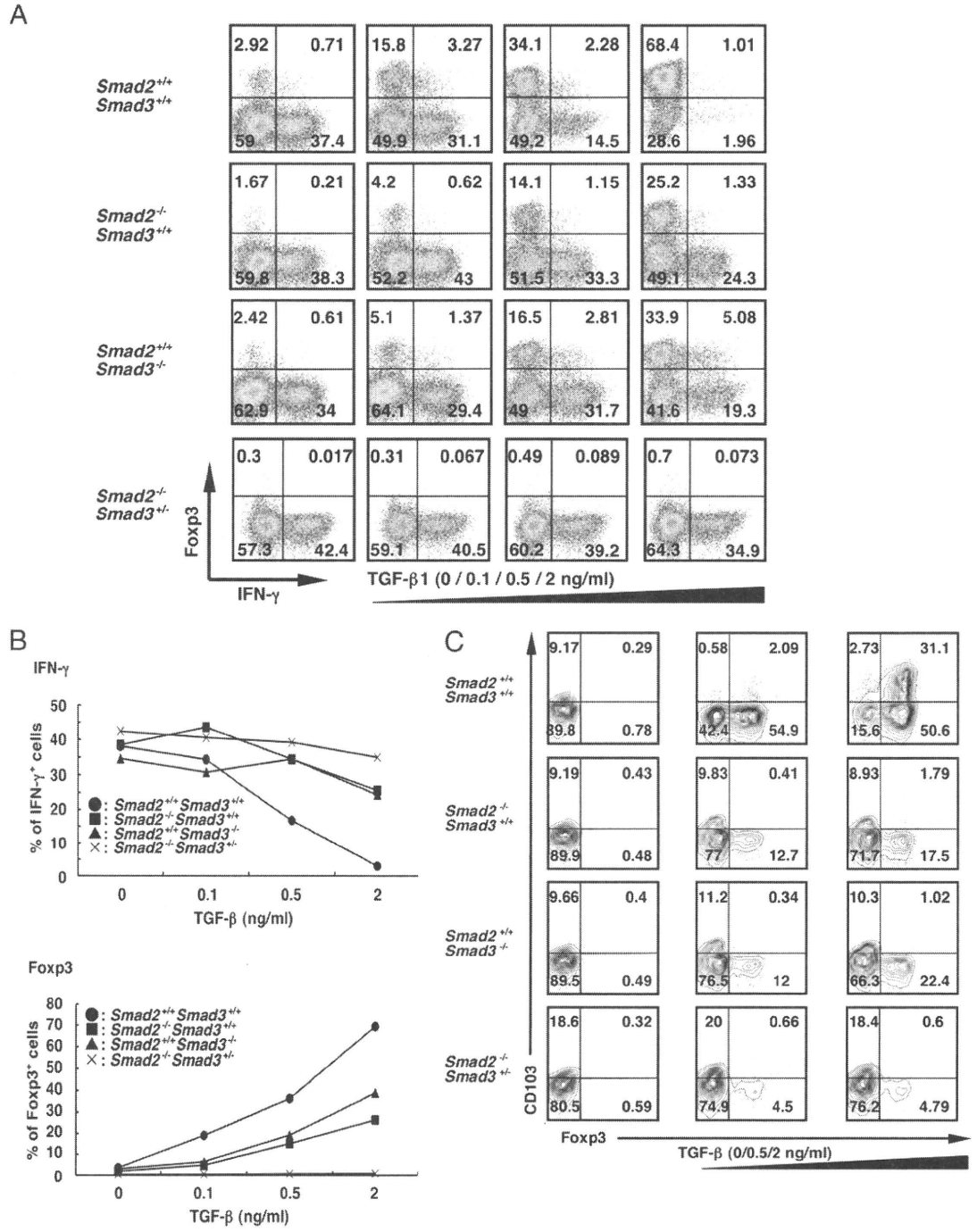

FIGURE 5. Relationship between Th1 cell development and Foxp3 induction in TGF-β-mediated Th1 cell regulation. *A*, FACS-sorted naive CD4$^+$ T cells from control (*Smad2*$^{+/+}$*Smad3*$^{+/+}$), *Smad2*-cKO (*Smad2*$^{-/-}$*Smad3*$^{+/+}$), *Smad3*-KO (*Smad2*$^{+/+}$*Smad3*$^{-/-}$), and 2cKO/3hetero (*Smad2*$^{-/-}$*Smad3*$^{+/-}$) mice were stimulated under the Th1-skewing condition with or without TGF-β1 at various concentrations. On day 4, cells were restimulated for 5 h with PMA and ionomycin. Brefeldin A was added to cultures <1 h after restimulation began. Cells were assessed for IFN-γ production and Foxp3 expression by intracellular staining. Data shown are representative of three independent experiments in triplicate with similar results. The percentage of IFN-γ$^+$ cells (*left panel*) and Foxp3$^+$ cells (*right panel*) of triplicate samples is shown in *B*. Each graph shows means ± SD. Data shown are representative of three independent experiments with similar results. *C*, FACS-sorted *Smad2*$^{+/+}$*Smad3*$^{+/+}$, *Smad2*$^{-/-}$*Smad3*$^{+/+}$, *Smad2*$^{+/+}$*Smad3*$^{-/-}$, and *Smad2*$^{-/-}$*Smad3*$^{+/-}$ naive CD4$^+$ T cells were cultured with anti-CD3e/CD28 and 10 ng/ml of IL-2 in the absence or presence of TGF-β at various concentrations for 4 d. Cells were stained for surface CD103 and intracellular Foxp3 and assessed by flow cytometry. Data shown resulted from experiments that were repeated two times with consistent results.

data suggest that Smad2/3 positively regulates Th17 differentiation by suppressing Th17-inhibitory cytokine production.

Next, to confirm a dispensable effect of Smad2/3 on Th17 differentiation, we examined TGF-β+IL-6–mediated induction of RORγt, a master regulator of Th17 cells (29). As expected from microarray analysis (Fig. 3*A*), the early induction of RORγt normally occurred in *Smad2*$^{-/-}$*Smad3*$^{+/+}$, *Smad2*$^{+/+}$*Smad3*$^{-/-}$, or *Smad2*$^{-/-}$*Smad3*$^{-/-}$ CD4$^+$ T cells in the Th17-skewing condition (Fig. 7*F*). This suggests that both Smad2 and Smad3 were not involved in the induction of RORγt by TGF-β. These results indicate that TGF-β mediates RORγt induction via the Smad-independent pathway. However, the TGF-β–Smad pathway is still required for

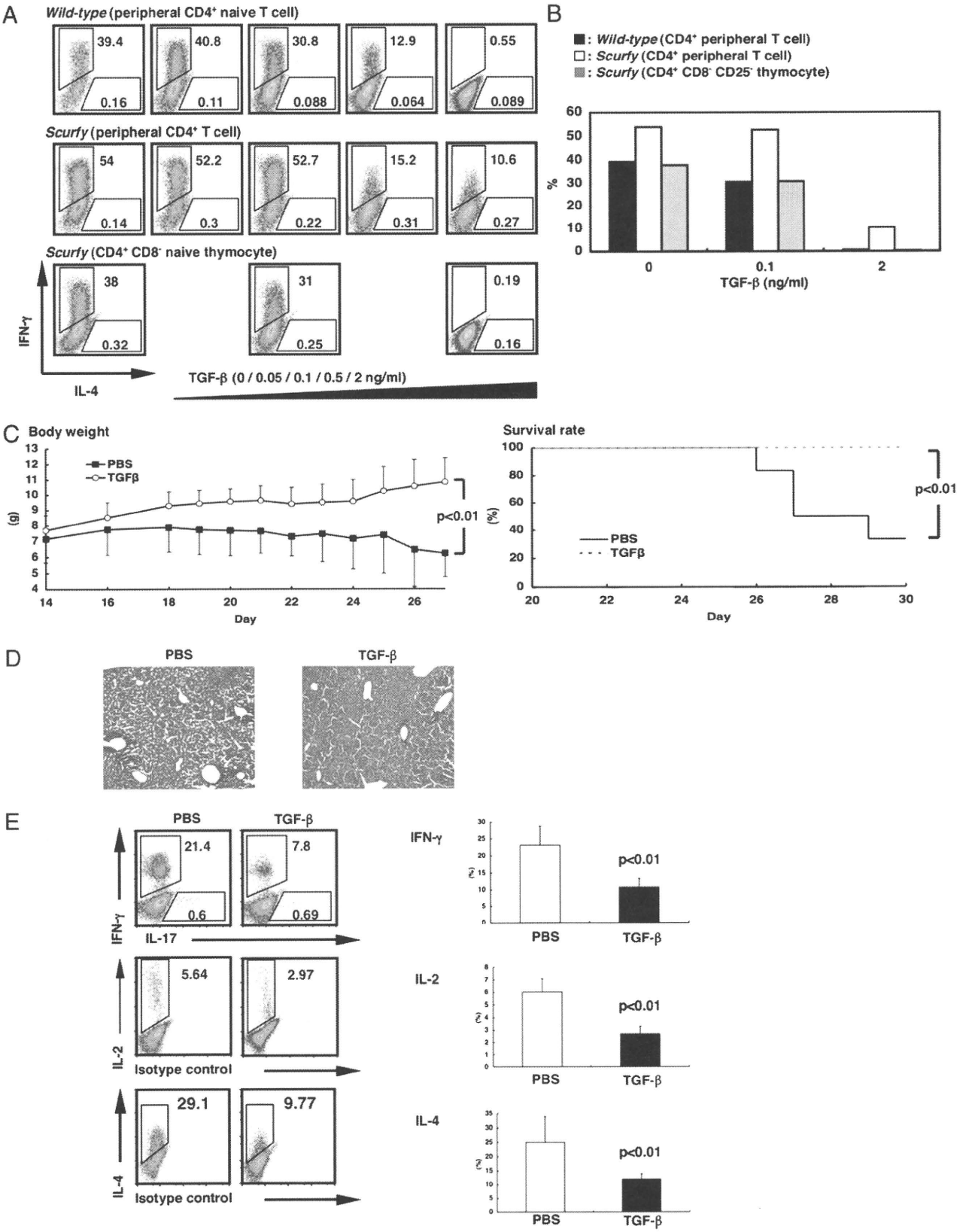

FIGURE 6. Foxp3-independent TGF-β–mediated suppression of Th1 cell development *A*, FACS-sorted peripheral naive CD4$^+$ T cells or thymic naive CD4$^+$ T cells from C57BL/6 (WT) or *scurfy* (*scurfy*) mice were cultured in the Th1-skewing condition with or without TGF-β1 at various concentrations for 4 d. Cells were then restimulated for 5 h with PMA and ionomycin. Brefeldin A was added to cultures <1 h after restimulation began. Cells were assessed for IFN-γ and IL-4 expression by intracellular staining. Data shown are representative of two independent experiments in triplicate with similar results. *B*, A bar graph shows the mean percentage of IFN-γ–producing cells in total CD4$^+$ T cells cultured as described in *A*. *C*, Male *scurfy* mice at 2 wk of age were daily injected i.p. and s.c. with PBS (closed square, solid line, $n = 10$) or 100 ng/ml recombinant hTGF-β1 (open circle, dotted line, $n = 12$) twice per day

efficient Th17 cell development by suppressing Th1 and Th2 cytokines. This finding is consistent with the recent finding that Smad4 is not required for the induction of RORγt (47).

Discussion

In this study, we demonstrated for the first time that Smad2 and Smad3 are essential for TGF-β–mediated regulation of T cell immunity. *Smad2*-cKO mice exhibited some immunologic disturbance, but not at lethal levels. When certain inflammatory events occurred, such as dextran sodium sulfate-induced colitis, *Smad2*-cKO mice failed to maintain the immunologic homeostasis. Furthermore, we showed that Smad2 and Smad3 have functional redundancy, sharing common roles in T cells. One Smad partially compensated for the deficiency of another in particularly important functions, such as Foxp3 induction or maintenance or Th1 cell suppression. Consistent with this idea, Smad2–three-dimensional–KO mice because of severe inflammation, as did *TGF-β1* KO mice. We demonstrated in this study that TGF-β–mediated induction of Foxp3 is also dependent on both Smad2 and Smad3 signaling. Furthermore, we demonstrated that both Smad2 and Smad3 play an important role in the maintenance of Foxp3 expression in nTregs. Like *TGF-β1* KO mice, Smad2–three-dimensional–KO mice possess nTregs, but their number was decreased especially at the periphery. We showed that nTregs lost the expression of Foxp3 in vitro, but TGF-β partially suppressed the loss of Foxp3. Conversion of Foxp3+ cells to Foxp3− cells was much more drastic in $Smad2^{-/-}$ $Smad3^{+/-}$ nTregs, and TGF-β–mediated Foxp3 maintenance was severely impaired in $Smad2^{-/-}Smad3^{+/-}$ nTregs. Molecular basis for such conversion remains to be investigated.

Currently, it is not known whether similar mechanisms were used for Foxp3 maintenance by TGF-β in nTregs and Foxp3 induction by TGF-β in naive $CD4^+$ T cells. Recently, it has been shown that Foxp3 expression is not stable and can be extinguished in Tregs that divert from their original phenotype and become Th17 cells (48, 49) or follicular helper T (Tfh) cells (50) both in vitro and in vivo. Zhou et al. (51) revealed that the loss of Foxp3 in Tregs under autoimmune conditions can result in the conversion of suppressor T cells into highly autoaggressive lymphocytes called *exFoxp3 cells*, which do not currently express Foxp3 but expressed it previously. In addition to the loss of the phenotypic characteristics of Tregs, exFoxp3 cells acquire some new features, including the ability to produce effector cytokines, such as IFN-γ and IL-17. In this study, both Smad2 and Smad3 were essential for the TGF-β–mediated maintenance of Foxp3 expression in Tregs. Our preliminary data also suggest that TGF-β prevents such conversion of Tregs into IFN-γ–producing cells in vitro, which could be one of the mechanisms of immune tolerance induced by TGF-β. It is possible that Smad2/3 interacts with the Foxp3 promoter, thereby maintaining Foxp3 expression and inhibiting pathogenic conversion of nTregs.

It is intriguing that Smad2 and Smad3 shared similar functions in T cells, because Smad3 binds to DNA, whereas Smad2 generally lacks any DNA-binding activity (18). Because Smad2 and Smad3 function redundantly for the regulation of >60% of genes in $CD4^+$ T cells, it is likely that Smad2 and Smad3 have a common partner and bind to a similar region. Our data suggest that Smad3 and Smad2 are involved in the TGF-β–mediated Foxp3 induction. However, it remains unclear how Smad2 regulates the gene expression of Foxp3. The cellular protein content of Smad2 is much higher than that of Smad3. Therefore, Smad2 may activate Foxp3 promoter by binding to the same Smad-binding site as Smad3, even though Smad2 has much lower affinity than Smad3. Another possibility is that Smad2 interacts with other transcription factors, thereby regulating Foxp3 expression by binding to the region of the Foxp3 enhancer different from the Smad3-binding element. We could not rule out the possibility that uncharacterized transcription factors induced by Smad2 contribute to the Foxp3 induction. Further study is necessary to determine the molecular mechanism of Smad2-mediated Foxp3 induction.

Smad4 is the most universal coregulator of canonical TGF-β signaling. However, there are significant differences between T cell-specific *Smad4*-KO mice and *Smad2/3*-DKO mice. For example, Smad4 deficeincy in T cells partially abrogated TGF-β–mediated Foxp3 induction (47), whereas it was completely diminished in $Smad2^{-/-}Smad3^{-/-}$ $CD4^+$ T cells. Thus, there may be a common partner for Smad2 and Smad3 in addition to Smad4. Tob, a member of an anti-proliferative gene family, was shown to bind to Smad2, thereby inhibiting IL-2 production (52). However, the interaction between Tob and Smad3 was not observed. Runx1/3, NF-AT, AP-1, and NF-kB also play essential roles in cytokine production from $CD4^+$ T cells, and they can be potential interaction partners of Smad2 and/or Smad3 (53). It is notable that an essential NF-AT binding site is present, adjacent to the Smad binding elements in the Foxp3 promoter (22). However, the interaction of these transcription factors for both Smad2 and Smad3 has not been reported. Finding a common interaction partner of Smad2 and Smad3 could be the next step in understanding the molecular basis for the immunoregulatory effects of TGF-β.

Smad2 exists in two isoforms, one of which is an alternatively spliced variant of Smad2 with a deletion of exon 3 and has a functional DNA binding domain (54). Because this spliced variant is thought to be important in certain aspects of mouse embryo development (55), we measured the expression of this variant in T cells. We did not observe expression of this spliced form of *Smad2* in naive $CD4^+$ T cells (data not shown), which makes it unlikely that this spliced form functions in $CD4^+$ T cells.

Recent studies demonstrated that TGF-β–induced Foxp3 antagonizes RORγt, which is also induced by TGF-β, to inhibit Th17 cell differentiation (13, 14). However, it has not yet been determined how TGF-β induces the distinct transcription factor Foxp3 or RORγt. We show that the induction of Foxp3 was completely dependent on Smad signaling, but the induction of RORγt was independent of it. However, we have found that Smad signaling indirectly promotes the inducing of Th17 cell differentiation by suppressing its inhibitory cytokine production. These data indicate that TGF-β plays an important role in Th17 cell development in a Smad-dependent and Smad-independent

for 2 wk. Body weight change and survival rate were monitored every day. Each graph shows means ± SD. Data shown are representative of two independent experiments with consistent results. Statistical differences were verified by paired Student *t* test. *D*, Histologic analysis by H&E staining of livers from PBS-treated ($n = 5$) and TGF-β–treated *scurfy* mice ($n = 5$) 10 d after each treatment began (original magnification ×20). Data shown are representative from one of the three tissue samples with similar results. *E*, Cytokine profiles of freshly isolated $CD4^+$ T cells from cervical lymph nodes of PBS- ($n = 5$) or TGF-β–treated ($n = 5$) *scurfy* mice 10 d after treatment began. A bar graph shows the mean percentage of indicated cytokine-producing cells in total $CD4^+$ T cells. Freshly isolated cells were restimulated for 5 h with PMA and ionomycin, and cytokines were detected with intracellular immunostaining (*right*) or ELISA (*left*). Statistical differences were verified by paired Student *t* test. Data shown are representative of two independent experiments. WT, wild-type.

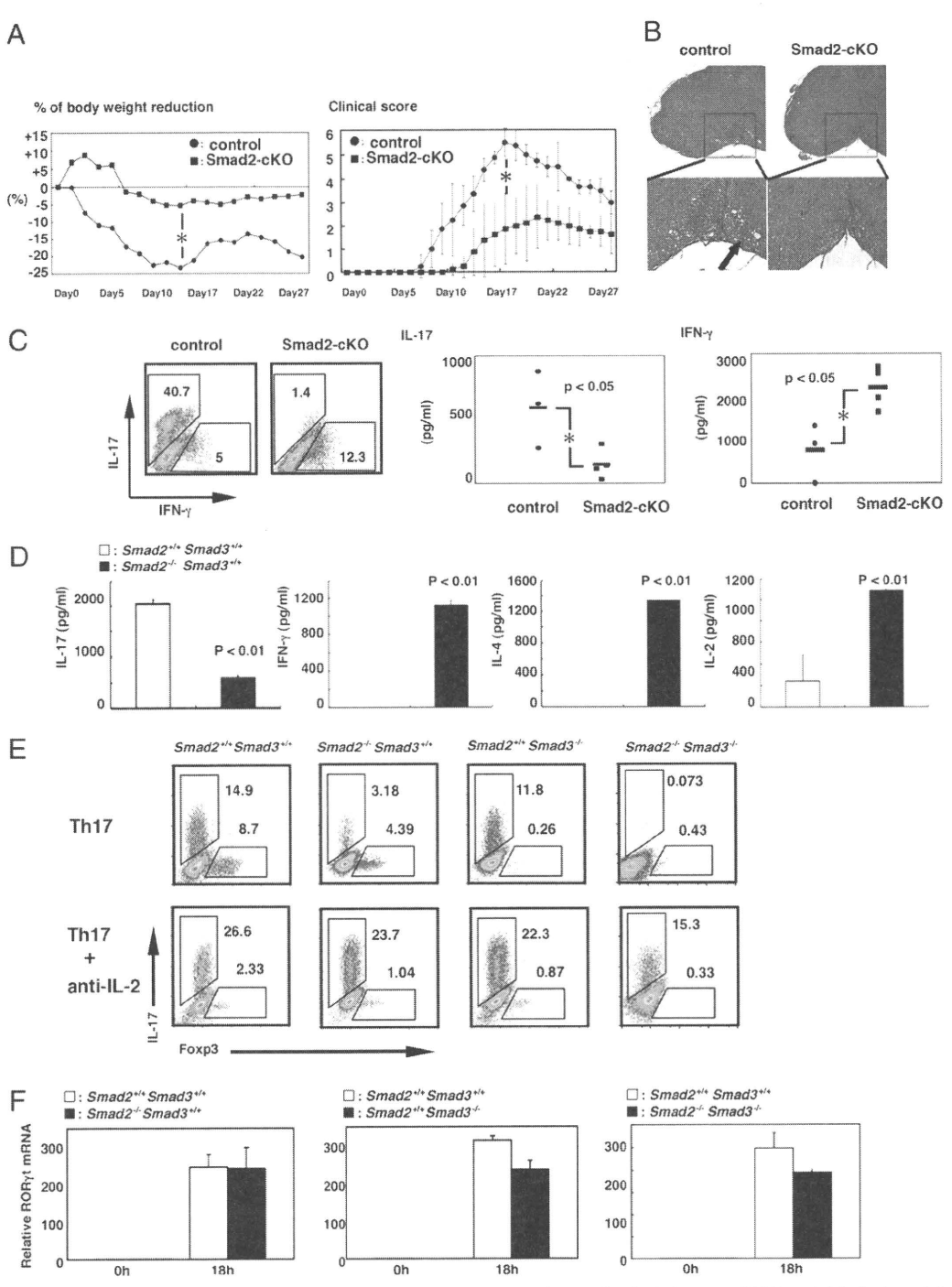

FIGURE 7. TGF-β–Smad signaling is essential for effective Th17 cell development, but not for the induction of RORγt. *A*, The body weight loss and clinical scores of neurologic impairment were recorded daily for control ($n = 8$) and *Smad2*-cKO ($n = 8$) mice after the induction of EAE. The experiments were repeated twice, with five to eight mice in each group, and similar results were obtained each time. The graph shows means ± SD. Statistical differences were verified by paired Student *t* test. $*p < 0.01$ between groups. *B*, Histologic analysis by H&E staining of spinal cords ($n = 5$) of mice 30 d after the induction of EAE (original magnification ×20 and ×200). Black arrows indicate demyelination in the subpial regions. Data shown are representative of two independent experiments with similar results. *C*, Ten days after the immunization, $CD4^+$ T cells were isolated from the draining lymph nodes in control ($n = 3$) and *Smad2*-cKO ($n = 4$) mice. Cells were restimulated for 5 h with PMA and ionomycin for intracellular immunostaining (*left panels*). ELISA of cytokines in supernatants of Ag-specific restimulated $CD4^+$ T cells of draining lymph nodes (*right panels*). Cells were cocultured for 24 h with irradiated splenocytes in the presence of 20 μg/ml MOG. These experiments were repeated twice with similar results. *D*, $Smad2^{+/+}Smad3^{+/+}$ or $Smad2^{-/-}Smad3^{+/+}$ $CD4^+$ T cells were cultured under the Th17-skewing condition for 5 d and then harvested and restimulated with 5 μg/ml plate-bound anti-CD3e Abs for 12 h. The levels of indicated cytokines in the supernatants were determined by ELISA. Experiments were repeated twice with similar results. The graph shows means ± SD. Statistical differences were verified by paired Student *t* test. *E*, FACS-sorted naive $CD4^+$ T cells from control ($Smad2^{+/+}Smad3^{+/+}$), *Smad2*-cKO ($Smad2^{-/-}Smad3^{+/+}$), *Smad3*-KO ($Smad2^{+/+}Smad3^{-/-}$), and *Smad2/3*-DKO ($Smad2^{-/-}Smad3^{-/-}$) mice were cultured in the Th17-skewing condition in the absence or presence of 10 μg/ml of anti-IL-2 Abs for 5 d. Cells were restimulated for 5 h with PMA and ionomycin. Cells were fixed, stained for intracellular Foxp3 and IL-17, and assessed by flow cytometry. Experiments were repeated three times with similar results. *F*, FACS-sorted naive $CD4^+$ T cells with indicated genotypes were stimulated under the Th17-skewing condition for 18 h, and mRNA expression of RORγt was assessed by real-time RT-PCR. The experiment was performed in duplicate. Data shown were normalized to the expression of a reference gene, HPRT. The expression for RORγt gene of $Smad2^{+/+}Smad3^{+/+}$ $CD4^+$ T cells at 0 h was set as 1. The graph shows means ± SD. Data represent two independent experiments with similar results.

manner. Furthermore, $CD4^+$ T cells deficient in Smad2/3 could be useful objects of study for identifying the TGF-β signaling pathway for RORγt induction.

Surprisingly, LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/-}$ mice in which T cells express only one fourth of Smad2/3 transcription factors can survive without any severe inflammatory diseases. In these mice, most of the $CD4^+CD25^-$ T cells showed memory phenotypes in vivo, and TGF-β hardly induced Foxp3 and suppressed IFN-γ production in naive $CD4^+$ T cells in vitro. It would be interesting to know how the immunologic tolerance was maintained in LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/-}$ mice. This may be due to more nTregs in these mice (T. Takimoto, unpublished data).

$CD103^+$ $Foxp3^+$ Tregs have been shown to possess potent immunosuppressive activity (39); therefore, we examined the induction of CD103 in iTregs. We demonstrated that CD103 expression was strongly induced by TGF-β in a Smad-dependent manner in iTregs. We could not detect CD103 in *Smad2*$^{-/-}$ *Smad3*$^{+/-}$ iTregs. However, we noticed that the number of CD103-expressing $CD4^+$ T cells in 2cKO/3hetero mice was similar to that in control *Smad2*$^{+/+}$*Smad3*$^{+/+}$ mice (data not shown). Thus, CD103 expression in vivo may be regulated by a mechanism other than TGF-β. One candidate is IL-2, because a previous report has shown that IL-2, rather than TGF-β, upregulates CD103 expression in vivo (38). Expression of CD103 in nTregs in a TGF-β–independent mechanism might explain the lack of inflammatory phenotypes in 2cKO/3hetero mice.

In this study, we discovered several Smad2/3-independent genes that were upregulated or downregulated by TGF-β. We found that *eomes* is downregulated by TGF-β in a Smad2/3-independent mechanism. It has been reported that *eomes*, a paralogue of T-bet, plays an important role for IFN-γ production (56). TGF-β might suppress IFN-γ production in $CD4^+$ T cells by inhibiting the expression of *eomes* as well as T-bet. We observed that CD73 and CCR8 was induced by TGF-β in a Smad-independent manner. CD73, an ectonucleotidase, has been shown to be highly expressed in nTregs and could be an immunosuppressive factor (57). CCR8 may be involved in migration of iTregs or Th17 cells into inflammatory cites in Smad2/three-dimensional KO mice. However, further investigation is necessary to clarify the role of these Smad-independent gens in TGF-β–mediated immune tolerance.

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Disclosures

The authors have no financial conflicts of interest.

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Corrections

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

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Summary

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

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

Introduction

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

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

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

Materials and methods

Patients

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

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

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

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

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

Total RNA extraction and RNA amplification

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

Microarray analysis

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

Accession number

GSE17975 (Gene Expression Omnibus).

Pathway analysis of microarray results

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

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

Quantitative real-time RT–PCR

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

Flow cytometry

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

Results

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

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

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

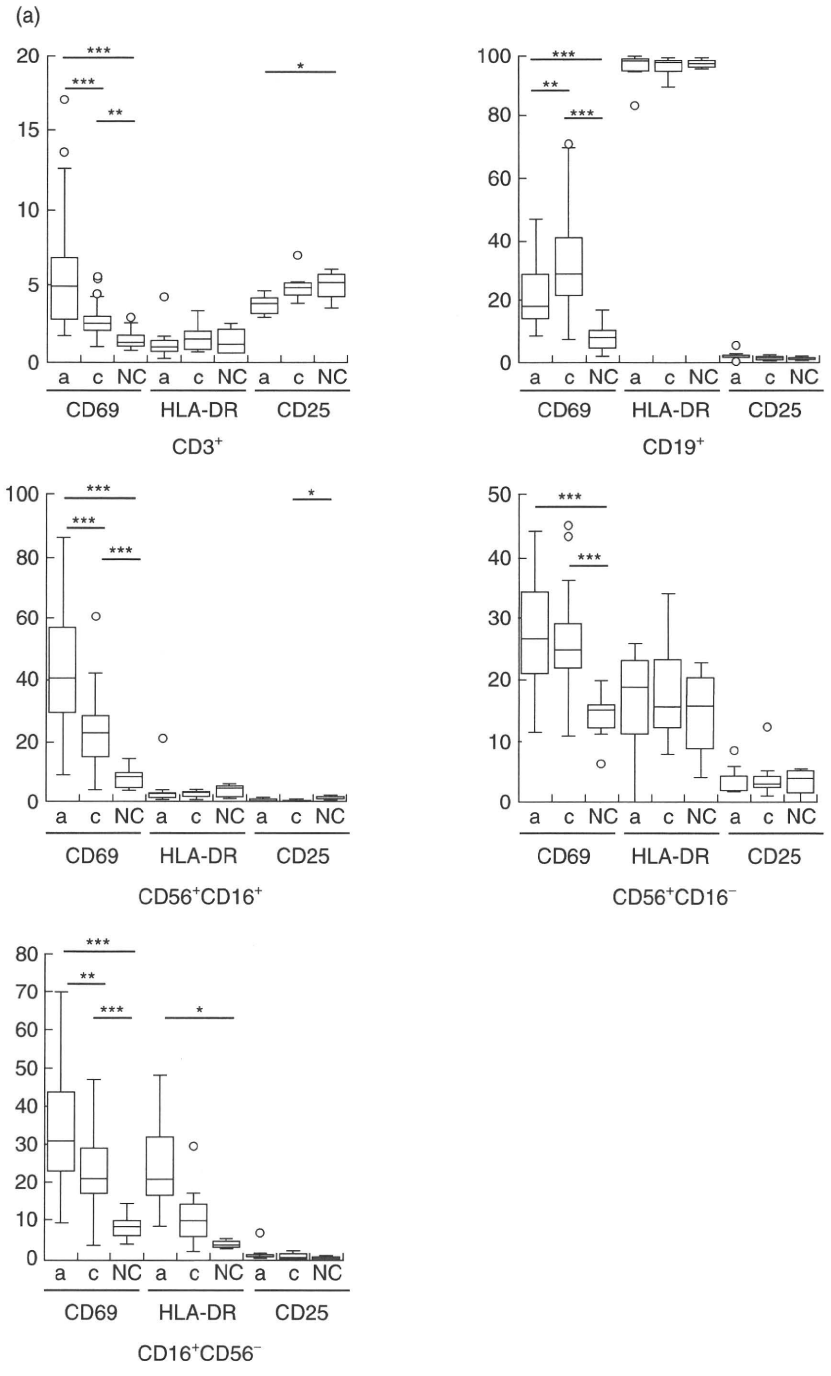

Fig. 1. Flow cytometric analysis of the activation markers on T, B and natural killer (NK) cells at acute phase of Kawasaki disease (KD). (a) The proportions of activated T, B and NK cells in the peripheral blood of seven patients with KD and 15 healthy control subjects were analysed by flow cytometry.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

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

Microarray analysis of the gene expression in PBMCs from KD patients

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

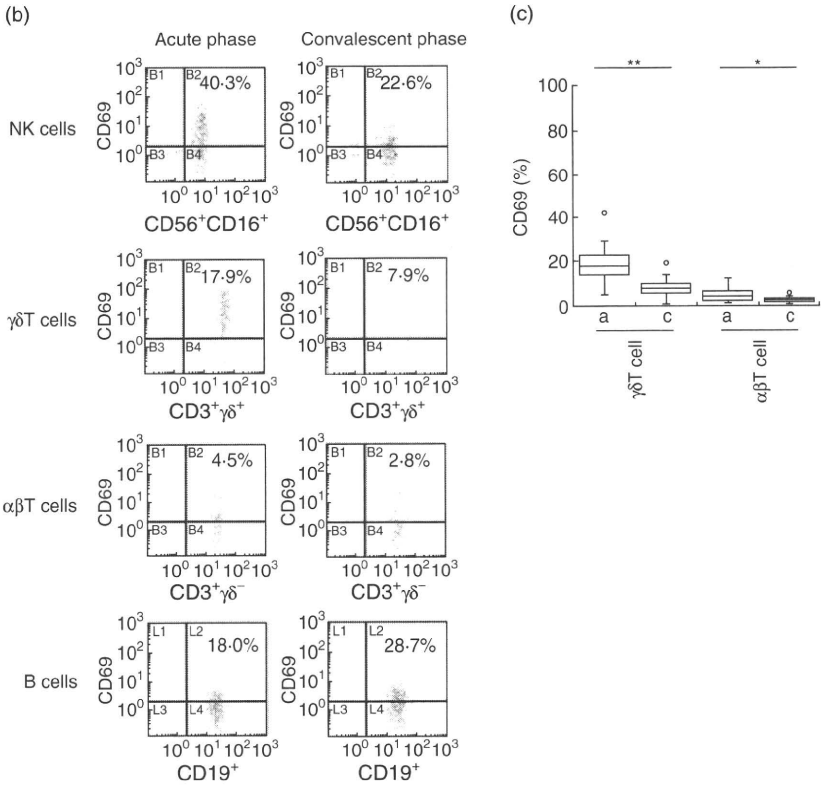

Fig. 1. *Continued*

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

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

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

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

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

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

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

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

Cytokine analyses in KD patients

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

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

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

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

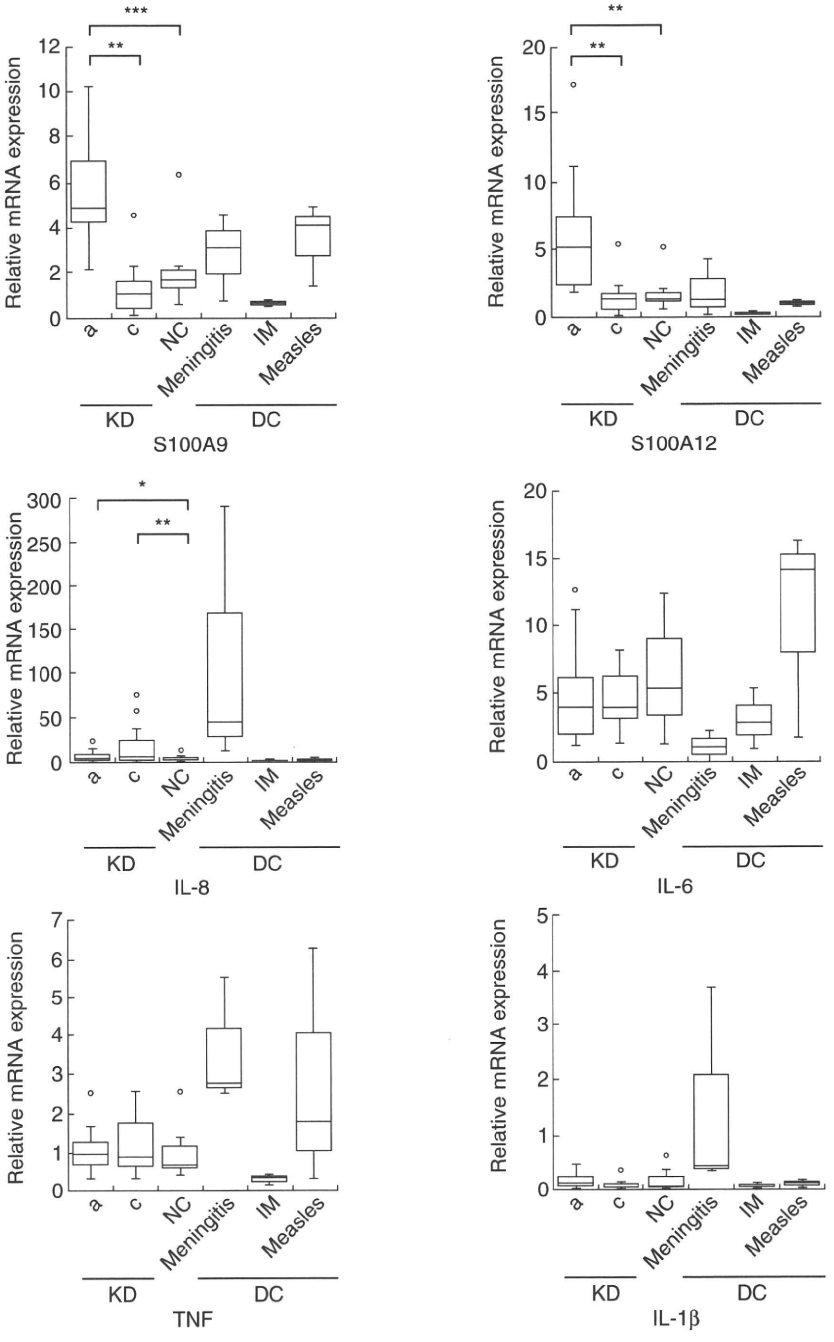

Fig. 2. Relative expression levels of *S100A9*, *S100A12*, *IL8*, *IL6*, *TNF* and *IL1B* genes in peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). The gene expression levels of these cytokines were determined by the reverse transcription–polymerase chain reaction (RT–PCR)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

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

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

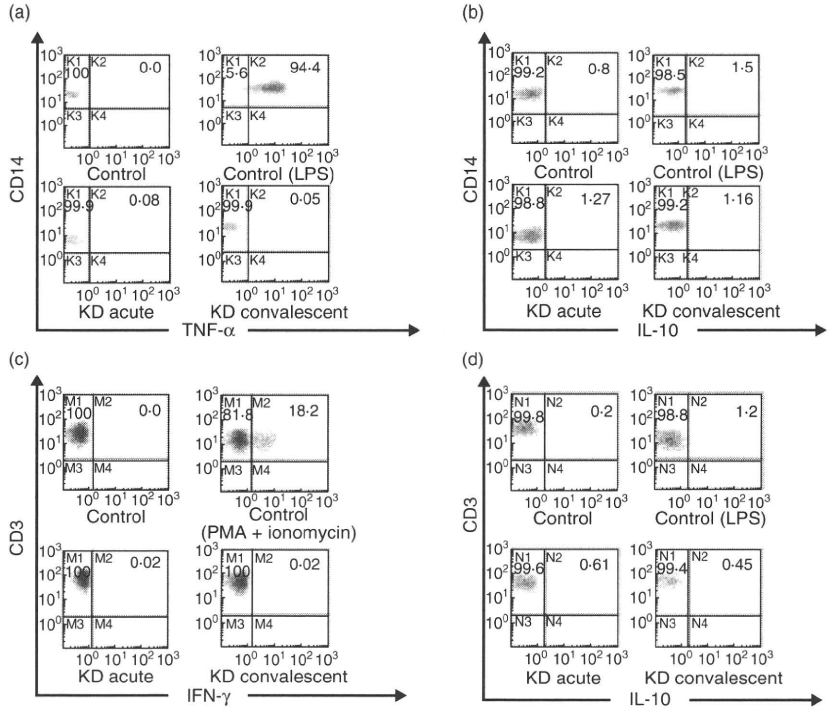

Fig. 3. Flow cytometric analysis of intracellular cytokine production of peripheral blood mononuclear cells (PBMCs) at acute phase of Kawasaki disease (KD). Intracellular cytokine production in PBMCs at acute and convalescent phases of KD was analysed by flow cytometry. Representative data of tumour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

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

Discussion

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

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

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

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

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

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

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Disclosure

None.

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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

be considered to be a process of the disease rather than a disease itself due to hypercytokinemia [29]. It can be difficult to diagnose patients as having MAS at a given time because MAS is a disease in which a series of events such as thrombocytopenia, endothelial cell damage, coagulation abnormalities, mitochondrial permeability transition and multiple organ failure occurs, fades away, and worsens in a couple of days.

Macrophage activation syndrome is clinically characterized by the rapid development of fever, hepatosplenomegaly, lymphadenopathy, purpura and mucosal bleeding. In our experience, an exact diagnosis will be made when precise laboratory examinations are performed during the course of the process. Laboratory studies primarily indicate the presence of hematocytopenia, and then, combinations of serum β2-microglobulin and ferritin, elevated tissue-derived enzymes such as mitochondrial aspartate aminotransferase (mAST), lactate dehydrogenase (LDH) and creatine phosphokinase (CK), hypoalbuminemia, increased levels of fibrin degradation products (FDP-E, D-dimer) and elevated triglycerides. A bone marrow examination, if performed with proper timing, may show active phagocytosis by macrophages and histiocytes [30]. Accompanying the progression of the process, finally, increases in creatinine, alanine aminotransferase (ALT) and amylase levels are present, indicating multiple organ failure.

The pathogenesis of MAS remains to be established. The first report described the pathogenic role of TNF-α in MAS [2]. The increased levels of IFN-α, TNF-α and other proinflammatory cytokines correlate with the rapid development of clinical symptoms and the progression of abnormal laboratory parameters [31]. In addition, since systemic JIA patients display decreased levels of perforin in NK cells and diminished NK cell function, the recent investigation suggested that perforin gene (*PRF1*) mutations also play a role in the development of MAS in systemic arthritis patients [5]. Thus, MAS would be the transition form of the disease process from IL-6 cytokinemia in systemic JIA to multiple proinflammatory cytokinemia for the background of *PRF1* gene mutation and diminished NK cell function.

Biologic function of IL-6 & tocilizumab

IL-6 is one of the most pleiotropic cytokines known that is involved in regulating a wide variety of inflammatory and immune functions, B-cell differentiation, T-cell growth, acute-phase reactions and hematopoiesis [32,33].

The first step in the induction of the transduction signals by IL-6 is the binding to its IL-6R, which is either localized at the cell surface or present in a soluble form in serum. The association of the IL-6/IL-6R complex with another receptor, gp130, forms a high-affinity complex that triggers specific transduction signals. Three members of the janus kinase family, JAK1, JAK2 and TYK2, are closely related to gp130 and are rapidly activated in the presence of IL-6 [34]. These kinases phosphorylate the tyrosine residues of the gp130 cytoplasmic domain, which allows the recruitment and phosphorylation of transcriptional factors of the signal transducers and activators of transcription family (STAT1 and STAT3) [35]. Once activated, the STAT proteins may activate different genes. Thus, the blockade of IL-6R by tocilizumab can result in invalidity of the formation of phosphorylated STAT proteins, which inhibits inflammatory responses [36].

Pathogenesis of systemic JIA & MAS

The pathogenic role of proinflammatory cytokines in systemic JIA has long been investigated. IL-6 is reported to be markedly elevated in blood and synovial fluid [37]. The IL-6 level increases before each fever spike and correlates with the systemic activity of the disease, arthritis and an increase in acute-phase reactions [38]. Abnormalities in the regulation of IL-6 are also responsible for the thrombocytosis and anemia seen in this disease [7]. *In vitro* studies have documented increased production of IL-6 by peripheral blood mononuclear cells from patients with systemic JIA [39]. An imbalance in IL-6 homeostasis is suggested by the observations that sIL-6R concentrations are significantly increased in children with systemic JIA. Growth retardation was found in IL-6 transgenic mice overexpressing human IL-6, similar to that in children with systemic JIA [8]. In contrast to IL-6, TNF-α levels are not increased in systemic JIA. Taken together, IL-6 and IL-6R might play a central role in the induction and progression of systemic disease and its complications. However, direct evidence in humans is not yet available.

During the course of recurrent inflammatory episodes of systemic JIA, MAS often follows a viral infection, such as Epstein–Barr virus or influenza virus [40]. Changes in medications, as well as the introduction of nonsteroidal anti-inflammatory drugs, gold compounds or methotrexate, were reported to trigger the syndrome [41]. However, it seems likely that changes in medications were coincidental, that is, occurring in a child who was susceptible to MAS and who required additional therapy for uncontrolled systemic JIA. The histopathologic features of skin biopsy specimens are the presence of microthrombi and endothelial cell proliferation [42], indicating that due to overwhelming proliferation of various proinflammatory cytokines such as IFN-γ and TNF-α, continuing damage to endothelial cells and the resultant vasculitis induce disseminated intravascular coagulation (DIC) and, subsequently, multiple organ failure. This is the whole spectrum of clinical MAS.

Clinically, MAS starts with thrombocytopenia and leukocytopenia, and then abrupt improvements in erythrocyte sedimentation rate (ESR) and CRP levels can be seen. Fibrin degradation products (FDP-E, D-dimer) and hypofibrinogenemia are present, indicating DIC due to activated and destroyed endothelial lining of the vasculature by combinatorial effects of proinflammatory cytokines [43]. Markedly increased levels of cytokine-induced proteins, serum ferritin by TNF-α [44] and β2-microglobulin by IFN-γ [45] can be observed during this stage. Subsequently, rising levels of serum mAST, LDH and CK indicate apoptosis due to mitochondrial permeability transition by TNF-α [46], which can solely be protected by cyclosporine [47]. In the late phase of MAS, increased levels of triglycerides and decreased levels of total cholesterol are present due to inhibited lipoprotein lipase activity by TNF-α [48]. Finally, multiple organ failure along with DIC will progress.