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nature genetics

ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms

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Kawasaki disease is a pediatric systemic vasculitis of unknown etiology for which a genetic influence is suspected. We identified a functional SNP (itpkc_3) in the inositol 1,4,5-trisphosphate 3-kinase C (*ITPKC*) gene on chromosome 19q13.2 that is significantly associated with Kawasaki disease susceptibility and also with an increased risk of coronary artery lesions in both Japanese and US children. Transfection experiments showed that the C allele of itpkc_3 reduces splicing efficiency of the *ITPKC* mRNA. ITPKC acts as a negative regulator of T-cell activation through the Ca^{2+}/NFAT signaling pathway, and the C allele may contribute to immune hyper-reactivity in Kawasaki disease. This finding provides new insights into the mechanisms of immune activation in Kawasaki disease and emphasizes the importance of activated T cells in the pathogenesis of this vasculitis.

Kawasaki disease (OMIM 300530) is an acute, self-limited vasculitis of infants and children characterized by prolonged fever unresponsive to antibiotics, polymorphous skin rash, erythema of the oral mucosa, lips and tongue, erythema of the palms and soles, bilateral conjunctival injection and cervical lymphadenopathy[1]. Coronary artery aneurysms develop in 15–25% of those left untreated[2], making Kawasaki disease the leading cause of acquired heart disease among children in developed countries. Treatment with intravenous immunoglobulin (IVIG) abrogates the inflammation in approximately 80% of affected individuals and reduces the aneurysm rate to less than 5%. Cardiac sequelae of the aneurysms include ischemic heart disease, myocardial infarction and sudden death[3]. Epidemiological features such as seasonality and clustering of cases suggest an infectious trigger, although no pathogen has been isolated and the etiology remains unknown.

Several lines of evidence suggest the importance of genetic factors in disease susceptibility and outcome. First, the incidence of Kawasaki disease is 10–20 times higher in Japan than in Western countries[4]. Second, the risk of Kawasaki disease in siblings of affected children is 10 times higher than that in the general population ($\lambda_s = 10$), and the incidence of Kawasaki disease in children born to parents with a history of Kawasaki disease is twice as high as that in the general

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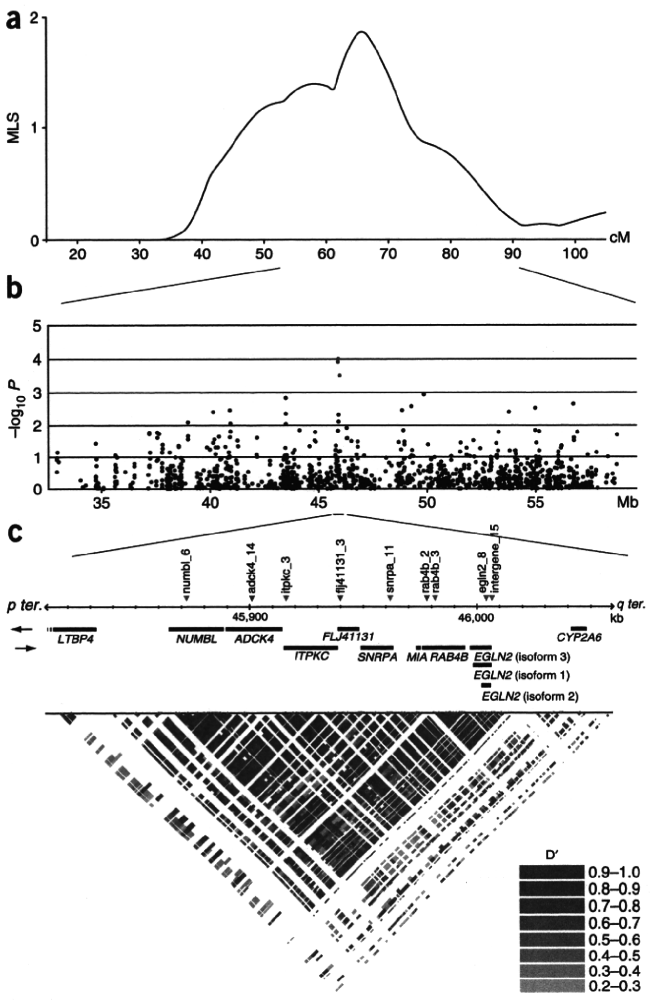

Figure 1 Results of SNP screening of chromosome 19 and structure of the linkage disequilibrium (LD) block in Japanese individuals showing SNPs significantly associated with Kawasaki disease. (**a**) Maximum lod score plot of affected sib-pair analysis conducted on 78 Japanese families. MLS, maximum lod score. (**b**) Case-control association analysis of 1,222 SNPs in 94 individuals with Kawasaki disease and 564 controls. *x* and *y* axes indicate the position from the *p* terminus of the chromosome and –log of *P* value for allele frequency comparison, respectively. The three most significant SNPs are marked by red dots. (**c**) Genes oriented *q* terminus to *p* terminus are in upper row, with genes in the opposite orientation shown below. Arrowheads indicate the position of SNPs significantly associated with Kawasaki disease: red arrowheads indicate the original three SNPs found by association studies, and blue arrowheads indicate the six SNPs from resequencing that were in LD with original three SNPs.

population[5,6]. Familial aggregation of the disease has also been observed[7]. Although association studies have identified candidate genes that may influence Kawasaki disease susceptibility, a systematic genetic approach has not been previously applied to study this disease.

Recently, we conducted affected sib-pair analysis of Kawasaki disease[8] that demonstrated linkage to several chromosomal regions, including chromosome 19. Here we show the results of linkage disequilibrium (LD) mapping carried out on 19q13.2, through which we identified a functional SNP in intron 1 of *ITPKC* that is significantly associated with risk of Kawasaki disease and with formation of coronary artery aneurysms. We also characterized ITPKC as a negative regulator of the Ca^{2+}/NFAT signaling pathway in T cells.

RESULTS

Linkage disequilibrium mapping

Through linkage analysis of 78 Japanese sib pairs concordant for Kawasaki disease, we identified a peak in the maximum lod-score plot at 19q13.2–13.3, located about 65.4 cM (48 Mb) from the *p* terminus of the chromosome[8] (**Fig. 1a**). An initial screening of 1,222 SNPs in 94 individuals with Kawasaki disease and 564 controls (see **Supplementary Methods** online) identified 131 candidates ($P < 0.05$; **Supplementary Table 1** online). Through association analysis of these 131 SNPs in an independent cohort of 276 Japanese individuals with Kawasaki disease and 282 controls, we found a cluster of three SNPs that were highly significant ($P < 0.01$; **Fig. 1b** and **Table 1**). The three SNPs (adck4_14, flj41131_3 and rab4b_2) were in strong linkage disequilibrium ($r^2 > 0.85$) within a single LD block identified by the HapMap database (**Fig. 1c**). In this LD block spanning about 150 kb, eight genes had been mapped: Numb (*Drosophila*) homolog like (*NUMBL*), aarF domain containing kinase 4 (*ADCK4*), *ITPKC*, hypothetical protein LOC284325 (*FLJ41131*), small nuclear ribonucleoprotein polypeptide A (*SNRPA*), melanoma inhibitory activity

Table 1 Results of association analyses between three independent sets of Japanese Kawasaki disease and control subjects

				94 KD vs. 564 controls[b]		276 KD vs. 282 controls		267 KD vs. 752 controls	
				Allele 1 vs. Allele 2		Allele 1 vs. Allele 2		Allele 1 vs. Allele 2	
SNPs	dbSNP ID	Allele 1/2	Chromosome position[a]	χ^2	*P*	χ^2	*P*	χ^2	*P*
numbl_6	–	C/T	45872187	–	–	3.9	0.049	13.7	0.00022
adck4_14	rs2288450	C/T	45901017	14.7	0.00012	5.0	0.026	15.7	7.4×10^{-5}
itpkc_3	rs28493229	G/C	45916044	–	–	5.0	0.026	16.3	5.4×10^{-5}
flj41131_3	rs3745213	C/T	45939849	15.1	0.00010	7.6	0.0060	17.7	2.6×10^{-5}
snrpa_11	rs17713068	T/G	45961895	–	–	7.3	0.0068	21.1	4.4×10^{-6}
rab4b_2	rs2287691	C/G	45978003	12.9	0.00032	7.3	0.0068	17.8	2.5×10^{-5}
rab4b_3	rs2287692	G/A	45981596	–	–	7.9	0.0050	19.1	1.2×10^{-5}
egln2_8	rs10416308	G/A	46004101	–	–	9.7	0.0019	13.0	0.00031
intergene_15	rs10405596	C/T	46006560	–	–	9.6	0.0020	13.2	0.00028

SNPs in bold are those identified in initial screening.
[a]Based on Build 36 NCBI reference sequence. [b]Genotype data for the 564 controls were available only for the three SNPs in bold.

Table 2 Results of association analysis with combined Japanese Kawasaki disease and control samples and TDT[a] analysis of US samples

			Japanese (case-control association analysis)[b]									United States (TDT)				
			Genotype			Allele 1 vs. Allele 2		Genotype 11 vs. 12 + 22								
SNPs	Allele 1/2	Subjects	11	12	22	χ^2	*P*	χ^2	*P*	OR	95% CI	T:U[c]	χ^2	*P*	OR	95% CI
numbl_6	C/T	KD	378	235	23	27.4	1.6×10^{-7}	30.5	3.3×10^{-8}	1.80	1.46–2.22	64:31	11.5	0.00071	2.06	1.34–3.17
		Control	748	259	25											
adck4_14	C/T	KD	374	235	26	31.7	1.8×10^{-8}	34.6	4.0×10^{-9}	1.87	1.52–2.30	64:31	11.5	0.00071	2.06	1.34–3.17
		Control	752	254	27											
itpkc_3	G/C	KD	376	234	27	32.4	1.2×10^{-8}	35.8	2.2×10^{-9}	1.89	1.53–2.33	64:30	12.3	0.00045	2.13	1.38–3.29
		Control	756	249	29											
flj41131_3	C/T	KD	372	237	28	37.8	7.8×10^{-10}	39.9	2.7×10^{-10}	1.95	1.58–2.41	66:32	11.8	0.00059	2.06	1.35–3.15
		Control	757	250	26											
snrpa_11	T/G	KD	375	235	27	40.8	1.7×10^{-10}	45.1	1.9×10^{-11}	2.05	1.66–2.53	55:40	2.4	0.12	1.38	0.91–2.07
		Control	771	235	28											
rab4b_2	C/G	KD	376	235	26	37.3	1.0×10^{-9}	41.7	1.1×10^{-10}	1.99	1.61–2.46	66:50	2.2	0.14	1.32	0.91–1.91
		Control	766	239	28											
rab4b_3	G/A	KD	374	235	27	39.5	3.2×10^{-10}	44.3	2.9×10^{-11}	2.04	1.65–2.51	57:39	3.4	0.066	1.46	0.97–2.20
		Control	767	235	29											
egln2_8	G/A	KD	374	232	27	36.7	1.4×10^{-9}	39.6	3.2×10^{-10}	1.96	1.59–2.42	68:51	2.4	0.12	1.33	0.93–1.92
		Control	763	243	27											
intergene_15	C/T	KD	375	237	25	36.5	1.6×10^{-9}	41.0	1.5×10^{-10}	1.98	1.60–2.44	63:49	1.8	0.19	1.29	0.89–1.87
		Control	764	243	27											

[a]Transmission disequilibrium test. $n = 209$. [b]637 KD (94 + 276 + 267) and 1,034 controls (282 + 752). [c]'T' and 'U' indicate transmitted and untransmitted allele 2 of each SNP, respectively.

(*MIA*), Ras-related GTP-binding protein 4b (*RAB4B*) and EGL nine (*C. elegans*) homolog 2 (*EGLN2*). Resequencing the 150-kb region from 12 Japanese individuals with Kawasaki disease and 12 healthy controls, we identified 109 SNPs and four deletion polymorphisms (**Supplementary Table 2** online). We discovered one previously unknown and five known SNPs that were in the same LD group ($r^2 > 0.80$) with the initial three SNPs (**Table 1**). We confirmed the association of these nine SNPs with Kawasaki disease in an independent case-control set (267 individuals with Kawasaki disease and 752 healthy controls; **Tables 1** and **2**). The association of these SNPs remained significant after Bonferroni correction for multiple testing ($n = 1,222$, $P < 0.001$). Meta-analysis of these two independent sets by the Mantel-Haenszel method confirmed significance (**Supplementary Fig. 1** online).

Transmission disequilibrium test (TDT) analysis of 209 US multiethnic trios showed asymmetric transmission of four of the nine SNPs (numbl_6, adck4_14, itpkc_3 and flj41131_3; **Table 2**). Of the 209 US trios, 106 were European Americans, and asymmetric transmission of these same four SNPs was again observed in this subgroup (data not shown). The results of a combined analysis of Japanese case-control and US TDT studies are summarized in **Supplementary Figure 1**. The significance of these SNPs in two different ethnic populations provided further evidence that genetic variation at this locus influences Kawasaki disease susceptibility.

LD analysis of the European American subgroup ($n = 106$) showed that the 150-kb region containing the nine SNPs was separated into three LD blocks: the four significant SNPs on the *p*-terminal side, the three in the middle and the other two on the *q*-terminal side (**Supplementary Fig. 2** online). Hence, the difference in haplotype structure in the European American and Japanese populations

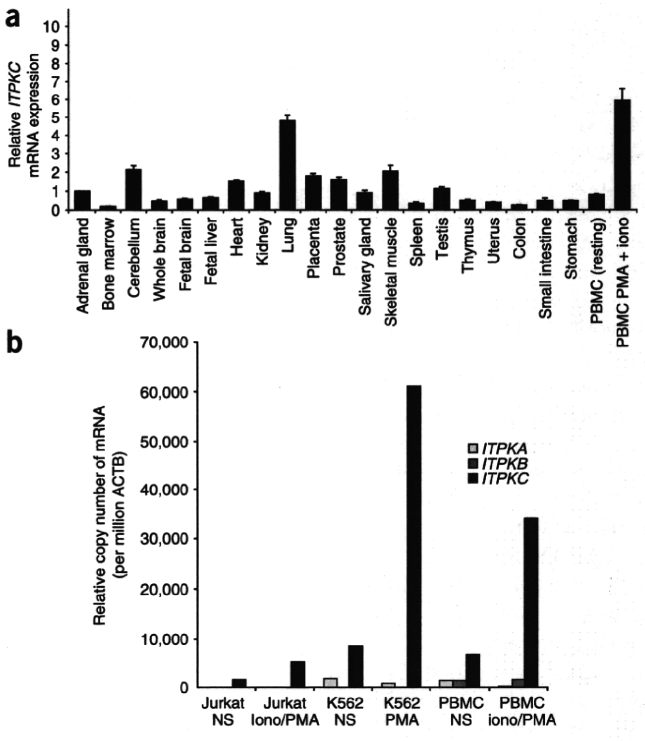

Figure 2 Comparison of relative mRNA expression of *ITPKC* in different tissues and cell lines. **(a)** Quantitative RT-PCR was carried out on RNA extracted from different human tissues, and the results were normalized to β-actin transcripts. RNA from both resting PBMCs and PBMCs stimulated with ionomycin (iono) and PMA was also analyzed. Results are mean ± s.d. of triplicate assays. **(b)** Expression pattern of ITPK isoforms in leukemic cell lines and PBMCs. Bars indicate relative mRNA copy number of *ITPKA* (yellow), *ITPKB* (red) and *ITPKC* (black), respectively. Expression was evaluated both in resting state and activated state. NS, no stimulation.

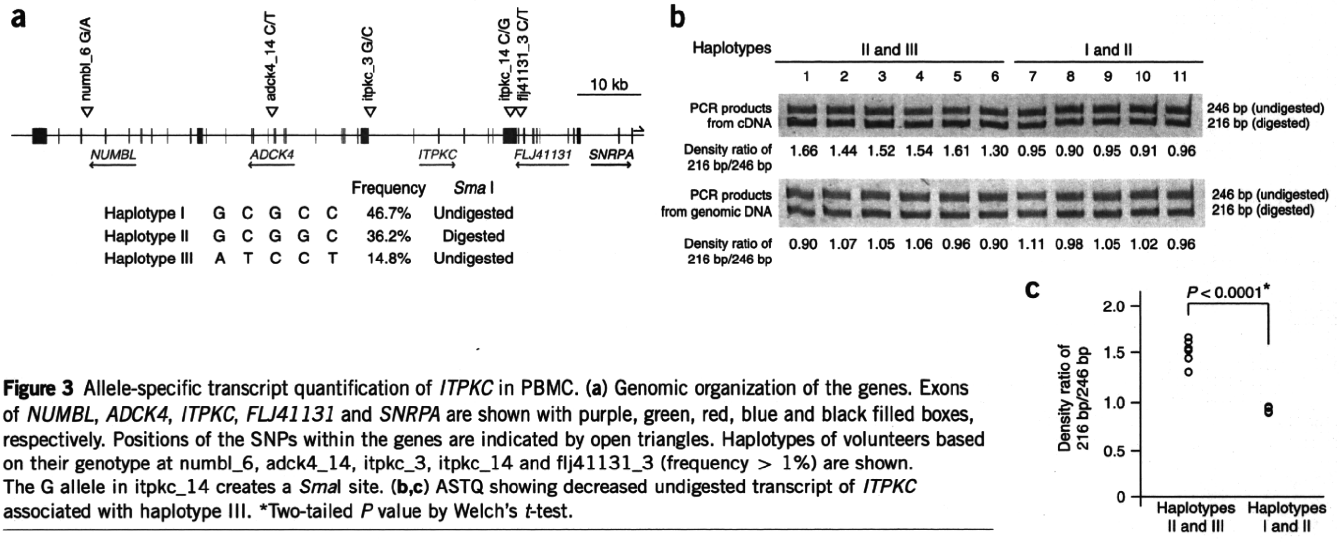

Figure 3 Allele-specific transcript quantification of *ITPKC* in PBMC. (**a**) Genomic organization of the genes. Exons of *NUMBL*, *ADCK4*, *ITPKC*, *FLJ41131* and *SNRPA* are shown with purple, green, red, blue and black filled boxes, respectively. Positions of the SNPs within the genes are indicated by open triangles. Haplotypes of volunteers based on their genotype at numbl_6, adck4_14, itpkc_3, itpkc_14 and flj41131_3 (frequency > 1%) are shown. The G allele in itpkc_14 creates a *Sma*I site. (**b,c**) ASTQ showing decreased undigested transcript of *ITPKC* associated with haplotype III. *Two-tailed *P* value by Welch's *t*-test.

suggested that these four SNPs were the likely candidates influencing Kawasaki disease susceptibility. The SNPs were located within introns of *NUMBL*, *ADCK4*, *ITPKC* and *FLJ41131*, respectively (**Fig. 1c** and **Supplementary Table 2**).

Identification of *ITPKC* as the most plausible candidate gene

To determine the most likely candidate gene out of the four, we first carried out multivariate analysis of the four SNPs to assess whether a single causal SNP or some synergistic interaction of the SNPs within the locus conferred the disease risk. However, the likelihood ratio test applied to each single SNP showed a similar trend of association in simple contingency table analyses (P = 0.00027, 0.000061, 0.000081 and 0.000067 for numbl_6, adck4_14, itpkc_3 and flj41131_3, respectively). Moreover, no epistasis worthy of note was shown between any two of the four SNPs or in any combination of each significant SNP and the other SNPs of the same gene region that were not independently associated with Kawasaki disease (data not shown). It seemed likely that the strong LD of the locus made the association of these SNPs equivalent. Thus, we considered that further biological evidence would be needed to identify the causal SNP and the gene responsible for the association.

We then reviewed the function of the four positional candidate genes. Although none of these had been previously recognized to have a role in immune activation, we postulated that *ITPKC* was the most likely candidate for such a role. ITPKC is one of the three isoenzymes of inositol 1,4,5-trisphosphate 3-kinase (ITPK) that phosphorylate inositol 1,4,5-trisphosphate (IP3), a key second messenger in many cell types. ITPK has been postulated to have a critical role in T-cell receptor (TCR) signaling, as IP3 kinase activity in Jurkat cells is rapidly upregulated after TCR stimulation[9], although the relative importance of the three known isoenzymes, ITPKA, ITPKB and ITPKC, has not yet been determined. Because individuals with Kawasaki disease have marked activation of the immune system, we hypothesized that the ITPKC might have a role in regulation of the immune response.

To study the role of ITPKC, we first analyzed the tissue distribution of *ITPKC* expression by RT-PCR. We detected expression in all tissues sampled, with the highest constitutive expression in cerebellum, lung and skeletal muscle. In the latter two tissues, high expression had been shown by RNA blot analysis in a previous report[10] (**Fig. 2a**). Low expression was detected in immune-related organs such as bone marrow, spleen, thymus and resting peripheral blood mononuclear cells (PBMCs). However, expression was notably induced in PBMCs when stimulated with phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+} ionophore ionomycin (**Fig. 2a**). We compared the mRNA expression of the three isoenzymes in PBMCs and two leukemic cell lines (Jurkat and K562). Before stimulation, the expression of all three isoenzymes was low; after stimulation, only the expression of the *ITPKC* isoenzyme was induced (3- to 7-fold increase; **Fig. 2b**). This result prompted us to pursue *ITPKC* as the most likely candidate gene in the associated haplotype block.

To determine whether any of the four SNPs in *ITPKC* or adjacent loci affected transcript abundance of *ITPKC in vivo*, we carried out allele-specific transcript quantification (ASTQ; **Fig. 3**). The RT-PCR product from mRNA isolated from PBMCs of individuals with haplotype II (G allele in itpkc_14), but not haplotypes I and III, could be digested with *Sma*I (**Fig. 3a**). The *Sma*I-treated RT-PCR product from six individuals with haplotypes II and III had a higher ratio of digested to undigested forms, suggesting lower transcript abundance from haplotype III (containing alleles associated with Kawasaki disease susceptibility; **Fig. 3b**, lanes 1–6). Five individuals with haplotypes I and II (containing alleles not associated with Kawasaki disease susceptibility and the C- or G-allele at itpkc_14, respectively; **Fig. 3b**, lanes 7–11) had an equal ratio of digested to undigested PCR product, suggesting that the difference between haplotypes II and III was due to the SNPs. The mean ratio was 1.51 for the former group and 0.93 for the latter ($P < 0.0001$; **Fig. 3c**). This finding further encouraged us to consider *ITPKC* as the most plausible candidate gene in the locus.

Regulatory role of ITPKC in T-cell activation

The increase in *ITPKC* expression after cell stimulation prompted us to study the role of IPTKC in immune activation (**Fig. 4**). IP3 is generated by the hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase C when activated by various external stimuli[11]. In T cells, IP3, released by stimulation of the TCR complex, increases intracellular Ca^{2+} through IP3 receptors (IP3Rs) expressed on endoplasmic reticulum[12]. Subsequent Ca^{2+} influx across the plasma membrane leads to nuclear translocation of nuclear factor of activated T cells (NFAT) and activates transcription of interleukin-2 (*IL2*) and other cytokines[13,14].

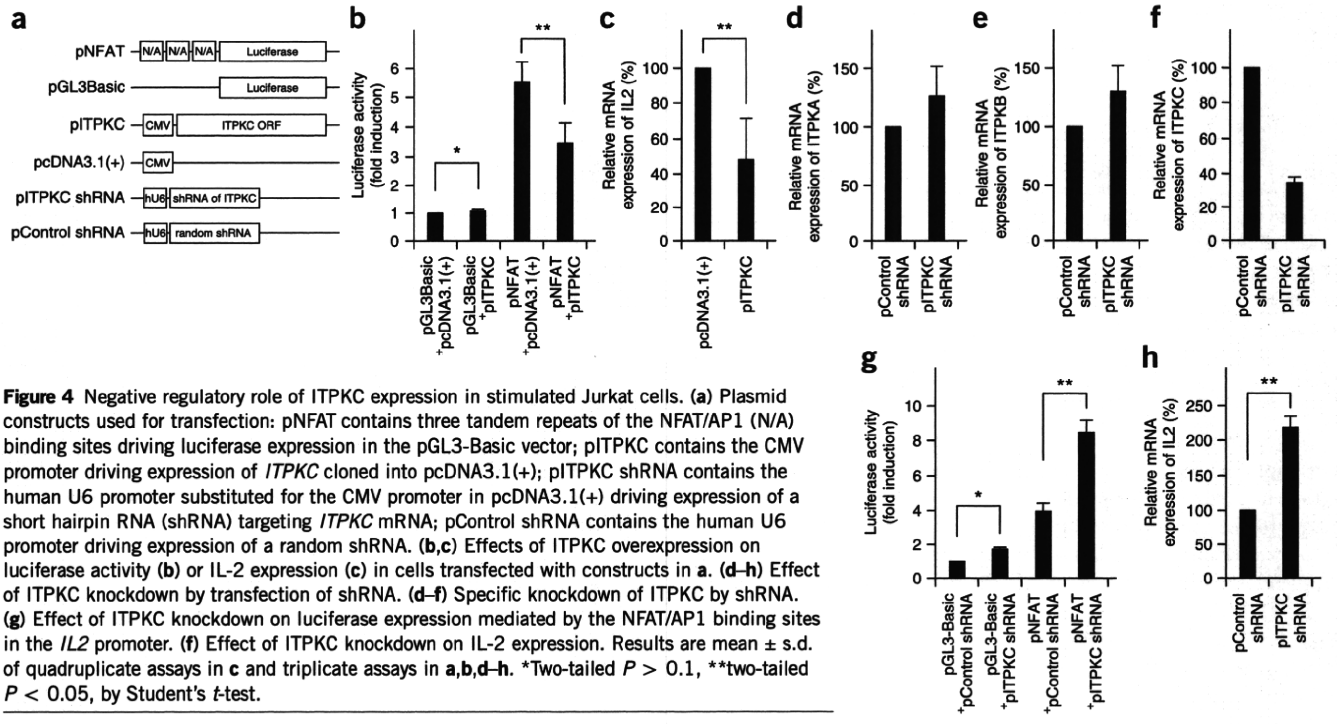

Figure 4 Negative regulatory role of ITPKC expression in stimulated Jurkat cells. (**a**) Plasmid constructs used for transfection: pNFAT contains three tandem repeats of the NFAT/AP1 (N/A) binding sites driving luciferase expression in the pGL3-Basic vector; pITPKC contains the CMV promoter driving expression of *ITPKC* cloned into pcDNA3.1(+); pITPKC shRNA contains the human U6 promoter substituted for the CMV promoter in pcDNA3.1(+) driving expression of a short hairpin RNA (shRNA) targeting *ITPKC* mRNA; pControl shRNA contains the human U6 promoter driving expression of a random shRNA. (**b**,**c**) Effects of ITPKC overexpression on luciferase activity (**b**) or IL-2 expression (**c**) in cells transfected with constructs in **a**. (**d**–**h**) Effect of ITPKC knockdown by transfection of shRNA. (**d**–**f**) Specific knockdown of ITPKC by shRNA. (**g**) Effect of ITPKC knockdown on luciferase expression mediated by the NFAT/AP1 binding sites in the *IL2* promoter. (**f**) Effect of ITPKC knockdown on IL-2 expression. Results are mean ± s.d. of quadruplicate assays in **c** and triplicate assays in **a**,**b**,**d**–**h**. *Two-tailed $P > 0.1$, **two-tailed $P < 0.05$, by Student's *t*-test.

We postulated that ITPKC regulates NFAT by modulating the abundance of IP3. When *ITPKC* was overexpressed in Jurkat cells, NFAT-mediated activation after stimulation with phytohemagglutinin (PHA) and PMA was significantly reduced (**Fig. 4b**). Next, we assessed NFAT-mediated activation when expression of *ITPKC* was decreased. In contrast to overexpression, knockdown of *ITPKC* using plasmids expressing short hairpin RNA (shRNA) resulted in enhanced NFAT-mediated activation in response to the same stimulation (**Fig. 4f,g**). ITPKA and ITPKB also catalyze phosphorylation of IP3, and their expression was observed in PBMCs, even though the expression was much lower than that of *ITPKC* (**Fig. 2b**). Thus, we assessed mRNA expression of these two genes to exclude the possibility that the shRNA designed for *ITPKC* also silenced *ITPKA* and *ITPKB*, thereby accounting for the effect on NFAT activation. We observed no suppression and actually saw a slight increase in transcript concentrations for both genes (**Fig. 4d,e**). Consistent with these results, *IL2* transcription in stimulated Jurkat cells decreased in response to *ITPKC* overexpression and increased following *ITPKC* knockdown (**Fig. 4c,h**). Given that NFAT mediates the expression of many proteins beside IL-2 that have important roles in T-cell regulation, ITPKC, and not ITPKA or ITPKB, may act as a key negative regulator of T-cell function.

Functional significance of itpkc_3

As none of the four significant SNPs was located in a protein coding region of *ITPKC* (**Fig. 3a**), we investigated the role of these SNPs in

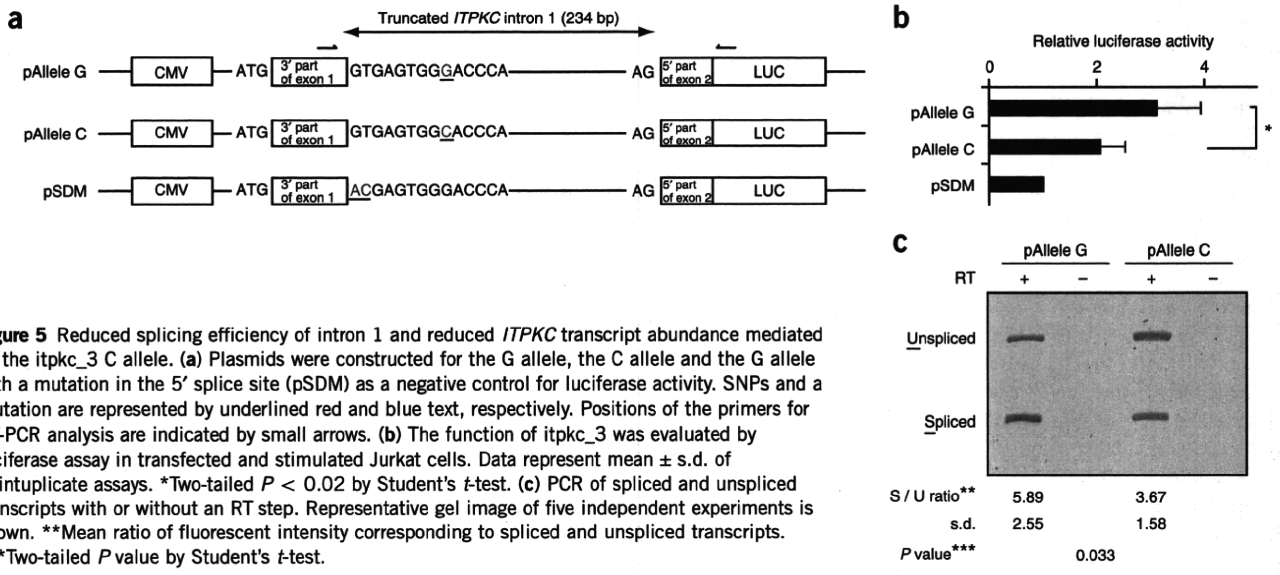

Figure 5 Reduced splicing efficiency of intron 1 and reduced *ITPKC* transcript abundance mediated by the itpkc_3 C allele. (**a**) Plasmids were constructed for the G allele, the C allele and the G allele with a mutation in the 5′ splice site (pSDM) as a negative control for luciferase activity. SNPs and a mutation are represented by underlined red and blue text, respectively. Positions of the primers for RT-PCR analysis are indicated by small arrows. (**b**) The function of itpkc_3 was evaluated by luciferase assay in transfected and stimulated Jurkat cells. Data represent mean ± s.d. of quintuplicate assays. *Two-tailed $P < 0.02$ by Student's *t*-test. (**c**) PCR of spliced and unspliced transcripts with or without an RT step. Representative gel image of five independent experiments is shown. **Mean ratio of fluorescent intensity corresponding to spliced and unspliced transcripts. ***Two-tailed *P* value by Student's *t*-test.

Table 3 Association analysis of itpkc_3 with stratified samples

	Japanese (case-control association analysis)[d]							United States (TDT)					
	Genotype												
Samples	GG	GC	CC	χ^2	*P*	OR	95% CI	*n*[e]	T:U[f]	χ^2	*P*	OR	95% CI
KD linked to 19q13.2[a]	21	18	1	8.2	0.0042	2.46	1.30–4.65	–	–	–	–	–	–
KD with family history[b]	53	44	4	19.1	0.000012	2.46	1.63–3.73	–	–	–	–	–	–
KD with CALs[c]	61	44	2	12.4	0.00044	2.05	1.37–3.08	108	37:11	14.1	0.00018	3.36	1.72–6.59
KD without CALs	172	94	12	13.4	0.00025	1.68	1.27–2.21	100	27:18	1.8	0.18	1.50	0.63–2.72
Control	756	249	29										

[a]Probands of 78 sib pairs in previous linkage analysis whose IBD allele number were estimated to be >1.0 at itpkc_3 (63cM). [b]Probands of 93 affected sib pairs, 4 parent-child pairs, 1 monozygotic twin, 1 dizygotic twin, 1 first cousin pair and 1 second cousin pair. [c]KD cases having coronary dilatation or aneurysms during the acute phase. [d]Genotype frequency comparison in dominant model of inheritance. [e]Number of affected individuals. [f]'T' and 'U' indicate transmitted and untransmitted C allele of itpkc_3, respectively.

transcriptional regulation. Using the TFSEARCH program (see URLs section in Methods), we predicted binding of the AP-1 transcription factors to the sequence containing flj41131_3 and lowered the score with a nucleotide substitution at the SNP (from 91.8 to 79.4; **Supplementary Fig. 3a** online). The SNP is located within intron 7 of *FLJ41131*, but because of close tail-to-tail gene arrangement (**Fig. 1c**), the distance between the SNP and the 3′ end of the *ITPKC* gene is only 1.2 kb. We tested the hypothesis that flj41131_3 affects the expression of *ITPKC* by altering activity of an enhancer element outside the gene. However, we observed no significant difference in luciferase assays using constructs corresponding to the two alleles of flj41131_3 (**Supplementary Fig. 3b**). Moreover, we did not observe higher concentrations of the digested transcripts in ASTQ analysis of an individual who was heterozygous at flj41131_3 and homozygous for major alleles at itpkc_3, adck4_14 and numbl_6 (data not shown). These findings led us to examine the functional significance of SNPs other than flj41131_3. No transcription factor was clearly predicted to bind to any alleles of numbl_6, adck4_14 and itpkc_3, and luciferase assays with constructs for these SNPs showed no functional effects (**Supplementary Fig. 3c**). Thus, we explored other possible mechanisms by which these SNPs might alter *ITPKC* expression.

Differences in splicing efficiency associated with nucleotide changes within introns have previously been observed[15,16]. Of the four significant SNPs, only itpkc_3 was located in an intron of *ITPKC* (**Fig. 3a**). Its location near the 5′ splice site further encouraged us to investigate the role of this SNP in regulating splicing. We constructed a minigene containing a truncated intron 1 with portions of exons 1 and 2 at either end and the luciferase gene fused in-frame downstream of exon 2 (**Fig. 5a**). When transfected into Jurkat cells, the plasmid containing the C allele had significantly lower luciferase activity compared to the plasmid containing the G allele (**Fig. 5b**). RT-PCR with primers designed to amplify cDNAs generated from transcripts of these plasmids yielded two bands. The lower and upper bands corresponded to spliced and unspliced transcripts, respectively. As expected, we observed a lower spliced/unspliced ratio of the transcripts for the C allele (**Fig. 5c**). Because no amplification was observed from the templates without a reverse transcriptase step, a possible plasmid DNA contamination in the cDNA templates as the source of the 'unspliced' bands was excluded. To our knowledge, no splice variants of this gene using a different 5′ splice site, which could rescue splicing inefficiency[17], have been reported in the literature or public databases. Furthermore, RT-PCR of the transcripts in PBMCs from individuals with the C allele did not detect such variants (data not shown). Therefore, we speculate that reduced splicing associated with the C allele could result in lower *ITPKC* transcript concentrations that might, in turn, lead to increased T-cell activation.

Association analysis with stratified samples

To further explore the effects of the proposed risk allele, we stratified the samples by the following two factors: family history of Kawasaki disease and presence of coronary artery lesions (CALs). Among the 78 Japanese affected sib pairs, 40 pairs shared more than one allele near itpkc_3. In this subset, the itpkc_3 C allele was over-represented compared to controls (n = 40, odds ratio (OR) = 2.46, 95% confidence interval (CI) = 1.30–4.65; **Table 3**). We observed the same trend in Japanese probands with a positive family history of Kawasaki disease (n = 101, OR = 2.46, 95% CI = 1.63–3.73; **Table 3**). These data strongly corroborate the association between itpkc_3 and Kawasaki disease. This allele also seemed to confer an increased risk of developing CALs (Japanese individuals with Kawasaki disease: n = 106, OR = 2.05, 95% CI = 1.37–3.08; US individuals with Kawasaki disease: n = 108 OR = 3.36, 95% CI = 1.72–4.96; **Table 3**).

DISCUSSION

We identified a SNP that contributes to Kawasaki disease susceptibility and disease outcome, starting from an LD mapping strategy for the chromosome 19q13.2–13.3 region for which evidence of linkage was observed in a previous sib-pair analysis[8]. We showed for the first time that ITPKC in humans is inducible in PBMCs and modulates NFAT activation. We further defined a role of ITPKC as a negative regulator of T-cell activation by showing that the itpkc_3 C allele results in increased *IL2* transcript abundance.

To our knowledge, alteration of splicing efficiency as a result of a single base substitution at nine nucleotides from the 5′ splice site has been rarely observed[18]. The SNP position was outside the limit of the consensus donor site sequence (+6)[19], and no cryptic splice site was generated by the nucleotide change. One possible explanation for this finding could be that a GGG motif might act as an intronic splicing control element, and the alteration of the motif to GGC reduced this activity. In an analysis of mammalian genomes, G nucleotides and G triplets were over-represented at the ends of introns[20,21]. Cumulative evidence suggests that these G-rich sequence elements have an important role in pre-mRNA splicing[15,16,22,23]. Change in the secondary structure of the pre-mRNA by a nucleotide substitution outside the consensus sequence[24,25] is another possible mechanism that could influence splicing. When the structure of pre-mRNAs in this region was predicted using the Mfold program (see URLs section in

Methods), the C-allele transcript was found to be likely to form a more stable stem-loop structure than the G-allele transcript (**Supplementary Fig. 4a** online).

An electrophoresis mobility shift assay (EMSA) using RNA oligonucleotides including itpkc_3 and nuclear extracts from either HeLa or Jurkat cells showed specific binding of an unknown protein to the G allele (**Supplementary Fig. 4b**). Identification of the RNA-binding nuclear factor may reveal the precise mechanism through which this SNP alters transcript abundance.

The biological impact of this SNP in Kawasaki disease pathogenesis requires further study. The weaker negative regulatory effect of itpkc_3 C allele on *IL2* is consistent with the significant elevation of IL-2 in acute Kawasaki disease compared to other febrile illnesses[26]. Autopsy studies in children who die during the acute phase of Kawasaki disease show infiltration of T cells, particularly $CD8^+$ cytotoxic T cells (CTL), into the coronary artery wall[27]. This suggests that T-cell activation and infiltration into selected compartments are critically involved in the pathogenesis of Kawasaki disease. Increased activation of T cells influenced by the *ITPKC* polymorphism may be responsible for a greater and more prolonged expansion of pro-inflammatory T cells during the acute phase, thus affecting Kawasaki disease susceptibility and leading to greater disease severity.

The association of the itpkc_3 C allele with Kawasaki disease may have direct clinical implications. In both Japan and the United States, approximately 10–20% of individuals with Kawasaki disease are resistant to IVIG therapy, and these individuals are at highest risk of developing CALs. Although the sample size was limited, the C allele also conferred an increased risk of IVIG resistance in the US cohort for which information regarding IVIG response was available ($n = 37$, OR = 4.67, 95% CI = 1.34–16.24; **Supplementary Table 3** online). Clinical scoring systems have been devised to identify this subgroup, but there is room for improvement in sensitivity and specificity to make them truly useful clinical tools[28–31]. Identifying a genetic signature for the subgroup of IVIG-resistant individuals would permit the use of more intensified therapy (for example, anti-cytokine therapy or plasmapheresis) to prevent the development of CALs. Cyclosporin A (CsA) mediates immunosuppression through blocking calcineurin, which is an important downstream molecule in the Ca^{2+}/NFAT signaling pathway[32]. A single case report describes the successful use of CsA in an individual with Kawasaki disease resistant to IVIG[33]. If further study confirms the importance of the Ca^{2+}/NFAT pathway in T-cell activation in acute Kawasaki disease, then a clinical trial of CsA in IVIG-resistant individuals may be warranted.

Because IP3 also acts as a second messenger in B cells, macrophages and neutrophils[11,34], the function of this SNP should be examined in these effector cells in Kawasaki disease. ITPKC is also expressed in the myocardium. The potential importance of IP3 and Ca^{+2} influx in the myocardium is also relevant to individuals with Kawasaki disease in whom subclinical myocarditis is a common feature of the acute illness. The potential role of this SNP in other inflammatory disorders of the vascular wall and myocardium, including other forms of systemic vasculitis, myocarditis and atherosclerosis, should also be considered.

METHODS

Subjects. The 564 control samples in the initial screening were members of the general Japanese population with various common diseases of adulthood unrelated to Kawasaki disease. Genotype data relating to 1,222 SNPs for this population was obtained from a database at our institute. We recruited 637 Japanese individuals with Kawasaki disease and 1,034 healthy control subjects from several medical institutes in Japan. The ethical committee of RIKEN approved the study, and all the parents of the patients gave written informed consent. All Japanese individuals with Kawasaki disease (male/female ratio = 384:253) were diagnosed by pediatricians based on the Japanese criteria for the disease[35]. Mean age of disease onset was 29.3 months (range 2–127 months).

Trios of Kawasaki disease–affected children and their biological parents ($n = 209$) were recruited at Rady Children's Hospital San Diego and at Boston Children's Hospital. Details regarding this cohort of US individuals with Kawasaki disease have been previously described[36]. Genomic DNA from whole blood, lymphoblastoid cell lines or mouth wash samples was extracted according to standard procedures.

SNP genotyping. We genotyped SNPs using the Invader and TaqMan assays as described previously[37].

Statistical analysis. The case-control association study was analyzed using a χ^2 test. We carried out a multivariate logistic regression analysis for the association between Kawasaki disease and multiple SNPs using forward-backward stepwise procedures to select SNPs and their interactions. For each step of the forward or backward process, we carried out a log likelihood test (0.05 significance threshold) to change the set of SNPs or their interactions. Meta-analysis of data from different case-control sets was conducted by Mantel-Haenszel methodology. The transmission disequilibrium test was performed using TDT software[38] integrated in Haploview version 3.32 (see URLs section below). Integration of the case-control and TDT data was conducted as previously described[39].

RNA extraction and quantitative RT-PCR. Total RNA from normal human tissues (except PBMCs) was purchased from Clontech. We isolated PBMCs from healthy human volunteers from venous blood using the Lymphoprep reagent (Axis-Shields). To assess the induction of *ITPKC* in stimulated white blood cells, we treated Jurkat cells and PBMCs with ionomycin (1 mg ml^{-1}) and PMA (50 ng ml^{-1}) for 8 h. K562 cells were treated only with PMA (50 ng ml^{-1}) for 8 h. We extracted total RNA from cell lines and PBMCs using the NucleoSpin RNA II kit (Macherey-Nagel). 1 μg of each RNA was reverse transcribed with Superscript III reverse transcriptase and oligo dT primers (Invitrogen). We quantified transcripts for *ITPKA, ITPKB, ITPKC* and *IL2* with TaqMan probe and primers (Hs00176658_m1, Hs00176666_m1 and Hs00363893_m1 for *ITPKA, ITPKB* and *ITPKC*, respectively, and Hs00174114_m1 for *IL2*). Amplification and detection were done using a Mx3000P thermal cycler (Stratagene). Results were normalized to the transcript levels of β-actin.

Allele-specific transcript quantification (ASTQ). ASTQ was carried out as described previously[40]. Genomic DNAs and cDNAs were amplified for 31 cycles with these primers. At the last cycle, we added forward primer labeled with Alexa Fluor 488 at the 5′ end. Amplicons were digested with *Sma*I according to manufacturer's instructions. Separation was conducted on 12% polyacrylamide gels in 25 mM Tris and 250 mM glycine. Quantification was carried out by using FLA-7000 analyzer (Fujifilm).

URLs. JSNPs, http://snp.ims.u-tokyo.ac.jp/index_ja.html; TFSEARCH, http://mbs.cbrc.jp/research/db/TFSEARCH.html; Mfold, http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi; International HapMap Project, http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B36/; Haploview version 3.32, http://www.broad.mit.edu/mpg/haploview/.

GenBank accession number. Inositol 1,4,5-trisphosphate 3-kinase C (*ITPKC*) mRNA, NM_025194.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y.O., A.H. and Yusuke N. designed the study. Y.O., J.C.B., C.S., J.W.N., F.K., K.H., M.T., Y.S., K.O., T.S., A.N., Y.K., T.Y., K.S., Takeo T., T.N., H.C. and A.F. collected most of the samples. M.Y., Yoshikazu N., H.Y. and T.K. provided information regarding the Japanese nation-wide survey of Kawasaki disease. K.W. and Y.F. established lymphoblastoid cell lines of individuals with Kawasaki disease. Tatsuhiko T. and A.S. supported the initial SNP screening by providing genotyping data for the control population. Y.O. performed genotyping and statistical analyses. R.N. and Tatsuhiko T. performed logistic regression analyses. Y.O. and T.G. performed functional assays. Y.O., J.C.B., C.S., J.W.N. and A.H. wrote the paper.

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Elevated granulocyte colony-stimulating factor levels predict treatment failure in patients with Kawasaki disease

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Background: Kawasaki disease (KD) is an acute vasculitis in young children, frequently associated with coronary artery aneurysms. The intravenous infusion of high-dose IgG (IVIG) effectively reduces the systemic inflammation and the incidence of coronary artery lesions, although the precise underlying mechanisms are unknown.

Objective: We performed expression profiling of whole blood cells to investigate the mechanisms underlying the effect of IVIG and to identify biomarkers associated with unresponsiveness to IVIG.

Methods: We compared the transcript abundance among pre-IVIG and post-IVIG patients and febrile control patients. Then we analyzed the mRNA levels and the protein levels among the different cohort of patients with KD who were either responsive or nonresponsive to the initial IVIG.

Results: A total of 298 transcripts were overrepresented or underrepresented in the pre-IVIG patients compared with post-IVIG patients and febrile controls, of which 15 transcripts were differentially expressed in nonresponsive patients with KD compared with responsive patients before IVIG. The protein levels of polycythemia rubra vera 1, which was one of the most variably expressed transcripts in pre-IVIG patients, and the serum granulocyte colony-stimulating factor levels were significantly higher in nonresponsive patients than in responsive patients before the initial IVIG administration.

Conclusion: These findings suggest that the variable gene expression profiles were correlated to the responses of patients with KD to IVIG administration. Polycythemia rubra vera 1 and granulocyte colony-stimulating factor levels may be good biomarkers for predicting response to IVIG in patients with KD. (J Allergy Clin Immunol 2008;122:1008-13.)

Key words: *Kawasaki disease, vasculitis, neutrophil, PRV-1, G-CSF, microarray, inflammation, IVIG, biomarker*

Abbreviations used

CAL: Coronary artery lesion
CR1: Complement component 3b/4b receptor 1
GAS7: Growth arrest-specific 7
G-CSF: Granulocyte colony-stimulating factor
ITGAM: α-M integrin
IVIG: Intravenous infusion of high-dose IgG
KD: Kawasaki disease
MFI: Mean fluorescence intensity
PRV: Polycythemia rubra vera

Kawasaki disease (KD) is an acute systemic vasculitis in infants and young children.[1] It preferentially affects coronary arteries and is the leading cause of acquired heart disease in childhood in developed countries.[2] The intravenous infusion of high-dose IgG (IVIG) effectively reduces the systemic inflammation and the incidence of coronary artery lesions (CALs).[3] However, the precise underlying mechanisms of the effect of IVIG are unknown, and about 15% of patients with KD are nonresponsive to IVIG and develop CALs more frequently than responsive patients.[4] Many researchers have attempted to identify risk factors associated with nonresponsiveness to IVIG.[5-9] Among them, the patients' age, the white blood cell count, and the serum levels of aspartate aminotransferase and C-reactive protein were frequently shown to be useful in determining a risk classification instrument. However, because these factors were statistically determined by using available laboratory test data, the precise mechanism underlying the relationships between these factors and the clinical outcome of the patients remains uncertain.

In a previous study, we performed expression profiling of purified PBMCs and monocytes obtained before and after IVIG from patients with acute KD.[10] The results demonstrated that IVIG influenced the gene expressions in a broad functional range in both PBMCs and monocytes, favoring downregulation. This finding is consistent with the suppressive effects of IVIG that are clinically observed in acute patients. In this study, we performed expression profiling of whole blood cells, including neutrophils, to extend farther our understanding of the mechanisms underlying the effect of IVIG on patients with KD, in the hope of eventually identifying biomarkers associated with the unresponsiveness to IVIG. Our results suggested that the protein levels of polycythemia rubra vera (PRV)–1, one of the most enriched transcripts in pre-IVIG patients, and granulocyte colony-stimulating factor (G-CSF) were significantly elevated in nonresponsive patients.

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TABLE I. Demographic data of patients

	First-array patients			Second-array patients			
	KD-pre	KD-post	Control	Responsive-pre	Responsive-post	Nonresponsive-pre	Nonresponsive-post
Donors (n)	4	4	4	6	6	4	4
Age (mo after birth)	8-54 (median, 13)		12-39 (median, 20)	39-60 (median, 56)		12-38 (median, 33)	
Sex (male, female)	2, 2		2, 2	2, 4		2, 2	
Cardiac involvement (positive patients)	0	0		0	0	0	0
Blood drawn (d after onset)	3-7 (median, 6.5)	8-10 (median, 8.5)	3-7 (median, 5)	4-7 (median, 4.5)	7-9 (median, 8)	3-5 (median, 4)	6-8 (median, 7)
% Neutrophil	49-86 (median, 53.5)	14-67 (median, 36)	36-70 (median, 52.3)	80-86 (median, 83)	18-73 (median, 35)	81-91 (median, 84)	59-77 (median, 69)
P value*		.07	.56		.03	.67	.07
% Monocyte	4-16 (median, 7.1)	5-10 (median, 8.5)	2-13 (median, 7.5)	2.4 -7.0 (median, 3.7)	7-10 (median, 9)	1.3-10.4 (median, 5.0)	3-14 (median, 4.0)
P value		.99	.77		.03	.92	.99
% Lymphocyte	10-44 (median, 30)	21-67 (median, 47)	27-63 (median, 30.3)	10-14 (median, 11)	19-62 (median, 46)	6-14 (median, 8)	13-36 (median, 19)
P value		.07	.56		.03	.14	.07
C-reactive protein (mg/dL)	6.4-14.5 (median, 8.4)	0.8-12.1 (median, 3.3)	0.3-4.1 (median, 1.4)	7.8-16.1 (median, 9.9)	3.5-14.2 (median, 7.0)	5-19 (median, 8.8)	1.7-21 (median,12.3)
P value		.07	.08		.2	.67	.47

**P* values between KD-post vs KD-pre, or control vs KD-pre in the first-array patients, and responsive-pre vs nonresponsive-pre, or responsive-pre vs responsive-post in the second-array patients.

METHODS

Patients

The patients with KD (n =7 6) were treated at Chiba University Hospital or Chiba Kaihin Municipal Hospital between May 2005 and June 2007. All the patients fulfilled the Criteria for Diagnostic Guidelines for Kawasaki Disease (5th revision) published by the Kawasaki Disease Research Committee in Japan and were initially treated with IVIG (2.0 g/kg for 1-2 days) and oral aspirin (30 mg/kg daily).[11] Informed consent was obtained from the parents according to the guidelines of each medical center. Clinical data including age, sex, duration of illness, laboratory findings, response to IVIG, and coronary artery involvement were documented and are summarized in Table I and this article's Table E1 in the Online Repository at www.jacionline.org. Abnormal cardiac function was monitored by using 2-dimensional echocardiography, and the presence of CALs was diagnosed according to the Japanese Ministry of Health criteria. Two patients developed coronary aneurysms at 1 month after the onset of their diseases. Nonresponsiveness to IVIG was defined as a persistent or recrudescent fever (body temperature higher than 37.5°C) at 24 hours after the completion of the IVIG treatment.

Venous blood was drawn from each patient before IVIG treatment (3-7 days after the onset of fever) and within 2 days after the completion of IVIG. In addition, sera obtained from 8 nonresponsive patients with KD treated at Chiba University Hospital before 2005 were used in an ELISA. These patients were initially treated using IVIG (2.0 g/kg for 1-2 days). Four of the 8 patients developed coronary aneurysms at 1 month after disease onset. Control blood samples were obtained from 15 patients who had been febrile (body temperature higher than 38°C) for at least 3 days (Tables I and E1). The clinical diagnoses of these control patients were pneumonia (n = 7), acute upper respiratory infection (n = 3), toxic shock syndrome–like disease (n = 2), infectious mononucleosis (n = 1), cervical lymphadenitis (n = 1), and meningitis (n = 1).

Extraction of RNA and reduction of globin mRNA content

Venous blood samples (2.5 mL) were collected in PAXgene Blood RNA Tubes (BD Biosciences, San Jose, Calif), and RNA was purified by using the PAXgene kit (Qiagen, Valencia, Calif) according to the manufacturer's instructions. The quality and the quantity of RNA were examined using the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, Calif). The total RNA samples were further processed to reduce the α-globin and β-globin mRNA content according to the Globin Reduction Protocol (Affymetrix, Santa Clara, Calif).[12] Briefly, total RNA was hybridized with Globin Reduction Oligo Mix (Affymetrix) and digested by using RNase H at 37°C for 10 minutes. The digested total RNA was then purified by using an RNeasy Micro kit (Qiagen) and quantified.

RNA amplification and GeneChip expression analysis

Gene expression profiles were examined by using the Human Genome U133 Plus 2.0 array (GeneChip; Affymetrix) according to the manufacturer's protocols. Briefly, double-stranded cDNA were synthesized from 2 μg total RNA by using the One-cycle cDNA Synthesis kit (Affymetrix). The cDNA was subjected to *in vitro* transcription by using the IVT Labeling kit (Affymetrix) and hybridized with a U133 Plus 2.0 array. The fluorescence intensity of each transcript was quantified by using a Affymetrix GeneChip Scanner 3000 (Affymetrix), and the expression value was determined by using the GeneChip Operating Software (Affymetrix). The results of the microarray analysis can be found on our web site at http://www.nch.go.jp/imal/GeneChip/KAWASAKI2.htm.

The data were further analyzed with GeneSpring GX version 7.3 (Agilent Technologies). Each array was normalized (mean centered) to the median intensity array. To minimize the influence of background noise, only probes reliably detected in at least 2 out of all the samples were included in the analysis. A 1-way ANOVA analysis and unsupervised hierarchical clustering using Pearson correlation were performed with GeneSpring GX version 7.3 software.

Quantitative real-time PCR

The PCR primers were designed based on sequences from GenBank. The primer sequences were as follows: *haptoglobin* forward primer, 5′-TCGGCATGTCTAAGTACCAGGAA-3′; haptoglobin reverse primer, 5′-AGGTCGTGAACGGCAAAGG-3′; *prv-1* forward primer, 5′-GGCCCAACCTTCCAGCTT-3′; *prv-1* reverse primer, 5′-CTTCTCACGCGCAGAGAAGA-3′; *growth arrest-specific 7 (gas7)* forward primer, 5′-GCAGCTGCGGCATGAAA-3′; *gas7* reverse primer, 5′-TGGCCGGGTCCACTTTT-3′; α-*M*

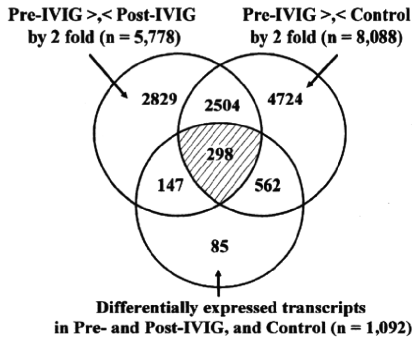

FIG 1. Variably expressed transcripts in the white blood cells obtained from the first cohort of patients with KD and febrile controls (Table I). Expression values of 1092 transcripts were significantly different among the 3 groups (1-way ANOVA, $P < .05$). Most variably expressed (>2.0x or <0.5x) transcripts (n = 298) in pre-IVIG patients compared with post-IVIG patients and controls were selected.

integrin (itgam) forward primer, 5′-CAGACTTGTGAGACCCTGAAACTA CA-3′; and *itgam* reverse primer, 5′-CGAAAGCAGACAATGGCGTT-3′. PCR was performed by using the ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, Calif) in a 25-μL reaction mixture containing iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, Calif). Samples were subjected to 40 cycles of amplification at 95°C for 15 seconds for denaturing, and at 60°C for 1 minute for annealing-extension. The expression of each target cDNA relative to glyceraldehyde-3-phosphate dehydrogenase was calculated for each sample by using a comparative cycle-threshold method described by the manufacturer (PE Applied Biosystems).

Flow cytometry

Cell surface molecules were measured by using mAbs in conjunction with 2-color immunofluorescence staining following a standard protocol. The mAbs used were anti-CD177 (PRV-1) in combination with fluorescein isothiocyanate–conjugated antimouse IgG_1 and phycoerythrin-conjugated anti-CD16b (BD Biosciences), or phycoerythrin-conjugated anticomplement component 3b/4b receptor 1 (CR1) in combination with fluorescein isothiocyanate–conjugated anti-CD16b (Beckman Coulter, Fullerton, Calif), respectively.

Measurement of serum G-CSF levels

An ELISA was performed to quantify the G-CSF concentrations in the sera. mAbs against human G-CSF, BVD13-3A5 (Beckman Coulter) as a capture antibody, and BVD11-37G10 (Beckman Coulter) as a detection antibody were used. The protein concentration was calculated using Microplate Manager III software (Bio-Rad Laboratories).

Statistical analysis

For the GeneChip microarray data, a nonparametric Mann-Whitney *U* test was performed by using the GeneSpring GX software version 7.3. For the real-time PCR, the flow cytometry, and the ELISA data, a 1-factor ANOVA and the Bonferroni/Dunn *F* test as a post hoc test were used to compare responsive and nonresponsive patients with KD and febrile control patients. A value of $P < .05$ was considered statistically significant.

RESULTS

Gene expression profiles of responsive patients with KD before and after IVIG

We first examined the gene expression profiles of patients with KD (n = 4) before and after IVIG therapy and age-matched febrile controls (n = 4). The demographic and laboratory data of the patients are summarized in Table I. Expression values of 1092 transcripts were significantly different among the 3 groups by a 1-factor ANOVA. To focus on the most variably expressed genes in the pre-IVIG patients, we selected 298 out of 1092 transcripts whose expression values in pre-IVIG patients were more than double or less than half of those in post-IVIG patients and in febrile controls (Fig 1; see this article's Table E2 in the Online Repository at www.jacionline.org). Among the 298 transcripts, 193 genes (225 transcripts) or 64 genes (67 transcripts) were more or less abundant in the pre-IVIG compared with the post-IVIG patients and control patients, respectively. Six genes (6 transcripts) were more abundant in control patients than in pre-IVIG and post-IVIG patients. The top 20 transcripts whose expression values were higher in the pre-IVIG patients compared with those in the post-IVIG patients are listed in Table II.

In an article examining the transcriptional program of terminal granulocytic differentiation, Theilgaard-Monch et al[13] reported that 6700 genes were differentially expressed among the highly purified bone marrow granulocyte precursors—that is, promyelocytes, myelocytes, and bands. According to their gene list, 154 of the 298 genes (52%) that showed variable expression values in our study were expressed during the granulocytic differentiation (Table E2). Interestingly, among the 225 more abundant transcripts in the pre-IVIG patients, 107 (48%) were specifically expressed in myelocytes or bands, whereas only 4 transcripts (2 %) were specific to promyelocytes. On the other hand, among the 67 less abundant transcripts in the pre-IVIG patients, only 4 transcripts (6%) were specifically expressed in myelocytes or bands, whereas 37 (55%) were specific to promyelocytes.

Gene expression profiles of nonresponsive patients with KD

We next questioned how these 298 transcripts were expressed in patients who were nonresponsive to IVIG. Because nonresponsiveness to IVIG is frequently associated with high percentages of neutrophils in patients with KD, we selected patients who had similar percentages of neutrophils before IVIG for a second microarray analysis. They included 6 responsive and 4 nonresponsive patients with KD, and blood samples were collected before and after IVIG (Table I). By an unsupervised hierarchical clustering of these 20 samples, the expression profiles of the 298 transcripts that were variably expressed in the first cohort of patients were significantly different between pre-IVIG and post-IVIG patients (data not shown). However, we were unable to discriminate between the responsive and the nonresponsive pre-IVIG patients in this clustering analysis. Thus, we directly compared the expression values of these 298 transcripts between responsive and nonresponsive patients before IVIG and found that 15 genes were differentially expressed between the 2 groups (Table III).

Elevated PRV-1 expression in nonresponsive patients with KD

The microarray analysis suggested that 15 genes were differentially expressed between responsive and nonresponsive patients with KD even before the initiation of IVIG. Among them, the biological functions of haptoglobin, CD177, and ITGAM genes are well characterized and are likely to be associated with the pathophysiological conditions of patients with KD. On the other hand, the transcripts of *gas7* gene had been enriched in PBMCs and purified monocytes obtained from pre-IVIG patients in our previous studies.[10] Thus, we selected these 4 genes and measured

TABLE II. The top 20 most variably changed transcripts in pre-IVIG patients

Probe ID	Gene symbol	Gene name	Fold change pre-IVIG/post-IVIG	Fold change pre-IVIG/control
219669_at	CD177	Polycythemia rubra vera 1	18.6	6.5
1557924_s_at	ALPL	Alkaline phosphatase, liver/bone/kidney	9.1	6.3
217552_x_at	CR1	Complement component (3b/4b) receptor 1	7.5	11.9
237618_at	—	Transcribed sequences	6.8	3.3
206548_at	FLJ23556	Hypothetical protein FLJ23556	6.5	3.0
244889_at	—	LOC388210 mRNA	6.2	8.0
231886_at	—	cDNA DKFZp434B2016	6.1	3.4
232465_at	—	cDNA FLJ11687	5.9	3.0
227250_at	KREMEN1	Kringle containing transmembrane protein 1	5.9	5.8
215783_s_at	ALPL	Alkaline phosphatase, liver/bone/kidney	5.5	5.2
237568_at	—	Transcribed sequences	5.3	2.6
228648_at	LRG1	Leucine-rich α-2-glycoprotein 1	5.0	2.9
218660_at	DYSF	Dysferlin, limb girdle muscular dystrophy 2B	4.8	3.3
240156_at	—	Transcribed sequences	4.8	4.3
216782_at	—	cDNA: FLJ23026	4.8	3.2
204713_s_at	F5	Coagulation factor V	4.6	5.0
227055_at	METTL7B	Integrin, α 7	4.6	3.5
236592_at	—	Transcribed sequences	4.5	3.6
228758_at	BCL6	B-cell/lymphoma 6	4.5	2.9
229296_at	—	LOC389793 mRNA	4.4	6.5

TABLE III. Differentially expressed transcripts between responsive and nonresponsive patients before IVIG

Probe ID	Gene symbol	Gene name	Fold change nonresponder/responder
208470_s_at	HP	Haptoglobin	4.44
239701_at	—	Transcribed sequence	2.21
219669_at	CD177	Polycythemia rubra vera 1	2.08
224818_at	SORT1	Sortilin 1	1.96
225499_at	—	Clone CDABP0105	1.87
232500_at	C20orf74	Chromosome 20 open reading frame 74	1.66
211974_x_at	RBPJ	Recombination signal binding protein for immunoglobulin κ J region	1.66
202191_s_at	GAS7	Growth arrest-specific 7	1.59
205786_s_at	ITGAM	Integrin, α M (complement component 3 receptor 3 subunit)	1.47
226080_at	SSH2	Slingshot homolog 2	1.42
229295_at	LOC150166	Hypothetical protein LOC150166	1.26
212516_at	CENTD2	Centaurin, delta 2	0.75
216841_s_at	SOD2	Superoxide dismutase 2	0.70
1558972_s_at	C6orf190	Chromosome 6 open reading frame 190	0.51
226682_at	LOC283666	Hypothetical protein LOC283666	0.42

mRNA levels using a real-time PCR in additional responsive (n = 18) and nonresponsive (n = 8) patients with KD who had similar percentages of neutrophils before IVIG administration (Table E1). Four responsive and 2 nonresponsive patients examined by the microarray were included in this analysis. The results indicated that mRNA levels of all 4 genes were decreased after IVIG in both responsive and nonresponsive patients. Before IVIG administration, the transcripts of *hp* and *prv-1* genes were more abundant in nonresponsive patients than in responsive patients, confirming the microarray results (Fig 2, *A*). However, the transcripts of *gas7* and *intam* genes were not significantly different between the 2 groups.

Because *prv-1* gene showed the most variable transcript abundance in the pre-IVIG patients in the first microarray analysis and the levels of PRV-1 mRNA were significantly higher in nonresponsive patients than in responsive patients, we next examined the protein levels of PRV-1 by immunostaining and flow cytometry. CR1 was also measured as a control. We examined 26 responsive patients with KD, 14 nonresponsive patients with KD, and 12 age-matched febrile control patients (Table E1). The mean fluorescence intensity (MFI) of PRV-1 and CR1 on the neutrophils was significantly elevated in pre-IVIG patients compared with those in febrile controls (mean ± SEM, 104.2 ± 7.8 vs 52.8 ± 8.7, $P = .002$; and 328.7 ± 28.0 vs 177.1 ± 40.9, $P = .007$, respectively), and these levels decreased after IVIG (104.2 ± 7.8 vs 67.0 ± 5.9, $P < .0001$; and 328.7 ± 28.0 vs 247.0 ± 29.6, $P = .02$, respectively; Fig 2, *B*). Importantly, MFI of PRV-1 on neutrophils was markedly increased in nonresponsive pre-IVIG patients compared with that in responsive pre-IVIG patients (138.3 ± 16.0 vs 87.2 ± 6.7; $P = .0004$). MFI of CR1 was not significantly different between the 2 groups.

Increased serum G-CSF levels in nonresponsive patients with KD

PRV-1 is known to be highly expressed in neutrophils from patients with polycythemia rubra vera.[14] Increased neutrophil PRV-1 mRNA levels are also associated with increased neutrophil production induced by acute bacterial infection and injection of recombinant G-CSF.[15] We speculated that elevated PRV-1 staining in nonresponsive pre-IVIG patients with KD may be caused

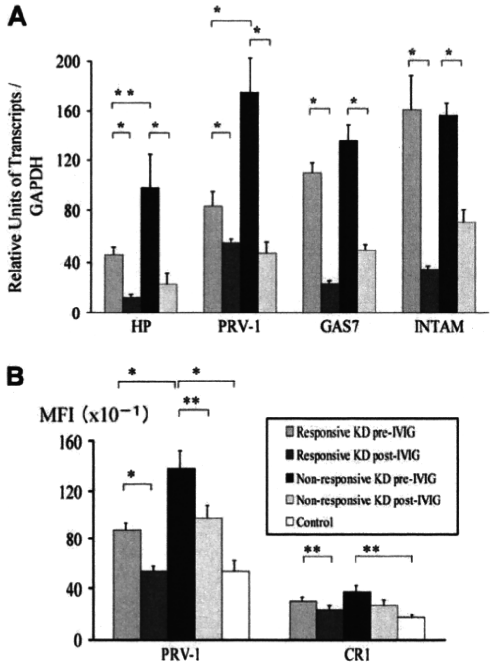

FIG 2. A, mRNA levels in responsive and nonresponsive patients before and after IVIG administration. Real-time RT-PCR results are presented as relative units of each transcript compared with GAPDH. *Error bars* indicate SEM. $*P = .001$, $**P = .01$ compared between pre- and post-IVIG patients (Wilcoxon signed-rank test), and between responsive and nonresponsive patients (Mann-Whitney *U* test). **B,** Immunostaining of PRV-1 and CR1 on neutrophils. Blood samples were double-immunostained and analyzed by flow cytometry. The results are expressed as MFI, and *error bars* indicate SEM. $*P = .001$, $**P = .01$ compared between pre-IVIG and post-IVIG patients (Wilcoxon signed-rank test), and among responsive and nonresponsive pre-IVIG patients and febrile controls (Bonferroni/Dunn *F* test). *HP,* Haptoglobin.

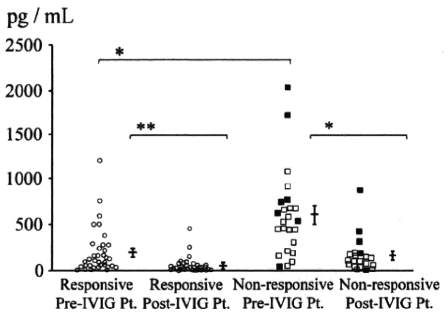

FIG 3. Elevated serum G-CSF levels in nonresponsive pre-IVIG patients. Concentration of G-CSF was measured by ELISA in responsive and nonresponsive patients before and after IVIG. *Solid and open squares* indicate patients with and without CAL, respectively. The *bar* indicates mean ± SEM for each group. $*P = .001$, $**P = .01$ compared using the Bonferroni/Dunn *F* test as a post hoc test.

by an elevation of serum G-CSF levels in these patients. Therefore, we measured the serum levels of G-CSF in patients with KD before and after IVIG. In addition to our patient group, we examined 8 nonresponsive pre-IVIG patients with KD who were treated at Chiba University Hospital between 2004 and 2005, 4 of whom developed CAL 1 month after the onset of their illnesses.

Fig 3 shows that the serum G-CSF levels in nonresponsive pre-IVIG patients with KD (n = 24) were markedly elevated compared with those in responsive pre-IVIG patients (n = 35; 592.6 ± 97.8 pg/mL vs 183.0 ± 40.8 pg/mL; $P < .0001$). After IVIG, the G-CSF levels were decreased in both responsive and nonresponsive patients, but the levels were still higher in nonresponsive patients than in responsive patients (158.2 ± 43.9 pg/mL vs 52.8 ± 15.0 pg/mL; $P < .0001$). Importantly, patients who developed CAL *(closed symbols)* had significantly higher pre-IVIG levels of G-CSF than patients without coronary aneurysms (918.4 ± 264.9 pg/mL vs 273.1 ± 39.5 pg/mL; $P < .0001$).

DISCUSSION

An increased white blood cell count is a hallmark of active KD and has been suggested to be a risk factor for the development of CAL by several investigators.[6,9] The production of active oxygen species by neutrophils has been postulated to contribute to endothelial cell damage in KD.[16] Recently, Takahashi et al[17] reported that neutrophils had infiltrated a coronary aneurysm in pathological specimens obtained from a patient who died early during the acute phase of KD. In this study, in the first cohort of patients, we demonstrated that a group of 298 transcripts were overrepresented in the patients with KD before IVIG compared with the age-matched febrile controls and post-IVIG patients with KD, and 154 of 298 transcripts were known to be expressed in the late granulocyte differentiation.[13]

In the second cohort of KD patients who had similar percentages of neutrophils before IVIG (Table I), the pre-IVIG patients also showed a distinct gene expression profiles of these granulocyte-derived transcripts from the post-IVIG patients after an unsupervised hierarchical clustering (data not shown). However, because this analysis failed to distinguish responsive and nonresponsive patients before the initiation of IVIG, we directly compared the gene expression values before IVIG between responsive and nonresponsive patients and found that 15 transcripts were differentially expressed. Among them, it is reported that haptoglobin and PRV-1 were mainly expressed in myelocytes, whereas sortilin 1, ITGAM, slingshot homolog 2, centaurin delta 2, and superoxide dismutase 2 were mainly expressed in bands in the bone marrow.[13] Thus, these results are consistent with the clinical observations and suggest that stimulation of granulocyte differentiation and recruitment from the bone marrow may be associated with the unresponsiveness of a patient with KD against IVIG therapy.

In the next step, we examined the microarray results by real-time PCR and flow cytometry in our third cohort of patients with KD. Among the 4 transcripts examined, the mRNA levels of haptoglobin and PRV-1 but not of GAS7 or INTAM were significantly higher in nonresponsive patients than those in responsive patients. The reason the transcripts of GAS7 and INTAM showed inconsistent results between microarray and real-time PCR analysis remains to be investigated. In our previous microarray studies, the *gas7* and *intam* genes but not the *hp* and *prv-1* genes were constitutively expressed by the resting neutrophils as well as the monocytes and the lymphocytes[18] (supplemental data in http://www.nch.go.jp/imal/GeneChip/public.htm). Therefore, the expression by these resting cells might affect the expression profiles of the *gas7* and *intam* genes differently from the *hp* and *prv-1* genes during the acute phase of KD. The *hp* and *prv-1* genes are both expressed mainly in myelocytes at steady state, but the transcription of these genes is extremely upregulated during an emergency situation such as acute bacterial infection.[15,19,20] Cytokines such as IL-6 and G-CSF stimulate haptoglobin synthesis during granulocyte differentiation through the induction of CCAAT/enhancer-binding protein-β in

granulocyte progenitors.[19,20] On the other hand, PRV-1, a surrogate marker of a patient with polycythemia rubra vera, is also induced in severe sepsis and in healthy volunteers injected with recombinant G-CSF.[15] Accordingly, in our patients with KD, the serum levels of G-CSF were positively correlated with the intensity of PRV-1 staining (data not shown) and higher in nonresponsive patients than in responsive patients both before and after IVIG. These findings suggest that G-CSF may be involved in the proliferation and the recruitment from the bone marrow of the granulocyte precursors in the acute phase of KD. More importantly, among the nonresponsive patients, the serum G-CSF levels were higher in the CAL(+) patients than in CAL(−) patients. Elevated G-CSF levels in patients with KD and their association with CAL have been reported previously by other investigators.[21-23] Because vascular endothelial and smooth muscle cells are known to produce G-CSF under inflammatory conditions,[24-26] it is of interest to examine whether IVIG works directly on vascular endothelial and smooth muscle cells to reduce the production of G-CSF or indirectly on tissue macrophages and hepatocytes to reduce the production of proinflammatory cytokines, such as IL-1β, which stimulates G-CSF production by vascular endothelial cells.

The prevention of CAL is one of the most important end points in the treatment of patients with KD. Because CAL often accompanies IVIG nonresponsiveness, many studies have attempted to identify risk factors associated with IVIG nonresponsiveness by using patients' demographic and laboratory test data.[5-9] Our finding that the PRV-1 protein levels differed significantly between the responsive and nonresponsive patients with KD before the initiation of IVIG may be interesting in this point. Because the measurement of PRV-1 using flow cytometry is simple and fast, this method may be of clinical relevance during the initial IVIG administration. It would be interesting to determine whether the measurement of PRV-1 levels by flow cytometry is useful in predicting a patient's response against initial IVIG administration so as to prepare more carefully for the possible nonresponsiveness in a high-risk patient.

In conclusion, using a DNA microarray technology, we found that a group of transcripts related to the late differentiating granulocytes was overrepresented in patients with acute KD and decreased after IVIG in responsive patients, but not in nonresponsive patients. The protein levels of PRV-1 on neutrophils and G-CSF concentrations were significantly higher in nonresponsive patients than in responsive patients. These findings suggest that the G-CSF stimulation of granulocytes may be an important risk factor in the pathogenesis of KD.

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Clinical implications: These findings suggest that the elevated serum levels of G-CSF and the PRV-1 expression on circulating granulocytes are useful biomarkers for nonresponsiveness to IVIG in patients with KD.

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