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Lack of association between E148Q *MEFV* variant and Kawasaki disease

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ABSTRACT

We investigated a possible association between Kawasaki disease (KD), a systemic vasculitis of unknown etiology, or its coronary artery lesions (CAL) and *MEFV* gene variants including E148Q, the most common and mild mutation in the *MEFV* gene for familial Mediterranean fever or vasculitis-related disorders. The study population comprised a total of 138 Japanese patients with KD, including 45 patients with CAL and 93 patients without CAL and 170 normal controls. Sequence variations for the *MEFV* gene were detected by direct sequencing, followed by the TaqMan SNP genotyping assay. The genotype and allele frequencies of *MEFV* gene variants (E148Q, L110P, R202Q, P369S, R408Q) were compared between KD patients with and without CAL or between KD patients with CAL and controls. E148Q heterozygotes and homozygotes were observed in 37.1 and 5.5% of healthy controls, 33.3 and 5.1% of KD patients, and 37.8 and 4.4% of KD patients with CAL. No significant differences were observed in the genotype and allele frequencies of other *MEFV* gene variants (L110P, R202Q, P369S, R408Q) between KD patients with and without CAL or between KD patients with CAL and controls. No associations were detected between the *MEFV* gene variants and the development of KD or CAL formation in KD.

1. Introduction

Kawasaki disease (KD) is an acute, self-limited systemic vasculitis that occurs predominantly in infants and young children. Coronary artery aneurysm or ectasia develops in <5 to 25% of untreated children with the disease [1,2]. Its etiology remains unknown; however, clinical and epidemiological features strongly indicate that it is caused by one or several widely distributed infectious agents [2]. It is likely that KD results from an abnormal immunologic response to certain microbial agents in genetically susceptible individuals. The higher rate of KD in the siblings of KD patients and the racial difference in its incidence support this consideration [2]. Recently, several host genetic factors have been identified in the development of KD and coronary artery lesions (CAL) [3–6].

Familial Mediterranean fever (FMF) is an inherited inflammatory disease that is common in Arabs, non-Ashkenazi Jews, Armenians, and Turks, whereas it is uncommon in east Asia, including Japan. FMF is characterized by self-limited periodic fever and various symptoms such as peritonitis, arthritis, rash, pleurisy, and pericarditis. The *MEFV* gene is responsible for FMF [7,8]. Among the *MEFV* mutations, the role of E148Q (c.442 G>C) is still controversial. Although some reports indicated that E148Q was only one of the gene polymorphisms, other reports indicated that E148Q was associated with the mildest disease with a low penetrance or usually required another additional *MEFV* mutation to cause the classical manifestation of FMF [9,10]. Although FMF is an uncommon disorder in Japan, the frequency of E148Q is higher in Japanese than in European or Arab populations [11–13]. *MEFV* was predominantly expressed in granulocytes and monocytes [7], both of which play major roles in the pathophysiology of KD at the acute phase [2]. Several reports revealed that *MEFV* mutations were associated with vasculitis-related disorders such as Behçet's disease, Henoch–Schönlein purpura, and polyarteritis nodosa [14–16], suggesting that *MEFV* gene mutations contribute to the development of a broader spectrum of vasculitis. Furthermore, it was reported that *MEFV* mutations might increase the baseline of inflammation, induce the development of rheumatic diseases, and affect the clinical course of inflammatory disorders [17].

To clarify the role of the *MEFV* gene in the development of KD as one of the host genetic factors, we investigated the associations between KD and *MEFV* gene variants, particularly E148Q, which is common in Japanese populations.

2. Subjects and methods

One hundred thirty-eight KD patients who were treated with oral aspirin plus intravenous immunoglobulin (IVIG:1–2 g/kg/total in CAL$^-$ patients and 3–4 g/kg/total in CAL$^+$ patients) at Kyushu University Hospital or its affiliated hospitals from 1991 through 2003 were enrolled. Informed consent was obtained from their parents, and the Ethical Committees of Kyushu University approved the study. All patients were Japanese and met the appropriate diagnostic criteria for KD [18]. The study population consisted of 92 boys and 46 girls; the median age at diagnosis was 19 months

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Table 1
Clinical and laboratory data of KD patients

Variables	With CAL ($n = 45$) Median (range)	Without CAL ($n = 93$) Median (range)	p value*
Age (months)	19 (1–151)	19 (2–105)	0.614
Admission (day of illness)	4 (1–13)	4 (1–9)	0.552
Start of IVIG (day of illness)	5 (2–15)	5 (1–9)	0.993
Duration of fever (days)	10 (4–27)	7 (3–15)	7.60×10^{-7}†
Peak of white blood cell ($\times10^3/\mu l$)	16.7 (7.3–35.9)	15.6 (7.9–31.0)	0.357
Peak of C-reactive protein (mg/dl)	15.5 (3.3–32.4)	9.6 (2.0–33.3)	0.00534†

IVIG = intravenous immunoglobulin therapy.
*Mann–Whitney U test.
†Significant difference.

(range: 1 to 151 months). Forty-five patients developed CAL and 93 patients did not. According to the criteria of the Japanese Ministry of Health, Labour, and Welfare, the coronary artery was considered abnormal if the diameter of the initial lumen was >3 mm in a child younger than 5 years or >4 mm in a child at or over 5 years of age or if the initial diameter of a segment was at least 1.5 times larger than that of an adjacent segment [19]. Clinical and laboratory data are shown in Table 1.

Peripheral blood was collected from KD patients and 170 randomly selected healthy Japanese volunteers. The donors and their families had no episodes of periodic fever similar to FMF. Genomic DNA was extracted from whole-blood leukocytes with a QIAamp blood kit (Qiagen GmbH, Hilden, Germany).

We screened coding regions of the *MEFV* gene for polymorphisms from genomic DNA of KD patients by direct sequencing with an ABI Prism 3100 Genetic Analyzer (PerkinElmer, Foster City, CA), as described previously [7,20]. The forward and reverse oligonucleotide primers used to amplify each exon were as follows (all oligonucleotide sequences are given 5′ to 3′): exon 1 forward, AACCTGCCTTTTCTTGCTCA; exon 1 reverse, CACTCAGCACTGGATGAGGA; exon 2a forward, ATCATTTTGCATCTGGTTGTCCTTCC; exon 2a reverse, TCCCCTGTAGAAATGGTGACCTCAAG; exon 2b forward, GGCCGGGAGGGGGCTGTCGAGGAAGC; exon 2b reverse, TCGTGCCCGGCCAGCCATTCTTTCTC; exon 3 forward, GAACTCGCACATCTCAGGC; exon 3 reverse, AAGGCCCAGTGTGTCCAAGTGC; exon 4 forward, TTGGCACCAGCTAAAGATGGC; exon 4 reverse, TCTCCCTCTACAGGGATGAGC; exon 5 forward, TATCGCCTCCTGCTCTGGAATC; exon 5 reverse, CACTGTGGGTCACCAAGACCAAG; exon 6 forward, TCCAGGAGCCCAGAAGTAGAG; exon 6 reverse, TTCTCCCTATCAAATCCAGAG; exon 7 forward, AGAATGTAGTTCATTTCCAGC; exon 7 reverse, CATTTCTGAACGCAGGGTTT; exon 8 forward, GCATGCTCACTTCCTCCCTA; exon 8 reverse CTTTGCTCCAGGTGTTTGGT; exon 9 forward, TTAGACCACAGTCCCCAACA; exon 9 reverse, CAGGAAACAGGGACAGGGTA; exon 10a forward, CCAGAAGAACTACCCTGTCCC; exon 10a reverse, AGAGCAGCTGGCGAATGTAT; exon 10b forward, GAGGTGGAGGTTGGAGACAA; exon 10b reverse, TCCTCCTCTGAAATCCATGG. Exons 2 and 10 were amplified in two overlapping polymerase chain reaction (PCR) fragments. The PCR conditions were as follows: initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for exons 1, 3, 4, and 5 and each part of exon 10, 58°C for exons 6, 7, 8, and 9, 55°C for each part of exon 2 for 30 seconds and extension at 72°C for 30 second, followed by a final extension step at 72°C for 5 minutes.

Genotyping of each variant was carried out using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA): *MEFV* L110P (rs11466018, Applied Biosystems code c_11186727_10), *MEFV* R202Q (rs224222, c_2394721_10), *MEFV* P369S (rs11466023, c_2394737_10), and *MEFV* R408Q (rs11466023, c_45171223_10). Detection of probes and primers for *MEFV* E148Q (rs3743930) was performed using Custom TaqMan SNP genotyping assays, and each oligonucleotide sequence was as follows (given 5′ to 3′); forward primer, CCAGCCTGCGGTGCA; reverse, GCCTTCTCTCTGCGTTTGCT;

Table 2
Polymorphisms of the *MEFV* genes in KD patients with and without CAL and control subjects

Gene	Genotype/ allele	Ctrl ($n = 170$)	KD total ($n = 138$)	KD CAL (+) ($n = 45$)	KD CAL (−) ($n = 93$)	Ctrl vs KD total			KD CAL (+) vs Ctrl			KD CAL (+) vs CAL (−)		
						p value	OR	95% CI	p value	OR	95% CI	p value	OR	95% CI
E148Q	GG	98 (0.58)	85 (0.62)	26 (0.58)	59 (0.63)									
	GC	63 (0.37)	46 (0.33)	17 (0.38)	29 (0.31)	0.496			0.824			0.512		
	CC	9 (0.05)	7 (0.05)	2 (0.04)	5 (0.05)									
	Allele G	259 (0.76)	216 (0.78)	69 (0.77)	147 (0.79)									
	Allele C	81 (0.24)	60 (0.22)	21 (0.23)	39 (0.21)	0.540	0.89	0.608–1.30	0.923	0.97	0.562–1.68	0.655	1.15	0.628–2.10
L110P	TT	139 (0.82)	115 (0.83)	36 (0.80)	79 (0.85)									
	TC	29 (0.17)	22 (0.16)	9 (0.20)	13 (0.14)	0.598			0.445			0.321		
	CC	2 (0.01)	1 (0.01)	0	1 (0.01)									
	Allele T	307 (0.90)	252 (0.91)	81 (0.90)	171 (0.92)									
	Allele C	33 (0.10)	24 (0.09)	9 (0.10)	15 (0.08)	0.667	0.89	0.510–1.54	0.933	1.03	0.475–2.25	0.593	1.27	0.532–3.02
R202Q	GG	163 (0.96)	132 (0.96)	43 (0.96)	89 (0.96)									
	GA	7 (0.04)	6 (0.04)	2 (0.04)	4 (0.04)									
	AA	0	0	0	0									
	Allele G	333 (0.98)	270 (0.98)	88 (0.98)	182 (0.98)									
	Allele A	7 (0.02)	6 (0.02)	2 (0.02)	4 (0.02)	0.855	1.05	0.351–3.18	0.751	1.08	0.221–5.30	0.688	1.03	0.186–5.75
P369S	CC	153 (0.90)	120 (0.87)	38 (0.84)	82 (0.88)									
	CT	17 (0.10)	18 (0.13)	7 (0.16)	11 (0.12)									
	TT	0	0	0	0									
	Allele C	323 (0.95)	258 (0.93)	83 (0.84)	175 (0.94)									
	Allele T	17 (0.05)	18 (0.07)	7 (0.16)	11 (0.06)	0.417	1.33	0.670–2.62	0.307	1.60	0.643–3.99	0.557	1.34	0.502–3.59
R408Q	GG	157 (0.92)	123 (0.89)	40 (0.89)	83 (0.89)									
	GA	13 (0.08)	15 (0.11)	5 (0.11)	10 (0.11)									
	AA	0	0	0	0									
	Allele G	327 (0.96)	261 (0.95)	85 (0.94)	176 (0.95)									
	Allele A	13 (0.04)	15 (0.05)	5 (0.06)	10 (0.05)	0.340	1.45	0.676–3.09	0.466	1.48	0.513–4.27	0.825	1.04	0.343–3.12

CI = confidence interval; Ctrl = Control subjects; OR = odds ratio.
Numbers in parentheses indicate the percentages of the genotype or allele frequencies. After Bonferroni's correction of multiple comparison, $p < 0.0166$ was considered statistically significant.
All evaluated SNPs in controls were under Hardy–Weinberg disequilibrium.

reporter 1 (VIC), CAGCCCGAGGCCG; reporter 2 (FAM), CAGCCCCAGGCCG. Genotyping using the TaqMan method was performed with an ABI Prism 7700 sequence detection system.

A χ^2 test was used to compare the genotype and allele frequency distributions of each variant between the KD patients and controls, between the KD patients with and without CAL, and between KD patients with CAL and controls. We also used the Mann–Whitney *U* test to compare the clinical and laboratory data between patients with and without minor alleles of E148Q or other *MEFV* variants.

3. Results

We first analyzed the allelic frequency of E148Q (c.442 G>C), which is a common SNP in Japanese populations [21] compared with other populations, in 138 KD patients and 170 controls by Taqman genotyping assay. E148Q heterozygotes and homozygotes were observed in 37.1 and 5.5% of healthy controls, 33.3 and 5.1% of KD patients, and 37.8 and 4.4% of KD patients with CAL (Table 2). The genotype and allele frequencies of E148Q variant between KD patients and healthy controls, KD patients with and without CAL, or KD patients with CAL and controls did not differ.

Because it might be still possible that other *MEFV* gene variants were associated with the development of KD in combination with E148Q, we investigated other *MEFV* gene variants by direct sequencing of PCR-amplified genomic DNA from 53 KD patients who had at least one Q148 allele. We found four types of nonsynonymous variants, L110P (c.329T>C), R202Q (c.606G>A), P369S (c.1105C>T), and R408Q (c.1223G>A), in 23, 1, 9, and 6 of the 53 patients, respectively. Then, we performed genotyping of the four gene variants for the 138 KD patients and 170 controls using TaqMan SNP genotyping assays. As a result, the genotype and allele frequencies of each variant indicated no significant differences between all KD patients and healthy controls or between KD patients with and without CAL (Table 2). We performed haplotype analysis of the five *MEFV* variants, but the assembly of these alleles indicated no significant differences between KD and healthy controls (data not shown). Clinical and laboratory data were analyzed by the comparison of subgroups with E148/E148, Q148/Q148, E148/Q148 or Q148/Q148, (E148/Q148 or Q148/Q148) and (L110/P110 or P110/P110), (E148/Q148 or Q148/Q148), and (P369/S369) (Table 3). The median serum C-reactive protein concentration (18.2 mg/dl) tended to be higher in Q148 homozygous or heterozygous groups (E/Q or Q/Q) than that in wild type group (9.3 mg/dl; E/E) among KD patients with CAL ($p = 0.059$), but there were no significant differences between the other groups in other variables.

Several limitations must be acknowledged and addressed regarding the present study. The sample sizes in this study did not have a sufficient power of statistical analysis, especially for the L110P, R202Q, P369S, and R408Q, because their minor allele frequencies in the controls were as low as 0.10 or less. Based on the minor allele frequencies of E148Q (0.24), L110P (0.10), R202Q (0.02), P369S (0.05), R408Q (0.04), or the cohort size, the estimated smallest detectable risks of their variants were calculated to 1.45, 1.67, 2.54, 1.89, and 2.07 for KD development (control: $2N = 340$ vs. KD: $2N = 276$); 1.68, 2.01, 3.40, 2.34, and 2.45 for CAL formations (control: $2N = 340$ vs. KD CAL(+): $2N = 90$), respectively. Alternatively, the study would require 81, 432, >1000, >1000, and >1000 KD patients to reach statistical significance ($p < 0.05$), respectively, if the relative risks for KD development were set to 1.50.

4. Discussion

Ozen *et al.* [17] reported that among 70 individuals with *MEFV* gene mutations, 28 (40.0%) had some form of rheumatic complaints and 15 (21.4%) developed rheumatic diseases or vasculitis, including Behçet's disease. They also reported that 30.5% of the children with rheumatic diseases and 25.4% of patients with juvenile idiopathic arthritis had mutations of the *MEFV* gene [17]. FMF and Behçet's disease are not rare disorders in the eastern Mediterranean and it was suggested that the *MEFV* mutation played a role in

Table 3
Clinical and laboratory data of all KD patients or KD patients with CAL, subgrouped by combined genotypes of MEFV gene

Population	All KD patients								
Subgroup	(1)	(2)	(3)	(4)	(5)	Comparison (*p* values)			
Genotype									
E148Q	E/E	Q/Q	E/Q or Q/Q	E/Q or Q/Q	E/Q or Q/Q				
L110P	–	–	–	L/P or P/P	–				
P369S	–	–	–	–	P/S				
Comparison						(1) vs (2)	(1) vs (3)	(1) vs (4)	(1) vs (5)
Number	85	7	53	23	9				
Variables									
Age (months)	18 (2–151)	22 (7–66)	22 (1–96)	20 (2–88)	40 (2–96)	0.473	0.38	0.669	0.338
Admission (day of illness)	4 (1–13)	3 (2–8)	4 (1–10)	4 (2–10)	4 (1–6)	0.348	0.382	0.243	0.870
Start of IVIG (day of illness)	5 (2–10)	4 (3–8)	5 (1–15)	4 (1–15)	4 (3–6)	0.552	0.518	0.485	0.218
Duration of fever (days)	7 (3–27)	6 (5–12)	7 (4–20)	7 (4–17)	7 (5–20)	0.781	0.574	0.451	0.55
Peak of white blood cell ($\times 10^3/\mu l$)	16.2 (7.3–35.9)	12.5 (10.8–21.3)	15.7 (8.8–31.0)	15.4 (8.8–22.6)	15.6 (12.5–19.1)	0.381	0.992	0.328	0.931
Peak of C-reactive protein (mg/dl)	9.3 (2.5–33.3)	12.2 (3.5–23.9)	13.4 (2.0–32.4)	5.4 (2.0–22.6)	10.7 (9.2–13.3)	0.664	0.157	0.518	0.495
	KD patients with CAL								
Subgroup	(1)	(2)	(3)	(4)	(5)	Comparison (*p* values)			
Genotype									
E148Q	E/E	Q/Q	E/Q or Q/Q	E/Q or Q/Q	E/Q or Q/Q				
L110P	–	–	–	L/P or P/P	–				
P369S	–	–	–	–	P/S				
Comparison						(1) vs	(1)	(1)	(1) vs (5)
Number	26	2	19	9	2				
Variables									
Age (months)	17 (2–151)	(13, 66)	38 (1–96)	17 (5–77)	(40, 96)	0.459	0.186	0.752	0.152
Admission (day of illness)	4 (2–13)	(3, 3)	4 (1–10)	4 (3–10)	(3, 4)	0.345	0.600	0.779	0.629
Start of IVIG (day of illness)	5 (2–10)	(3, 6)	5 (3–15)	5 (3–15)	(3, 5)	0.694	0.757	0.779	0.459
Duration of fever (days)	10 (4–27)	(6, 12)	11 (5–20)	12 (5–17)	(11, 20)	0.629	0.990	0.690	0.247
Peak of white blood cell ($\times 10^3/\mu L$)	16.2 (7.3–35.9)	(10.8, 21.3)	16.8 (10.8–22.6)	16.9 (9.9–22.6)	(16.7, 19.1)	0.636	0.843	0.607	0.776
Peak of C-reactive protein (mg/dl)	9.3 (3.9–29.3)	(11.8, 12.7)	18.2 (3.3–32.4)	10.7 (9.2–13.3)	(12.5, 13.7)	0.784	0.059	0.836	0.717

Patients who had Q148 + Q202 and Q148 + Q408 were so rare ($n = 1$ and 6) that we did not analyze this group.

the pathogenesis of Behçet's disease [16]. Furthermore, Rozenbaum and Rosner reported that the prognosis of 3 juvenile idiopathic arthritis patients who had a M694V mutation of the *MEFV* gene was extremely poor [22]. In addition, Tunca *et al.* demonstrated an increased acute phase response in carriers for the *MEFV* gene [23]. These reports indicated that *MEFV* gene mutation was also related to enhanced inflammatory responses and severity in these disorders. On the other hand, we reported that *MEFV* gene variants in Japanese subjects were not related to the development of KD or CAL formation in KD and played no major role in the inflammatory responses in KD.

Most Japanese FMF patients have compound heterozygous *MEFV* mutations [21,24,25]. Among them, E148Q is the most common and is reported to have a weak effect that serves only to enhance the development and severity of FMF that was primarily induced by another mutation [25]. We reported that Q148 was not associated with the development of KD that occurs in a higher frequency in Japanese populations. It is possible that the functional effect of Q148 might be too small to accelerate the inflammation for the development of KD or to exacerbate vasculitis in KD. Because Q148 was not significantly associated with enhanced inflammatory responses in KD patients, it is less likely that these Q148 homozygotes will develop FMF, rheumatic disease, or vasculitis in the future. Rather, the high frequency of Q148 variant and the low incidence of FMF, as well as the presence of many asymptomatic Q148 homozygotes in Japanese populations strongly suggest that E148Q is one of the genetic polymorphisms of the *MEFV* gene with little functional significance. Further long-term evaluation of Q148 homozygotes would be necessary to clarify a role of Q148 in the pathogenesis of rheumatic or vasculitis disorders.

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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

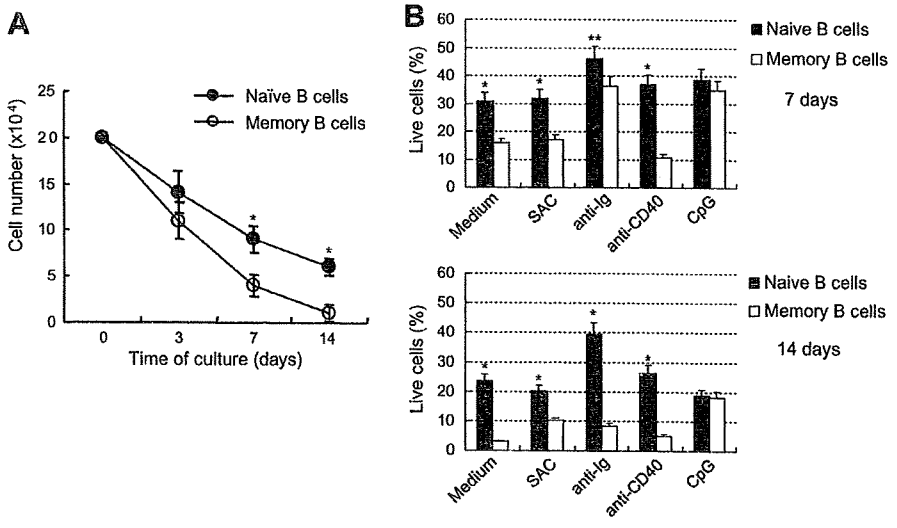


Fig. 1. Cell viability of na and memory B cells. Purified adult $CD27^-$ naive and $CD27^+$ memory B cells were separated by means of flow cytometry. The purity of both types of B cells was more than 95%. B cells (2×10^5) were cultured (A) without stimulation or (B) with SAC (0.01%), anti-Ig (5 μg/ml), 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.

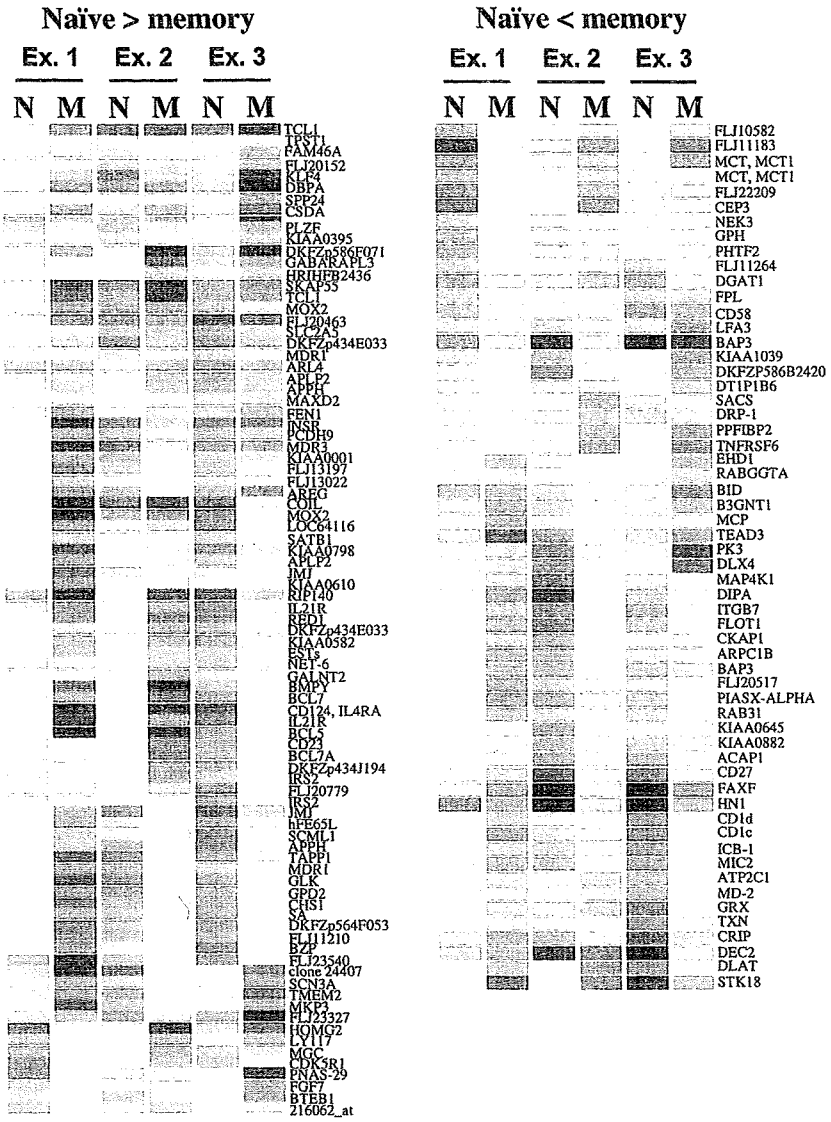


Fig. 2. Comparison of gene profiles between resting na and memory B cells. Human circulating $CD27^-$ na and $CD27^+$ memory B cells were obtained by sorting ($n = 3$). Total RNA was extracted and used to conduct GeneChip analysis. Each experiment contained three independent donors. Shown are only those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

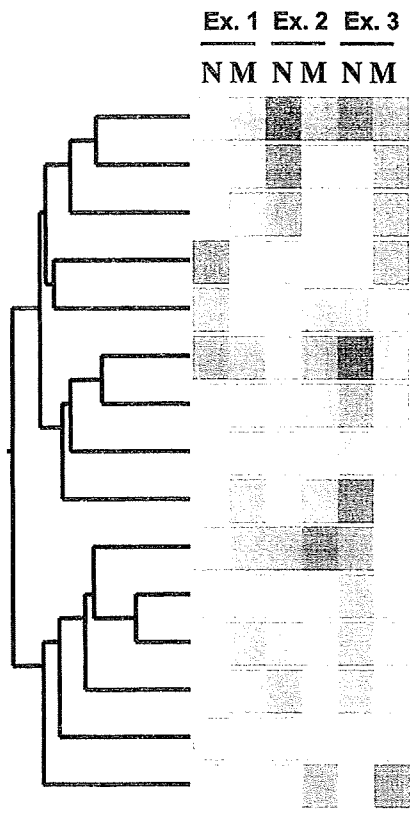
(BHLHB3, 14.3-fold), thioredoxin (TXN, 2.5-fold), cystein-rich protein 1 (CRIP1, 3.8-fold) and NIMA-related kinase 3 (NEK3, 2.0-fold), in addition to the well-known memory B-cell specific molecules, CD27 (3.6-fold) and FAS (2.9-fold).

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.

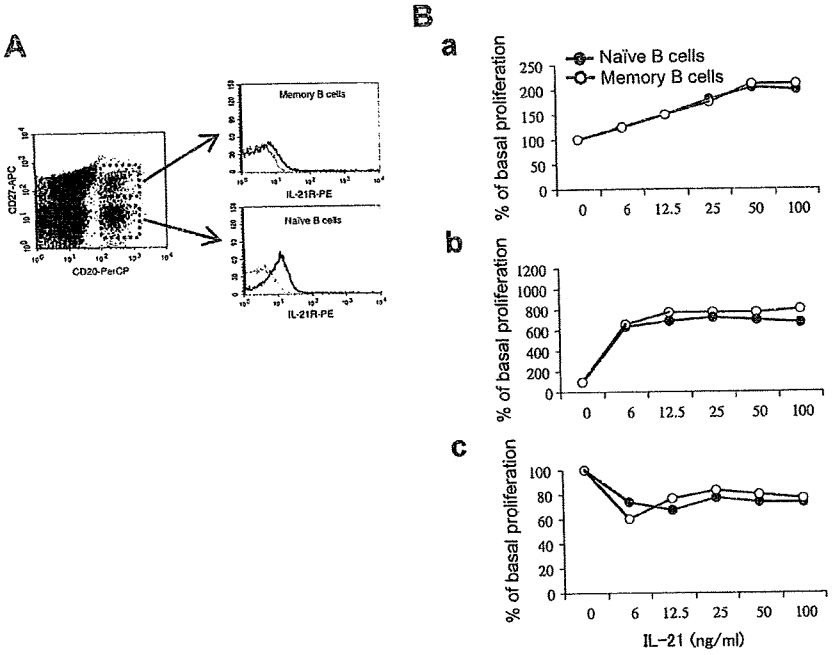


Fig. 4. Different response of B-cell subsets by IL-21. (A) Human circulating MNCs were stained with anti-IL-21R-PE, anti-CD20-PerCP and biotin conjugated anti-CD27 followed with avidin-APC. Expression of IL-21R on na or memory B cells is shown with a log scale (solid line). Isotype-matched PE-labeled 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.

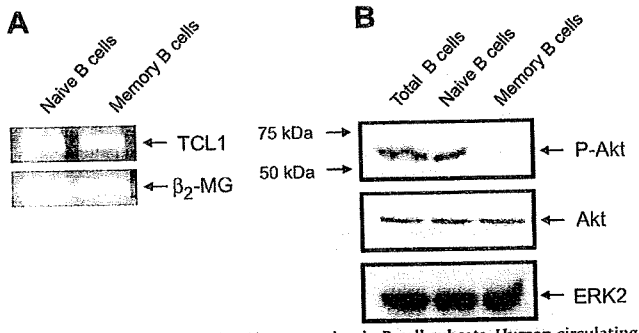


Fig. 5. TCL1, Akt and phospho-Akt expression in B-cell subsets. Human circulating $CD27^-$ na and $CD27^+$ memory B cells were obtained by sorting. (A) After extraction of total RNA from each B-cell subset, RT-PCR was performed as described in Section 2. The β_2-microglobulin (β_2-MG) was used as a positive 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.

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AN ELEVATED VALUE OF HIGH MOBILITY GROUP BOX 1 IS A POTENTIAL MARKER FOR POOR RESPONSE TO HIGH-DOSE OF INTRAVENOUS IMMUNOGLOBULIN TREATMENT IN PATIENTS WITH KAWASAKI SYNDROME

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Abstract: We examined the serum values of high mobility group box 1 (HMGB1) in 36 patients with Kawasaki syndrome (KS) (29 responders and 7 poor-responders to initial intravenous immunoglobulin treatment). A mean value of HMGB1 of poor-responders was significantly elevated compared with those of responders ($P = 0.0042$). Among the 6 factors showing significant differences between responders and poor-responders including HMGB1 (admission illness day, white blood cell counts, C-reactive protein, aspartate aminotransferase, lactate dehydrogenase), values of HMGB1 showed the widest area under the receiver operating characteristic curve. In conclusion, an elevated HMGB1 value could be a potential marker for poor-responders.

Key Words: Kawasaki syndrome, high mobility group box 1, immunoglobulin treatment, treatment failure

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Kawasaki syndrome (KS) is an acute febrile vasculitis of childhood.[1,2] The most serious problem in KS patients is a coronary artery abnormality.[1,2] Activated monocytes/macrophages appear to have an important role in KS[2]; however, the relevance of these

cells and/or many cytokines to the pathophysiology of inflammation in KS is not fully understood.

High mobility group box 1 (HMGB1) is an abundant protein present in nuclei and cytoplasm and is involved in maintaining the nucleosome structure and the regulation of gene transcription.[3,4] Once released into the extracellular milieu, HMGB1 is a potent activator of monocytes/macrophages cytokine synthesis releasing multiple proinflammatory cytokines.[3–5] Additionally, monocytes/macrophages stimulated with TNF-α or IL-1β also induce HMGB1 release.[4] These mechanisms mediate a delayed and prolonged phase of monocytes/macrophages activation. Therefore, HMGB1 may be a good candidate as a prognostic marker in diseases that combine inflammation and cell (tissue) injury. The inflammation related to HMGB1 is similar to that of KS in many respects; such as marked cytokine cascade stimulation, monocytes/macrophages activation, and cell (tissue) injury. Taking these similarities into consideration, investigating the relevance of HMGB1 in patients with KS would clarify the usefulness of HMGB1 values for evaluating the severity of KS. Therefore, we examined the serum values of HMGB1 in KS along with many inflammatory cytokines.

PATIENTS AND METHODS

Patients with KS who were referred to and then admitted to the Kagoshima City Medical Association Hospital between January 2003 and December 2006, and ultimately fulfilled the diagnostic criteria of the Japanese Kawasaki Disease Research Committee[6] during the course of the disease, were the subjects for this study. All KS patients were treated with a single dose of 2 g/kg intravenous immunoglobulin (IVIG) and oral administration of 30 mg/kg aspirin. Patients who were treated with IVIG after the 7th day of illness were excluded. Patients who were treated without IVIG were also excluded. Coronary artery abnormalities estimated by echocardiography were assessed using the z score as described by Kurotobi et al.[7] KS patients were classified into 2 groups: responders and poor-responders. A responder was defined as a patient who showed resolution of fever (<37.5°C) within 48 hours after initial IVIG, and a poor-responder as a patient who needed additional IVIG (s) to resolve the fever. Patients who were referred to and admitted to Kagoshima City Medical Association Hospital and were diagnosed with bacterial infection during the same period were used as controls. Informed consent was obtained from each child's parents before routine blood sampling. Illness day 1 was determined as the first day of fever.

The values of HMGB1 in the sera of patients with KS and controls were measured using an ELISA kit (HMGB1 ELISA kit II; Shino-test Corporation, Tokyo, Japan). The levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p70) were measured using a Human Inflammation Kit (BD Biosciences, San Diego, CA).

Statistical Analysis. The Mann-Whitney *U* test was used to compare the mean ranks between the groups. Relationships between the value of HMGB1 and other factors were estimated using Spearman's rank correlation. To compare correlation coefficients, the z test was applied after Fisher's z transformation of each correlation coefficient. Cut-off point analysis using receiver operating characteristic curves was applied to maximize the sensitivity and accuracy for discriminating the poor-responders from the responders. Data were processed using a statistical program, SPSS 14.0 (SPSS Inc., Chicago, IL.). $P < 0.05$ was considered statistically significant.

RESULTS

We examined 36 patients with KS in the acute phase (Table 1). Twenty-six patients were used as controls; 18 patients had

TABLE 1. Laboratory and Cytokine Findings and Treatments and Outcomes

	Control	Kawasaki Syndrome		
		All Patients	Responder	Poor-Responder
Number (M/F)	26 (11/15)	36 (21/15)	29 (16/13)	7 (5/2)
Age (yrs)	2.1 (1.5; 0.6/2.0)	2.0 (1.7; 0.9/2.5)	1.8 (1.5; 1.0/2.2)	2.9 (3.3; 1.0/3.5)
Admission day	3.7 (3.0; 2.0/5.5)	4.0 (4.0; 3.0/5.0)	4.2 (4.0; 3.8/5.0)*	3.4 (3.0; 3.0/3.8)*
WBC ($10^2/\mu L$)	127 (115; 91/141)*	152 (144; 114/183)*	138 (136; 109/167)†	212 (192; 184/270)†
Plt ($10^4/\mu L$)	32 (31; 24/40)	35 (34; 30/42)	37 (35; 31/43)	30 (30; 24/31)
CRP (mg/dL)	10.1 (7.7; 6.0/12.1)	9.4 (8.2; 6.0/10.2)	8.5 (6.8; 5.9/9.9)*	12.8 (9.5; 8.4/16.6)*
AST (IU/L)	35 (31; 26/36)†	124 (51; 30/107)†	64 (48; 29/92)*	372 (291; 52/603)*
ALT (IU/L)	16 (13; 10/17)‡	109 (57; 15/136)‡	70 (45; 13/118)	268 (108; 51/395)
LD (IU/L)	439 (345; 284/476)‡	710 (632; 563/715)‡	620 (630; 534/680)†	1,086 (906; 654/1408)†
HMGB1 (ng/mL)	2.7 (0.8; 0/2.8)	2.4 (1.4; 0.6/2.5)	1.5 (1.2; 0.5/1.9)†	6.0 (3.0; 2.5/8.5)†
Number	25	33	26	7
TNF-α (pg/mL)	0.5 (0; 0/0)	0.7 (0; 0/0)	0.8 (0; 0/0)	0.4 (0; 0/0.9)
IL-1β (pg/mL)	2.9 (0; 0/5.7)†	0.8 (0; 0/0)†	0.8 (0; 0/0)	1.0 (0; 0/0)
IL-6 (pg/mL)	306 (47; 21/137)	136 (80; 40/126)	129 (61; 37/126)	162 (92; 66/276)
IL-8 (pg/mL)	106 (48; 25/83)	70 (48; 21/88)	63 (38; 20/77)	97 (99; 40/149)
IL-10 (pg/mL)	143 (15; 7/46)	29 (15; 7/34)	20 (13; 7/28)	60 (45; 15/96)
IL-12p70 (pg/mL)	2.7 (1.4; 0/4.3)	2.4 (0; 0/2.0)	2.9 (0; 0/2.6)	0.8 (0; 0/0)
Treatments and outcomes of patients with Kawasaki syndrome				
Day of initial IVIG		4.6 (5.0; 4.0/5.0)	4.7 (5.0; 4.0/5.0)	4.3 (4.0; 3.3/5.0)
Dose of initial IVIG (g/kg)		1.9 (2.0; 1.9/2.1)	1.9 (2.0; 1.9/2.1)*	2.2 (2.1; 2.0/2.2)*
Duration of fever (day)		6.3 (6.0; 5.0/7.0)	5.6 (5.0; 5.0/6.0)‡	10.7 (9.5; 9.0/10.0)‡
Coronary z score		2.4 (1.9; 1.1/2.7)	1.7 (1.6; 1.1/2.4)*	5.5 (3.2; 2.3/8.1)*

Data are expressed as mean (median; 25th percentile/75th percentile).

*$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

Admission day indicates day of illness at admission; Plt, platelet counts; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LD, lactate dehydrogenase; TNFα, tumor necrosis factor α; IL, interleukin; Day of initial IVIG, day of illness when initial intravenous immunoglobulin was given; coronary z score, the maximum coronary artery size during the first month of illness evaluated using echocardiography.

respiratory tract infection, 3 had urinary tract infection, 3 had bacterial meningitis, and 2 had enteritis. All control patients were successfully treated with antibiotics.

There were no significant differences in the mean ranks of age or values of C-reactive protein between patients with KS and controls at admission (Table 1). Significant elevated values of white blood cell counts (WBC), aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase (LD) were observed in the acute phase of KS compared with controls. Mean rank of value of HMGB1 did not differ between the acute phase of KS and controls. Extremely elevated values of HMGB1 in KS were observed at the third day of illness. In controls, 2 control patients showed striking elevation of HMGB1 (34.3 ng/mL, acute pneumonia; 9.2 ng/mL, urinary tract infection). Most of the values of inflammatory cytokines were higher in the controls than in the acute phase of KS; they did not differ significantly but IL-1β (Table 1).

Correlation coefficients between HMGB1 and laboratory findings of KS patients and control are examined. Correlations with values of HMGB1 showed different patterns between KS and controls. A significant positive correlation with HMGB1 was observed for WBC in KS ($\rho = 0.671$, $P < 0.0001$), whereas it was not observed in controls ($\rho = -0.063$). A similar correlation was observed between HMGB1 and neutrophil counts (KS, $\rho = 0.571$, $P = 0.0044$; control, $\rho = 0.162$). A significant positive correlation with LD ($\rho = 0.694$, $P = 0.0012$) was observed in controls, whereas it was not observed in KS ($\rho = 0.011$). Correlation coefficients in WBC or LD were significantly different between KS and control (WBC, $P = 0.001$; LD, $P = 0.003$).

Of 36 patients with KS, 7 needed additional IVIG (poor-responders) (Table 1). A mean rank of admission illness day in the poor-responders was significantly earlier than that in responders. Although the mean rank of initial IVIG dose in the poor-responders was slightly larger than that in responders, the duration of fever in the poor-responders was significantly longer than that in responders. Values of WBC, C-reactive protein, aspartate aminotransferase, LD, and HMGB1 were significantly elevated in the poor-responders compared with those in responders. Among these 5 factors and admission illness day, HMGB1 values showed the widest area under the receiver operating characteristic curve (0.852). A cut-off point analysis revealed an optimal point of 2.4 ng/mL of HMGB1 to screen for poor-responses (sensitivity, 86%; specificity, 86%; and accuracy, 86%).

DISCUSSION

It was interesting that the correlation coefficients between the values of HMGB1 and laboratory findings in the acute phase of KS showed different patterns from those in controls. Especially for WBC, a positive correlation was observed in KS but not in controls. Elevated WBC is commonly observed in KS, and highly elevated WBC is one of the risk factors for poor-responders.[8] In bacterial infections, WBC is usually elevated in severe inflammation, but is lowered in some extremely severe cases.[9] This difference in WBC might be the reason for the difference in the correlation coefficient between WBC and HMGB1 values.

It has been reported that HMGB1 induces only minimal cytokine production by macrophages unless bound to proinflammatory mediators when it acquires markedly enhanced cell-activating properties.[10] Under conditions of high levels of inflammatory mediators like bacterial infection or KS, marked secretion of HMGB1 may occur after the release of HMGB1 that resulted from tissue injury. It was, therefore, understandable for controls to show a positive correlation between values of HMGB1 and LD, which represent tissue injury. However, this correlation was not observed in KS, which suggests that mild to moderate elevations of LD observed in many KS patients[1,2] does not simply represent tissue injury related with HMGB1.

To examine as a prognostic marker in acute phase of KS, clinical profiles and laboratory findings were compared between responders and poor-responders. Among the many factors that show a significant difference between both groups, HMGB1 may be the most attractive candidate as a prognostic marker in KS. Prediction methods for poor-responders have already been reported; Kobayashi et al reported high sensitivity (86%) and specificity (68%) for the prediction of poor-responders.[8] Of 7 poor-responders in the present study, 5 were classified as high-risk patients using this method.[8] The remaining 2 poor-responders in the present study were classified as low-risk patients (both had scores of 1), and showed elevated levels of HMGB1 (5.9 and 18.8 ng/mL). On the other hand, one poor-responder with 0.7 ng/mL HMGB1 could have been screened as a high-risk patient (Kobayashi's score of 5). Therefore, in addition to the prediction methods for poor-responders reported previously, combinational use of HMGB1 values might increase sensitivity and specificity. Since this study was examined on the small number of specific patients (untreated or delayed treated patients were excluded), further studies will be needed to confirm a new combination method for prediction of poor-responders.

In conclusion, inflammation in acute phase of KS was different from the usual bacterial infections with respect to HMGB1. HMGB1 may be a useful tool for evaluating the severity of KS; thus an elevated value of HMGB1 would be a potential marker for poor-response to IVIG treatment in KS patients.

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bjh research paper

Platelet vascular endothelial growth factor is a useful predictor for prognosis in Kawasaki syndrome

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Summary

Kawasaki syndrome (KS) is an acute febrile vasculitis of childhood. Coronary artery abnormalities (CAA) are a significant problem in KS patients. High dose intravenous immunoglobulin (IVIG) is effective for reducing the occurrence of CAA. Clinical and histopathological findings suggest that vascular endothelial growth factor (VEGF) is involved in CAA. In circulating blood, newly activated platelets are the major source of VEGF, which is released in large amounts in vascular inflammation. The present study analysed 80 KS patients (69 IVIG responders and 11 IVIG non-responders) and evaluated the role of platelet VEGF in KS vasculitis. Serum VEGF and platelet VEGF levels were significantly higher in KS patients than controls ($P < 0{\cdot}001$). Platelet VEGF reflected the reactivity of IVIG treatment and was decreased in responders ($P < 0{\cdot}001$), but remained increased in non-responders ($P = 0{\cdot}01$). Platelet VEGF levels, but not serum VEGF levels, before IVIG were significantly correlated with the maximum CAA z-score ($r = 0{\cdot}524$, $P = 0{\cdot}02$). Our findings demonstrate that platelet VEGF may reflect the severity of vasculitis related to the pathological development of CAA in KS. Platelet VEGF may be an important feature of KS pathophysiology.

Keywords: vascular endothelial growth factor, Kawasaki syndrome, platelet, coronary artery abnormalities, mean platelet volume.

Kawasaki syndrome (KS) is an acute febrile illness with systemic vasculitis, which may cause coronary artery abnormalities (CAA) (Kawasaki *et al*, 1974; Kato *et al*, 1975). Inflammation of KS initially involves increased elevation of various cytokines, such as interleukin (IL)-1β, IL-6, IL-18 and tumour necrosis factor-α, which can cause endothelial cell activation and microvascular damage (Leung *et al*, 1989; Furukawa *et al*, 1992; Nomura *et al*, 2004).

Vascular endothelial growth factor-A (VEGF-A; hereafter termed VEGF) acts mostly on endothelial cells. It has also been shown to act on hematopoietic stem cells, monocytes, osteoblasts and neurons (Ferrara *et al*, 2003; Storkebaum *et al*, 2004). VEGF induces major angiogenesis, microvascular permeability, hematopoietic stem cell mobilisation from the bone marrow and stimulates inflammatory cell recruiting (Ferrara *et al*, 2003; Storkebaum *et al*, 2004). In KS, elevated levels of circulating VEGF are observed and are associated with clinical features, including oedema or skin rash, and cardiovascular events, such as CAA (Maeno *et al*, 1998; Ohno *et al*, 2000; Terai *et al*, 2003). High dose intravenous immunoglobulin (IVIG) treatment is effective in resolving inflammation and reducing the occurrence of CAA (Newburger *et al*, 1986). However, approximately 10–20% of KS patients have sustained or recurrent fever after IVIG and are at risk of developing CAA (Wallace *et al*, 2000).

It has been reported that platelets are the major source of VEGF in circulating blood and newly produced platelets contain an abundant amount of VEGF in their cytoplasm, known as platelet VEGF (Möhle *et al*, 1997; Maloney *et al*, 1998; Hashiguchi *et al*, 2000; Arisato *et al*, 2003; Kimura *et al*, 2007). Serum VEGF levels are similar to VEGF that is released from platelets in collected blood (Hashiguchi *et al*, 2000; Kimura *et al*, 2007). Serum VEGF levels are influenced not only by platelet counts but also by platelet VEGF content (Kimura *et al*, 2007). Plasma VEGF is immediately scavenged from the circulating blood and is trapped by its specific receptors, Flt-1 and KDR (Ferrara *et al*, 2003). Based on these findings, we calculated the amount of VEGF contained in a

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single platelet from serum VEGF levels, hematocrit and platelet counts. Highly accumulated platelet VEGF may reflect its biological property as an important mediator of intimal repair and may contribute to microvascular leakage and angiogenesis that is associated with wound repair in vascular injury sites (Möhle *et al*, 1997; Maloney *et al*, 1998). Thus, platelet VEGF could be closely related to pathological development associated with systemic vasculitis, such as in KS.

Platelet reactivity is associated with a high rate of platelet turnover, which showed positive correlation with mean platelet volume (MPV) in coronary artery disease (Cesari *et al*, 2008). It has also been reported that MPV indicates the platelets' biological property and higher thrombotic potential (Karpatkin & Strick, 1972). Large platelets contain more dense granules and produce more thromboxane A2 levels, aggregate more rapidly with collagen, and express more glycoprotein Ib and IIb/IIIa receptors (Karpatkin, 1969; Karpatkin & Strick, 1972; Jakubowski *et al*, 1983; Giles *et al*, 1994). Therefore, we evaluated the physiological activity of platelets in KS vasculitis through the examination of platelet VEGF levels and MPV.

This study showed that platelet VEGF levels highly reflect the severity of vasculitis and are a useful predictor for prognosis in KS.

Methods and materials

Patients

Clinical records of consecutive patients with definite KS who were referred and then admitted to Kagoshima Medical Association Hospital between April 2003 and June 2008 were used in this study. KS was defined using the Japanese criteria (Ayusawa *et al*, 2005). The first day of illness was defined as the first day of fever. KS patients were all treated with IVIG 1 g/kg per day for one or two consecutive days or 2 g/kg per day for 1 day. Patients also received aspirin (30 mg/kg), and the dose of aspirin was decreased to 5 mg/kg per day after the normalisation of C-reactive protein (CRP) values. Patients, who had persistent fever after more than 24 h of initial IVIG treatment, or recurrent fever associated with KS symptoms after an afebrile period, were treated with additional IVIG treatment; these patients were defined as IVIG non-responders. Patients who did not receive IVIG were excluded from this study. Control subjects consisted of febrile patients with bacterial infections such as bronchitis, pneumonia, or bacterial enterocolitis.

Echocardiography

Two-dimensional echocardiography was performed to evaluate cardiac function and the presence of CAA. Examination was performed before and after IVIG treatment at 2–3 d intervals during hospitalisation and once a week at outpatient clinics during the first month of illness. All coronary diameters (in mm) in KS patients were transformed to CAA *z*-score using the Japanese normal values of coronary artery dimensions as previously described (Kurotobi *et al*, 2002). The CAA *z*-score is based on the following equation: CAA *z*-score = (observed diameter − mean normal diameter)/(standard deviation of the normal diameter). The maximum CAA *z*-score among the right, left main and left anterior descending arteries during the first month of illness was used for evaluating CAA.

Serum samples and measurement of serum VEGF levels

Serum samples were collected before the first treatment with IVIG and the following day after completion of the initial IVIG (median, 3 d after IVIG), and were separated by centrifugation at 7 *g* for 15 min. All samples were stored at −40°C until time of assay. Serum VEGF levels were measured in duplicate using a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). Optical density at 450 nm was measured using an ImmunoMini NJ-2300 (Nippon Inter Med, Tokyo, Japan) and VEGF levels were determined by linear regression from a standard curve using the VEGF supplied with the kit as standard. The clinical protocols to measure serum VEGF levels were approved by the Institutional Review Board at Kagoshima University Hospital.

Plasma samples and measurement of plasma VEGF levels

Plasma samples were separated from whole blood immediately after the blood was drawn by centrifugation at 7 *g* for 15 min. Plasma VEGF levels were measured as described above.

Quantitative analysis of VEGF from a single platelet

The amount of VEGF contained in a single platelet was calculated using serum VEGF levels, peripheral hematocrit and platelet counts. The amounts of total VEGF (pg) were obtained using the equation: Serum VEGF (pg/ml) × serum volume (ml) = Serum VEGF (pg/ml) × Blood volume (ml) × (100-hematocrit)/100. Total platelet counts were obtained using the equation: Platelet counts ($\times 10^4/\mu l$) × blood volume (ml) × 10^3. From these calculations, VEGF released from a single platelet (platelet VEGF) ($pg \times 10^{-8}$) were obtained using the equation: Serum VEGF (pg/ml) × serum volume (ml)/total platelet counts, as described previously (Hashiguchi *et al*, 2000; Kimura *et al*, 2007).

MPV values

Venous blood samples were collected into EDTA tubes and analysed by the sheath flow direct current (DC) detection method in an automated haematology analysis system (Sysmex XE-2100, Kobe, Japan) (Jerelyn & Patricia, 2000).

Statistical analysis

Mean values of laboratory findings between KS patients and control patients were compared using the unpaired Student's

t-test. Values of laboratory findings between the IVIG responder and non-responder groups were compared using the Mann–Whitney *U*-test. Changes in serum or platelet VEGF levels before and after IVIG treatment between the IVIG responder and non-responder groups were compared by the paired Student's *t*-test. A simple linear regression analysis was used to evaluate the correlation between parameters. Correlations between platelet VEGF levels and platelet counts, CAA *z*-score, or MPV were studied by correlation coefficients. All statistical analyzes were processed using Stat View 5.0 software (Abacus Concepts, Berkeley, CA, USA). A *P* value of less than 0·05 was considered statistically significant.

Results

Comparison between serum VEGF levels and plasma VEGF levels

Plasma VEGF data were obtained from 41 KS patients before IVIG treatment and 10 febrile patients with bacterial infections. Serum VEGF levels in KS patients were markedly higher than mean plasma VEGF levels before IVIG in KS patients (964 ± 551 pg/ml vs. 95 ± 44 pg/ml). Serum VEGF levels in febrile patients with bacterial infections were also markedly higher than mean plasma VEGF levels (439 ± 119 pg/ml vs. 39 ± 10 pg/ml).

Comparison of clinical characteristics and laboratory findings between KS patients and controls

During the study period, 80 KS patients (aged 3 months to 8 years; median 25 months) and 26 febrile patients with bacterial infections (aged 6 months to 4 years; median 23 months) were evaluated. Clinical characteristics and laboratory findings in the KS patients and controls are shown in Table I. No significant differences were observed between sex and laboratory data, except for serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LD) levels. Although baseline values of white blood cell (WBC) counts and CRP values were not different, serum VEGF and platelet VEGF levels were significantly higher in KS patients than in controls.

In addition, platelet VEGF levels and platelet counts before treatment showed a significant negative correlation in KS patients ($r = -0{\cdot}528$, $P < 0{\cdot}001$, Fig 1), while there were no correlations in controls ($r = 0{\cdot}243$). Serum VEGF levels and platelet counts before treatment showed a significant positive correlation in KS patients ($r = 0{\cdot}266$, $P = 0{\cdot}008$).

Comparison of clinical features and laboratory findings between IVIG responder and non-responder groups

Clinical characteristics and laboratory findings in the IVIG responder group and IVIG non-responder group (14% of total KS patients) are shown in Table II. No significant differences were observed between age, sex, and days of illness at IVIG initiation. However, baseline levels of WBC counts, neutrophil counts, red blood cell counts, haemoglobin, and AST and CRP values were significantly different between the groups.

Serum and platelet VEGF levels before IVIG were higher in the non-responder group than those in the responder group, but these differences were not statistically significant. Serum and platelet VEGF levels after IVIG were higher in the non-responder group than those in the responder group ($P = 0{\cdot}470$, $P = 0{\cdot}170$, respectively). Serum VEGF levels after IVIG treatment were increased in both groups compared to

Table I. Comparison between KS patients and control patients.

Group	Kawasaki syndrome ($n = 80$)	Control ($n = 26$)	*P* value
Male/female	43/37	16/10	
Age at onset (years)	2·1 ± 1·8	1·9 ± 1·1	
White blood cell count ($\times 10^9$/l)	13·9 ± 4·9	13·4 ± 6·5	
Neutrophil count ($\times 10^9$/l)	9·5 ± 4·2	9·2 ± 4·5	
Haemoglobin (g/l)	114 ± 11	118 ± 12	
Platelet count			
($\times 10^9$/l)	350 ± 110	365 ± 134	
($\times 10^4$/μl)	35·0 ± 11·0	36·5 ± 13·4	
Aspartate aminotransferase (i/u per l)	112 ± 241	33 ± 11	0·049
Alanine aminotransferase (i/u per l	91 ± 153	19 ± 16	0·009
Lactate dehydrogenase (i/u per l)	533 ± 427	315 ± 62	0·005
Sodium (mmol/l)	135 ± 2·8	136 ± 3·3	
C-reactive protein (mg/l)	70 ± 48	57 ± 38	
VEGF (pg/ml)	964 ± 551	439 ± 189	<0·001
Platelet VEGF ($\times 10^{-8}$ pg)	18·8 ± 10·1	8·1 ± 3·0	<0·001

Data are expressed as mean ± SD.
VEGF, vascular endothelial growth factor; Platelet VEGF, vascular endothelial growth factor in a single platelet.

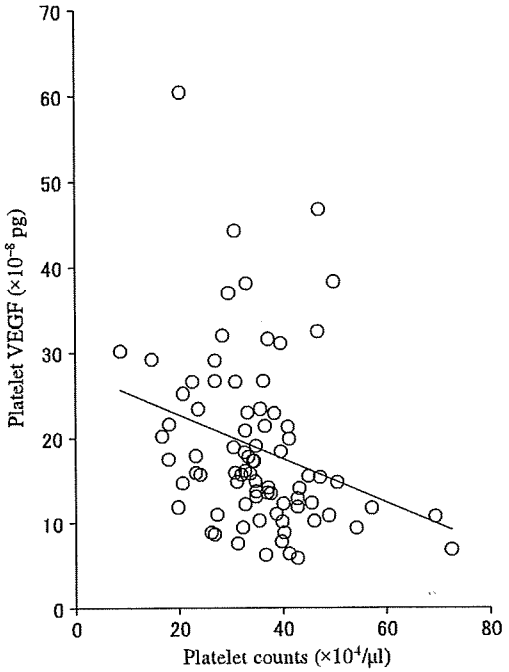

Fig 1. Correlation between platelet counts and platelet VEGF levels. There was a significant negative correlation between platelet counts before IVIG treatment and levels of platelet VEGF in KS ($r = -0{\cdot}528$, $P < 0{\cdot}001$).

those before IVIG. Platelet VEGF levels after IVIG were significantly decreased in the responder group, while they were significantly increased after IVIG (median, 3 d after initial IVIG) in the non-responder group (Fig 2).

Relationship between platelet VEGF and CAA

We examined platelet VEGF levels before IVIG and the CAA *z*-score. Platelet VEGF levels were significantly higher in patients who showed the maximum CAA *z*-score (>3) than in those who showed a lower score (<3) ($27{\cdot}5 \pm 13{\cdot}6 \times 10^{-8}$ pg vs. $16{\cdot}1 \pm 6{\cdot}9 \times 10^{-8}$ pg, $P < 0{\cdot}001$), while there were no significant differences in serum VEGF levels ($P = 0{\cdot}29$). Platelet VEGF levels showed a significant correlation with the CAA *z*-score ($r = 0{\cdot}524$, $P = 0{\cdot}02$), but there was no correlation with serum VEGF levels ($r = -0{\cdot}212$, Fig 3).

Relationship between platelet VEGF and MPV

Mean platelet volume data were obtained from 59 KS patients before and after IVIG treatment. MPV levels in KS patients were significantly higher than those of controls ($P = 0{\cdot}020$). MPV levels before IVIG were not statistically significant between the responder and non-responder groups in KS patients ($10{\cdot}4 \pm 1{\cdot}1$ fl vs. $9{\cdot}7 \pm 0{\cdot}9$ fl). MPV levels after IVIG in KS patients were significantly higher in the non-responder group

Table II. Comparisons between IVIG responder and non-responder group.

	Responder ($n = 69$)	Non-responder ($n = 11$)	*P* value
Male/female	39/30	6/5	
Age (years)	$2{\cdot}2 \pm 1{\cdot}7$	$1{\cdot}6 \pm 2{\cdot}1$	
White blood cell count ($\times 10^9$/l)	$13{\cdot}3 \pm 4{\cdot}1$	$18{\cdot}3 \pm 7{\cdot}4$	0·001
Neutrophil count ($\times 10^9$/l)	$8{\cdot}9 \pm 3{\cdot}4$	$13{\cdot}5 \pm 6{\cdot}7$	<0·001
Red blood cell count ($\times 10^{12}$/l)	$4{\cdot}4 \pm 0{\cdot}4$	$4{\cdot}1 \pm 0{\cdot}4$	0·011
Haemoglobin (g/l)	114 ± 11	110 ± 10	
Hematocrit (%)	$34{\cdot}8 \pm 2{\cdot}8$	$33{\cdot}8 \pm 2{\cdot}8$	
Platelet count			
($\times 10^9$/l)	351 ± 101	346 ± 160	
($\times 10^4$/μl)	$35{\cdot}1 \pm 10{\cdot}1$	$34{\cdot}6 \pm 16{\cdot}0$	
Aspartate aminotransferase (i/u per l)	107 ± 248	147 ± 198	0·049
Alanine aminotransferase (i/u per l)	90 ± 159	98 ± 117	
Lactate dehydrogenase (i/u per l)	517 ± 429	635 ± 421	
C-reactive protein (mg/l)	64 ± 44	109 ± 59	0·002
Total protein (g/l)	66 ± 5	64 ± 3	
Albumin (g/l)	38 ± 4	36 ± 6	
Sodium (mmol/l)	$134{\cdot}9 \pm 2{\cdot}7$	$133{\cdot}8 \pm 0{\cdot}3$	
VEGF (before IVIG) (pg/ml)	923 ± 454	1224 ± 957	0·460
VEGF (after IVIG) (pg/ml)	1052 ± 459	1726 ± 737	0·005
Platelet VEGF (before IVIG) ($\times 10^{-8}$ pg)	$17{\cdot}8 \pm 8{\cdot}1$	$25{\cdot}2 \pm 17{\cdot}6$	0·170
Platelet VEGF (after IVIG) ($\times 10^{-8}$ pg)	$14{\cdot}1 \pm 5{\cdot}9$	$38{\cdot}0 \pm 16{\cdot}5$	<0·001

Data are expressed as mean ± SD.
IVIG, intravenous immunoglobulin; responder, IVIG responder; non-responder, IVIG non-responder; CAA, coronary artery abnormalities; VEGF, vascular endothelial growth factor; Platelet VEGF, vascular endothelial growth factor in a single platelet.

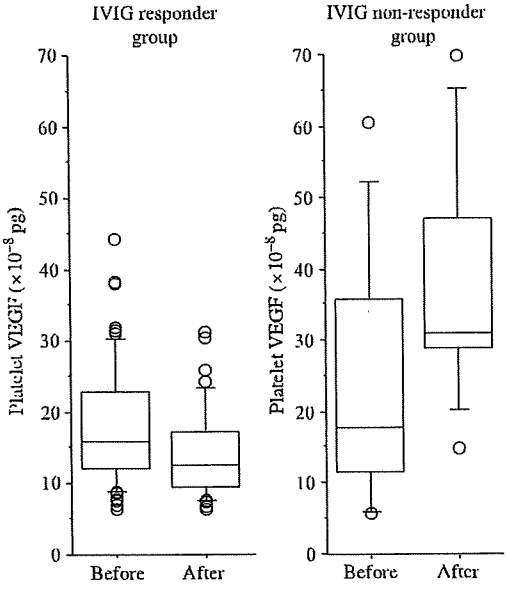

Fig 2. Platelet VEGF levels before and after IVIG treatment. Left, IVIG responder group (n = 69); right, IVIG non-responder group (n = 11). Platelet VEGF levels were significantly decreased after IVIG ($P < 0{\cdot}001$) in the responder group, while they were significantly increased after IVIG in the non responder group ($P < 0{\cdot}001$).

than those in the responder group (10·9 ± 0·8 fl vs 9·3 ± 1·5 fl, $P = 0{\cdot}003$). Platelet VEGF levels before IVIG and after IVIG in KS patients were strongly correlated with MPV (Fig 4). Platelet VEGF levels before treatment in controls were also strongly correlated with MPV (n = 26, $r = 0{\cdot}768$, $P < 0{\cdot}001$).

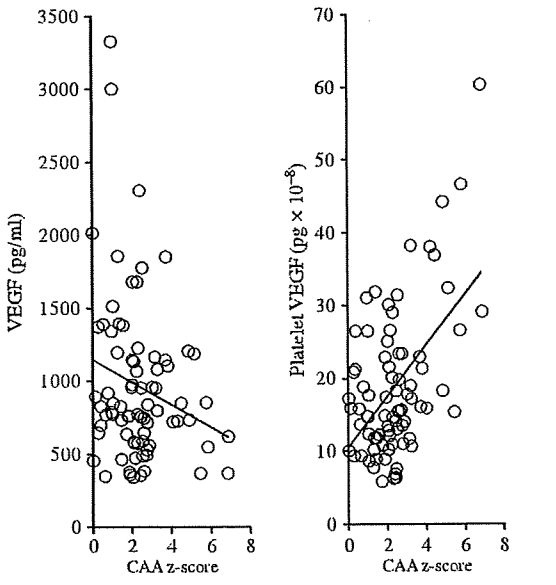

Fig 3. Correlation between serum/platelet VEGF levels and CAA-z score. There was a significant positive correlation between platelet VEGF levels and CAA z-score ($r = 0{\cdot}524$, $P < 0{\cdot}001$), and a negative correlation between serum VEGF levels and CAA z-score ($r = 0{\cdot}21$, $P = 0{\cdot}06$).

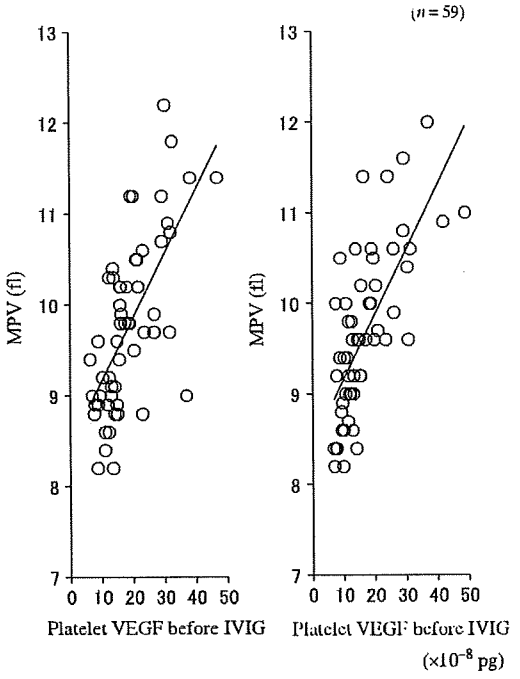

Fig 4. Correlation between platelet VEGF and MPV levels. Platelet VEGF levels before IVIG and after IVIG were strongly correlated with MPV (n = 59, $r = 0{\cdot}752$, $r = 0{\cdot}714$, respectively, $P < 0{\cdot}001$).

Discussion

Vascular endothelial growth factor is released from platelets under physiological conditions, which might be related to the pathological mechanism of vasculitis in KS. Our findings demonstrated that platelet VEGF may reflect the reactivity of IVIG treatment and the severity of vasculitis related to the pathological development of CAA in KS. Platelet VEGF may be one of the main features of KS pathophysiology.

Although plasma VEGF levels showed a slight correlation with serum VEGF levels, serum VEGF levels were much higher than plasma VEGF levels before IVIG in KS patients and controls. Platelets and megakaryocytes contain an abundant amount of VEGF in their cytoplasm, and platelets are the major source of VEGF in circulating blood (Möhle *et al*, 1997; Hashiguchi *et al*, 2000). Platelets are easily activated by stimulation, like collection of blood or centrifugation; plasma must include 'leaking VEGF' from platelets. Furthermore serum VEGF concentration could be influenced not only by platelet counts but also by platelet VEGF contents (Kimura *et al*, 2007). In light of these considerations, we would like to emphasize that serum VEGF is a valid and more reliable marker than plasma VEGF for measuring the amount of VEGF contained in a single platelet.

In the current study, serum and platelet VEGF levels before IVIG treatment in KS patients were much higher than those in patients with infectious diseases. These markedly elevated levels of serum and platelet VEGF in KS patients could explain