but not naïve B cells, proliferate and differentiate into antibody-secreting cells in response to microbial products, such as unmethylated single-stranded DNA motifs (CpG DNA), which stimulate B cells via Toll-like receptor (TLR) 9 [5].

GeneChip analysis is an approach ideally suited for addressing the complexity of biological processes in which multiple pathways are sequentially activated. While an enormous amount of information regarding the characteristics of human naïve/memory B cells has been generated by GeneChip analysis [6], the functions of these cells, especially in proliferation and lifespan, remain to be explored. GeneChip analysis here has led us to the proposal that IL-21 and T-cell leukemia 1 (TCL1) play a critical role in maintaining B-cell homeostasis through different pathways.

Activated $CD4^+$ T cells [7] and activated NKT cells [8] secretes IL-21, and IL-21R is readily detectable on B cells, T cells, NK cells, and monocyte-derived dendritic cells [7,9]. The ligation of IL-21R induces the likes of Jak1 and Jak3, and STAT1 and STAT3 activation [10]. Whereas IL-21 co-stimulates human B-cell proliferation induced by anti-CD40, it inhibits proliferation induced by anti-IgM and IL-4 [7]. In contrast to human IL-21, murine IL-21 does not enhance the proliferation of anti-CD40-stimulated murine B cells [11]. Other studies showed that IL-21 induced both costimulation and apoptosis for anti-CD40-stimulated B cells, whereas IL-21 primarily costimulated B cells activated by anti-IgM or anti-IgM plus anti-CD40 in a different strain [12]. IL-21 induced phosphorylation of Akt in mouse $CD8^+$ T cells [13].

TCL1 was first identified as an oncogene overexpressed in T-cell leukemias [14], but is more widely expressed in B cells. In the

☆ Naïve/memory B cells are distinctly maintained by IL-21R/TCL1/Akt pathways.

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B-cell lineage, *TCL1* is expressed in pre-B cells, immature B cells, naïve B cells and germinal center (GC) B cells, but not in memory B cells or plasma cells [15–18]. Other recent studies showing that the TCL1 protein enhances the activation of the pro-survival kinase Akt have revealed a potential mechanism underlying the development of B-cell leukemias [19]. TCL1 increases the ability of Akt to phosphorylate its substrates [20–22].

The purpose of this study was to investigate the differences in lifespan between naïve B cells and memory B cells in humans, as well as the molecules involved in B-cell lifespan by GeneChip analysis. From these molecules, we found that the IL-21R and TCL1/Akt pathways might be required for the maintenance of naïve B cells, thus contributing to innate immunity in humans.

2. Materials and methods

2.1. Antibodies and reagents

Anti-CD27 mAb (8H5, IgG1) was provided by Dr. C. Morimoto (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Streptavidin–phycoerythrin (PE) was purchased from DAKO Japan (Tokyo, Japan). Purified anti-CD40 mAb (MAB89, IgG1) was purchased from Immunotech (Westbrook, MA). PerCP-conjugated anti-CD20 mAb was purchased from Becton Dickinson (Mountain View, CA). Conjugation of biotin to anti-CD27 mAb was performed by the standard technique using *N*-hydroxysuccinimidobiotin (Sigma Chemical Co., St. Louis, MO). PE-conjugated anti-IL-21R mAb was purchased from R&D systems (Minneapolis, MN). Anti-Akt, anti-phospho-Akt (Ser 473) and anti-β-actin Abs were purchased from cell signaling technology (Danvers, MA), anti-ERK2 Ab, from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and HRP-conjugated anti-rabbit Ig from Amersham Biosciences (Buckinghamshire, UK). *Staphylococcus aureus* Cowan strain (SAC) and propidium iodide (PI) were obtained from Sigma Chemical Co. IL-21 was purchased from Biosource (Camarillo, CA). The CpG DNA with the sequence 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′ was used.

2.2. Preparation of human peripheral B-cell subsets and CD32-transfectants

Human adult peripheral blood samples were obtained from healthy volunteers after obtaining informed consent. Pure B cells were obtained from peripheral blood along the following lines. Peripheral blood mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient. MNCs were further purified into B cells by positive selection with anti-CD19 mAb-coated immunomagnetic microbeads (Miltenyi Biotic, Bergisch Gladbach, Germany) for total B cells. The purity of B cells thus obtained exceeded 95% with anti-CD20 mAbs as determined by flow cytometry. We also separated B cells by human B-cell enrichment cocktail (RosetteSep™, IgG1: Stemcell Technologies Inc., Vancouver, Canada), which Abs are bound in bispecific Ab complexes which are directed against cell surface antigens on human hematopoietic cells (CD2, CD3, CD16, CD36, CD56) and glycophorin A on red blood cells for purifying naïve and memory B cells. The purity of negatively selected B cells exceeded 85%. $CD27^+$ and $CD27^-$ B cells were separated by cell sorting, using a FACS Vintage (Becton Dickinson), under sterile conditions. Both purified populations were more than 95% pure, as determined by flow cytometry. The B cells thus obtained did not show any signs of proliferation or activation.

CD32- (FcRII-) transfectants (CD32T) were prepared as described elsewhere [23–25].

2.3. Flow cytometric analysis

PBMNCs were stained with anti-IL-21R-PE, anti-CD20-PerCP, and biotin conjugated anti-CD27 followed with avidin-APC. Triple-color analysis of B-cell surface molecules was performed by a FACS Calibur (Becton Dickinson).

2.4. Investigations of cell-survival

Purified human B cells were cultured for 7 or 14 days. Dead cells and viable cells were discriminated by staining with PI and evaluated by means of flow cytometric analysis.

2.5. GeneChip expression analysis

Total RNA (0.1–3 μg) was extracted from approximately 10^5 to 10^6 cells. Double-stranded cDNA was synthesized, and the cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleoside triphosphates. The biotinylated cRNA was hybridized with a probe array for 16 h at 45 °C, and the hybridized biotinylated cRNA was stained with streptavidin–PE and then scanned with a Hewlett-Packard Gene Array Scanner (Palo Alto, CA). The fluorescence intensity of each probe was quantified using a computer program, GeneChip Analysis Suite 5.0 (Affymetrix, Santa Clara, CA). The expression level of a single mRNA was determined as the average fluorescence intensity among the intensities obtained by 11 paired (perfect-matched and single nucleotide-mismatched) probes consisting of 25-mer oligonucleotides. If the intensities of mismatched probes were very high, gene expression was judged to be absent even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The level of gene expression was determined as the average difference (AD) using GeneChip software. The percentages of the specific AD level versus the mean AD level of six probe sets for housekeeping genes (β-actin and GAPDH) were then calculated. Data were considered significant when (1) expression changed by at least 2-fold (activation program) and (2) increased gene expression included at least one "present absolute call" (Affymetrix algorithm). The expression levels of genes of the same cells analyzed twice showed a statistically significant correlation ($r = 0.997$). Under these criteria, the reproducibility of the differences that were seen between different cells under different conditions was confirmed.

2.6. B-cell proliferation assay

Highly purified adult naïve and memory B cells were cultured in the presence of SAC, anti-CD40 mAb cross-linked with CD32-transfectants (anti-CD40/CD32T), or CpG DNA with various concentrations of IL-21 at a final cell density of 1×10^5/ml in a volume of 200 μl per well. The cells were cultured in 96-well round-bottom plates (Nunc, Roskide, Denmark) for 3 days at 37 °C in a humidified atmosphere with 5% CO_2. The cultures were then pulsed with 0.5 μCi [^{3}H]thymidine. After 18 h of incubation, the cells were harvested by an automatic cell harvester (Packard, Meriden, CT), and [^{3}H]thymidine incorporation was measured on a liquid scintillation analyzer (Packard).

2.7. Western blotting

The cell pellets from purified adult naïve and memory B cells, and activated B cells were collected by centrifugation and then the cells were lysed with lysis buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 10% glycerol and protease inhibitors for 15 min on ice. Lysates were cleared of nuclear debris by centrifugation for 5 min at 4 °C. Total cell lysates

were separated on SDS–PAGE using 10% polyacrylamide gels and analyzed by Western blotting using anti-Akt and anti-phospho-Akt Abs conjugated with HRP. Anti-β-actin or anti-ERK2 Ab was used as controls. Proteins were then visualized by an ECL system (Amersham Biosciences).

2.8. RT-PCR

Total RNA from highly purified adult naïve and memory B cells was extracted by the acid–guanidine thiocyanate–phenol–chloroform method using an RNAzol rapid RNA purification kit (Biotex, Houston, TX). First-strand cDNA copies were synthesized by using Superscript II Reverse Transcriptase (Life Technologies, Grand Island, NY) with oligo (dT) (Life Technologies) as a primer in a total volume of 20 μl, and then PCR was performed. The following oligonucleotide primers were used for TCL1, 5′-CCACCAAACCCAA AAAAAGAGATCGAATTCATG-3′ and 5′-ATTCATAGATCTCTGCAGGT CGACGGATCCTCA-3′, sense and antisense, respectively. A total of 2 μl cDNA was amplified in PCR using each primer and Taq DNA polymerase (Life Technologies). The amplified products were analyzed on a 1.2% agarose gel containing ethidium bromide and visualized by UV light illumination. The β_2-microglobulin (β_2-MG) sense primer 5′-GCTATGTGTCTGGGTTTCAT-3′ and antisense primer 5′-ATCTTCAAACCTCCATGATG-3′ were used as controls.

2.9. Statistical analysis

Statistical significance between groups was determined with Student's *t*-test. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Cell viability of naïve and memory B cells

To clarify the lifespan of naïve and memory B cells, human circulating naïve and memory B cells were cultured in medium alone and counted. Under this condition, the cell viability of naïve B cells was higher than that of memory B cells (Fig. 1A). When the effects of various stimuli on B-cell subsets were examined after 7 days of culture, SAC, anti-Ig and anti-CD40 preferentially promoted survival of naïve B cells, while CpG DNA promoted survival of memory B cells to levels comparable to naïve B cells (Fig. 1B). In B cells cultured for 14 days, similar results were obtained in both naïve and memory B cells with or without stimuli (Fig. 1B). These findings demonstrate that although the survival of naïve and memory B cells responded differently to various stimuli, naïve B cells have the innate ability to survive longer than memory B cells without stimulation, or with BCR or CD40 signaling, but not with TLR9 cross-linkage.

3.2. Gene expression profile analysis

To delineate the specific gene expression profile in human naïve and memory B cells, total RNA samples from highly purified B-cell subsets were converted into labeled cRNAs and hybridized to Affymetrix arrays representative of 12,000 genes [26]. Totals of 87 and 58 genes were predominantly up-regulated in resting naïve and memory B cells, respectively (Fig. 2), indicating that each purified B-cell subset displays a distinct gene expression profile that is consistent among individuals. Next, to identify differences in gene expression associated with cell proliferation and survival, we compared the gene expression patterns from the two subsets by referring to the Gene Ontology Biological Process in Affymetrix' annotation table (http://www.affymetrix.com/index.affx). Nine naïve dominant and six memory dominant proliferation/cell-survival associated genes were identified (Fig. 3), namely: TCL1 (8.6-fold), Krüppel-like factor 4 (KLF4, 4.4-fold) which is important in maintaining cellular quiescence in humans [27] and could oppose proliferation and survival of several B-lymphoid cell lines in mice [28], dual-specific phosphatase 6 (DUSP6, 2.7-fold), fibroblast growth factor 7 (FGF7, 3.1-fold), cycline-dependent kinase 5 regulatory subunit 1 (CDK5R1, 2.6-fold), IL-21R (8.5-fold), MAX-interacting protein 1 (MXI1, 3.5-fold), amphiregulin (AREG, 2.1-fold) and B-cell CLL/lymphoma 6 (BCL6, 3.2-fold) in naïve B cells, and basic helix-loop-helix domain-containing protein class B, 3

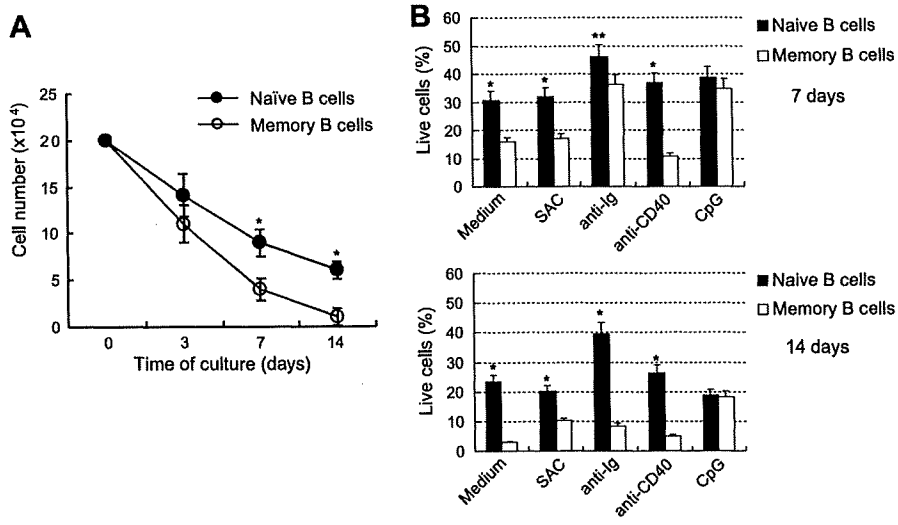


Fig. 1. Cell viability of na and memory B cells. Purified adult $CD27^-$ naive and $CD27^+$ memory B cells were separated by means of flow cytometry. The purity of both types of B cells was more than 95%. B cells (2×10^5) were cultured (A) without stimulation or (B) with SAC (0.01%), anti-Ig (5 μg/ml), anti-CD40 mAb (1 μg/ml), or CpG (1 μg/ml) at a final cell density of 1×10^5/ml per well in 96-well round-bottom plates for 14 days at 37 °C in a humidified atmosphere with 5% CO_2. The cells were collected at 0, 3, 7, or 14 culture day, stained with propidium iodide (PI), and then counted by means of flow cytometric analysis. Data are shown as living cell number or percentage of living cells per total cultured cells. These data represent means ± SD of five different experiments. *P* values <0.05 were considered statistically significant. $^*P < 0.01$, $^{**}P < 0.05$, compared na B cells versus memory B cells.

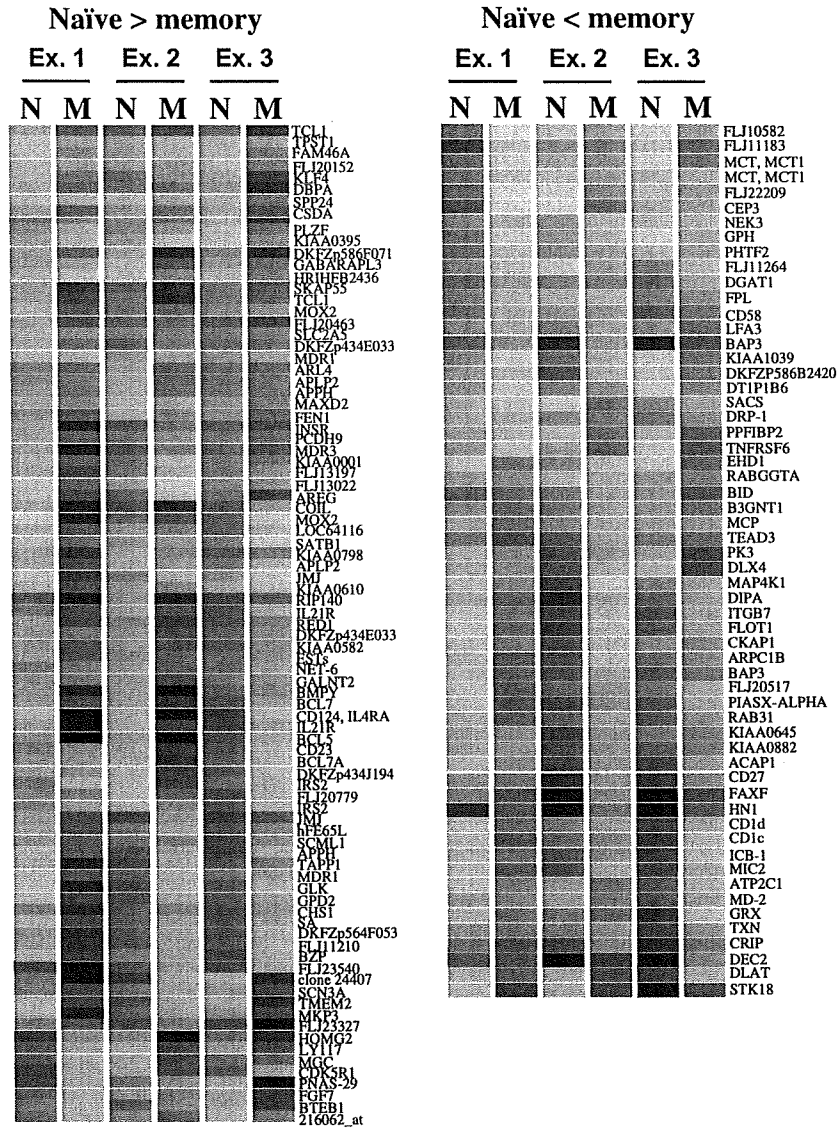


Fig. 2. Comparison of gene profiles between resting na and memory B cells. Human circulating $CD27^-$ na and $CD27^+$ memory B cells were obtained by sorting ($n = 3$). Total RNA was extracted and used to conduct GeneChip analysis. Each experiment contained three independent donors. Shown are only those gene segments that differ 2-fold or more.

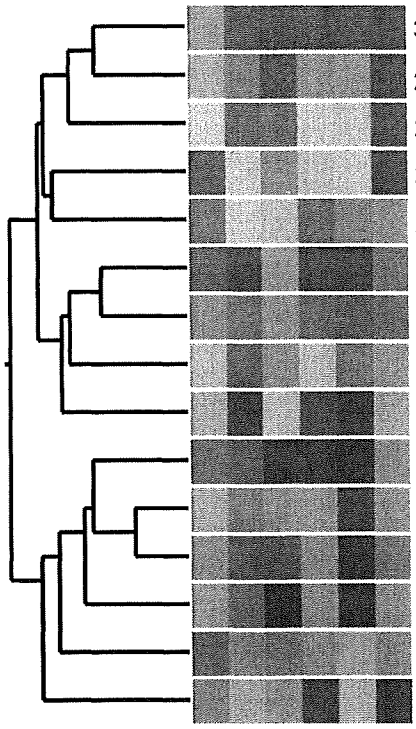
(BHLHB3, 14.3-fold), thioredoxin (TXN, 2.5-fold), cystein-rich protein 1 (CRIP1, 3.8-fold) and NIMA-related kinase 3 (NEK3, 2.0-fold), in addition to the well-known memory B-cell specific molecules, CD27 (3.6-fold) and FAS (2.9-fold).

3.3. Response of B-cell subsets to IL-21

In response to the remarkable difference noted in IL-21R gene expression (8.5-fold), we examined the effects of this molecule on naïve and memory B cells. Flow cytometric analysis showed that IL-21R protein expression was higher in naïve B cells compared with memory B cells (Fig. 4A). Functional studies demonstrated that IL-21 induced co-stimulation of SAC or anti-CD40-stimulated B cells, the responses of which were similar both in naïve B cells and in memory B cells. B-cell proliferation in both subsets increased in a dose-dependent manner of IL-21 in the presence of SAC, whereas the proliferation was substantial at low concentration of IL-21 in the presence of anti-CD40/CD32T (Fig. 4B). In contrast, IL-21 substantially inhibited proliferation of both CpG DNA-activated naïve and memory B cells (Fig. 4B). Taken together, these findings indicate that responses of naïve and memory B cells to IL-21 are similar, and likely increased expression levels of IL-21R on naïve B cells.

3.4. TCL1 and phospho-Akt expression in B-cell subsets

From comparisons of the gene expression profiles of naïve and memory B cells, the remarkable gene expression of TCL1 specifically in naïve B cells (8.6-fold) prompted us to investigate whether this molecule was involved in cell survival. RT-PCR analysis showed that TCL1 mRNA expression was significantly higher in naïve B cells compared to expression in memory B cells (Fig. 5A). Since TCL1 binds Akt, a central regulator of cell survival, and enhances Akt activation [19,29,30], we evaluated the expression and activation of Akt in both naïve and memory B cells. Akt was expressed equally in both resting naïve and memory B cells (Fig. 5B).



Ex. 1 N	Ex. 1 M	Ex. 2 N	Ex. 2 M	Ex. 3 N	Ex. 3 M	Probe set ID	Gene symbol	Average diff. Naïve B cells	Average diff. Memory B cells	Fold change
						39318_at	(TCL1)	2,232 ± 946	199 ± 40	8.6
						221841_s_at	(KLF4)	2,127 ± 932	490 ± 239	4.4
						208891_at	(DUSP6)	292 ± 137	119 ± 95	2.7
						205782_at	(FGF7)	96 ± 51	31 ± 17	3.1
						204995_at	(CDK5R1)	338 ± 112	125 ± 13	2.6
						219971_at	(IL21R)	126 ± 72	14 ± 8	8.5
						202364_at	(MXI1)	3,386 ± 1,096	938 ± 203	3.5
						205239_at	(AREG)	633 ± 189	315 ± 152	2.1
						203140_at	(BCL6)	1,271 ± 1,120	403 ± 344	3.2
						221530_s_at	(BHLHB3)	41 ± 37	438 ± 251	0.07
						208864_s_at	(TXN)	229 ± 61	557 ± 54	0.4
						205081_at	(CRIP1)	677 ± 225	2,476 ± 132	0.26
						206150_at	(CD27)	209 ± 100	681 ± 33	0.28
						213116_at	(NEK3)	314 ± 80	615 ± 97	0.5
						215719_x_at	(FAS)	72 ± 17	239 ± 135	0.35

Fig. 3. Cell proliferation and survival associated gene profiles. Gene profiles associated with cell proliferation and survival were selected from na/memory B-cell gene profiles as shown in Fig. 2 by using Affymetrix' annotation table (http://www.affymetrix.com/index.affx).

However, Western blots for Akt Ser-phosphorylation showed that phospho-Akt levels in naïve B cells were higher than memory B cells (Fig. 5B), indicating increased Akt activity. These results suggest that activated Akt could promote the survival of human B cells, particularly naïve B cells.

4. Discussion

Naïve- and memory-activated B-cell populations show independent homeostatic regulation [31]. To directly identify the phenotypic and homeostatic differences between these two B-cell subsets, several studies have compared the gene expression profiles of naïve and memory B cells; Klein et al. reported that osteoprotegerin ligand (OPGL) (9-fold), CD11b/Mac-1 (12.5-fold) and IL-2Rβ (95-fold) were up-regulated in memory B cells and protocadherin-9/VRA-11 (19-fold) in naive B cells in human tonsil samples [6]. However, in our experiments using highly purified human peripheral blood B cells, the above four genes were similarly expressed in both naïve and memory B cells. There are at least three explanations for these discrepancies. Firstly, the contents of B-cell subsets in peripheral blood differ from that in tonsils. Tonsillar B cells also include GC B cells which consist of centroblasts and centrocytes. Klein et al. indicated that IL-2Rβ is also expressed in late centrocytes that are committed to the memory lineage or differentiated memory B cells. This finding suggests that the memory B-cell pool includes distinct subsets in tonsils [6]. Secondly, the studies by Klein et al. used child samples, while our study used adult samples. The percentage of memory B cells increases gradually with age. Memory B cells do not appear in peripheral blood in newborn, while the cells are approximately 40% of peripheral blood B cells in adult. The third possibility is the purity of B cells. We isolated B-cell subsets by sorting such that the purity was more than 95%, but Klein et al. did not precisely describe the purity of the isolated B-cell fractions. A remarkable difference in mRNA expression in three genes was obtained by gene expression analysis: IL-21R (8.5-fold) and TCL1 (8.6-fold) dominant in naïve B cells, and BHLHB3 (DEC2) (14.3-fold) dominant in memory B cells (Fig. 3). BHLHB3 is highly expressed in skeletal muscle and the brain and is involved in regulation of proliferation and molecular clock [32]. Given that BHLHB3 affects cells outside of the immune system, detailed analysis regarding BHLHB3 was omitted from this study.

Good et al. reported that human splenic naïve B cells express higher levels than memory B cells of KLF4, KLF9, and promyelocytic leukemia zinc finger (PLZF), transcription factors important in maintaining cellular quiescence [27]. Enforced expression of KLF4, KLF9, and PLZF in memory B cells delayed their entry into division and reduced the number of proliferating cells, such that the behavior of transfected memory cells resembled that of naïve B cells [27]. It was also reported that KLF4 expression could oppose proliferation and survival of several B-lymphoid cell lines in mice [28]. KLF4 displayed a dramatic reduction in expression after activation and KLF4 expression leads to reduce c-Myc expression in activated mature B cells [28]. Pluripotent stem cells can be directly induced from mouse and adult human fibroblasts by the addition of only a few defined factors: Oct3/4, Sox2, c-Myc, and KLF4 [33–35]. Our data by GeneChip analysis also showed that KLF4 was expressed 4.4-fold in naïve B cells as much as in memory B cells in human peripheral blood (Fig. 3). However, since the value of KLF4 was half of that of IL-21R and TCL1, we omitted detailed analysis regarding KLF4 from this study.

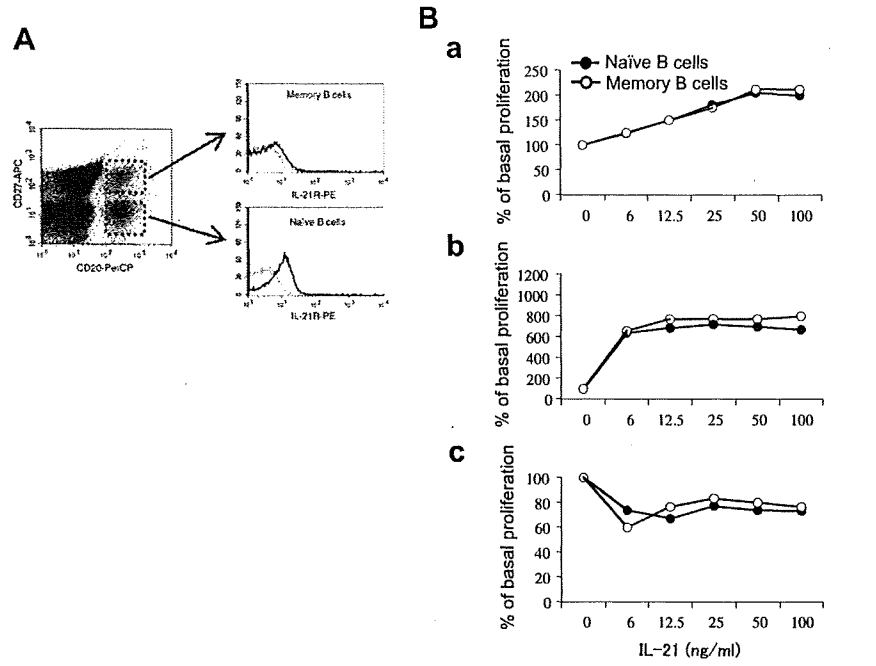


Fig. 4. Different response of B-cell subsets by IL-21. (A) Human circulating MNCs were stained with anti-IL-21R-PE, anti-CD20-PerCP and biotin conjugated anti-CD27 followed with avidin-APC. Expression of IL-21R on na or memory B cells is shown with a log scale (solid line). Isotype-matched PE-labeled mAb was used as the negative control (dotted line). (B) Purified na or memory B cells were cultured in 96-well round-bottom plates with various concentration of IL-21 (0, 6, 12.5, 25, 50, and 100 ng/ml) in the presence of (a) SAC (0.01%), (b) anti-CD40 mAb (1 μg/ml) cross-linked with CD32T (40%), or (c) CpG DNA (1 μg/ml) at a final cell density of 0.5×10^5/ml in a volume of 200 μl per well for 72 h at 37 °C in a humidified atmosphere with 5% CO_2. Proliferation assays were performed in triplicate. The proliferation in each concentration of IL-21 was shown as a percentage of the basal proliferation without IL-21 stimulation. The basal proliferation in na and memory B cells were (a) 20,873 and 7952, (b) 15,044 and 11,032, and (c) 30,179 and 51,807 c.p.m., respectively. The results depicted are representative of three independent experiments.

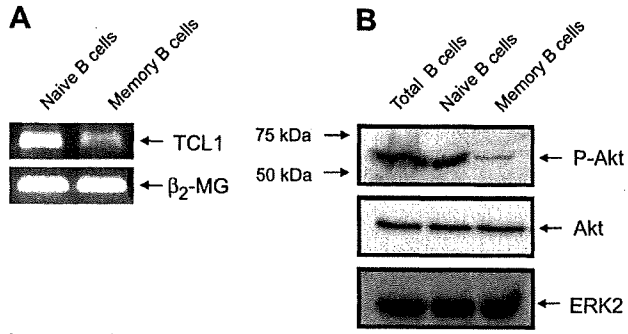


Fig. 5. TCL1, Akt and phospho-Akt expression in B-cell subsets. Human circulating $CD27^-$ na and $CD27^+$ memory B cells were obtained by sorting. (A) After extraction of total RNA from each B-cell subset, RT-PCR was performed as described in Section 2. The β_2-microglobulin (β_2-MG) was used as a positive control. (B) Cell lysates were analyzed using immunoblotting with anti-Akt Ab, anti-phospho-Akt Ab (P-Akt), and anti-ERK2 Ab. The results depicted are representative of three independent experiments.

IL-21 is closely related to IL-2 and IL-15, and all of their receptors share a common cytokine receptor gamma chain, γ_c, which is mutated in humans with X-linked severe combined immunodeficiency disease (XSCID). Mice lacking both IL-4 and IL-21R exhibit a significantly more pronounced phenotype, with dysgammaglobulinemia, characterized primarily by a severely impaired IgG response, indicating that IL-21 has a significant influence on the regulation of B-cell function in vivo [36]. In agreement with our findings, recent studies reported that naïve B cells express higher levels of IL-21R than memory B cells [37–39]. IL-21 substantially inhibited proliferation and induced Bim-dependent apoptosis for LPS or CpG DNA-activated B cells in mice [12]. In contrast, IL-21 induced both costimulation and apoptosis for anti-CD40-stimulated B cells, whereas IL-21 primarily costimulated B cells activated by anti-IgM or anti-IgM plus anti-CD40 [12]. Our experiments showed that IL-21 strongly enhances both of naïve and memory B-cell proliferation in the presence of SAC or anti-CD40/CD32T in humans. Good et al. suggested that CD40L/IL-21-stimulated naïve B cells underwent the same number of divisions as memory B cells and IL-21 is a powerful growth factor for naïve B cells [39]. Furthermore, *Streptococcus pneumoniae*-specific (SP-specific) IgM production in naïve B cells increased more than in memory B cells in the presence of IL-21 and CpG DNA (data not shown). IL-21 signaling is important for B-cell proliferation (Fig. 4B) and Ig secretion, as well as differentiation of both naïve and memory B cells into plasma cells [40]. IL-21 promotes the differentiation of memory B cells into plasma cells in the presence of B cell-activating factor of the tumor necrosis factor family (BAFF) signaling [41]. Recent studies reported that IL-21 induced phosphorylation of Akt in mouse $CD8^+$ T cells, albeit weakly [13]. Of γ_c-dependent cytokines, IL-2, IL-4, IL-7, and IL-15 also activate Akt [13,42].

The TCL1 proto-oncogene is highly expressed in many human B-cell leukemias, and two recent papers show that TCL1 overexpression causes rapid development of B-cell lymphomas in mice [43,44]. Normal expression of *TCL1* is limited to early embryos and lymphoid cells and, despite its name, *TCL1* is more widely expressed in B cells than in T cells. In the B-cell lineage, *TCL1* is expressed in pre-, immature, naïve, and GC B cells, but not in memory B cells or plasma cells [15–18]. In mature B cells, these data were consistent with our data that TCL1 was expressed significantly in naïve B cells but not in memory B cells. Akt-dependent signaling pathways augment the activation of NF-κB and S6 kinase, both factors that promote cell survival and growth. For B cells, Akt activity is a key determinant of survival versus apoptosis [45]. TCL1

increases the ability of Akt to phosphorylate its substrates both *in vitro* and when overexpressed in 293 human embryonic kidney cells [20–22]. These effects of TCL1 on cell survival and proliferation are at least in part mediated by Akt because they could be blocked by co-expressing a dominant-negative form of Akt. Similarly, a mutant form of TCL1 that could not bind Akt was unable to promote the survival of transfected cells [22]. Hoyer et al. extended these findings and showed that *TCL1* overexpression enhanced the survival of primary splenic lymphocytes [43]. The ability of TCL1 to increase the basal level of Akt activity in naïve B cells might tilt the balance in favor of a normal life span as opposed to a premature death [19], which was consistent with the data of TCL1 knockout mice [46]. These reports support our finding that phospho-Akt levels were highly in naïve B cells. TCL1/Akt signaling also involves in promoting the cell-survival and cell-cycle in ES cells [47]. BAFF also supports B-cell survival, metabolic fitness, and readiness for antigen-induced proliferation, which is controlled through protein kinase Cβ and Akt signaling [48]. Our findings show that TCL1 might maintain naïve B cells, perhaps resulting in expansion of the naïve B-cell pool.

The IL-21R and TCL1/Akt pathways reflect the different roles in naïve B-cell function; TCL1/Akt is important for prolonged lifespan of naïve B cells, while IL-21 promotes the proliferation both in naïve and memory B cells as well as the synthesis of Igs, such as antigen-specific IgM predominantly by naïve B cells (data not shown). Thus, both pathways may play an important role in innate immunity. It is commonly believed that it takes much longer to remove invading pathogens via naïve B cells than by memory B cells. However, our findings suggest that naïve B cells may also be beneficial in acute bacterial clearance by producing antigen-specific IgM (data not shown). Therefore, it is expected that further analysis of these molecules may be useful for treatment of patients with antibody deficiencies, such as common variable immunodeficiency, characterized by a lack of memory B cells and suffering from infectious diseases.

Acknowledgments

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Research article

Interleukin-21 stimulates B-cell immunoglobulin E synthesis in human beings concomitantly with activation-induced cytidine deaminase expression and differentiation into plasma cells

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ABSTRACT

Interleukin (IL)-21 downregulates immunoglobulin E (IgE) production in murine systems by inhibiting germline ε transcription in IL-4-stimulated B cells. We here sought to clarify the function of IL-21 in human B-cell IgE synthesis. IL-21 dramatically enhanced IgE production by human mononuclear cells, or purified total, naive, or memory B cells in the presence of IL-4 plus anti-CD40 mAb cross-linked with CD32-transfectants, and the production was strengthened with further addition of IL-10. It was concomitant to the enhancement of activation-induced cytidine deaminase (AID) mRNA expression, but no increase of germline ε transcription. We also observed that IL-21 promoted B-cell differentiation into plasma cells with increase of B-lymphocyte-induced maturation protein-1 (Blimp-1), but not X-box binding protein 1 (XBP-1), which was further accentuated by co-stimulation with IL-4 plus CD40 signaling. Thus, IL-21 is a strong inducer of IgE production in human beings concomitantly with AID expression and the differentiation into plasma cells. Our data suggest that IL-21 plays an important role in occurrence and the treatment of allergic disorders.

1. Introduction

Allergen-specific immunoglobulin (Ig) E plays a crucial role in the pathogenesis of allergic disorders. The processes involved in IgE production by B cells include germline ε transcript expression, IgE class switching, clonal expansion of B cells, and differentiation into IgE-secreting plasma cells [1]. A variety of cytokines, including interleukin (IL)-4, IL-10, interferon (IFN)-γ, and IL-13, control IgE production. IL-4 is an important cytokine that promotes IgE synthesis, in which there are two effects: a polarization of T-cell responses toward T-helper (Th) 2 cells, and germline ε transcripts in activated B cells [2]. Stimulation of CD40 in addition to IL-4 or IL-13 is also necessary for IgE synthesis, presumably by inducing class switch recombination (CSR). The crucial role of activation-induced cytidine deaminase (AID) in CSR has been established by the observation that CSR is markedly impaired both in AID-deficient mice [3] and in a subset of patients with autosomal-recessive hyper-IgM syndrome who had mutations in the AID gene and do not produce IgE *in vitro* [4]. B-lymphocyte-induced maturation protein-1 (Blimp-1) and X-box binding protein-1 (XBP-1) are also novel genes which are involved in the terminal differentiation of B cells into immunoglobulin secretary plasma cells [5, 6]. IL-10, which is a major regulatory cytokine involved in inflammatory responses, enhances B-cell IgE synthesis by promoting differentiation into plasma cells [7]. Several other cytokines, including IL-6, IL-7, and IL-9, enhance both IgE and IgG4 production in vitro assays. In contrast, IFN-γ inhibits IgE synthesis, by suppressing the IL-4 induction and germline ε transcripts and Th2 differentiation [8,9].

IL-21 is the most recently identified type I cytokine, with receptors that contain the common cytokine receptor γ chain (γc) also found in IL-2, IL-4, IL-7, IL-9, and IL-15 [10]. IL-21 is made by both $CD4^+$ T cells and NKT cells [11], but IL-21 has a much broader range of cell targets and functions; IL-21 has a role in the proliferation and maturation of natural killer (NK) cells, enhancement of the cytotoxic function of NK cells, proliferation of CD8+ T cells, production of Ig, and apoptosis of B cells. IL-21 receptor-deficient mice and IL-21 transgenic mice demonstrated that IL-21 does not have an essential role in the development or proliferation of B cells; nevertheless, IL-21 has been shown to regulate Ig production [12].

The suppressive function of IL-21 on murine B-cell IgE synthesis was extrapolated from the analysis that $IL\text{-}21R^{-/-}$ mice have a lower serum concentration of IgG1, which is analogous to human IgG4, but a considerably higher serum concentration of IgE than wild-type mice in response to antigen [12]. Another finding supporting this is that IL-21 blocks IgE production in murine systems by inhibiting germline ε transcription stimulated by IL-4 without altering signal transducer and activator of transcription 6 (STAT6) activation [13]. Regarding human systems, Caven *et al.* have shown that IL-21 together with IL-4 and CD40 signaling enhanced plasma

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cell differentiation and IgE production in low-density tonsillar B cells [14]. In marked contrast, it has been reported that IL-4 inhibited IL-21–induced differentiation into plasma cells by reducing the expression of Blimp-1[15]. Meanwhile, Pene *et al.* revealed that IL-21 differentially regulates IL-4–induced human IgE production by various conditions such as IL-21 concentrations and INF-γ production in the culture systems [16].

Despite this, however, the effects of IL-21 on human B-cell IgE synthesis have not been clearly evaluated. To clarify this, the present study investigated IgE synthesis by the stimulation of mononuclear cells (MNCs) and purified human B-cell populations with IL-21 *in vitro* assay, and found that IL-21markedly enhances IgE synthesis.

2. Subjects and methods

2.1. Antibodies and reagents

FITC-conjugated anti-human CD20 mAb (anti-CD20-FITC) and PE-conjugated anti-human CD38 mAb (anti-CD38-PE) were purchased from DAKO Japan (Tokyo, Japan). Anti-CD40 mAb (G28.5; IgG1) was obtained from American Type Culture Collection (Manassas, VA). Human IL-4 was obtained from PeproTech EC (London, UK). Human IL-10 was obtained from Genzyme (Cambridge, MA). Human IL-21 was obtained from BioSource International Inc. (Camarillo, CA).

2.2. Cell contents and preparation

Human adult peripheral blood was obtained from volunteers (27–48 years of age; male:female ratio, 3:7) with no history of allergic disorders (asthma, atopic dermatitis, and/or perennial rhinitis). Peripheral mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Piscataway, NJ). Human B cells were obtained by the negative selection, which was performed in using a RosetteSep-human B-cell cocktail (StemCell Technologies, Vancouver, Canada) which included anti-CD2, CD3, CD16, CD36, and CD56 mAb. Whole blood was incubated with RosetteSep-human B cells for 20 minutes at room temperature and centrifuged over Ficoll-Hypaque. The cells at the interface were washed twice with phosphate-buffered saline (PBS) and the resulting population of B cells was 85% ± 5%, mean ± SD with ≤1% of CD3 positive and ≤2% of CD56-positive cells. $CD20^+$ $CD27^-$ or $CD20^+$ $CD27^+$ B cells were isolated from the peripheral blood mononuclear cells by sorting with a FACStar Plus (Becton Dickinson, San Jose, CA) under sterile conditions. Both populations obtained were ≥98% pure.

2.3. Preparation and fixation of transfectants

CD32 (Fc γ II receptor)-transfectants (CD32T) were prepared for strengthening the CD40 signaling with CD40 mAb cross-linked with CD32 molecule by conventional methods as described previously [17–19]. For fixation, the transfectants were incubated with 1% paraformaldehyde in PBS for 5 min. After washing three times with PBS, the cells were cultured in RPMI-1640 containing 10% fetal calf serum for 30 minutes, and after washing were used for analysis.

2.4. Flow-cytometric analysis

Activated B cells were stained with anti-CD20-FITC and anti-CD38-PE, or with anti-CD38-PE and anti-IgE-FITC (Sigma) after permeabilize membranes with BD Cytofix/Cytoperm (Becton Dickinson). Two-color analysis of B-cell surface or cytoplasmic molecules was performed using a FACS Calibar cytometer (Becton Dickinson). The antibody-coated cells were gated on living cells by cell size and granularity and were then counted.

2.5. Cell stimulation and IgE assay

Either PBMCs or highly purified human B cells, which were aliquoted to a final cell density of 5–10×105 cells/ml in a volume of 200 μl/well, were cultured with medium in 96-well round-bottom plates (Nunc, Rosklide, Denmark) in the presence of 50 ng/ml of IL-4 alone, 50 ng/ml of IL-4 plus 1 μg/ml of CD40 mAb with or without CD32T, and IL-4 plus CD40 mAb/CD32T plus 50 ng/ml of IL-10 at 37°C in a humidified 5% CO_2 atmosphere. The density of CD32T was 40 × 103 cells/well which was 20% of total B-cell numbers. It was in these conditions that we stimulated the PBMCs and B cells by various concentrations of IL-21 (from 10 to 50 ng/ml). Cultured supernatants of PBMCs and purified B cells were harvested on day 14 for IgE assays and transferred to 96-well flat enzyme-linked immunoabsorbent assay (ELISA) plates (NUNC), which had been coated with anti-human IgE mAbs (CIA-E7.12 and CIA-E4.15) provided by Prof. A. Saxon, Division of Clinical Immunology/Allergy, University of California at Los Angeles. After 2 hours incubation at room temperature, the supernatants were discarded and the wells were washed with 0.05% Tween 20 in PBS. Alkaline phoshatase-labeled goat anti-human IgE (Sigma, St. Louis, MO) was added at a dilution of 1/5000. After 2 hours incubation at room temperature, color was detected in 3-[cyclohexylamino]-1-propanesulphonic acid (CAPS) buffer containing p-nitrophenyl phosphate (pNPP) (Sigma). The optical density was determined with an automated ELISA plate reader (Bio-Rad, Hercules, CA), and standard curves were constructed using serial 1/2 dilutions of human IgE (Chemicon, Temwcula, CA) from 8 ng /ml to 1000 ng/ml. The dose examination of IL-21 revealed that the 25 ng/ml of IL-21 strongly stimulated the production of IgE (data not shown).

2.6. Detection of germline ε, AID, Blimp-1, and XBP-1 transcripts

Highly purified human B cells were stimulated with medium, 50 ng/ml of IL-4 alone, or IL-4 plus 1 μg/ml of CD40 mAb with or without 25 ng/ml of IL-21 for 24 hours. Total RNA was extracted with a TRIzol rapid RNA purification kit (Life Technologies, Grand Island, NY). First-strand cDNA copies were synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo (dT) (Invitrogen, San Diego, CA) as the primer in a total volume of 20 μl. The following oligonucleotide primers were used: for germline ε, 5′-CATGCGGTCCACGACCAAGAC-3′ and 5′-CCACTGCACAGCTGGATGGAC-3′, and for AID, 5′-GAGGCAAGAAGACACTCTGG-3′ and 5′-GTGACATTCCTGGAAGTTGC-3′, for XBP-1, 5′-GGTGCGTAGTCTGGAGCTAT-3′ and 5′-GCAAAAGTGTCCTCCCAAGAAT-3′, and for Blimp-1, 5′-CAACCAACCAGTCCCAGCTTC-3′ and 5′-TCACAATGGGGTAGGACTGCG-3′ sense and antisense, respectively. The β 2-microglobulin (β2-MG) sense primer 5′-ACCCCCACTGAAAAAGA-3′ and antisense primer 5′-CTCCTAGAGCTACCTGTGGAGCA-3′ were used as controls. A quantity of 2 μl cDNA was amplified in using each primer and Taq DNA polymerase (Sigma) by 35 cycles of the following steps: denaturation (94°C, 30 seconds), annealing (57°C, 30 seconds) and elongation (72°C, 60 seconds). The final polymerization step was extended for 5 minutes more. The amplified products were analyzed on a 1.2 % agarose gel and visualized by ultraviolet light illumination. The relative integrated OD of the mRNA bands was estimated with Image Processing and Analysis in Java.

2.7. Immunoblot analysis

Extracts prepared from the 5-day cultured B cells were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride membranes. Membranes were incubated with the rabbit polyclonal antibodies to human XBP-1 (Santa Cruz Biotech., CA) or β-actin (Cell Signaling technology, Danvers, MA). Membranes were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Promega Corpora-