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Unique activation status of peripheral blood mononuclear cells at acute phase of Kawasaki disease

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Summary

Although Kawasaki disease (KD) is characterized by a marked activation of the immune system with elevations of serum proinflammatory cytokines and chemokines at acute phase, the major sources for these chemical mediators remain controversial. We analysed the activation status of peripheral blood mononuclear cells (PBMCs) by flow cytometry, DNA microarray and quantitative reverse transcription–polymerase chain reaction. The proportions of $CD69^+$ cells in both natural killer cells and γδT cells at acute-phase KD were significantly higher than those at convalescent-phase KD. Microarray analysis revealed that five genes such as *NAIP*, *IPAF*, *S100A9*, *FCGR1A* and *GCA* up-regulated in acute-phase KD and the pathways involved in acute phase KD were related closely to the innate immune system. The relative expression levels of damage-associated molecular pattern molecule (DAMP) (*S100A9* and *S100A12*) genes in PBMCs at acute-phase KD were significantly higher than those at convalescent-phase KD, while those of *TNFA*, *IL1B* and *IL6* genes were not significantly different between KD patients and healthy controls. Intracellular production of tumour necrosis factor-α, interlaukin-10 and interferon-γ in PBMCs was not observed in KD patients. The present data have indicated that PBMCs showed a unique activation status with high expression of DAMP genes but low expression of proinflammatory cytokine genes, and that the innate immune system appears to play a role in the pathogenesis and pathophysiology of KD.

Keywords: acquired immunity, cytokines, innate immunity, Kawasaki disease, peripheral blood mononuclear cells

Introduction

Kawasaki disease (KD) is an acute febrile illness of childhood with systemic vasculitis characterized by the occurrence of coronary arteritis. Although KD is characterized by a marked activation of the immune system with elevations of serum proinflammatory cytokines and chemokines at acute phase [1–3], no previous studies have demonstrated that peripheral blood mononuclear cells (PBMCs) serve as the major sources for these chemical mediators. Although the activation of monocytes/macrophages has been reported to have an important role at acute phase of KD [4], there were no significant differences in the expression levels of *IL6*, *IL8* and *TNFA* genes in separated monocytes before and after high-dose gammaglobulin therapy [5].

Activation status of PBMCs, especially T cells, at acute phase of KD is also controversial. In a previous report, it has been thought that most activated T cells moved to local tissues from peripheral blood at acute phase and returned from there at convalescent phase [3]. Although numerous immunological studies on T cells have been reported, no previous studies analysed T cells by separating them into two distinct populations, αβT cells and γδT cells, which are involved mainly in acquired and innate immunity, respectively.

To clarify the pathophysiology of KD, we analysed the activation status of PBMCs including αβT cells, γδT cells, natural killer (NK) cells and B cells by flow cytometry, DNA microarray and quantitative reverse transcription–polymerase chain reaction (RT–PCR). These analyses have shown consistently that the innate immune system might be involved in the pathogenesis and pathophysiology of KD, and that PBMCs were not a major source for proinflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF) in acute-phase KD sera.

Materials and methods

Patients

All patients enrolled in this study were admitted to the Kyushu University Hospital or Fukuoka Children's Hospital between April 2005 and February 2009. The patient group consisted of 51 KD patients who met the criteria for the Diagnostic Guidelines of Kawasaki Disease (http://www.kawasaki-disease.org/diagnostic/index.html). A coronary artery was defined as abnormal if the luminal diameter was greater than 3 mm in children aged less than 5 years (greater than 4 mm in children older than 5 years), if the internal diameter of a segment was at least 1·5 times as large as that of an adjacent segment, or if the lumen was irregular [6]. All patients received oral aspirin (30 mg/kg/day) and 1–2 g/kg of intravenous immunoglobulin (IVIG) as an initial treatment.

To analyse immunological profiles in KD by flow cytometry, we recruited 38 KD patients (median age, 2·0 years; range, 3 months–7·3 years) between September 2006 and August 2008. No patients had coronary artery lesions (CAL). We first analysed the proportions of activated T, B and NK cells in the peripheral blood of both seven patients with KD and 15 age-matched healthy controls by flow cytometry. CD69, human leucocyte antigen D-related (HLA-DR) and CD25 were used as activation markers. These cells were analysed before treatment with IVIG (median day of illness, day 5; range, days 3–6) and in the convalescent phase (median day of illness, day 13; range, days 13–18). To analyse further the immunological profiles in KD, the proportion of $CD69^+$ cells were investigated in αβT cells ($n = 23$), γδT cells ($n = 23$), NK cells ($n = 35$) and B cells ($n = 35$).

To analyse mRNA expression levels, blood samples were obtained prior to the treatment (on 4–5 days of illness) from three KD patients (median age, 4·7 years; range, 4·1–5·3 years) without CAL and from five healthy adults. PBMCs were separated from peripheral blood and were used for cDNA microarray analysis.

To analyse mRNA expression levels using quantitative real-time RT–PCR, blood samples were obtained from 10 to 16 KD patients (median age, 1·7 years; range; 4 months–7·2 years) in both acute and convalescent phase, and from 20 age-matched control subjects including nine patients (median age, 2·6 years; range, 5 months–13·1 years) with active infections [three patients with bacterial meningitis (one *Haemophilus influenzae* type b, one *Streptococcus pneumoniae* and one unknown), six patients with viral infection (three measles, three Epstein–Barr virus infection)] and 11 healthy children (median age, 5·0 years; range, 1·7–7·6 years).

All subjects gave written informed consent for this study, according to the process approved by the Ethical Committee of Kyushu University and Fukuoka Children's Hospital and Medical Center for Infectious Diseases, Fukuoka, Japan.

Total RNA extraction and RNA amplification

PBMCs were separated from peripheral blood by density-gradient centrifugation using lymphocyte separation medium (Cappel-ICN Immunobiologicals, Costa Mesa, CA, USA) containing 6·2 g Ficoll and 9·4 g sodium diatrizoate per 100 ml. Total RNA was extracted from these cells using an RNA extraction kit (Isogen; Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. Total RNAs from five healthy adults were mixed. An amino allyl message amp aRNA Kit (Ambion, Austin, TX, USA) was used to amplify the total RNA. Briefly, double-stranded complementary DNA (cDNA) was synthesized from total RNA using oligo-dT primer with a T7 RNA polymerase promoter site added to the 3′ end. Then, *in vitro* transcription was performed in the presence of amino allyl uridine-5′-triphosphate (UTP) to produce multiple copies of amino allyl-labelled complementary RNA (cRNA). Amino allyl-labelled cRNA was purified, and then reacted with N-hydroxy succinimide esters of Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for cRNA from PBMCs of healthy controls, and Cy5 (Amersham Pharmacia Biotech) for that from PBMCs of the acute-phase KD patients, according to the protocol of Hitachi Software Engineering (Yokohama, Japan).

Microarray analysis

Microarray analysis for PBMCs of acute-phase KD patients was performed using an AceGene Human Oligo Chip 30K (Hitachi Software Engineering) that contains approximately 30 000 genes. The arrays were scanned by FLA-8000 (Fuji Photo Film, Tokyo, Japan), and changed to the numerical values by ArrayVision (Amersham Biosciences). The numerical data were normalized using the LOWESS method. In the microarray analysis of PBMCs, data from three KD patients and those from five healthy controls were compared. Genes that were up-regulated consistently in KD patients compared with healthy controls, and that showed more than a threefold difference by the comparison between the two groups in the mean expression levels, were selected. The data with low signal-to-noise ratios ($S/N < 3$) were not used for further analysis. The data were analysed using Gene Spring software (Silicon Genetics, Redwood City, CA, USA).

Accession number

GSE17975 (Gene Expression Omnibus).

Pathway analysis of microarray results

To understand the underlying phenomenon in the acute phase of KD, a system biology approach was performed using microarray data. Genes were selected as follows: (i)

data with low signal-to-noise ratios (S/N < 3) were excluded; (ii) the mean expression ratio between three KD patients and five healthy controls was more than 1·0 $\log_2$, or less than −1·0 $\log_2$; and (iii) if two or more probes represented the same gene, probes with maximum mean fold-change values were selected. Selected genes were put into Pathway-Express in Onto-Tools (http://vortex.cs.wayne.edu). Pathway-Express searches the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.ad.jp/) for each input gene, and the impact analysis was performed in order to build a list of all associated pathways [7–9]. An impact factor (IF) is calculated for each pathway incorporating parameters, such as the normalized fold change of the differentially expressed genes, the statistical significance of the set of pathway genes and the topology of the signalling pathway [8]. The corrected gamma *P*-value is the *P*-value provided by the impact analysis. The differences were considered to be significant when the corrected gamma *P*-value was less than 0·05.

Quantitative real-time RT–PCR

Total RNA was extracted from cell pellets of PBMCs using the same method as used in the microarray analysis, followed by cDNA synthesis using a first-strand cDNA synthesis kit (GE Healthcare UK Ltd, Buckinghamshire, UK) with random hexamers. *S100A9*, *S100A12*, *TNFA*, *IL1B*, *IL8* and *IL6* mRNA expression levels were analysed by *Taq*Man® gene expression assays Hs00610058_m1, Hs00194525_m1, Hs00174128_m1, Hs99999029_m1, Hs99999034_m1 and Hs99999032_m1 (Applied Biosystems, Foster City, CA, USA). These products consisted of a 20× mix of unlabelled PCR primers and a *Taq*Man MGB probe (FAMTM dye-labelled). A *Taq*Man human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) control reagent kit (Applied Biosystems) was used as an internal control. These *Taq*Man probes were labelled with the quencher fluor-6-carboxy-tetramethyl rhodamine (emission I, 582 nm) at the 3′ end through a linker-arm nucleotide. The mRNA expression levels of the targeted and GAPDH genes were quantified by an ABI PRISM 7700 sequence detector (Applied Biosystems), as described previously [10]. A comparative threshold cycle (CT) was used to determine gene expression levels relative to those of the no-tissue control (calibrator). Hence, steady-state mRNA levels were expressed as an *n*-fold difference relative to the calibrator, as described previously [11]. To calculate the relative expression level in cells, the level of gene expression was divided by that of the GAPDH. All experiments were carried out in duplicate and repeated for confirmation.

Flow cytometry

Ethylenediamine tetraacetic acid (EDTA) blood samples were collected from both patients and controls. The proportions of $CD69^+$ cells were analysed within 12 h after sampling by using an EPICS XL (Beckman Coulter, Fullteron, CA, USA), as described previously [10]. The proportions of $HLA\text{-}DR^+$ or $CD25^+$ cells were also analysed within 24 h. The forward and side light-scatter gate was set to analyse viable cells and to exclude background artefacts. Multi-colour staining was carried out with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-cyanin 5·1 (PC5)-conjugated monoclonal antibodies against CD3, CD16, CD19, CD25, CD56, CD69, HLA-DR and T cell receptor (TCR)γδ (Beckman Coulter). Three-colour flow cytometric analysis was performed on cells within the lymphocyte light-scatter gate using forward and side scatters. Heparinized whole blood samples from five healthy controls were preincubated with or without lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h at 37°C under a 95% humidified air with 5% CO_2, and intracellular tumour necrosis factor (TNF)-α, IL-10 or interferon (IFN)-γ staining was performed using the Fastimmune Intracellular Staining System (BD Bioscience Pharmingen, San Diego, CA, USA) [12]. The analysis gate was set for monocytes or T cells by side scatter, and CD14 or CD3 expression. Intracellular TNF-α, IL-10 and IFN-γ staining in peripheral blood cells from seven KD patients was performed using the same system, without *in vitro* stimulation.

Results

Flow cytometric analysis of the activation markers on T, B and NK cells at acute phase of KD

We first analysed the proportions of activated T, B and NK cells in the peripheral blood of KD patients by flow cytometry. CD69, HLA-DR and CD25 were used as activation markers. As shown in Fig. 1a, the proportions of $CD69^+$ T cells were significantly higher at acute phase than those at convalescent phase of KD, while those of $CD69^+$ B cells were more prominent at convalescent phase than at acute phase of KD ($P < 0{\cdot}01$). The proportions of $CD69^+$ cells in $CD56^+CD16^+$ and $CD16^+CD56^-$ NK cells at acute phase of KD were significantly higher than those at convalescent phase of KD. The proportions of $CD69^+$ cells in $CD56^+CD16^-$ NK cells and the proportions of $CD25^+$ or $HLA\text{-}DR^+$ cells in T cells, B cells or all three NK cell subsets were not significantly different between the two phases of KD.

To analyse further T cell activation in KD, the proportion of $CD69^+$ cells were investigated through the separation of T cells to αβ and γδT cells, which are involved in acquired and innate immunity, respectively. As shown in Fig. 1b and c, the proportions of $CD69^+$ cells in γδT cells at acute phase of KD were significantly higher than those at convalescent phase of KD (median values: 17·9% at acute phase *versus* 7·9% at convalescent phase in γδ T cells, $P < 0{\cdot}0005$). Conversely, the activation of αβ T cells was minimal in terms of CD69

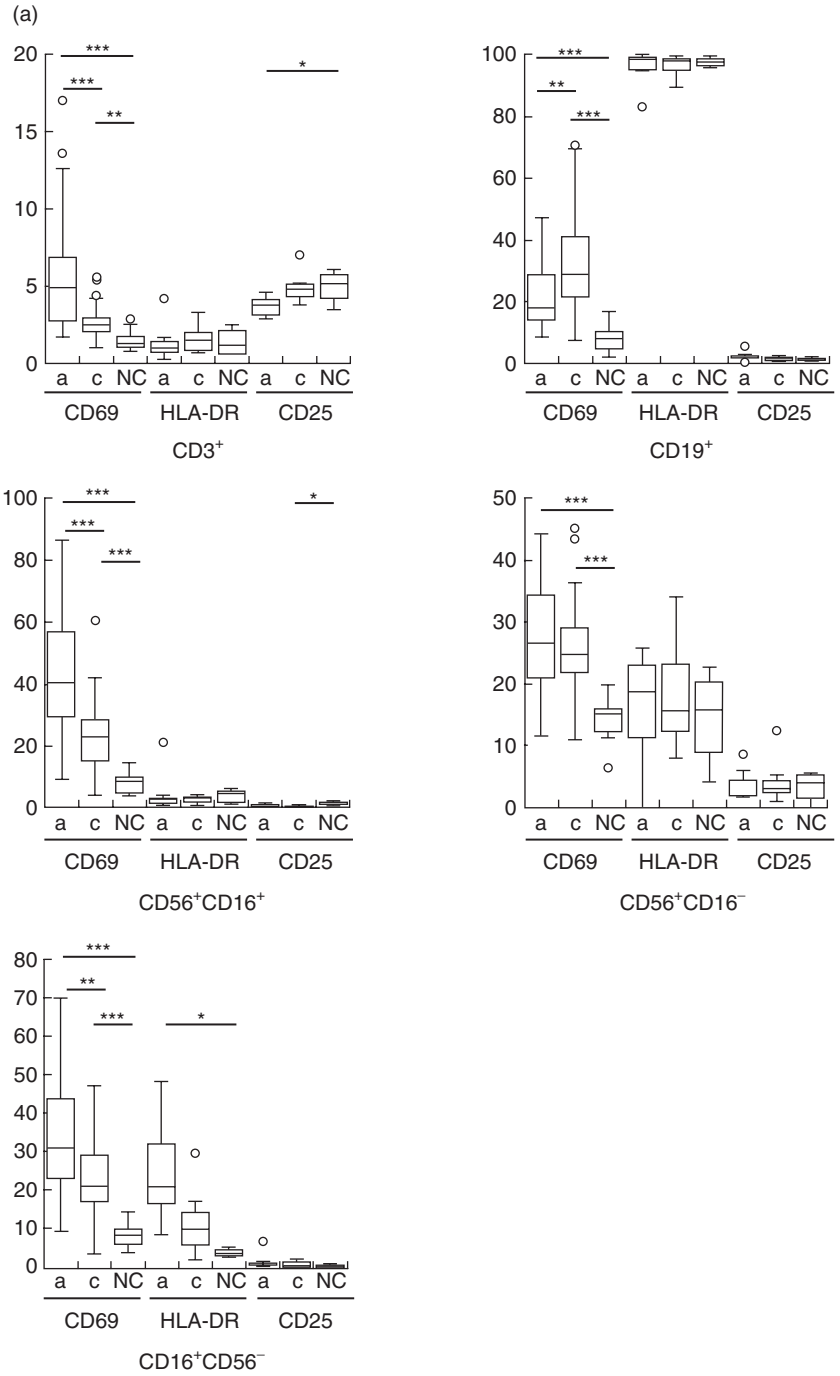

Fig. 1. Flow cytometric analysis of the activation markers on T, B and natural killer (NK) cells at acute phase of Kawasaki disease (KD). (a) The proportions of activated T, B and NK cells in the peripheral blood of seven patients with KD and 15 healthy control subjects were analysed by flow cytometry. CD69, human leucocyte antigen D-related (HLA-DR) and CD25 were used as activation markers. $*P \leq 0{\cdot}05$; $**P \leq 0{\cdot}01$; $***P \leq 0{\cdot}0001$. (a) Acute phase; (c) convalescent phase; NC, healthy controls. The form of box-plot is as follows. The bottom and the top of the box correspond to 25th and 75th percentile points, respectively. The line within the box represents the median, and the whiskers indicate the values of 10th and 90th percentiles. (b,c) Representative density plot of flow cytometric analysis of $CD69^+$ cells on NK, T and B cells (b) and the proportions of $CD69^+$ cells in αβ and γδ T cells (c) in KD patients. The proportions of $CD69^+$ cells were investigated in NK cells ($n = 35$), αβT cells ($n = 23$), γδT cells ($n = 23$) and B cells ($n = 35$). $**P \leq 0{\cdot}0005$; $*P \leq 0{\cdot}01$.

expression at acute phase of KD (median values: 4·5% at acute phase and 2·8% at convalescent phase).

Microarray analysis of the gene expression in PBMCs from KD patients

Pathway analysis. To assess the innate and acquired immunological status in KD more precisely, the gene expression profiles of PBMCs from the patients were analysed by microarray. Six hundred and fifty-eight genes in PBMCs from KD patients showed more than twofold higher expression levels compared with those from healthy controls. These 658 genes were put into Pathway-Express in Onto-Tools (http://vortex.cs.wayne.edu). Pathway-Express searched the KEGG pathways in the Onto-Tools database for each input gene, and built a list of pathways [7]. Thirty-six pathways, associated significantly with acute phase of KD, were selected and the top 12 pathways are listed in Table 1. Among the pathways extracted by Pathway-Express, all input genes in antigen processing and presentation, T cell receptor (TCR)

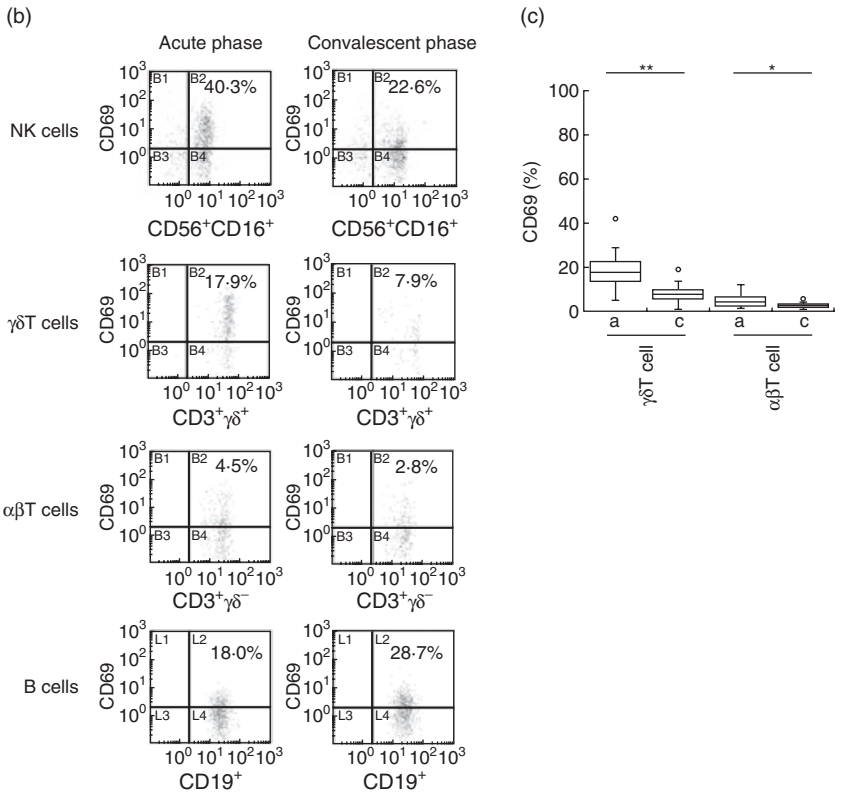

Fig. 1. *Continued*

Table 1. The results of the pathway impact analysis for a set of genes associated with acute phase of Kawasaki disease.

Pathway name	Input genes in pathway			Impact factor	Corrected gamma *P*-value
	Total	Up	Down		
Antigen processing and presentation	7	0	7	51·621	2·01E-21
Phosphatidylinositol signalling system	2	0	2	35·807	1·04E-14
Circadian rhythm	3	0	3	22·942	2·60E-09
T cell receptor signalling pathway	14	0	14	18·903	1·23E-07
Toll-like receptor signalling pathway	14	6	8	18·526	1·76E-07
Natural killer cell-mediated cytotoxicity	14	4	10	14·664	6·71E-06
Ribosome	11	0	11	13·743	1·59E-05
Apoptosis	10	3	7	13·426	2·13E-05
MAPK signalling pathway	17	4	13	10·964	2·07E-04
Cytokine–cytokine receptor interaction	16	7	9	9·511	7·78E-04
Fc epsilon RI signalling pathway	8	3	5	9·323	9·22E-04
B cell receptor signalling pathway	7	0	7	8·690	0·00163044

Pathway-Express was used for the pathway impact analysis in order to build a list of all associated pathways. An impact factor (IF) is calculated for each pathway incorporating parameters such as the normalized fold change of the differentially expressed genes, the statistical significance of the set of pathway genes and the topology of the signalling pathway. The corrected gamma *P*-value is the *P*-value provided by the impact analysis. Thirty-six pathways were significant at the 5% level on corrected *P*-values, and the top 12 pathways were selected. Up-regulated genes were as follows: (i) Toll-like receptor signalling pathway; extracellular-regulated kinase (ERK), CD14, Toll-like receptor (TLR)-8, MAP kinase kinase 6 (MKK6), MD2 and TLR-5. (ii) Natural killer cell-mediated cytotoxicity; tumour necrosis factor-related apoptosis inducing ligand (TRAIL), ERK, Fc epsilon RI gamma (FCER1G) and TRAILR3. (iii) Apoptosis; TRAIL, protein kinase A regulatory subunit 1A (PRKAR1A) and TRAILR3. (iv) Mitogen-activated protein kinase (MAPK) signalling pathway; ERK, CD14, interleukin (IL)-1R2 and MKK6. (v) Cytokine–cytokine receptor interaction; TRAIL, tumour necrosis factor receptor superfamily, member 17 (TNF-RSF17), IL-18RAP, IL-1R2, TNF-SF13B, TRAILR3, and hepatocyte growth factor (HGF). (vi) Fc epsilon RI signalling pathway; ERK, FCER1G, and MKK6.

Table 2. Microarray analysis of peripheral blood mononuclear cells (PBMCs) between Kawasaki disease (KD) patients and healthy controls.

Gene name	Gene ontology	Synonyms	GenBank	Fold difference*
NLR family, apoptosis inhibitory protein	Nucleotide binding	NAIP	NM_004536	7·2
Fc fragment of IgG, high-affinity Ia, receptor (CD64)	Immune response	FCGR1A	NM_000566	5·6
Haemoglobin, gamma A	Oxygen transport	HBG1	NM_000559	5·3
Haemoglobin, alpha 1	Oxygen transport	HBA1	NM_000558	5·1
Grancalcin, EF-hand calcium-binding protein	Calcium ion binding	GCA	NM_012198	4·5
Fibrinogen-like 2 (constitutively expressed in cytotoxic T-cells)	Signal transduction	FGL2	NM_006682	4·4
Ice protease-activating factor	Defence response to bacterium	NLRC4 (IPAF)	NM_021209	4·2
Placenta-specific 8		PLAC8	NM_016619	4·1
Immunoglobulin superfamily, member 6	Immune response	IGSF6	NM_005849	4·1
S100 calcium binding protein A9 (calgranulin B)	Inflammatory response	S100A9	NM_002965	3·9

*The difference of mean gene expression levels between 3 KD patients and controls (healthy donors) in microarray analysis is given. NLR: nucleotide-binding domain, leucine-rich repeat containing. Genes that showed more than threefold expressional differences between KD patients and healthy controls were selected and the top 10 genes were listed. Gene ontology was not applied in PLAC8. Hypothetical proteins were excluded. IgG: immunoglobulin G; EF hand: The EF-hand describes the nearly perpendicular arrangement of the E and F helices flanking the 12-residue Ca^{2+}-binding loop, in analogy to the stretched out right hand with the forefinger (E helix) and thumb (F helix) and the remaining fingers folded to form the Ca^{2+}-binding loop.

signalling pathway and B cell receptor (BCR) signalling pathway, which are involved in acquired immunity, were down-regulated. Conversely, TLR signalling and NK cell-mediated cytotoxicity pathways, related closely to innate immunity, were partly up-regulated.

Top 10 genes in microarray analysis. In microarray analysis, 47 genes in KD patients were up-regulated more than three-fold compared with those in healthy controls, and the top 10 genes are shown in Table 2. Among them, five genes such as nod-like receptor (NLR) family, apoptosis inhibitory protein (NAIP), NLRC4 (IPAF), S100A9 protein, Fc fragment of IgG, high-affinity Ia, receptor (FCGR1A, also known as CD64) and grancalcin (GCA, EF-hand calcium-binding protein) were related closely to innate immune responses [13–17], while three genes such as fibrinogen-like protein 2 (FGL2), placenta-specific 8 (PLAC8) and immunoglobulin super-family, member 6 (IGSF6) were related to both innate and acquired immunity [18–20].

Cytokine analyses in KD patients

Microarray analysis. Sixteen genes that have been reported to have a role in the pathophysiology of KD were selected from the microarray data, and the relative gene expression levels in PBMCs of KD patients compared with those of healthy controls are shown in Table 3. Expression levels of *S100A9* and *S100A12* genes, which encode the

Table 3. Cykokine- and chemokine-related genes expressed in peripheral blood mononuclear cells (PBMCs) of acute-phase Kawasaki disease (KD) patients.

Gene name	Gene ontology	Synonyms	GenBank	Fold difference*
Interleukin 1 beta	Immune response	IL-1B	NM_000576	0·3
Interleukin 2	Immune response	IL-2	NM_000586	0·7
Interleukin 4	Regulation of immune response	IL-4	NM_000589	0·4
Interleukin 6	Inflammatory response	IL-6	NM_000600	0·5
Interleukin 8	Immune response	IL-8	NM_000584	0·2
Interleukin 10	Immune response	IL-10	NM_000572	0·8
Tumour necrosis factor	Inflammatory response	TNF	NM_000594	0·9
Interferon gamma	Regulation of immune response	IFN-γ	NM_000619	0·9
Chemokine (C-C motif) ligand 2	Inflammatory response	CCL2 (MCP1)	NM_002982	1·1
Chemokine (C-C motif) ligand 4	Immune response	CCL4 (MIP1B)	NM_002984	0·6
Chemokine (C-C motif) ligand 5	Immune response	CCL5 (RANTES)	NM_002985	0·4
Colony stimulating factor 3 (granulocyte)	Immune response	CSF3	NM_172220	1·0
Vascular endothelial growth factor A	Cytokine activity	VEGFA	NM_001025366	0·4
Hepatocyte growth factor	Protein binding	HGF	NM_000601	2·8
S100 calcium binding protein A9 (calgranulin B)	Inflammatory response	S100A9	NM_002965	3·9
S100 calcium binding protein A12	Inflammatory response	S100A12	NM_005621	3·5

*The difference of mean gene expression levels between three KD patients and controls (healthy donors) in microarray analysis is given. Sixteen genes that have been reported to have a role in the pathophysiology of KD were selected from the microarray data.

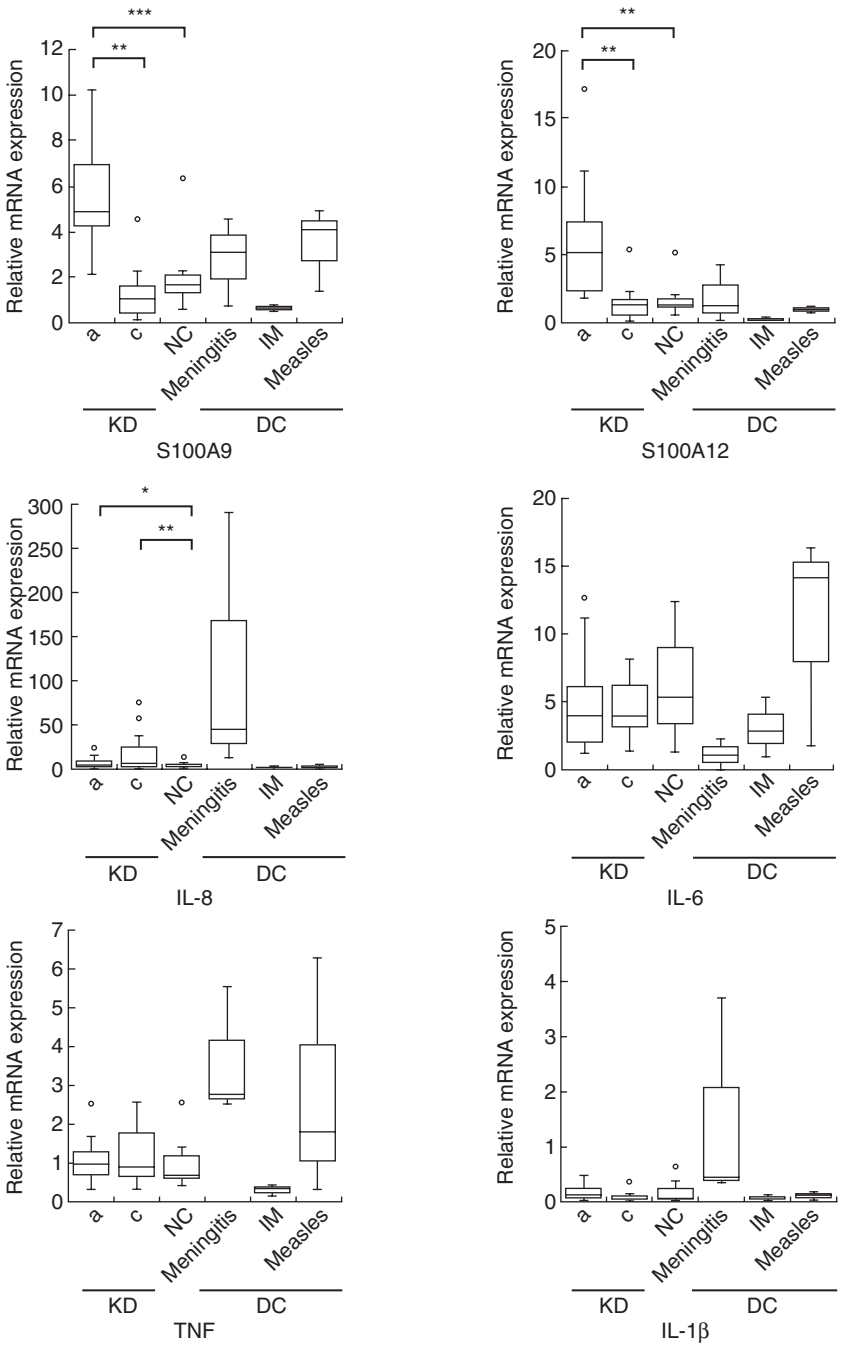

Fig. 2. Relative expression levels of *S100A9*, *S100A12*, *IL8*, *IL6*, *TNF* and *IL1B* genes in peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). The gene expression levels of these cytokines were determined by the reverse transcription–polymerase chain reaction (RT–PCR) method using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as an internal control. Gene expression levels of PBMCs from 10 KD patients, 11 healthy controls (NC), nine diseased control subjects [three patients with meningitis, three patients with acute infectious mononucleosis (IM) and three patients with measles] are shown. Only *IL8* gene expression levels were analysed in 16 KD patients. The form of box-plot was the same as Fig. 1. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. (a) Acute phase; (c) convalescent phase.

proinflammatory factors in innate immunity, as well as of the hepatocyte growth factor (*HGF*) gene, were more than twofold higher in KD patients than in healthy controls, while the expression levels of other cytokine, chemokine and growth factor genes were not elevated. Decreased gene expression levels of *IL4*, *IL10* and *IFNG* in KD patients were consistent with our previous data obtained by quantitative RT–PCR [21].

Quantitative RT–PCR analysis. To confirm the microarray data, the gene expression levels of six major cytokines, S100A9, S100A12, IL-8, IL-6, TNF-α and IL-1β, were analysed in KD patients and controls by quantitative RT–PCR. As shown in Fig. 2, the relative expression levels of *S100A9* and *S100A12* genes in PBMCs at acute-phase KD were significantly higher than those at convalescent-phase KD, consistent with previous reports [5,22]. Expression levels of the *IL8* gene at both acute and convalescent phases of KD were slightly but significantly higher than those of healthy controls. The expression levels of *TNF*, *IL1B* and *IL6* genes at either acute or convalescent phases of KD were not significantly different from those in healthy controls.

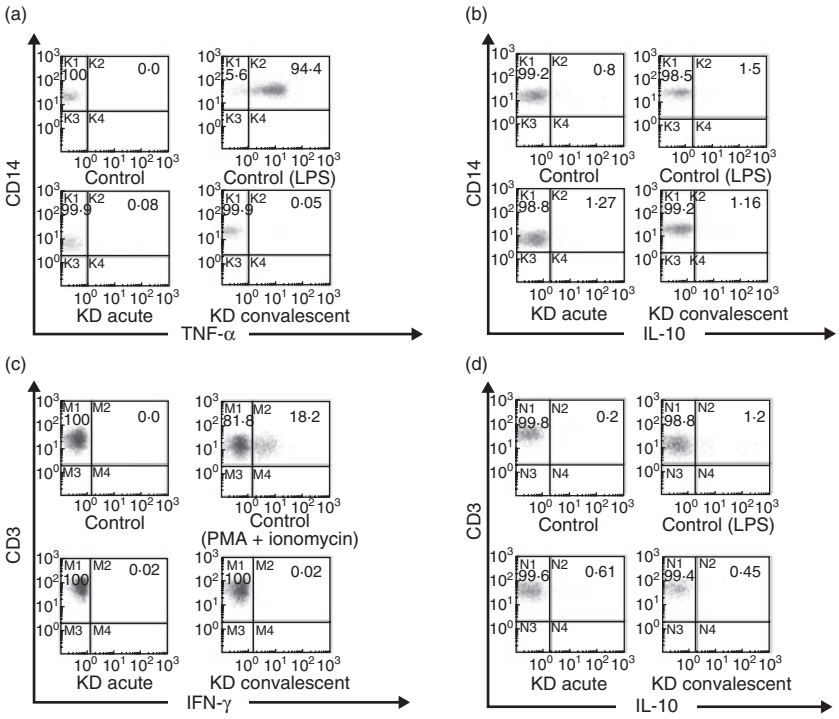

Fig. 3. Flow cytometric analysis of intracellular cytokine production of peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). Intracellular cytokine production in PBMCs at acute and convalescent phases of KD was analysed by flow cytometry. Representative data of tumour necrosis factor (TNF)-α (a) and interleukin (IL)-10 (b) staining in monocytes, and those of interferon (IFN)-γ (c) and IL-10 (d) staining in T cells are shown. As positive and negative controls, representative data of TNF-α (a) and IL-10 (b) staining in monocytes with and without crude lipopolysaccharide (LPS) (1 μg/ml), and IFN-γ (c) and IL-10 (d) staining in T cells with and without phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) plus ionomycin (1 μg/ml) are shown. The figure shows representative results of seven KD patients and three healthy controls.

Intracellular cytokine analysis. We analysed intracellular cytokines in the freshly isolated PBMCs at acute and convalescent phases of KD by using flow cytometry. Intracellular TNF-α or IL-10 production in monocytes and IFN-γ or IL-10 production in T cells were analysed in the peripheral blood of KD patients. As shown in Fig. 3, the percentages of both TNF-α or IL-10-producing cells in monocytes and IFN-γ or IL-10-producing cells in T cells were not significantly different between acute phase (TNF-α-producing cells: median 0·08%, range 0·04–0·09%; IL-10-producing cells: median 1·27%, range 0·47–1·31% in monocytes; IFN-γ-producing cells: median 0·02%, range 0·00–0·03%; IL-10–producing cells: median 0·61%, range 0·35–0·69% in T cells) and convalescent phase (TNF-α-producing cells: median 0·05%, range 0·00–0·08%; IL-10-producing cells: median 1·16%, range 0·79–2·43% in monocytes; IFN-γ-producing cells: median 0·02%, range 0·00–0·07%; IL-10-producing cells, median 0·45%, range 0·40–0·70% in T cells), further suggesting little intracellular production of such cytokines by peripheral blood cells at acute-phase KD.

Discussion

Massive releases of cytokines, chemokines and growth factors play a pivotal role in the immunopathogenesis of KD [1]. Although numerous immunological studies on peripheral blood leucocytes have been reported, the status of peripheral T cell activation remains controversial [3]. In this regard, no previous studies have analysed T cells by separating them into two distinct populations, αβT cells and γδT cells, which are involved mainly in acquired and innate immunity, respectively. A predominant activation of γδT cells as well as NK cells in the present study, together with previous observations that neutrophils and monocytes are activated in KD [3,23,24], has suggested that innate immunity is involved actively in acute-phase KD. Although a recent report has shown no expansion of $CD69^+CD4^+$ or $CD69^+CD8^+$ cells in the peripheral blood of KD [25], it might have been difficult to detect the increases of $CD69^+$ T cells in the peripheral blood without the separation into αβ and γδT cells, because a major $CD69^+$ T cell population resided in $CD4^-CD8^{-/dim+}$ γδT cells in KD.

In KD, it has been thought that most activated T cells moved to local tissues from peripheral blood at acute phase and returned from there at convalescent phase [3]. However, because significant proportions of activated γδT cells and NK cells with a small proportion of activated αβT cells were detected constantly in the peripheral blood at acute-phase KD, we performed DNA microarray analysis of PBMCs to check the activation status of these cells. Pathway analysis revealed that the pathways involved in acquired immunity such as antigen processing and presentation, TCR signalling and BCR signalling were all down-regulated, and that innate immunity pathways such as TLR signalling and NK cell-mediated cytotoxicity were partly activated, with a large part of them down-regulated. These findings suggested that a small proportion of αβT cells and a considerable proportion of γδT cells were activated not through TCR signalling pathway by either conventional antigen or superantigen but directly through innate immunity receptors and/or cytokine signalling pathways.

Among the top 10 genes whose expression was more than threefold higher in KD than in normal controls, five genes were related to innate immunity and two of the five were molecules associated with the NLR signalling pathway. Popper *et al.* reported that the expression levels of genes involved in innate immunity, proinflammatory responses and neutrophil activation and apoptosis were up-regulated and those related to NK cells and $CD8^+$ lymphocytes were down-regulated at acute-phase KD by DNA microarray analysis of peripheral whole blood cells, including neutrophils [26]. Verma *et al.* have also reported the up-regulated expression of the genes related to innate immunity such as the TLR signalling pathway, complement activation and matrix-adhesion molecule at acute-phase KD [27]. These studies demonstrated consistently the importance of innate immunity in the pathophysiology of acute-phase KD.

Although monocytes in the peripheral blood are considered to be activated *in vivo* in KD [3], there have been few reports showing that monocytes are actually producing such cytokines as IL-6, IL-8 and TNF *in vivo*, which are elevated in sera of KD patients. Abe *et al.* [5] demonstrated that there were no significant differences in the expression levels of *IL6*, *IL8* and *TNF* genes in separated monocytes before and after high-dose gammaglobulin therapy. Rather, monocytes are actively producing unique cytokines such as damage-associated molecular pattern molecules (DAMPs) (S100A9, S100A12) [5], one of which was reported to be produced by monocytes through the interaction with TNF-activated endothelial cells [14]. In our study, no significant differences of *IL6*, *IL1B* or *TNFA* mRNA levels in PBMCs were detected among patients with acute-phase KD, those with convalescent-phase KD and controls by microarray and quantitative RT–PCR. In the *IL8* gene expression, however, quantitative RT–PCR analysis of samples from a larger number of patients showed that slightly increased expression levels of the *IL8* gene at both acute and convalescent phases of KD, suggesting a weak activation of monocytes among PBMC. Although a previous study showed that 1–2% of PBMCs were positive for intracellular IL-6, TNF-α or TNF-β by immunofluorescent microscopy [28], our analysis of blood samples shortly after drawing revealed no expansion of intracellular TNF-α, IL-10 or IFN-γ-positive cells in acute-phase KD by flow cytometry.

We confirmed that the inositol 1, 4, 5-trisphosphate 3-kinase C (ITPKC) gene was associated with the development of KD [29] in our KD samples (data not shown), but presumably ITPKC acts mainly as a regulator of innate immune cells or non-immune cells (endothelial cells) rather than of $\alpha\beta$T cells, because (i) only a small fraction of $\alpha\beta$T cells showed an activation marker *in vivo*; (ii) the pathways involved in acquired immunity were all down-regulated (Table 1); and (iii) we have found a significant association between an innate immunity receptor gene and KD development, and have established a new KD mouse model with coronary arteritis by an innate immunity receptor ligand (unpublished observations).

In conclusion, the present data have indicated that PBMC showed a unique activation status with high expression of DAMP genes but low expression of proinflammatory cytokine genes, and that the innate immune system appears to play a role in the pathogenesis and pathophysiology of KD. Further studies are needed to elucidate the mechanism responsible for the development of KD and coronary arteritis in terms of the activation of the innate immune system both *in vitro* and *in vivo*.

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Disclosure

None.

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