included psychomotor retardation with or without seizures (n = 5), brain atrophy (n = 1), hearing difficulty (n = 1), short stature (n = 5), and impaired sexual development (n = 1).

DISCUSSION

No underlying immunodeficiency has yet been identified for idiopathic EBV-HLH, which has been recognized to be distinct from familial or inherited disease-related HLH like FHL. However, EBV also acts as a trigger in the development of HLH episodes in FHL patients. Therefore, caution must be exercised in the differentiation of the two types of HLH disease. Strict use of the renewed diagnostic criteria for the registered cases in Japan enabled an analysis of the SCT results of 43 FHL and 14 EBV-HLH patients. The data first revealed a high survival rate in UCBT recipients in either type of HLH, indicating that CB could be preferable BM as the unrelated donor source in SCT for pediatric patients with refractory HLH. In addition, SCT in FHL patients was more problematic than that in EBV-HLH, where it was associated with a high incidence of post-transplant early death rate as well as late sequelae including neurological deficits. The EBV-HLH patients showed no apparent sequelae even if they had CNS involvement at diagnosis.

Information concerning SCT for HLH patients has been accumulated mostly in FHL, but little has been published in EBV-HLH except for sporadic case reports [10,11]. Previously published major studies on SCT in FHL patients are summarized in Table III. Because of the historical changes in the available genetic analyses, supportive care practices, donor sources and conditioning, the pre-2000 studies [23–27] might not be comparable to the current data. Henter et al. [21] showed the improved survival of patients treated with HLH-94 followed by BMT, in which the 3-year post-BMT survival was 62%. Horne et al. [28] noted significant TRM due to venoocclusive disease (VOD) after myeloablative conditioning, and that an active disease status at SCT was associated with a poor prognosis. Ouachee-Chardin et al. [29] reported 59% of OS in a series of 48 patients including 60% of haploidentical SCT, and indicated a high TRM due to VOD associated with young age. Recently, Baker et al. [30] reported that BU/CY/VP16 plus or minus ATG-conditioning provided a cure in 53% of patients after unrelated donor BMT, but a high mortality rate at day 100 (32 of 50 [64%] deceased patients). The present study showed a comparably high OS rate (69%) and similarly high incidence of early death until day 100 (7 of 13 [54%] deaths after allogeneic SCT) in Japan. Probably, the major distinction of the current study from the other reports is a higher usage of UCBT (50%) and RIC (26%). Unfortunately, the combined usage of RIC-UCBT was applied only in eight cases (14%) in this study, which was insufficient to fully evaluate its effectiveness. With regard to RIC-SCT with or without UCBT for FHL, Cooper et al. [31] reported a high disease free survival (75%) in 12 HLH patients (including 5 FHL) who underwent RIC-SCT from matched family/unrelated or haploidentical donor, in which 3 of 9 survivors had mixed chimerism but remain free of disease. The most recent report by Cesaro et al. [32] analyzed 61 cases including an appreciable number of RIC (18%) and UCBT (10%), but did not document the superiority of RIC-UCBT. In the present study, UCBT had a tendency to yield a more favorable outcome than UBMT, although the difference was not statistically significant. FHL infants received SCT early; however the fact that survival of FHL patients who underwent SCT at <2 years of age was not better than later SCT might reflect the difficulty in determining the optimal timing of SCT

TABLE III. Reports on the Clinical Outcome of Patients With HLH Who Underwent Allogeneic Hematopoietic Stem Cell Transplantation

No. pts	Median age at SCT (months)	FH (%)	Major conditioning regimen	Donor	Source	OS (%)	Engraft. (%)	Causes of death	Refs.
9	13	45	Myeloab	VP16/BU/CY ±anti-LFA1	MRD/MMRD/haplo	BM	44.0	100	TR, HLH	[24]
29	NR	48	Myeloab	NR	MRD/MUD/haplo	BM	66.0	72	TR, HLH	[25]
20	9	30	Myeloab	VP16/BU/CY ±ATG	MSD/URD (80%)	BM	45.0	90	TR, HLH	[26]
14	14	36	Myeloab	VP16/BU/CY, ATG/BU/CY	MMRD/MUD	BM (T cell depleted)	64.3	65	TR, HLH	[27]
12	18	42	Myeloab	VP16/BU/CY	MSD/URD (67%)	BM	100	100	No	[33]
17	NR	NR	Myeloab	VP16/BU/CY ±ATG, TBI	MRD/URD/haplo	BM, CB (2), PB, CD34	58.0	94	TR, HLH, lymphoma	[8]
65[a]	13	31	Myeloab	VP16/BU/CY ±ATG	MRD/URD/haplo	BM, CB (5), PB, CD34	62.0	89	TR, HLH, AML	[21]
86[a]	13	34	Myeloab	VP16/BU/CY ±ATG, TBI	MRD/URD/haplo	BM, CB (7)	64.0	90	TR, HLH, 2nd AML	[28]
48	6	35	Myeloab	VP16/BU/CY, ATG/BU/CY	MSD/URD/haplo	BM, PB	58.5	78	HLH	[29]
12	14	17	RIC	FLU/MEL ± BUS, FLU/2GyTBI	MRD/URD/haplo	BM, CD34	75.0	100	TR	[31]
91	12	NR	Myeloab	VP16/BU/CY ±ATG	URD	BM, PB, CB (9)	45.0	83	TR, HLH	[30]
61	13	20	RIC (18%)	VP16 or MEL/BU/CY ±ATG	MRD/MMRD/URD	BM, PB, CB (6)	63.9	78	TR (68%), HLH (27%)	[32]
42	17	55	RIC (26%)	VP16/BU/CY ±ATG, TBI	MRD/MMRD/URD	BM, PB, CB (21)	69.0	78	TR (79%), HLH (21%)	Ours

AML, acute myelogeneous leukemia; BM, bone marrow; BU, busulfan; CB, cord blood; CY, cyclophosphamide; FHL, familial hemophagocytic lymphohistiocytosis; FH, family history; FLU, fludarabine; MEL, melphalan; MMRD, HLA-mismatched related donor; MRD, HLA-matched related donor; MSD, HLA-matched sibling donor; MUD, HLA-matched unrelated donor; NR, not recorded; PB, peripheral blood; RIC, reduced intensity conditioning; TBI, total body irradiation; TR, transplantation-related events; URD, unrelated donor; VP16, etoposide. [a]Sixty four of 65 patients studied by Henter et al. [21] were included in 86 patients by Horne et al. [28].

or introducing appropriate RIC regimens in young infants. In UCBT, a major obstacle was thought to be early graft failure, but once engrafted no late graft failure could not be seen [29]. We confirmed this finding in our UCBT cases.

Dürken et al. [33] reported that six HLH patients with CNS disease underwent allogeneic BMT and three of them had no persistent neurological problems after transplant. More recently, SCT is thought to be preferable for FHL patients at the early stage of CNS disease with variable presentation [34,35]. Fludarabine-based RIC has been preferred in SCT for FHL patients in order to reduce late sequelae [36,37]. Since CNS disease itself had no impact on the OS in the current study, but nearly half of the long-term survivors of FHL had late sequelae associated with growth and development, further prospective studies should be focused on how to reduce late sequelae in SCT for FHL patients.

In the treatment of refractory EBV-HLH, no consensus has yet been reached concerning the treatment of patients who fail to respond to the HLH-2004 protocol type immunochemotherapy. Several reports documented that SCT led to a complete remission in such cases [8,10,11,28,38,39]. The present study revealed that use of pre-SCT combination chemotherapy might be associated with a better therapeutic impact on subsequent SCT in patients with EBV-HLH. Furthermore, long-term survival, that is, a probable cure, could be obtained even after autologous SCT [22] or identical twin donor BMT, suggesting that a reconstitution of allogeneic hematopoietic stem cells was not essential in the successful SCT for EBV-HLH patients as described in the autologous PBSCT success for lymphoma-associated HLH [40]. In addition, long-term survival even after graft failure or post-transplant relapse in EBV-HLH patients might suggest the possibility of resetting the adaptive immune response to the virus as postulated in autologous SCT for the treatment of autoimmune diseases [41,42]. Moreover, successful syngeneic SCT may imply that EBV-HLH is not a monogenic disease, since Chen et al. [43] observed that a primary infection of EBV incited HLH in a pair of the twins, but not in the identical twin counterpart. These observations implied that the genetic influence in patients with EBV-HLH might be distinct from that in patients with FHL on precipitating the excessive immune activation. Further prospective studies should therefore be directed toward not only the optimization of UCBT-RIC to improve survival of FHL patients, but to better understanding of the pathological interaction between cytotoxic granule disorders and EBV.

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Short Report

NEMO mutation as a cause of familial occurrence of Behçet's disease in female patients

Takada H, Nomura A, Ishimura M, Ichiyama M, Ohga S, Hara T. *NEMO* mutation as a cause of familial occurrence of Behçet's disease in female patients.
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Behçet's disease is a chronic, relapsing, multisystem inflammatory disease of unknown etiology. Nuclear factor κB (NF-κB) essential modulator (NEMO) that is required for the activation of NF-κB plays an important role in inflammation. To investigate the role of NEMO in the pathogenesis of Behçet's disease, we analyzed *NEMO* gene and its expression pattern in tissues in a family with Behçet's disease. We found a heterozygous mutation (1217A>T, D406V) in a 6-year-old girl and her mother. Skewed X-chromosome inactivation was not observed in the peripheral blood mononuclear cells as well as in oral and intestinal mucosa of the patients. Accordingly, there was a significant proportion of peripheral blood monocytes that did not produce sufficient intracellular tumor necrosis factor-α with the stimulation of lipopolysaccharide. Heterozygous *NEMO* mutation is a cause of familial occurrence of Behçet's disease in female patients.

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Key words: Behçet's disease – nuclear factor κB essential modulator Incontinentia pigmenti – X-linked anhidrotic ectodermal dysplasia with immunodeficiency

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Nuclear factor κB (NF-κB) essential modulator (NEMO) is required for the activation of the transcription factor NF-κB (1). The *NEMO* gene has been mapped to the chromosome location Xq28 (1). Large genomic rearrangements or amorphic mutations of *NEMO* cause incontinentia pigmenti, a disorder that is usually prenatally lethal in males, and contribute to abnormalities of skin, hair, nails, teeth and central nervous system in female heterozygotes (2). On the other hand, hypomorphic *NEMO* mutations cause X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) in male, characterized by immunodeficiency associated with an impaired development of skin adnexa (hair, sweat glands, and teeth) (3).

Behçet's disease is a chronic, relapsing, multisystem inflammatory disease of unknown etiology characterized by mucocutaneous, ocular, articular, vascular, urogenital, neurological, and gastrointestinal involvements, such as ulcerative colitis and congestive gastritis (4, 5). We found heterozygous *NEMO* mutation in two female patients with Behçet's disease.

Materials and methods

Patient 1 was a 6-year-old girl who suffered from ulcers in oral cavity and perianal area for 7 months. Her elder brother was diagnosed as XL-EDA-ID and died of gastrointestinal bleeding when he was 9 years old. On admission, her skin showed hypopigmented lesions without atrophy in the abdominal area and extremities (Fig. 1a), which had been observed since early infancy. A small ulcer was observed in oral

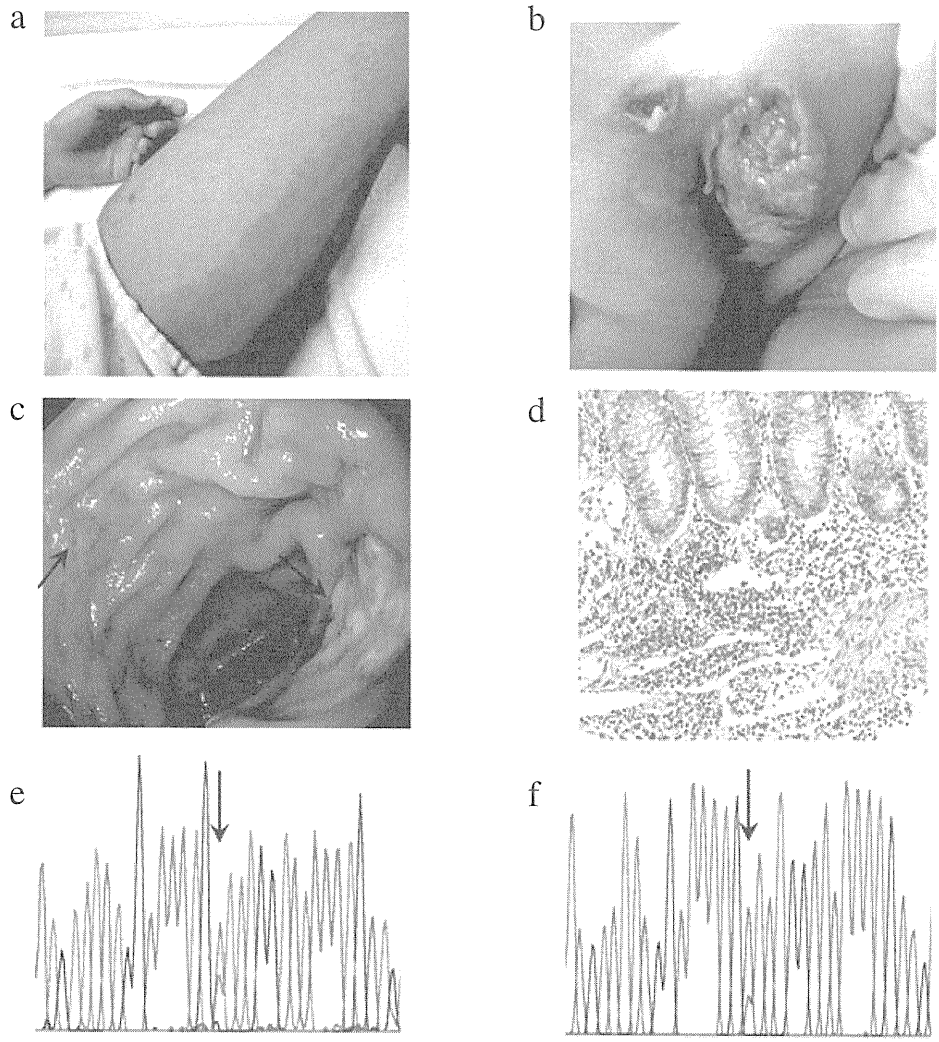

Fig. 1. Clinical manifestations and *NEMO* mutation in the patients. The hypopigmented skin lesions on the lower extremities observed along the curvilinear lines of Blaschko (**a**) and the ulcerative lesions in perianal area (**b**) are shown. The endoscopic finding with ulcerative lesions (arrows) in ascending colon and the histology (hematoxylin–eosin staining, ×100) of the ulcerative lesion are shown in (**c**) and (**d**), respectively. Sequencing results of the peripheral blood cells from patients 1 and 2 on *NEMO* are shown in (**e**; patient 1) and (**f**; patient 2).

cavity. She had large and deep painful ulcerative lesions in perianal area (Fig. 1b). The laboratory examinations showed a white blood cell count of 13.2×10^9/l with 80.9% neutrophils, hemoglobin of 12.3 g/dl, and erythrocyte sedimentation rate of 69 mm/h. Human leukocyte antigen (HLA) typing showed A2/A24, B61/B54, Cw1, Cw15, DR4, and DR12. Endoscopic examination showed multiple ulcerative lesions in colon, lacking reactive change in their marginal area (Fig. 1c). Histologically, chronic active inflammation was observed (Fig. 1d). These findings met the diagnostic criteria for Behçet's disease (entero-Behçet type) (6).

Patient 2 was a 42-year-old mother of the patient 1, who also suffered from ulcers in oral cavity and perianal area since she was 8 years old. She was diagnosed as having Behçet's disease when she was 12 years old. She also had hypopigmented skin lesions in the abdominal area and extremities, which had been observed since early infancy.

Genomic DNA and cDNA were amplified by polymerase chain reaction (PCR) as reported previously (2). The direct sequencing was performed using ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA).

Intracellular tumor necrosis factor (TNF)-α staining was performed using the Fastimmune Intracellular Staining System (BD Bioscience Pharmingen, San Diego, CA, USA) (7). Flow cytometric analysis was performed using EPICS XL (Beckman Coulter, Miami, FL, USA).

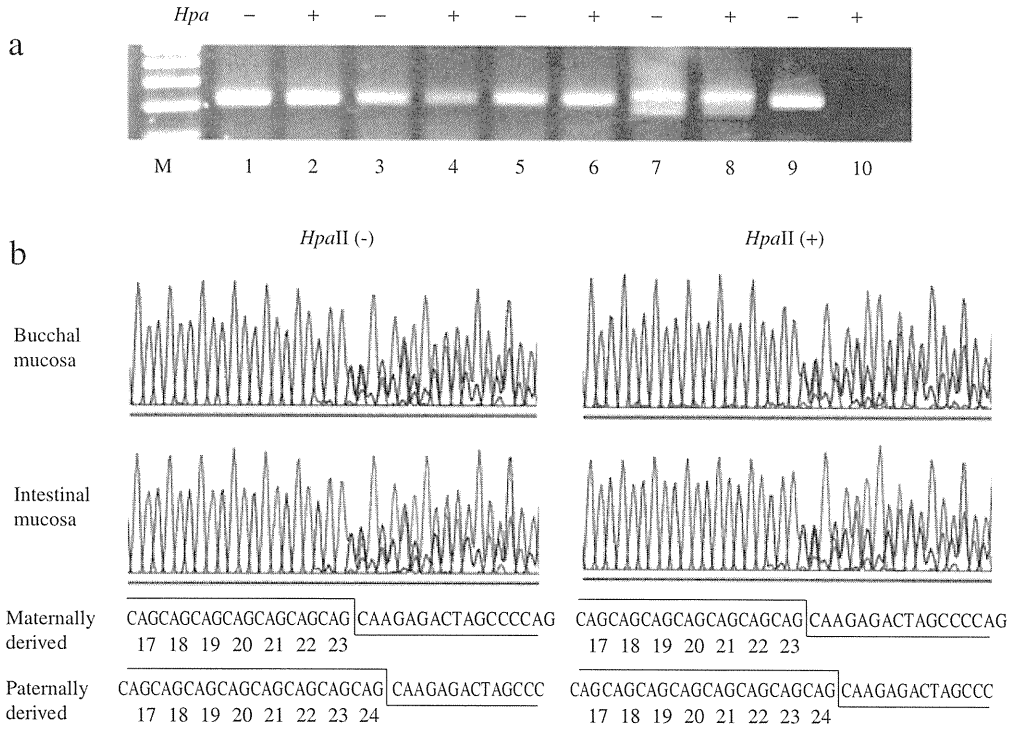

Fig. 2. X-chromosome inactivation of the patients. (**a**) Exon 1 of the HUMARA locus that contains CAG repeats was amplified by PCR after the digestion by methylation-sensitive *Hpa*II. Lanes 1 and 2; PBMNC, lanes 3 and 4; buccal mucosa, lanes 5 and 6; intestinal mucosa of patient 1, lanes 7 and 8; PBMNC of patient 2, lanes 9 and 10; PBMNC of the father of patient 1. (**b**) Sequencing results of the PCR products of the HUMARA locus from PBMNC, and buccal and intestinal mucosa of the patient 1 with and without *Hpa*II treatment are shown. The CAG repeats in the HUMARA locus of the maternally derived and paternally derived X-chromosome were 23 and 24 in number, respectively.

X-chromosome inactivation was analyzed as previously described (8). In brief, DNA was digested with the methylation-sensitive *Hpa*II (New England BioLabs, Beverly, MA, USA), amplified by the PCR at the exon 1 of human androgen receptor (HUMARA) gene locus that contains a highly polymorphic trinucleopeptide repeat (CAG), and sequenced.

Results

The cDNA and genomic DNA were obtained from peripheral blood mononuclear cells (PBMNC), and *NEMO* gene was amplified by PCR and sequenced. Heterozygous mutation (1217A→T, D406V) was observed in patients 1 and 2 (Fig. 1e,f), and elder brother of patient 1 had the same mutation (data not shown). We then investigated X-chromosome inactivation pattern of PBMNC, buccal mucosa, and intestinal mucosa by analyzing the effect of methylation-sensitive *Hpa*II on the HUMARA locus. Although we could not detect the difference of CAG repeat number in HUMARA locus between maternally and paternally derived X-chromosomes by electrophoresis of PCR products due to the minimal (1 repeat) difference in repeat number between them in patient 1 (Fig. 2a), the lack of extreme skewing was confirmed in PBMNC of patient 2 (Fig. 2a). The sequencing of these PCR products showed the lack of extreme skewing in all these tissues in patient 1 (Fig. 2b).

We analyzed lipopolysaccharide (LPS)-induced monocytic TNF-α production using flow cytometer to investigate individual cell function caused by the *NEMO* mutation and X-chromosome inactivation. As shown in Fig. 3, there was a significant proportion of monocytes that did not produce sufficient intracellular TNF-α with the stimulation of LPS, which functionally supported the lack of extensive X-inactivation skewing in the patient.

Discussion

A familial aggregation of Behçet's disease has been reported previously (9–14). Although *Familial Mediterranean fever* (*MEFV*) gene mutation is reported to be one of the genetic backgrounds of Behçet's disease (15), most of the patients as well as our patients did not have *MEFV* mutation (data not shown). There are several

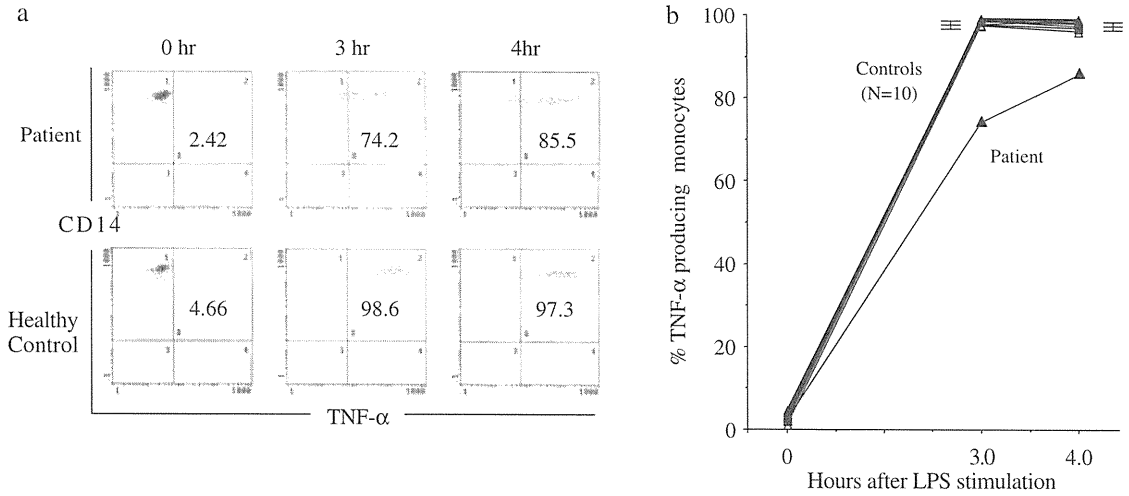

Fig. 3. A population of peripheral blood monocytes with insufficient production of intracellular TNF-α by the stimulation of LPS. A representative data of intracellular TNF-α staining (**a**) and the percentage of TNF-α producing cells in monocytes (**b**) without and with LPS stimulation are shown. Horizontal bars indicate the mean value and standard deviation in healthy controls.

reports of the association of Behçet's disease and incontinentia pigmenti (16–18). All the patients were females and developed Behçet's disease in childhood (16–18), which further supports our results.

Several clinically unique features were observed in our patients. The first was the occurrence of incontinentia pigmenti and XL-EDA-ID in a family. The second was the hypopigmented skin lesions since early infancy, because they are usually observed in early teens to adulthood (19). The third was the lack of extremely skewed X-chromosome inactivation (Fig. 2). Most of the patients with incontinentia pigmenti showed skewed X-chromosome inactivation in PBMNC and hepatocytes, which spared any apparent phenotype of these cells (20). This *NEMO* mutation was reported previously only in one patient with XL-EDA-ID (21), not in females. The D406V mutation locates in zinc finger domain, which is important in phosphorylation of NEMO, binding with ubiquitin, and full NF-κB activation (22–24). The development of Behçet's disease may be restricted only in a small proportion of the patients with incontinentia pigmenti caused by some particular type of *NEMO* mutation. Alternatively, it is possible that there are unrecognized patients with Behçet's disease and atypical or mild skin lesions caused by *NEMO* mutations.

NEMO-deficient mice developed intestinal inflammation by the impaired intestinal integrity caused by increased sensitivity to TNF-induced cell death, diminished expression of antimicrobial peptides such as defensins, and recruitment of inflammatory cells into damaged tissues (25). It is possible that this occurs in female patients with heterozygous *NEMO* mutation if they do not have skewed X-chromosome inactivation in the intestine. Immunocompetent inflammatory cell fraction in these patients can be recruited and it accelerates the inflammatory reaction in the intestine. The latter seems to be more important for the development of the Behçet's disease, because low-dose corticosteroid treatment was effective in both patients. This mechanism would also be applied to the lesions in oral mucosa and perianal tissues where continuous bacterial stimulation and infection occur.

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Conflict of interest

Nothing to declare.

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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

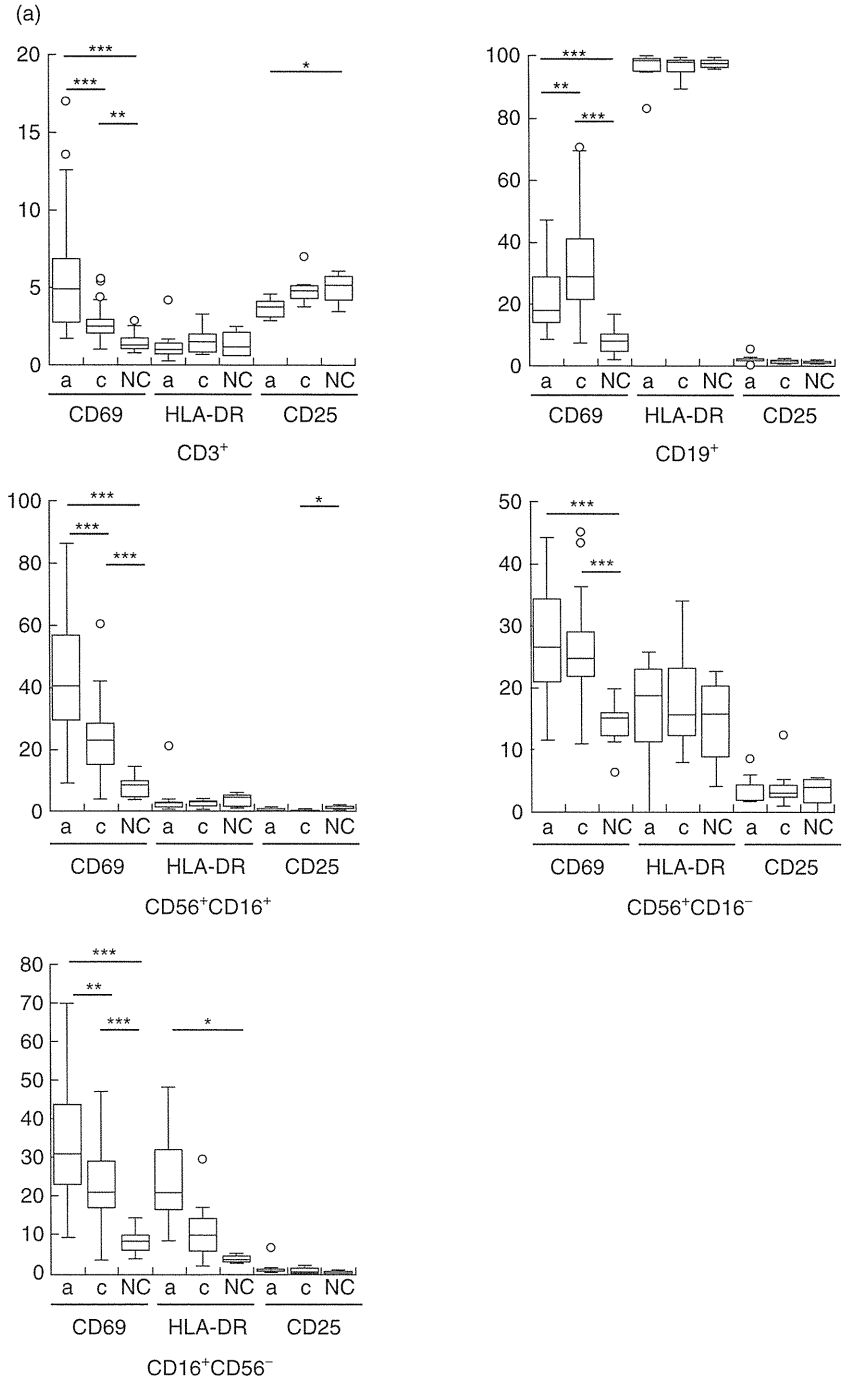

Fig. 1. Flow cytometric analysis of the activation markers on T, B and natural killer (NK) cells at acute phase of Kawasaki disease (KD). (a) The proportions of activated T, B and NK cells in the peripheral blood of seven patients with KD and 15 healthy control subjects were analysed by flow cytometry.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

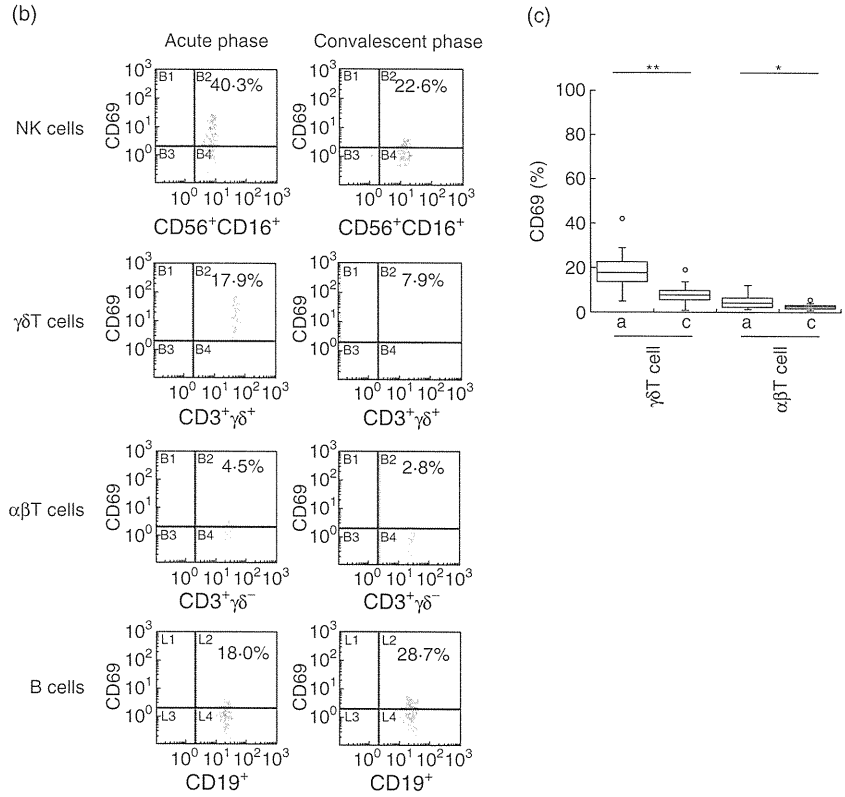

Fig. 1. *Continued*

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 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.

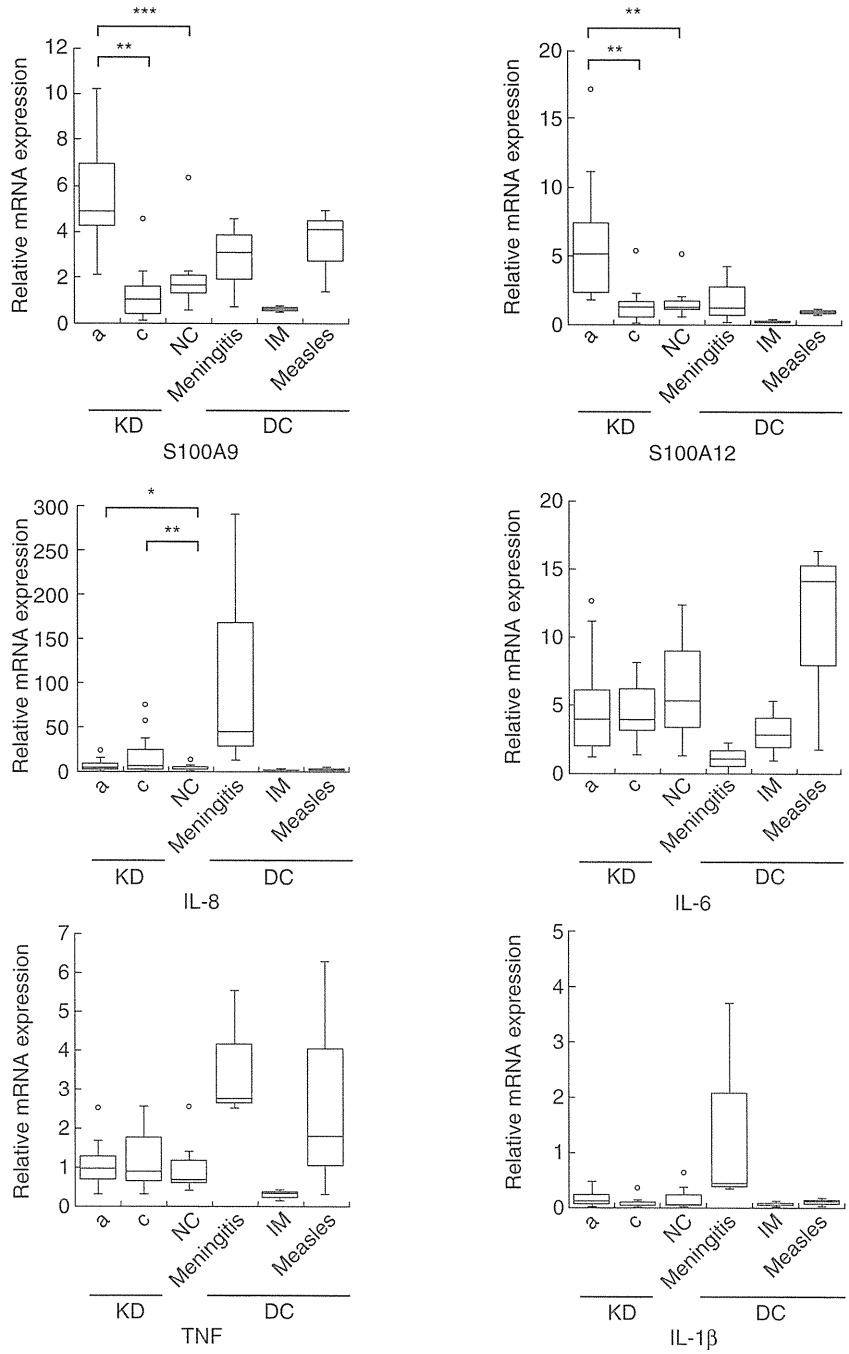

Fig. 2. Relative expression levels of *S100A9*, *S100A12*, *IL8*, *IL6*, *TNF* and *IL1B* genes in peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). The gene expression levels of these cytokines were determined by the reverse transcription–polymerase chain reaction (RT–PCR)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

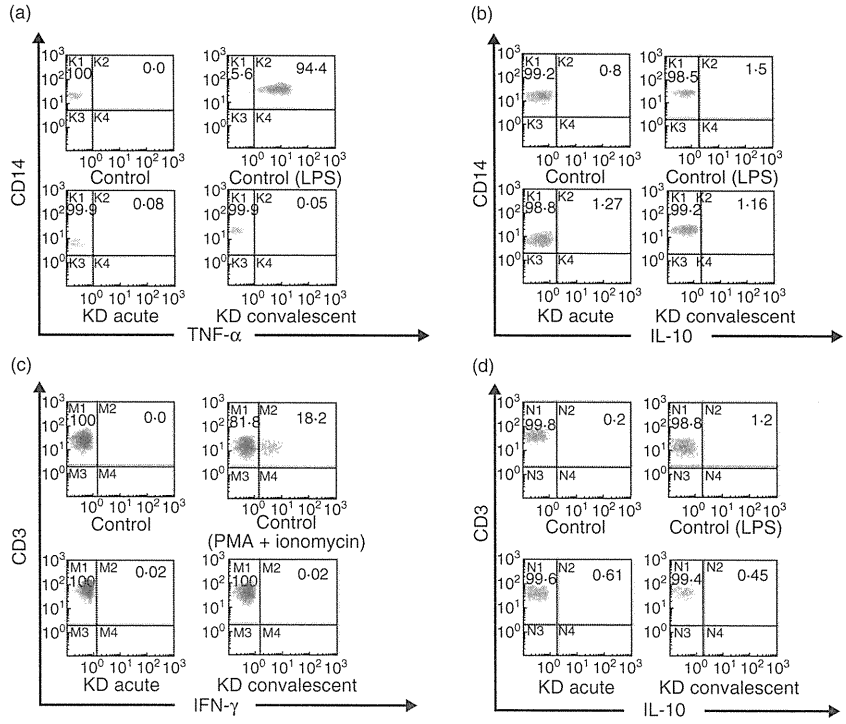

Fig. 3. Flow cytometric analysis of intracellular cytokine production of peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). Intracellular cytokine production in PBMCs at acute and convalescent phases of KD was analysed by flow cytometry. Representative data of tumour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

Tocilizumab: molecular intervention therapy in children with systemic juvenile idiopathic arthritis

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Systemic juvenile idiopathic arthritis (JIA) is a subtype of chronic childhood arthritis of unknown etiology, manifested by long-lasting systemic inflammation and complicated by joint destruction, functional disability and growth impairment. Macrophage activation syndrome is the most devastating complication, which is associated with serious morbidity. IL-6 has been hypothesized to be a pathogenic factor of this disease. The anti-IL-6 receptor monoclonal antibody, tocilizumab, was developed, and we investigated the safety and efficacy of tocilizumab in children with this disorder. The Phase II trial revealed that high-grade fever abruptly subsided and that inflammatory markers were also normalized. The dose of tocilizumab for systemic JIA was revealed to be 8 mg/kg at 2-week intervals. The Phase III trial, a placebo-controlled, double-blind study, indicated that patients in the tocilizumab group had sustained clinical measures of effectiveness and wellbeing, whereas most of those in the placebo group needed rescue treatment. The most common adverse events were symptoms of mild infections and transient increases of alanine aminotransferase. Serious adverse events were anaphylactoid reaction and gastrointestinal hemorrhage. Clinical and laboratory improvement in fever, sickness behavior, C-reactive protein gene expression and chronic inflammatory anemia in children with systemic JIA treated with tocilizumab indicated the possible roles played by IL-6 in this inflammatory disease. Thus, tocilizumab is generally safe and well tolerated. It might be a suitable treatment in the control of this disorder, which has so far been difficult to manage.

Keywords: biologic response modifier • C-reactive protein • IL-6 • IL-6 receptor • systemic juvenile idiopathic arthritis • tocilizumab

Systemic juvenile idiopathic arthritis (JIA), a systemic inflammatory disease of unknown etiology, is one of the most common physically disabling conditions of childhood [1]. The long-lasting inflammation also causes anemia, impairment of growth and development, and amyloidosis. Moreover, the acute complication known as macrophage activation syndrome (MAS) is associated with serious morbidity and sometimes death [2].

This severe inflammatory disease is refractory to various cytotoxic and immunosuppressive medications. High doses and a long duration of corticosteroids have been inevitably chosen as regimens for suppressing disease activity. Consequently, corticosteroid therapy leads to iatrogenic Cushing-like syndrome, osteoporosis and compression fractures, growth impairment, cataracts and increased susceptibility to overwhelming infection [3].

The pathogenesis of systemic JIA remains obscure. However, several studies have provided evidence implicating the circulating levels of IL-6 and soluble IL-6 receptor (sIL-6R), but not TNF-α, as playing an essential role as inflammatory mediators. The oldest cytokine, IL-1β, has also been recognized as an important pathogenic player in systemic JIA [4]. The impaired natural killer (NK) cell function correlated with perforin gene (*PRF1*) mutation [5] and defective phosphorylation of IL-18 receptor-β [6] was also reported.

Serum IL-6 and IL-6R levels in children with systemic JIA have shown correlations with both disease activity and the extent and severity of

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joint involvement [7]. A human *IL-6* gene transgenic study in mice indicated that overproduction of IL-6 leads to severe inflammatory responses and growth retardation, similar to that found in children with systemic JIA [8]. Taken together, this information indicates that overfunction of the IL-6 signaling system may play a central role in the induction and progression of systemic JIA and its complications. This disease is starting to be regarded as an autoinflammatory disease rather than an autoimmune disease [9].

Recently, the molecular mechanisms of inflammatory responses have been precisely described, and proinflammatory cytokines are known to contribute to variable physiologic and pathophysiologic processes of inflammation. Among their physiologic functions, cytokines may regulate the central mechanisms of fever and sickness behavior such as prolonged sleep, lethargy and anorexia observed in experimental animals [10]. The combination of IL-1β and IL-6 plays an essential role in the anemia of chronic inflammatory diseases [11]. IL-6 can function as the key hepatocyte-stimulating factor to induce, at least in rodents, acute-phase reactants including fibrinogen, α-2-macroglobulin and α-1-acid glycoprotein [12]. Serum amyloid A (SAA) [13] and C-reactive protein (CRP) [14] are also products of the IL-6 plus IL-1β action in human cell line experiments. Overproduction of IL-6 has been implicated in the disease pathology of several inflammatory autoimmune disorders, rheumatoid arthritis [15], Castleman's disease [16] and adult Still's disease [17]. However, direct evidence in humans is not yet available to show that the inflammatory changes of clinical manifestations and laboratory findings are correlated with cytokine functions.

Tocilizumab (Actemra®, Roche, Basel, Switzerland) is a recombinant humanized anti-IL-6R monoclonal antibody that acts as an IL-6 antagonist [18]. The hypothesis that inhibition of IL-6 signaling with tocilizumab can result in a significant improvement in the signs and symptoms of systemic JIA appears to have been substantiated in Phase II [19] and Phase III [20] clinical trials for children with systemic JIA, which demonstrated a marked reduction in inflammatory responses and an improvement in osteoporosis and growth retardation. The results of these clinical trials indicate that tocilizumab treatment generally has a good safety profile and improves health-related quality of life in children with systemic JIA. Tocilizumab appears to provide an additional option for those children who have recurrent inflammatory episodes. In addition, the blockade of IL-6R by the monoclonal antibody tocilizumab has a distinct mechanistic action on the IL-6 signaling pathway, that is, molecular intervention. Thus, the alterations in clinical manifestations and laboratory findings during tocilizumab treatment can be attributable to the normalization of IL-6 and sIL-6R levels, indicating that clinical inflammatory manifestations such as fever, sickness behavior, osteoporosis and growth retardation, and laboratory abnormalities such as increased levels of acute-phase proteins and chronic anemia are direct or indirect functions of the IL-6 signaling pathway.

Overview of current therapy

Children with systemic JIA have a higher rate of etanercept failure than other chronic arthritis subtypes, indicating that TNF-α is not the only cytokine implicated in the pathogenesis of the disease [21]. Although serum concentrations of IL-1 are not increased in this disease, dysregulation of IL-1 might play a part in the pathogenesis [22]. Case reports and an early uncontrolled study have suggested that treatment with anakinra, an IL-1 receptor antagonist, might be effective in patients with this illness, but MAS still occurred despite treatment with anakinra [23]. Recently, a trial of anakinra for patients with systemic JIA was carried out in France, and less than half of the patients achieved a marked and sustained improvement [24]. Anakinra has not been approved for patients with systemic JIA by the government in either Japan or the USA. Thus, tocilizumab is the only approved drug for children with systemic JIA in Japan. Fortunately, trials of tocilizumab for patients with systemic JIA are now making progress in the EU and the USA, and thus, in the near future, tocilizumab will hopefully be approved and available worldwide.

Clinical & laboratory features of systemic JIA

Systemic JIA

Children with JIA represent a clinical heterogeneity of phenotypes. According to the ILAR classification criteria (Edmonton, 2001) [25], the systemic type of JIA is one of the JIA subtypes, which is unique among the chronic arthritides of childhood in several ways. In particular, the range and severity of characteristic extra-articular features mark this disease as a systemic inflammation with arthritis [1].

Systemic inflammatory manifestations with recurrent quotidian fever, fatigue, anorexia, skin rash and polyarthritis are present and are sometimes accompanied by serositis, lymphadenopathy and hepatosplenomegaly. Laboratory investigation shows markedly increased levels of CRP, SAA and other acute-phase reactants [1].

In the long-term course of the disease, severe arthritis progresses in half of the affected children, is resistant to treatment and can eventually result in significant disability [26]. Moreover, growth retardation, severe osteoporosis and compression are seen in most patients, and x-ray examinations and laboratory experiments suggest that enchondral ossification may be disturbed by the long-lasting inflammation. *In vitro* examination of IL-6 on ATDC5 cells, which are chondrogenic progenitor cells, indicated that IL-6 inhibits the early chondrogenesis of these cells. It was suggested that IL-6 might affect committed stem cells at a cellular level during chondrogenic differentiation of growth plate chondrocytes [27]. In these children, laboratory examination will frequently indicate anemia, hypoalbuminemia and hypergammaglobulinemia of chronic inflammatory disease. Consequently, children with recurrent inflammatory episodes develop amyloidosis [28]. Thus, the emerging consensus in the field of pediatric rheumatology is that since the clinical abnormalities and pathogenesis of systemic JIA are attributable to a breakdown of proinflammatory cytokine homeostasis, this disease should be viewed as an autoinflammatory syndrome rather than an autoimmune disease [9].

Macrophage activation syndrome

The most devastating complication of systemic JIA is MAS [2]. Approximately 7% of affected children progress to MAS, which is associated with serious morbidity and sometimes death. It can