行された。

flow cytometry 法を用いて川崎病における免疫学的 profile を検討する目的で、38例の川崎病患者について解析を行った。冠動脈病変合併例は認められなかった。まず始めに、川崎病 7 症例、正常対照(age-matched)15 例について、末梢血における活性化 T, B, NK 細胞の割合を解析した。CD69, HLA-DR, CD25 を活性化マーカーとして用いた。これらの細胞は IVIG 治療前(中央値 day5; range, day3-6)および回復期（中央値 day13 : range, day13-18）で採取した。川崎病における免疫学的 profile をさらに解析するために、αβT 細胞、γδT 細胞、NK 細胞、B 細胞について CD69 陽性細胞の割合を解析した。

mRNA 発現レベルを解析するために、5 名の正常対照および CAL を合併していない川崎病 3 症例(中央値 4.7 歳; range, 4.1-5.3 歳）から治療前（day 4-5）に末梢血を採取した後、PBMNCs を分離し、cDNA microarray を行った。

定量的 RT-PCR 法を用いて mRNA 発現レベルを解析する目的で、川崎病患者 10-16 症例(急性期および回復期。中央値 1.7 歳；range 5 か月-13.1 歳)、対照症例 20 例(age-matched)[急性期感染症 9 例（中央値 2.6 歳；range, 5 か月-13.1 歳）: 細菌性髄膜炎 3 例、麻疹 3 例、EB ウイルス感染症 3 例)、正常対照 11 例(中央値 5.0 歳；range; 1.7-7.6 歳)]から検体を採取した。

本研究は、九州大学病院および福岡市立こども病院倫理委員会の承認を得た書面に従い、全研究対象者に対して書面によるインフォームドコンセントを行った。

【手法】

1．Microarray 解析

3 万遺伝子を搭載した AceGene Human Oligo Chip 30K (Hitachi Software Engineering)を用いて、急性期川崎病患者 PBMNCs について Microarray 解析を行った。

2．pathway 解析

急性期川崎病における免疫病態を解析するために、Microarray data を用いてシステム生物学的 approach を行った。正常対照に比較して、川崎病急性期 PBMNC での遺伝子発現が 2 倍以上もしくは 1/2 以下の遺伝子を選択した。選択された遺伝子を Pathway-Express に input し、Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway を用いて各 pathway の有用性を解析した。

3．定量的 RT-PCR

S100A9, S100A12, TNFA, IL1B, IL8, IL6 mRNA 発現レベルは、TaqMan® gene expression assays(Applied Biosystems)を用いて解析を行った。TaqMan human GAPDH (Applied Biosystems)を内在性コントロールとして用いた。全ての実験は、duplicate で行った。

4．flow cytometry

EDTA 採血により血液検体を採取した。EPICS XL (Beckman Coulter, Fullteron, CA, USA)を用いて、採取後 12 時間以内に CD69 陽性細胞の割合について解析を行った。HLA-DR, CD25 陽性細胞の割合については、採取後 24 時間以内で解析を行った。

細胞内サイトカイン染色では、LPS もし

くは PMA + ionomycin で 4 時間刺激(37℃、5% CO_2)を行ったものを positive control とした。川崎病患者末梢血の細胞内サイトカイン染色は、in vitro での刺激を行わずに解析した。

C. 研究結果

1. Flow cytometry 法を用いた、川崎病急性期における T, B, NK 細胞の活性化マーカーの解析

flow cytometry 法を用いて川崎病患者末梢血における活性化 T, B, NK 細胞について解析した。NK 細胞は 3 つの subtype に分け、活性化マーカーには CD69, HLA-DR, CD25 を用いた。Figure 1a に示すように、$CD69^+$T 細胞の割合が川崎病回復期に比較して急性期で有意に高値を示したが、$CD69^+$B 細胞の割合は急性期に比較して回復期で有意に上昇していた。また、$CD56^+CD16^+$, $CD16^+CD56^-$ NK 細胞における CD69 陽性細胞の割合が川崎病回復期に比較して急性期で有意に高値だった。

川崎病における T 細胞の活性化をさらに解析するために、T 細胞を αβ、γδT 細胞へ分けて CD69 陽性細胞の割合を解析した。Figure 1b, 1c に示すように、γδT 細胞における CD69 陽性細胞の割合が回復期に比較して急性期で有意に高値だった(中央値 急性期 17.9%、回復期 7.9%, $p<0.0005$)。一方、αβT 細胞については、γδT 細胞に比較して活性化は弱い傾向を示した(中央値 急性期 4.5%、回復期 2.8%)。

2. cDNA Microarray 法を用いた、川崎病患者 PBMNCs の遺伝子発現解析

【pathway 解析】

川崎病における自然免疫、獲得免疫の関与をより詳細に検討するために、cDNA Microarray 法を用いて、川崎病患者 PBMNCs の遺伝子発現 profile について解析した。解析の結果、正常対照に比較して、川崎病急性期患者 PBMNCs で 2 倍以上発現の高い遺伝子が 658 存在した。その 658 遺伝子を用いて Pathway 解析を行ったところ、川崎病急性期と有意に相関のある pathway が 36 存在し、そのうち上位 12pathway を示した(Table 1)。獲得免疫に関与している antigen processing and presentation, T cell receptor(TCR) signaling pathway, B cell receptor(BCR) signaling pathway は、down-regulated されていた。一方、自然免疫に関与している Toll-like receptor(TLR) signaling pathway、NK 細胞関連細胞傷害性 pathways は、一部 up-regulated していた。

【マイクロアレイ解析における上位 10 遺伝子について】

マイクロアレイ解析の結果、正常対照に比較して川崎病患者 PBMNCs で 3 倍以上発現の高い遺伝子が 47 存在し、そのうち上位 10 遺伝子を示した(Table2)。10 遺伝子のうち、NLR family, apoptosis inhibitory protein(*NAIP*), *NLRC4*(*IPAF*), *FCGR1A*, grancalcin(*GCA*), *S100A9* などの自然免疫系に関与しているものが 5 遺伝子存在した。一方、fibrinogen-like protein 2 (*FGL2*), placenta-specific 8 (*PLAC8*), immunoglobulin superfamily, member 6 (*IGSF6*)などの自然免疫、獲得免疫系の双方に関連のあるものが 3 遺伝子存在した。

3. 川崎病患者におけるサイトカイン解析

【マイクロアレイ解析】

これまでに川崎病との関連が報告されている16遺伝子について、マイクロアレイ解析の結果からデータを抽出し、正常対照と川崎病患者 PBMNCs における遺伝子発現の比較を行った(Table 3)。自然免疫と関連のある *S100A9* や *S100A12* 遺伝子、また hepatocyte growth factor(*HGF*)遺伝子は、正常対照に比較して川崎病急性期で2倍以上、発現が高値だった。一方、ほかのサイトカイン、ケモカイン遺伝子の発現レベルは上昇していなかった。*IL4*, *IL10*, *IFNG* 遺伝子発現レベルの減少は、定量的RT-PCR法を用いた我々の過去の報告と一致していた。

【定量的RT-PCR法による解析】

マイクロアレイ解析のデータを裏付けるために、6つの主要なサイトカイン(S100A9, S100A12, IL-8, IL-6, TNF-α, IL-1β)の遺伝子発現について、定量的RT-PCR法を用いて川崎病患者、正常対照PBMNCsにおいて解析を行った。*S100A9*や*S100A12*遺伝子発現レベルは、川崎病回復期に比較して急性期で有意に高値を示した(Figure 2)。*IL8* 遺伝子発現レベルは、正常対照に比較して、川崎病急性期および回復期でわずかながら上昇していた。*TNF*, *IL1B*, *IL6*遺伝子の発現レベルは、正常対照に比較して、川崎病急性期や回復期で有意な上昇は認められなかった。

4. 細胞内サイトカイン染色

flow cytometry法を用いて、川崎病患者(急性期・回復期)PBMNCsの細胞内サイトカイン染色を行った。川崎病患者末梢血において、単球におけるTNF-α, IL-10産生、T細胞におけるIFN-γ, IL-10産生について解析を行った。Figure 3に示すように、単球におけるTNF-αもしくはIL-10産生細胞の割合や、T細胞におけるIFN-γもしくはIL-10産生細胞の割合は、急性期(単球における TNF-α 産生細胞の割合：中央値 0.08%, range 0.04%-0.09%; 単球における IL-10 産生細胞の割合：中央値 1.27%, range 0.47%-1.31%; T細胞における IFN-γ 産生細胞の割合：中央値 0.02%, range 0.00%-0.03%; T細胞における IL-10産生細胞の割合：中央値 0.61%, range 0.35-0.69%)と回復期(単球におけるTNF-α産生細胞の割合：中央値 0.05%, range 0.00%-0.08%; 単球における IL-10 産生細胞の割合：中央値 1.16%, range 0.79%-2.43%; T細胞における IFN-γ 産生細胞の割合：中央値 0.02%, range 0.00%-0.07%; T細胞における IL-10産生細胞の割合：中央値 0.45%, range 0.40-0.70%)で有意な変化は認められなかった。川崎病急性期末梢血におけるこれらのサイトカインの細胞内産生は非常に少ないことが示唆された。

D. 考案

サイトカイン、ケモカインおよび成長因子の大量産生が川崎病の免疫病態に重要な役割を果たしている。末梢血白血球についての数多くの免疫学的研究が報告されてきたが、末梢血T細胞の活性化状態についてはいまだ議論が分かれるところである。T細胞を、自然免疫系、獲得免疫系にそれぞれ主に関与している、αβT細胞、γδT細

胞に分けて解析した論文はこれまでに報告されていない。本研究では、NK細胞と同様にγδT細胞の有意な活性化が認められたが、これまでの報告にあるように好中球や単球が川崎病において活性化していることを合わせると、川崎病急性期において自然免疫系が密接にかかわっていることが示唆された。

川崎病において、活性化したT細胞が急性期に末梢血から炎症局所へ移動して、回復期に末梢血へ戻ってくると考えられてきた。しかし、川崎病急性期患者の末梢血において、γδT細胞とNK細胞が有意に活性化していること、また活性化したαβT細胞の割合がそれほど多くないことから、これらの細胞の活性化状態を検討するために、cDNA microarray法を用いてPBMNCs遺伝子発現解析を行った。pathway解析の結果から、獲得免疫系に関与しているTCR, BCR signaling pathwayがdown-regulateされており、自然免疫系に関連のあるTLR signaling pathway、NK細胞関連細胞傷害性pathwaysは、一部up-regulatedしていたが、大部分はdown-regulateされていた。これらの結果から、少ない割合のαβT細胞や多くの割合のγδT細胞は、TCR signaling pathwayを介してconventionalな抗原もしくはスーパー抗原により刺激されるのではなく、自然免疫系の受容体を直接介して、ないしはサイトカインsignaling pathwayを介して活性化されることが示唆された。

Microarray解析の結果、正常対照に比較して川崎病患者PBMNCsにおいて3倍以上発現の高かった上位10遺伝子のうち、5遺伝子が自然免疫系に関与しており、そのうち2遺伝子はNLR signaling pathwayに関連している分子だった。Popperらは、川崎病急性期患者から採取した、好中球を含んだ末梢血全血でのDNA Microarray解析において、自然免疫系、炎症反応、好中球の活性化、apoptosis等に関与している遺伝子の発現レベルがup-regulateされており、NK細胞やCD8陽性リンパ球に関連する遺伝子発現がdown-regulateされていたと報告した。Vermaらは、川崎病急性期において、TLR signaling pathwayなどの自然免疫系や，補体の活性化、基質-接着分子に関連する遺伝子の発現がup-regulateされていたと報告した。

末梢血単球は川崎病において活性化していると考えられているが、川崎病患者血清中で上昇しているIL-6, IL-8, TNF-αなどのサイトカインを実際に単球が産生していることを示した論文は非常に少ない。Abeらは、川崎病患者から分離した単球における*IL6*, *IL8*, *TNF*遺伝子の発現レベルは大量γグロブリン療法前後で有意な変化は認められなかったと報告した。一方、単球はdamage-associated molecular pattern molecules(DAMPs)(S100A9, S100A12)などのユニークなサイトカインを産生しており、DAMPsのうちの一分子は、TNFによって活性化された内皮細胞との相互作用を介して、単球から産生されることが報告されている。本研究でのMicroarray、定量的RT-PCR法による解析により、川崎病急性期、回復期、および正常対照から採取したPBMNCsにおける*IL6*, *IL1B*, *TNFA* mRNAレベルは3群間で有意差は認められなかった。しかしながら、*IL8*遺伝子発現レベルについては、より多くのサンプルで定量的RT-PCR法によ

り解析をした結果、川崎病急性期および回復期で *IL8* 遺伝子の発現レベルが軽度上昇していた。このことから、末梢血単核球のうち、単球は弱く活性化していることが推測された。過去の報告では、免疫蛍光顕微鏡により細胞内 IL-6, TNF-α, TNF-β を解析したところ、PBMNCs のうち 1-2%がサイトカイン産生細胞だったことが示されたが、我々の研究では、血液採取後速やかに解析を行った flow cytometry 法により、細胞内 TNF-α, IL-10, IFN-γ 陽性細胞は、川崎病急性期において殆ど検出されなかった。

我々は、当研究室で独自に収集した川崎病検体において、inositol 1, 4, 5-trisphosphate 3-kinase C (ITPKC) 遺伝子が川崎病の発症と関連があることを確認したが(data not shown)、ITPKC は恐らく αβT 細胞というよりはむしろ、自然免疫系の細胞や非免疫系の細胞（内皮細胞）の regulator として役割を果たしていることが予想される。理由としては(i)αβT 細胞のうち活性化マーカーが陽性だった細胞の割合が比較的小さかったこと、(ii) 獲得免疫系に関与している pathway が全て down-regulate されていたこと、(iii) 我々の研究室において、自然免疫系受容体遺伝子と川崎病の発症に有意な関連があることを発見し、さらに自然免疫系受容体 ligand により惹起される新しい川崎病冠動脈炎マウスモデルを確立したこと (unpublished observation) が挙げられる。

E. 結論

川崎病急性期における PBMNCs は、DAMP 遺伝子の高発現や、炎症性サイトカイン遺伝子の低発現といったユニークな活性化状態を示し、自然免疫系が川崎病の病因、病態に重要な役割を果たしていることが示唆された。

F. 健康危険情報

特になし。

G. 研究発表

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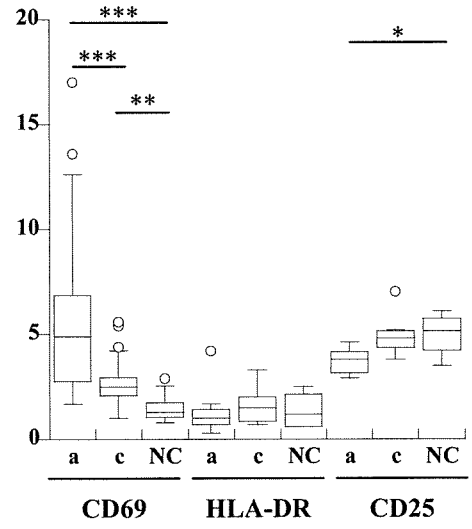
H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得
   なし

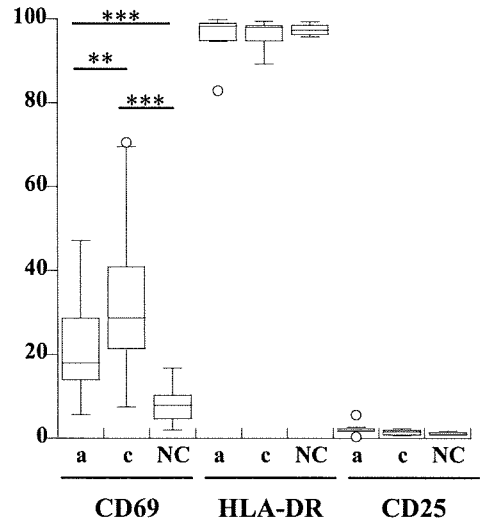
2. 実用新案登録
   なし

3. その他
   なし

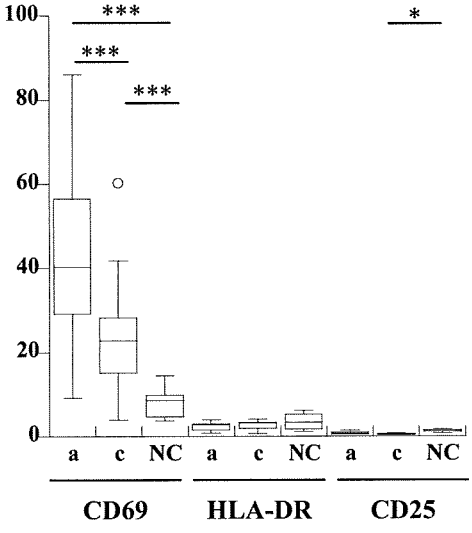
A



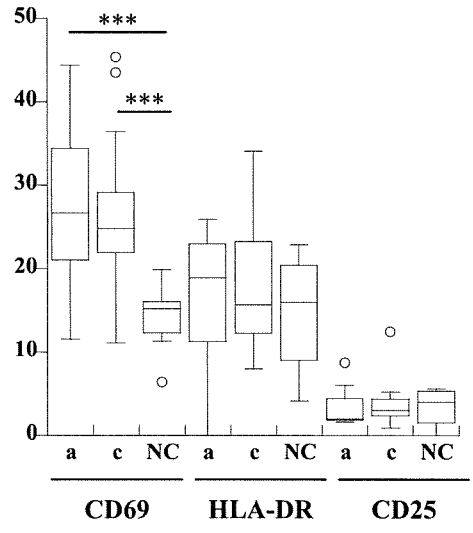
CD3+



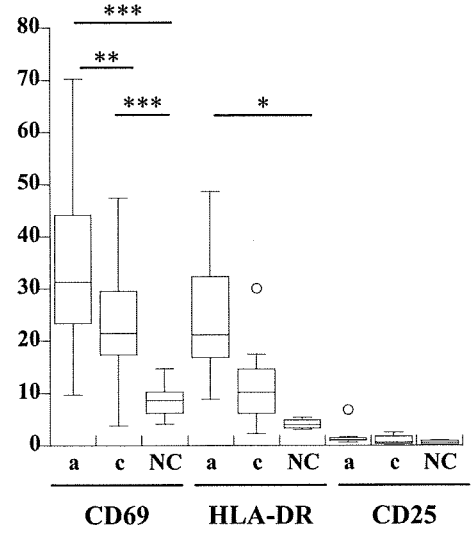
CD19+



CD56+CD16+



CD56+CD16-



CD16+CD56-

Figure 1.

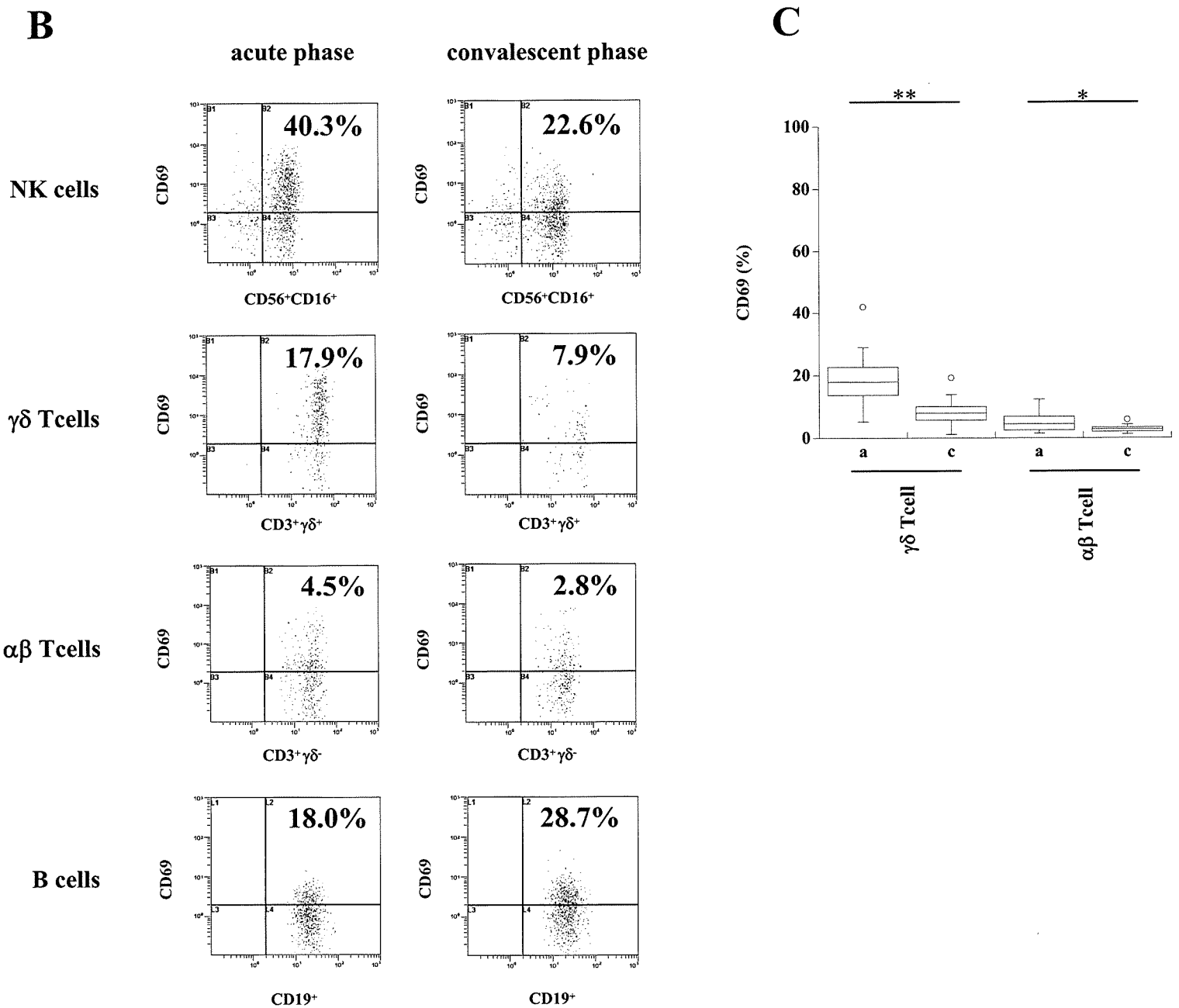
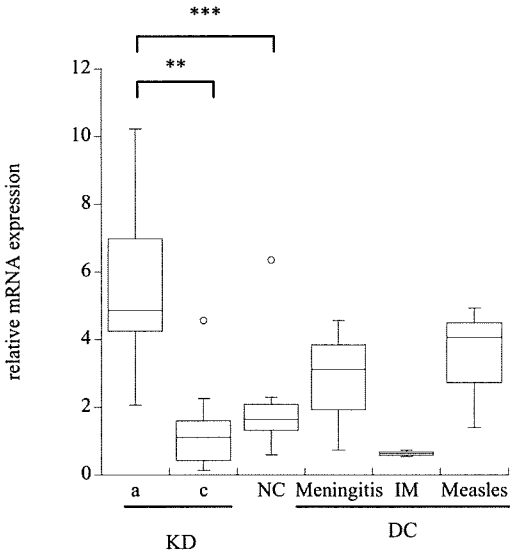
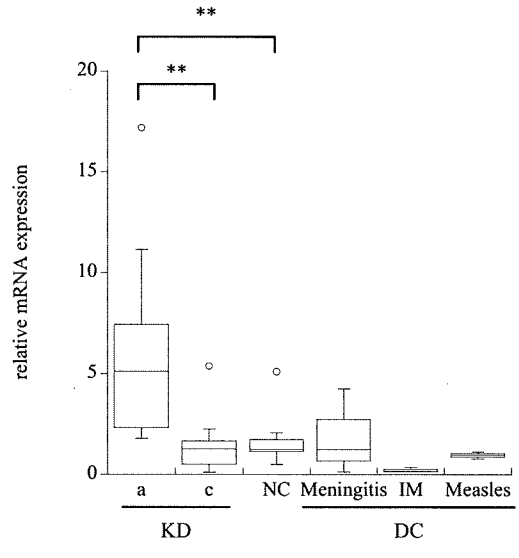

B
acute phase
convalescent phase
NK cells
γδ Tcells
αβ Tcells
B cells
40.3%
22.6%
17.9%
7.9%
4.5%
2.8%
18.0%
28.7%
CD69
CD56+CD16+
CD3+γδ+
CD3+γδ-
CD19+
C
CD69 (%)
100
80
60
40
20
0
**
*
a
c
γδ Tcell
αβ Tcell

Figure 1.



S100A9



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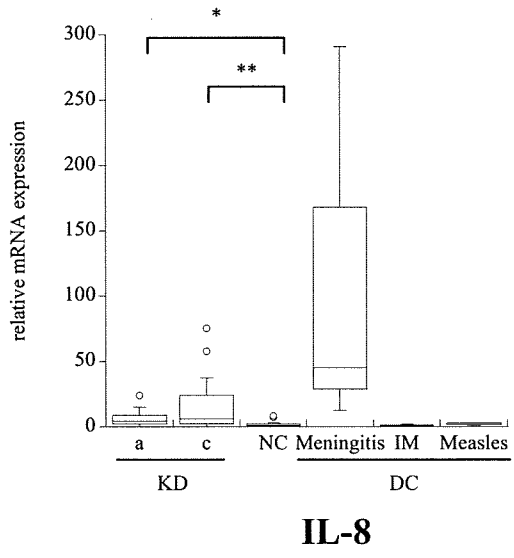

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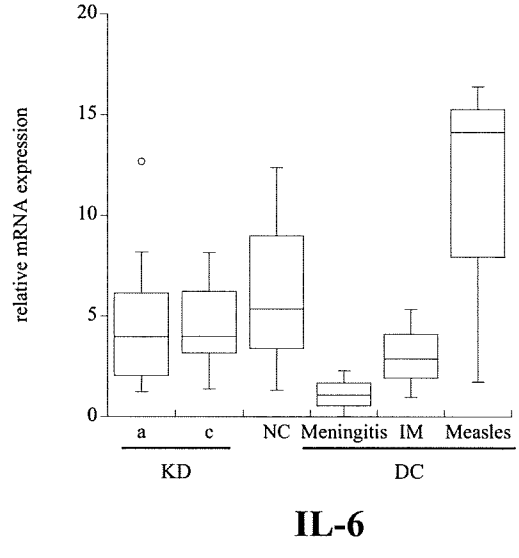

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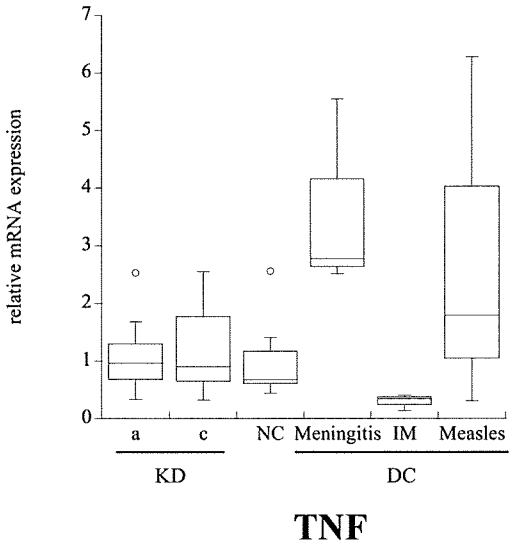
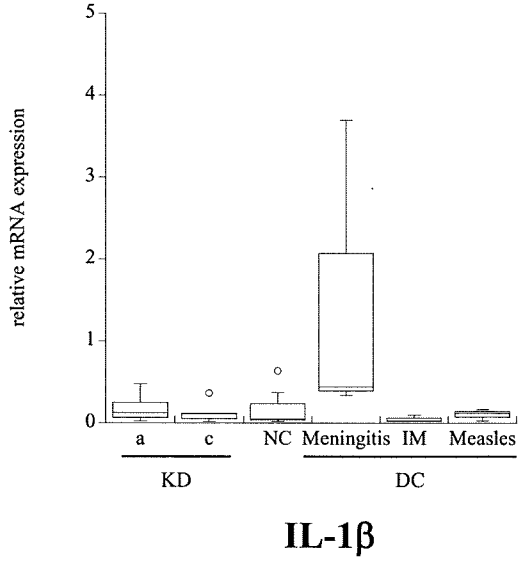


TNF



IL-1β

Figure 2.

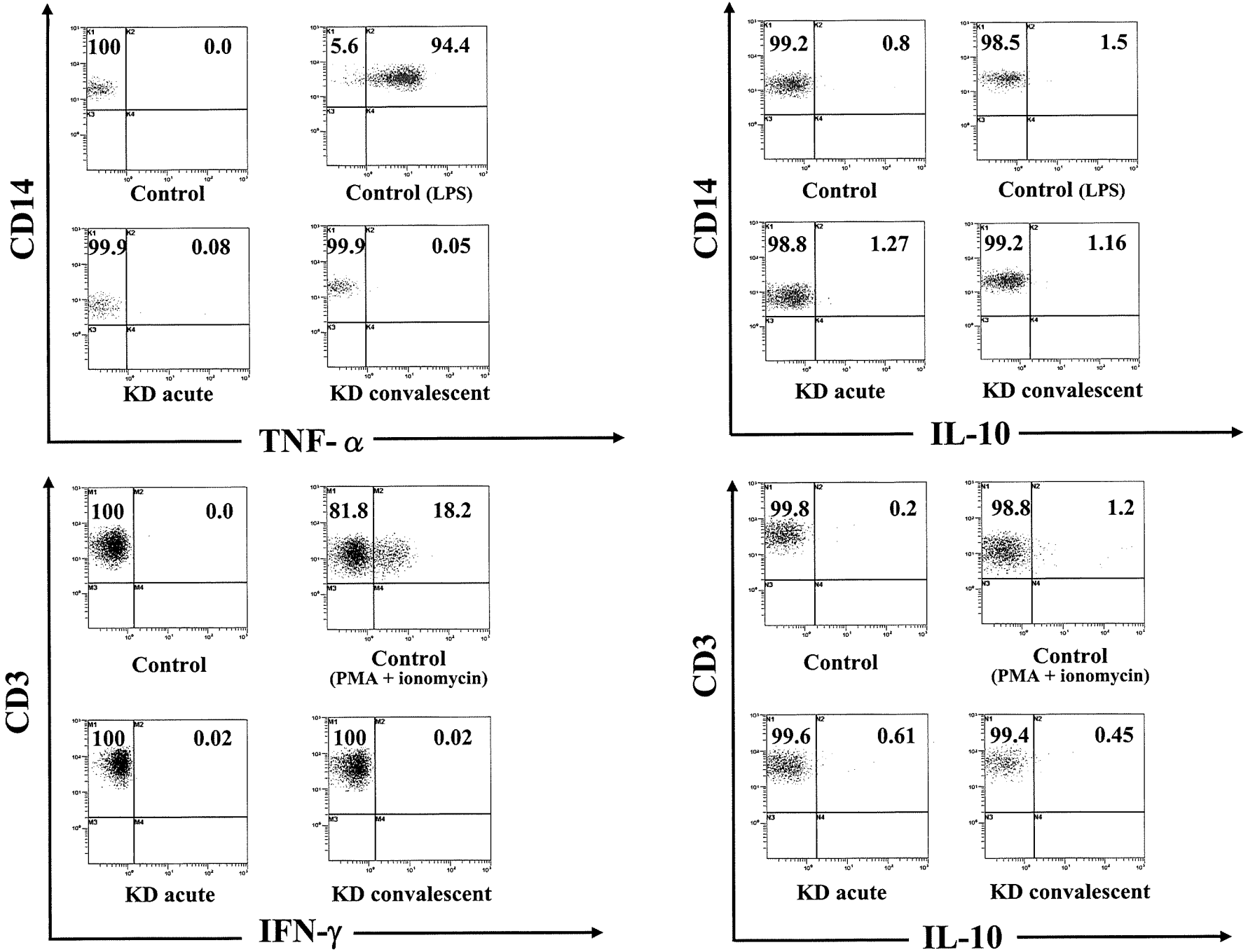

CD14
100
0.0
Control
5.6
94.4
Control (LPS)
99.9
0.08
KD acute
99.9
0.05
KD convalescent
TNF- α
CD14
99.2
0.8
Control
98.5
1.5
Control (LPS)
98.8
1.27
KD acute
99.2
1.16
KD convalescent
IL-10
CD3
100
0.0
Control
81.8
18.2
Control
(PMA + ionomycin)
100
0.02
KD acute
100
0.02
KD convalescent
IFN-γ
CD3
99.8
0.2
Control
98.8
1.2
Control
(PMA + ionomycin)
99.6
0.61
KD acute
99.4
0.45
KD convalescent
IL-10

Figure 3.

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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 $\alpha\beta$T cells ($n = 23$), $\gamma\delta$T 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

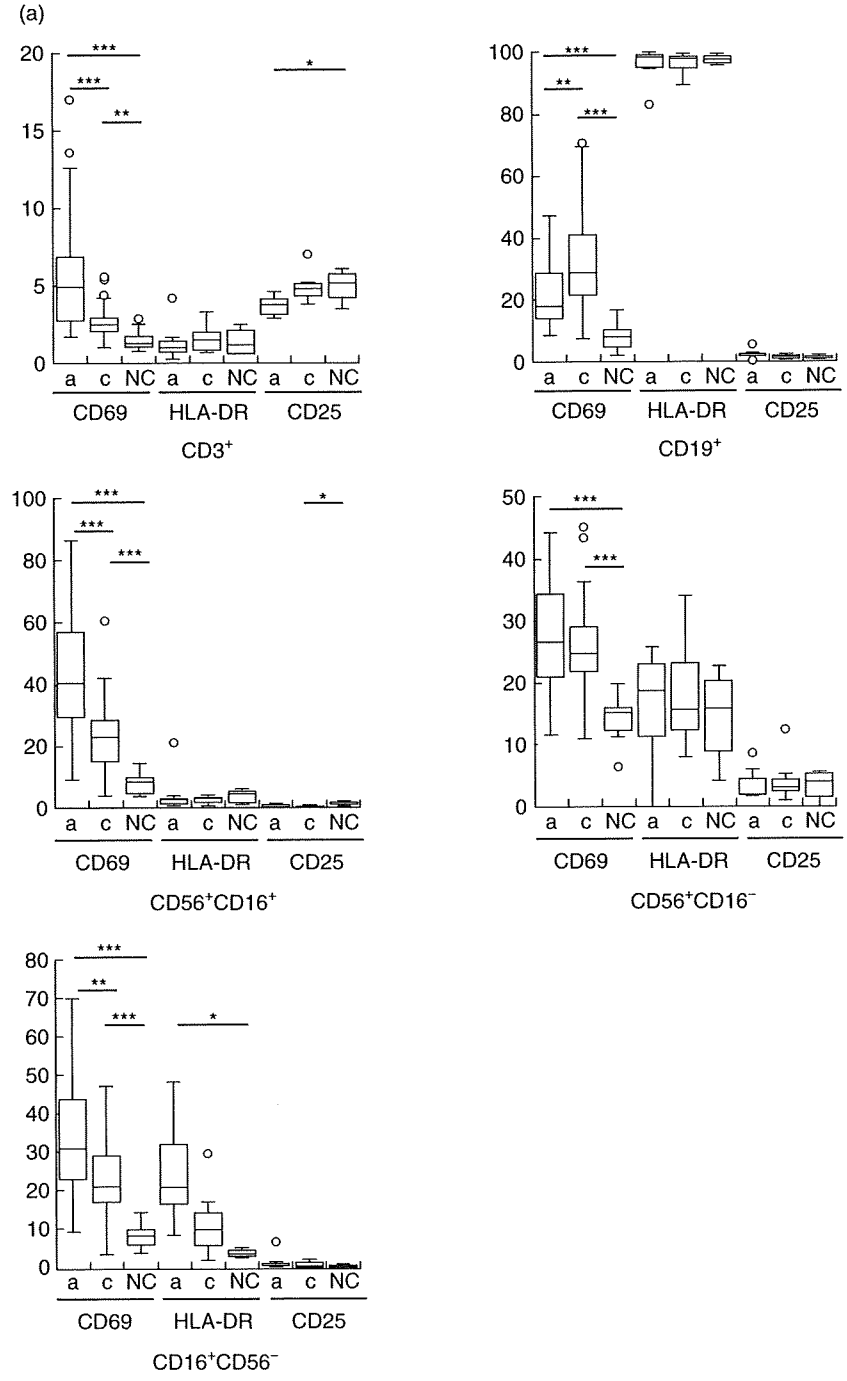

Fig. 1. Flow cytometric analysis of the activation markers on T, B and natural killer (NK) cells at acute phase of Kawasaki disease (KD). (a) The proportions of activated T, B and NK cells in the peripheral blood of seven patients with KD and 15 healthy control subjects were analysed by flow cytometry. 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)

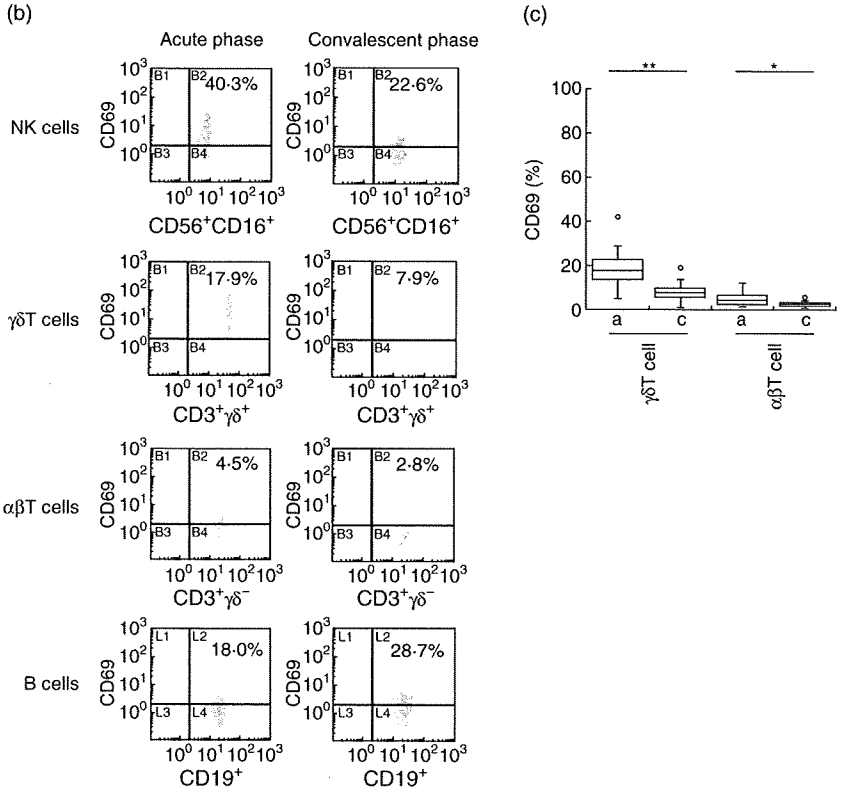

Fig. 1. *Continued*

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 superfamily, 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.

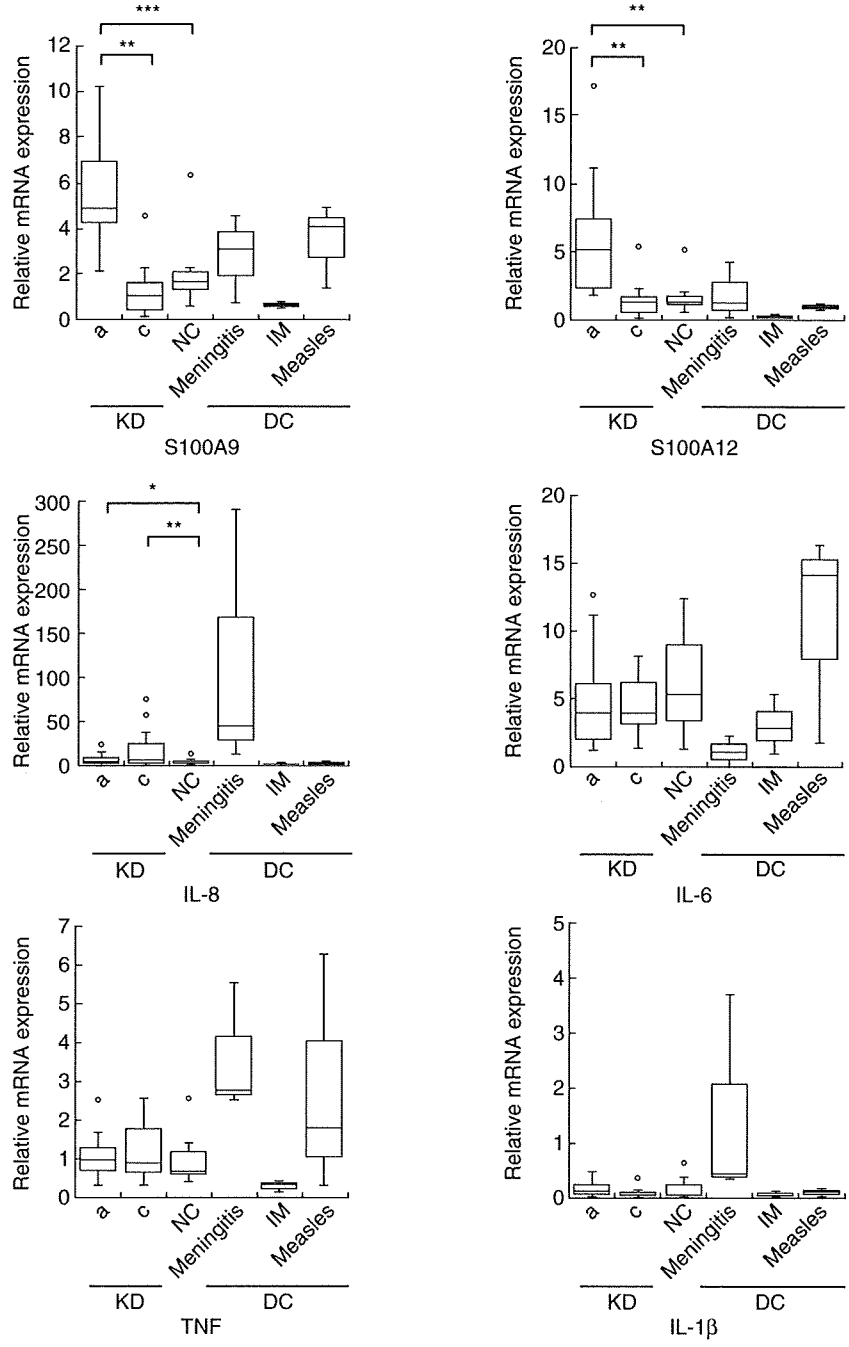

Fig. 2. Relative expression levels of *S100A9*, *S100A12*, *IL8*, *IL6*, *TNF* and *IL1B* genes in peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). The gene expression levels of these cytokines were determined by the reverse transcription–polymerase chain reaction (RT–PCR) 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.

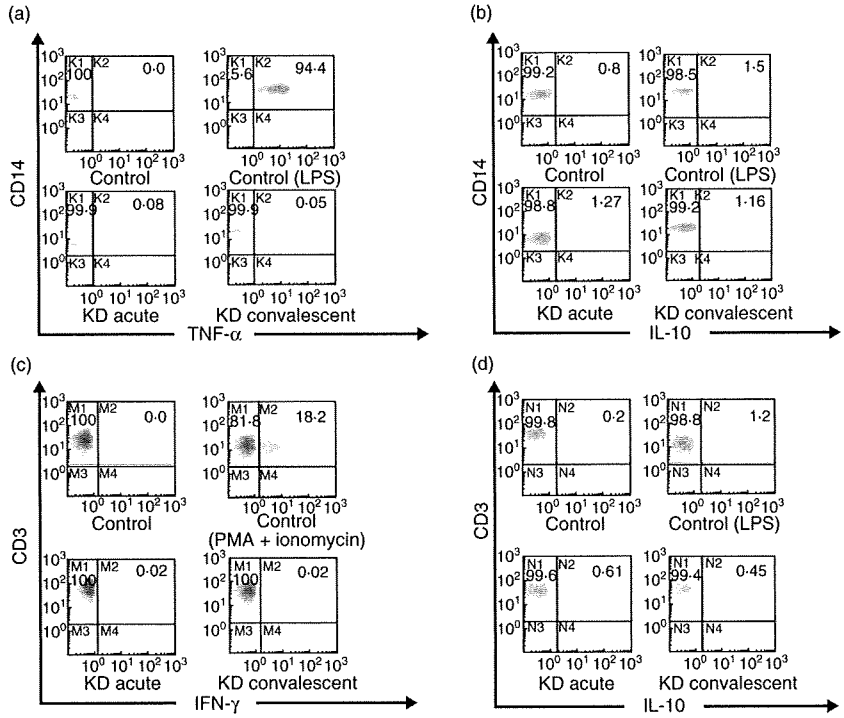

Fig. 3. Flow cytometric analysis of intracellular cytokine production of peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). Intracellular cytokine production in PBMCs at acute and convalescent phases of KD was analysed by flow cytometry. Representative data of tumour 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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