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Increased TLR2 and TLR4 Expression in Peripheral Neutrophils Isolated from Kawasaki Disease

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Background: Since neutrophils are important in innate immunity against invading microorganisms, this study investigated the expression of TLR2 and TLR4 in neutrophils derived from infants and children aged 12 years or younger as a marker of such immune functions.
Methods: We enrolled 70 patients aged 12 years or younger: 18 patients with microbial infections, 14 patients with Kawasaki disease (KD), and 38 patients without infection, who first presented to the pediatric department at Toho University Omori Medical Center, Tokyo, Japan. The expression of TLR2 and TLR4 in neutrophils was assayed by flow cytometry, using phycoerythrin-conjugated antihuman monoclonal TLR2 or TLR4.
Results: TLR2 and TLR4 expression in neutrophils derived from patients aged 1–5 years old without infection were significantly higher than in patients younger than 1 year and 6 years or older. In addition, TLR2 expression was greater than TLR4 expression in patients aged 12 years or younger with and without infections. The coefficients between expression of TLR2 and TLR4 in patients with and without infections were 0.8730 and 0.8393 respectively. Similar findings were obtained in patients with KD, and the coefficient was 0.9095. C-reactive protein (CRP) serum concentration and TLR2 and TLR4 expression in neutrophils gradually decreased during the follow-up period in patients with KD.
Conclusions: The function of neutrophils matured between the ages of 1 and 5 years. The pattern of decrease in CRP levels was correlated to levels of toll-like receptors (TLRs) expression, especially TLR2, in KD.

Introduction

NEUTROPHILS ARE EXTREMELY important in the mechanisms of both infection response and innate immunity, and provide early defenses against invading microorganisms. Neutrophils are the main component of peripheral blood leukocytes, and transit rapidly to the sites of infections, where they act to limit the infection and allow recruitment and activation of other immune cells through the release of inflammatory mediators and antimicrobial products, resulting in clearance of the invading pathogen and, ultimately, the initiation of an adaptive immune response.[1] Neonates have immature immune system, which may explain the susceptibility of neonates to infection, reflected in the finding that approximately one third of neonatal deaths worldwide is caused by infectious diseases.[2–4] Human defenses against pathogens are partly based on leukocytes, such as granulocytes and monocytes, which express pattern recognition receptors (PRRs) that recognize specific structures present on microorganisms, termed pathogen-associated molecular patterns (PAMPs).[5,6] Toll-like receptors (TLRs) such as the PRRs recognize microbial PAMPs and alert the host's immune system to the presence of invading microbes.[7] TLRs can be located on the surfaces or internally in cells, and are expressed by various cells and tissues in the body including leukocytes.[7,8] Neutrophils from humans are now known to express all TLRs except TLR3 and TLR7. Neutrophil TLR ligation modulates various neutrophil responses, including migration, phagocytosis, programmed cell death, degranulation, and ROS generation. In particular, the role of TLR2 and TLR4 has been studied in infections in detail. It is well known that TLR2 is the receptor for peptidoglycan, lipoteichoic acid, and lipoprotein, and that TLR4 is the receptor for lipopolysaccharide. TLR expression by neonatal neutrophils has not been clearly characterized, as most studies have focused on adults. Neonatal leukocytes function poorly. In particular, monocytes and neutrophils have reduced inflammatory responses.[1] In addition, there is little direct evidence that the function of neutrophils in infants and children is insufficient.

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Kawasaki disease (KD) is an acute febrile illness that predominantly affects infants and children, and is characterized by systemic vasculitis, including coronary artery involvement.[9,10] The clinical features of KD are well known, but the underlying immunopathogenetic mechanisms remain unclear, particularly the agent responsible for the development of coronary artery lesions. KD has been suggested to be an autoimmune disorder rather than an infectious disease.[11] Immunological abnormalities during the acute phase of KD are characterized by marked activation of the immune system, including functional activation of neutrophils and monocytes, and excessive production of inflammatory mediators such as cytokines, proteases, and toxic oxygen radicals.[11–13] These findings suggest that circulating neutrophils may be primed by particular factor(s) during the early phase of KD and, as a result, have increased susceptibility to IVIG (intravenous immunoglobulin)-induced apoptosis. On the other hand, other studies have proposed that KD is caused by an infectious agent, with suggestions ranging from bacteria such as staphylococci to viruses such as adenovirus.[14,15] In fact, antibodies to bacterial heat shock proteins were detected in plasma derived from patients with KD.[14] Surface enhancement of TLR2, but not of TLR4, on circulating CD14+ monocytes is augmented in KD patients,[15] and LPS-bound neutrophils were detected in the acute phase of KD,[12] indicating that infection may be associated with the occurrence of KD.

The present study first evaluated the expression of TLR2 and TLR4 as a functional marker of the activity to recognize PAMPs in neutrophils derived from infants and children aged 12 years or younger without infections to clarify the maturation age, and, second, investigated the possibility of infection as a causative factor in KD patients by comparing the expression of TLR2 and TLR4 in patients with infections and KD.

Materials and Methods

Reagents

VersaLyse was purchased from Beckman Coulter, Inc. (Brea, CA). Human monoclonal CD66abce antibodies conjugated to allophycocyanin, and human monoclonal TLR2 antibody conjugated to phycoerythrin and human monoclonal TLR4 antibody conjugated to phycoerythrin were purchased from Miltenyi Biotec, Inc. (Auburn, CA) and Acris Antibodies GmbH (Herford, Germany) respectively. Human CD66abce MicroBead Kit was purchased from Miltenyi Biotec Inc.

Study design and population

This study was reviewed and approved by the Toho University Ethics Committee on March 5, 2008, and July 25, 2012 (permission no. 1930 and no. 2403). We enrolled 18 patients aged 12 years or younger with microbial infections, 14 patients with KD who met the diagnostic criteria for KD established by the Japanese Kawasaki Disease Research Committee,[16] and 38 patients without infection, who first presented to the pediatric department at Toho University Omori Medical Center. Eight of the patients with KD were admitted to our hospital, so the expression levels of TLR2 and TLR4 in neutrophils and the concentration of C-reactive protein (CRP) in serum samples obtained from those inpatients could be followed up by the method described below. The inpatients received IVIG (2 g/kg/day for 1 day, Kenketu Venilon I; Teijin Pharma, Tokyo, Japan) and aspirin (30 mg/kg/day).

Assay of CRP

Blood samples (3 mL) were taken at the first visit from patients, and a portion (1 mL) was stored in plastic tubes to obtain serum samples. The other portion (2 mL) was stored in plastic tubes containing EDTA-2Na for neutrophil isolation. CRP serum concentrations were determined by a commercial chemiluminescence-immunoassay (CRPELISA Kits; Helica Biosystems, Inc., Santa Ana, CA).

Human neutrophil isolation

All steps were performed with ice water cooling according to the instructions provided by the manufacturer. VersaLyse was added to the sample tube containing 2 mL blood to a total volume of 10 mL, and then mixed gently. The mixture was allowed to stand for 10 minutes at room temperature, and turned several times during incubation. The mixture was centrifuged at 300 *g* for 10 minutes at room temperature, and then all supernatant was aspirated. Cells were washed twice with 10 mL of phosphate buffered saline (PBS), pH 7.4. The mixture was centrifuged at 300 *g* for 10 minutes at room temperature, and the supernatant carefully removed. The cell pellet was resuspended in 40 μL of PBS, and 10 μL of CD66abce-biotin was added. The mixture was gently agitated and incubated for 10 minutes in the refrigerator, and then 30 μL of PBS and 20 μL of anti-biotin MicroBeads were added, mixed gently, and incubated for 15 minutes in the refrigerator. Finally, 2 mL of PBS was added, and the cell suspension was centrifuged at 300 *g* for 10 minutes and all supernatant was aspirated. The cell pellet was resuspended in 500 μL of PBS for applying to the column of the following MicroBeads kit.

The human CD66abce MicroBeads kit was used for purification of neutrophils. The column was placed in the magnetic field of a suitable MACS Separator. A filter was placed on the top of the column, and the column was rinsed with 500 μL of PBS. Then the cell suspension was applied to the column. Unlabeled cells passed through the column, and the column was washed with 500 μL of buffer three times. The column was removed from the separator and placed on a suitable collection tube. One mL of PBS was applied to the column, and the magnetically labeled cells were immediately flushed out by firmly pushing the plunger into the column. Five mL of PBS was added to the eluted solution and divided into Eppendorf tubes (1 mL). The cell suspension was centrifuged at 300 *g* for 5 minutes, and supernatant was removed to leave 100 μL of liquid. The cell pellet was gently suspended in the remaining liquid, and used as isolated peripheral neutrophils.

Flow cytometry

Neutrophils isolated from peripheral blood were stained with phycoerythrin (PE)-conjugated antihuman monoclonal TLR2, PE-conjugated antihuman monoclonal TLR4, or appropriate isotype-matched immunoglobulin (BD PharMingen, San Diego, CA) as negative control for 30 minutes at 4°C. The sample was analyzed using CellQuest software

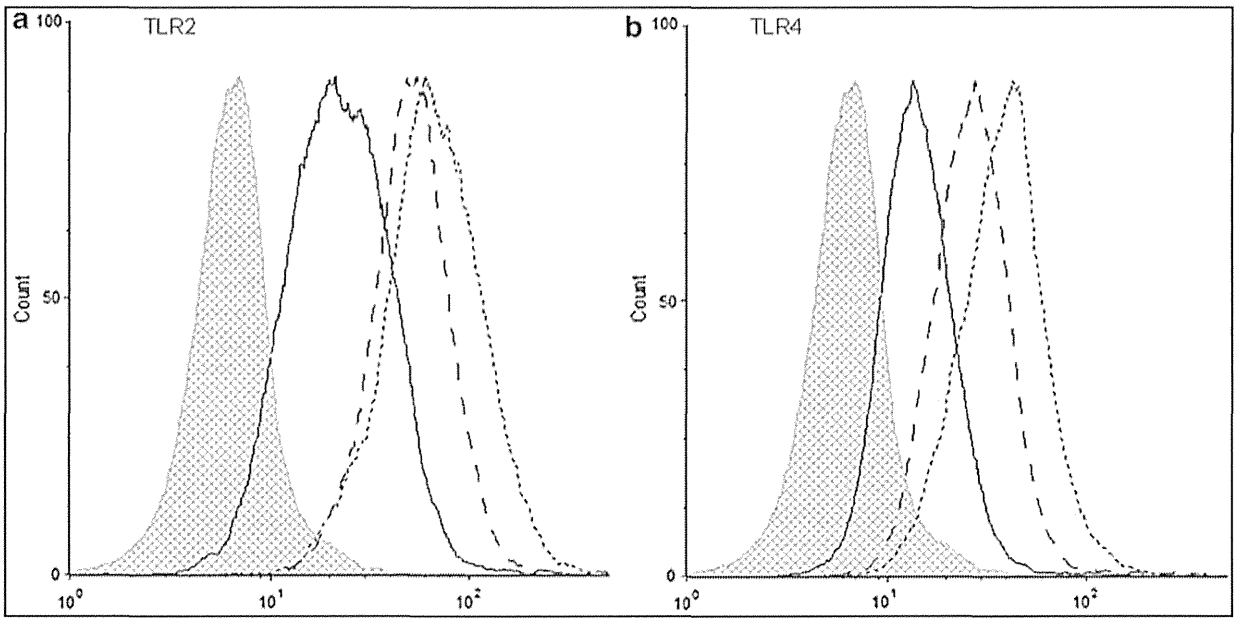

FIG. 1. Examples of toll-like receptor (TLR) expression on infant neutrophils derived from patients without and with infections or Kawasaki disease. Mesh, isotype-matched immunoglobulin as a negative control; solid line, without infection; dashed line, with infection; dotted line, Kawasaki disease (KD).

with a BFACSCalibur (BD PharMingen). Following the previous method,[17] the results are presented as the proportion of cells with expression of a particular receptor as well as mean fluorescence intensity (MFI). Isotype-matched control antibodies were used to determine the background levels of staining (Fig. 1).

Statistical analysis

Results are expressed as mean ± standard deviation (*SD*). Statistical analysis was performed using Student's *t*-test. The statistical significance level was $p < 0.05$.

Results

Patient characteristics and laboratory findings

The three groups showed no significant differences except for the following items (Table 1). Neutrophil count in the patients with infections and KD was significantly higher than in control patients. In addition, the percentage of neutrophils in the white blood cells (WBCs) derived from the patients with infections and KD was significantly higher than in the WBCs from control patients.

TLR2 and TLR4 expression in neutrophils derived from patients without infections

The expression of TLR2 and TLR4 was significantly higher in patients aged 1–5 years compared to patients younger than 1 year and 6 years or older (Table 2). The mean MFIs of expression of TLR2 and TLR4 in all infection-free patients were not significantly different from those in patients aged 1–5 years. These values were used as controls. The relationship between the expression of TLR2 and TLR4 was also analyzed (Fig. 2a). The coefficient of correlation between the expression of TLR2 and TLR4 was higher than 0.8393.

Expression of TLR2 and TLR4 in neutrophils derived from patients with KD and infections

The mean MFIs of expression of TLR2 and TLR4 in neutrophils derived from newly presenting patients with

TABLE 1. PATIENT CHARACTERISTICS AND LABORATORY FINDINGS

	Infection	*Kawasaki disease*	*Control (without infection)*
Number	18	14	38
Median age (month) (range, month)	52.8 (1–186)	16 (4–132)	25.5 (0–131)
Male/female	13/5	11/3	19/19
WBC (/μL)	11,319 ± 4139	11,979 ± 5317	8209 ± 1956
Neutrophils (/μL)	5297 ± 4840[a]	7922 ± 4703[a]	2621 ± 1131
Neutrophils/WBC (%)	49.0 ± 28.2[a]	59.6 ± 15.4[a]	32.5 ± 13.8

Causative organism: *Staphylococcus aureus* (four patients), *Staphylococcus epidermidis* (one patient), *Streptococcus pneumoniae* (four patients), *Streptococcus alagactiae* (one patient), *Streptococcus pyogenes* (one patient), *Haemophilus influenzae* (four patients), *Escherichia coli* (one patient), and *Mycoplasma pneumoniae* (two patients).

[a] $p < 0.05$; infection or Kawasaki disease vs. control.

WBC, white blood cells.

TABLE 2. TLR2 AND TLR4 EXPRESSION IN PATIENTS WITH INFECTIONS AND KAWASAKI DISEASE

Disease	Average of mean fluorescence intensity	
	TLR2 expression	*TLR4 expression*
Without infection		
Younger than 1 year old (7 patients)	17.3±5.9	11.1±2.4
1–5 years old (23 patients)	24.3±8.8[a]	15.2±5.1[a]
6 years old and older (8 patients)	15.6±5.6	10.0±2.6
Total (38 patients)	21.2±8.5	13.4±4.8
Infection (18 patients)	34.9±12.5[b]	25.9±20.0[d]
Kawasaki disease (14 patients)	62.1±32.7[c]	38.0±19.5[e]

Causative organism: *Staphylococcus aureus* (four patients), *Staphylococcus epidermidis* (one patient), *Streptococcus pneumoniae* (four patients), *Streptococcus alagactiae* (one patient), *Streptococcus pyogenes* (one patient), *Haemophilus influenzae* (four patients), *Escherichia coli* (one patient), *Mycoplasma pneumoniae* (two patients).

[a] $p < 0.05$; 1–5 years old patient without infection was higher than younger than 1 year old and 6 years old or older. [b] $p < 0.05$; infection vs. control. [c] $p < 0.05$; Kawasaki disease vs. infection and control. [d] $p < 0.05$; infection vs. control. [e] $p < 0.05$; Kawasaki disease vs. infection and control.

infections are shown in Table 2. Expression of TLR2 and TLR4 in neutrophils derived from the patients with infections was at least twofold higher than in the control patients, which was significantly higher than in control patients. Expression of TLR2 and TLR4 was correlated in the infection group (Fig. 2b). Expression of TLR2 and TLR4 in the patients with KD was 1.3-fold and at least threefold respectively, higher than in the control patients, and was significantly higher than in the patients with infections and control patients. Expression of TLR2 and TLR4 was correlated with a coefficient constant of 0.9095 (Fig. 2c).

Expression of TLR-2 and TLR-4 in patients with KD during follow-up

Expression of TLR2 and TLR4 gradually decreased depending on the follow-up period in the patients with KD (Fig. 3). The CRP serum concentration also decreased gradually in the patients with KD.

Discussion

Although the lifespan of neutrophils is relatively short, neutrophils perform many kinds of functions such as ingestion of microbes, production of destructive reactive oxygen species, and sense an array of microbial and endogenous ligands via a broad repertoires of PRRs. So, we thought it important to know when neutrophils mature. Neutrophils are programmed to undergo apoptosis, so peripheral neutrophils have a circulatory half-life of 6–10 hours *in vivo*, and about half isolated neutrophils die within the first 24 hours *ex vivo*.[18,19] These findings indicate that it is not maturation of neutrophils but the age of the human source that may be the most important in host defense. In support of this theory, the present study indicates that the higher immunological response in patients aged 1–5 years might be due to the higher frequency of various stimuli such as the colonization of microbial flora. The expression of TLR2 and TLR4 was at the same level in healthy adults as

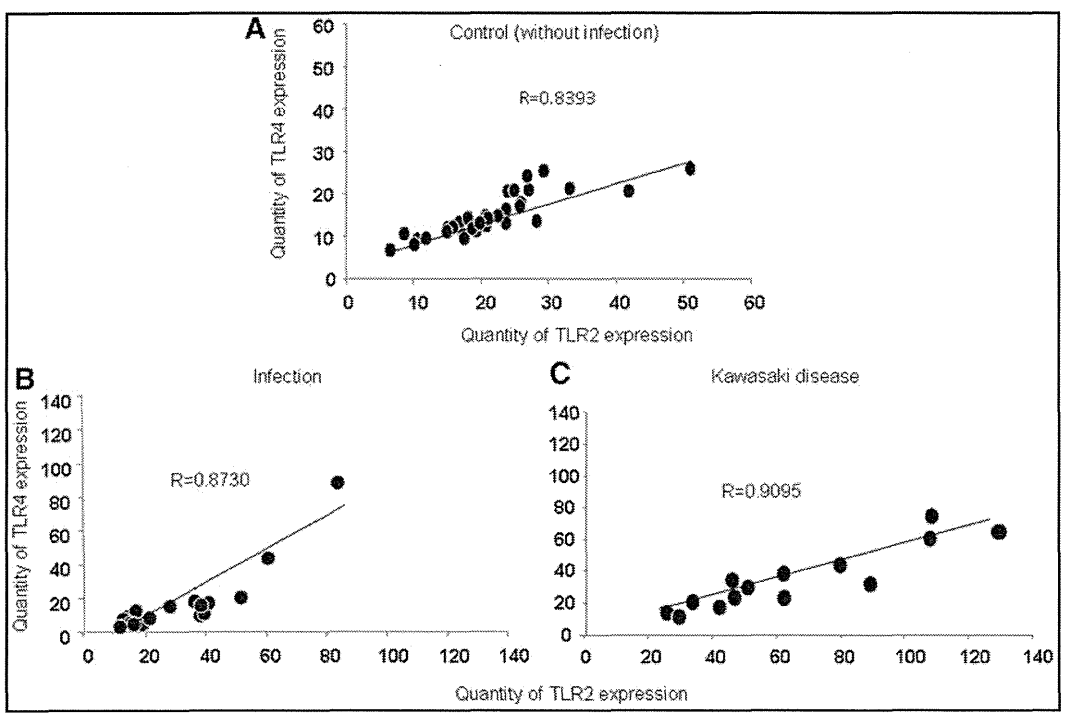

FIG. 2. Relationship between TLR2 and TLR4 expression in peripheral neutrophils derived from patients with and without infections: **(A)** patients without infection; **(B)** patients with infections; **(C)** patients with KD.

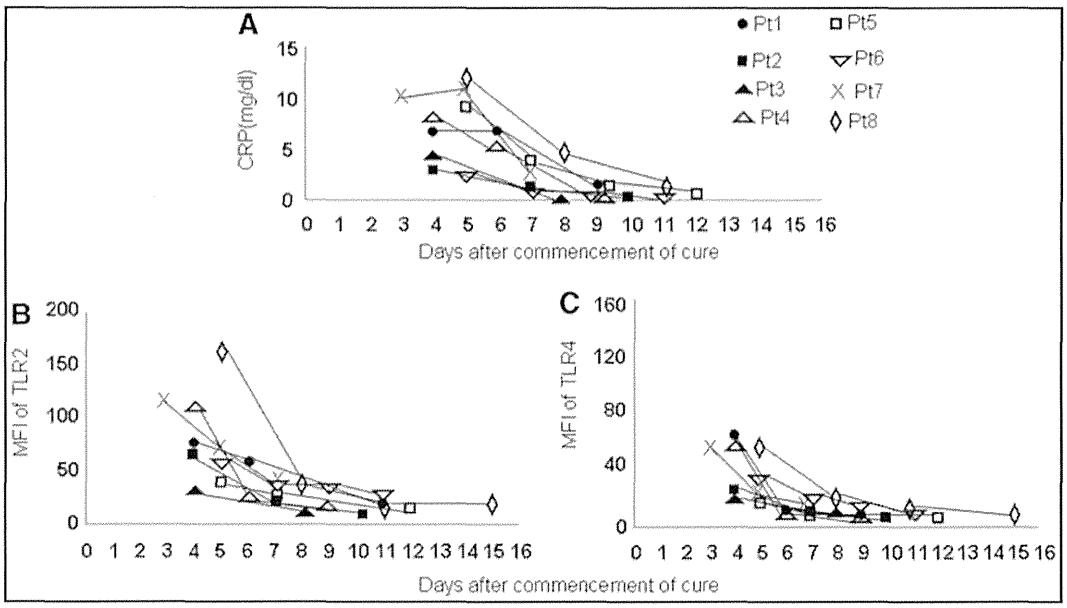

FIG. 3. Follow-up findings in patients with KD: **(A)** TLR2 expression; **(B)** TLR4 expression; **(C)** C-reactive protein serum concentration.

in infants and children without infections (unpublished data). Those data clarified that the function of neutrophils matures between the ages of 1–5 years.

Lin et al.[20] reported that monocytes with significantly elevated TLR2 expression were found in KD patients. Pathological study confirmed transient infiltration of neutrophils in the very early stage of acute KD before infiltration of mononuclear cells.[21] Although it was originally assumed that neutrophils are transcriptionally inert and their short lifespan limits their ability to respond to PRR agonists, the past 5 years has seen tremendous advances in neutrophil PRR signaling that have shifted this paradigm.[22] The present study confirmed that neutrophils showed the same result as in monocytes in KD patients and also expression of TLR4 being significantly higher than control patients.

TLR2 and TLR4 are originally described as recognizing PAMPs derived from bacteria and other organisms.[23] TLR2 and TLR4 are the most widely studied TLRs in adults with infections.[24] There are some reports describing that TLR2 or bacteria were closely related to KD.[14,20] On the basis of those data, the present study attempted to clarify the involvement of TLR2 and TLR4 in neutrophils being the first migration into the invaded microorganisms. Both TLRs are well known to recognize lipids as a PAMP, and both TLRs contain the CD14 and gp96 as an accessory molecule. However, the present study showed that TLR2 expression was significantly higher than TLR4 expression in patients with infections and found no relationship with the causative organisms. This finding suggests that, as TLR2 is expressed at apparently higher levels on neutrophils than TLR4, TLR2 may be the dominant receptor on neutrophils.[15] In addition, TLR2 protects against the early stage of infection by inducing innate immune responses (e.g., NK cells and neutrophil influx), but if infection is not controlled, TLR4 mediates subsequent inflammatory and adaptive responses (DCs and activated CD8+ T-cells).[25] There are two reports. One described that TLR2 augmentation on RAW 264.7 cells might be indicative of strong inflammation of monocytes/macrophages due to this augmentation was positively correlated with IL-6, NCP-1, and TNF-α upregulation.[26] These upregulation of proinflammatory cytokines/ chemokines could be suppressed *in vitro* by using not TLR4 but TLR2 neutralizing antibody.[20] Taken together, there is a possibility that TLR2 augmentation may serve an inflammatory marker in KD patients.

Since neutrophils provide early defenses against invading agents, we thought that the assay of TLRs expression in neutrophils might be one method to establish whether the infection might be involved in KD. The present study found that the mean values of TLR2 and TLR4 expression in newly presenting patients with KD were higher than in patients with various infections and control patients, and the comparative quantitative levels of TLR2 and TLR4 expression were similar to those in patients with infections. Moreover, both the expression of TLRs and the CRP serum concentration decreased gradually during the follow-up period in patients with KD after a single high dose of intravenous immunoglobulins. The patterns in the decrease of CRP levels and TLRs expression were similar, suggesting that expression of TLRs may be induced by microorganisms, and infection may be one of the causative factors of KD. There are some reports that TLR2 expression was augmented on monocytes in patients with KD,[20,27] which supported the present results.

Not TLR4 but TLR2 expression is augmented on monocytes in patients with KD, and TLR2 augmentation might be a candidate indicator of $CD14^+$ monocyte activation in human KD.[15,22] On the other hand, LPS binding and CD14 expression on neutrophils increased in KD patients.[12] On the basis of these findings, since neutrophils provide early defense against invading agents, we thought that expression of TLRs in neutrophils might also be involved in KD.

In conclusion, the present study found that maturation of neutrophils occurred between the ages of 1–5 years, and that augmented TLR2 and TLR4 expression on neutrophils might be important in KD, indicating that the infection might be associated with the occurrence of KD. Neutrophils are fragile with short half-life, so many activities are hard to assay simultaneously. The present findings will undergo more detailed analysis to investigate cytokine production in the near future.

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Author Disclosure Statement

No competing financial interests exist.

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Update on etio and immunopathogenesis of Kawasaki disease

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Purpose of review
This review first discusses the pathogenesis of Kawasaki disease based on the results of recently performed studies aimed at identifying Kawasaki disease-susceptibility genes and the results of analyses of the immune system. Following that, we discuss the findings generated using a murine Kawasaki disease arteritis model and speculate regarding the mechanism of Kawasaki disease onset based on immune function aberrations seen in that model.

Recent findings
Recent advances in gene analysis studies of Kawasaki disease are contributing not only to prediction of disease susceptibility but also to improving our understanding of the pathogenesis of Kawasaki disease and development of new improved therapies. In addition, Th17/Treg imbalance is observed in patients with acute-phase Kawasaki disease. Th17/Treg imbalance may be an important factor causing disturbed immunological function. IL-17 induced by Th17 cells have proinflammatory properties and act on inflammatory cells, thereby inducing expression of cytokines and chemokines and resulting in tissue inflammation.

Summary
Kawasaki disease vasculitis may be triggered by aberrant activation of inflammatory cytokines mediated by IL-17 that is produced by Th17 cells that have been activated by some infectious agent(s).

Keywords
genetic susceptibility, Kawasaki disease, microbiota, Th17 cell, TNF-α, vasculitis model

INTRODUCTION

Kawasaki disease is a systemic vasculitis that affects mainly children under 5 years of age [1,2]. Although over 45 years have passed since Dr Tomisaku Kawasaki first reported Kawasaki disease, its cause remains unclear. Kawasaki disease is diagnosed on the basis of characteristic clinical signs and symptoms. The principal symptoms of Kawasaki disease are a fever persisting for 5 days or longer, bilateral conjunctival injection, changes in the lips and oral cavity, polymorphous exanthema, changes in the peripheral extremities and acute nonpurulent cervical lymphadenopathy. At least five of those principal signs and symptoms should be present for a diagnosis of Kawasaki disease [3,4].

Coronary artery abnormalities (CAAs), including dilatation and aneurysms, are the most serious complications of Kawasaki disease. Although Kawasaki disease is a self-limiting disease in most patients, CAAs develop in about 25% of untreated patients. Therefore, Kawasaki disease is recognized as a leading cause of acquired heart disease in children in developed countries. Here, we review advances in our understanding of the pathogenesis of Kawasaki disease.

EPIDEMIOLOGIC ASPECTS OF KAWASAKI DISEASE

In Japan, nationwide epidemiologic surveys of Kawasaki disease have been conducted every 2 years since 1970. The epidemiological features of Kawasaki disease can be summarized as follows:

(1) The surveys documented three nationwide epidemics in Japan, in 1979, 1982 and 1986. Although there have been no nationwide

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KEY POINTS

- Analyses of genetic susceptibility to Kawasaki disease are contributing to the development of new treatments for Kawasaki disease.
- An imbalance in Th17/Treg cells occurs in acute-stage Kawasaki disease patients.
- Macrophages and neutrophils which are activated by various inflammatory cytokines mediated by IL-17 that is produced by Th17 cells involve in the vasculitis.
- The intestinal microbiota may strongly influence tissue inflammation via Th17 cells.
- Comparative studies of the intestinal microbiota in children will be important for elucidating the pathogenesis of Kawasaki disease.

Kawasaki disease epidemics since 1986, the number of patients and the incidence rate of Kawasaki disease have been increasing rapidly since 1990.

(2) Kawasaki disease shows seasonal variation, with the number of patients always increasing in winter.

(3) Siblings of a Kawasaki disease patient are at 10–30-fold higher risk of Kawasaki disease compared with the general population [5].

(4) The rate of a parental history of Kawasaki disease is twice as high for Kawasaki disease patients compared with the general population [6].

(5) The odds of having sibling cases are significantly increased in patients whose parents also had had Kawasaki disease [7].

(6) As a function of age, the incidence rate of Kawasaki disease is highest in children aged 6–11 months, after which it gradually decreases with age. In contrast, the incidence rate just after birth is extremely low [8▪].

Today in Japan, more than 12 000 individuals are newly diagnosed with Kawasaki disease each year, and in 2010, the annual incidence rate was 222.9 per 100 000 children aged 0–4 years [8▪]. International comparative research revealed that the annual incidence in Japan is more than 10-fold higher than in the US [9]. It is higher in other East Asian countries, as well, and Korea [10] and Taiwan [11] have the second and third highest annual incidences of Kawasaki disease in the world. In addition, epidemiological data from Hawaii suggest that the ethnic differences in the incidence of Kawasaki disease are related to genetic factors. Holman *et al.* [9] reported that the average annual incidence rate of Kawasaki disease among Japanese Americans in Hawaii was almost the same as that in Japan. Similarly, the incidence among Caucasian children in Hawaii was comparable to that on the US mainland.

Summarizing the epidemiological data, the seasonal variation, geographical localization of occurrence and age distribution of Kawasaki disease suggest that some infectious agent(s) might be involved in the cause of Kawasaki disease. On the contrary, the data also strongly suggest that the interindividual variability in susceptibility to Kawasaki disease and the different prevalences of Kawasaki disease among races or ethnicities involve genetic factors.

GENES RELATED TO KAWASAKI DISEASE SUSCEPTIBILITY

To date, numerous genetic studies have been performed to clarify Kawasaki disease susceptibility and disease severity. Considerable progress has been made in identification of disease-susceptibility genes since the start of genome-wide association studies (GWAS). Candidate Kawasaki disease-susceptibility genes that have recently generated interest are shown in Table 1 [12,13,14▪,15–17]. In 2012, two GWAS results were reported independently [14▪,15]. In both studies, significant associations with Kawasaki disease were observed in the FAM167A-BLK region on chromosome 8p23–p22. B-lymphoid kinase (BLK) is a src family tyrosine kinase expressed mainly in B cells and involved in B-cell receptor signal transduction. It has also been reported to be responsible for phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITAM) of the low-affinity immunoglobulin gamma Fc region receptor II-a (FCGR2A) molecule. In addition, it is notable that BLK is required in the development of interleukin (IL)-17-producing γδ T cells in mice [18] because high level of IL-17 and increased numbers of activated γδ T cells in the acute phase of Kawasaki disease have been reported [19,20]. On the contrary, the function of the FAM167A molecule remains unclear.

Single-nucleotide polymorphisms (SNPs) around CD40, located on chromosome 20q12–q13.2, were also significantly associated with Kawasaki disease in both Japanese and Taiwanese GWAS [14▪,15]. CD40 is a type I transmembrane protein belonging to the tumor necrosis factor (TNF) receptor superfamily. It is constitutively expressed on normal B cells, as well as on monocytes/macrophages, endothelial cells, epithelial cells, smooth muscle cells, dendritic cells, fibroblasts and adipocytes. Its receptor, CD40 ligand (CD40L), is a type II transmembrane protein belonging to the TNF family. Following activation, CD40L can be upregulated, predominantly on $CD4^+$ T cells and platelets, but also on $CD8^+$ T cells, natural killer cells, B cells, dendritic cells, monocytes/

Table 1. Susceptibility genes for Kawasaki disease recently reported

Gene	Methods	Reference
ITPKC	Linkage analysis	[12]
CASP3	Linkage analysis	[13]
BLK	GWAS	[14▪,15]
CD40	GWAS	[14▪,15]
FCGR2A	GWAS	[16]
TGFB2, TGFBR2, SMAD3	Association study	[17]
HLA	GWAS	[14▪]

BLK, B-lymphoid kinase; CASP3, caspase-3; FCGR2A, low-affinity immunoglobulin gamma Fc region receptor II-a; GWAS, genome-wide association study; HLA, human leukocyte antigen; ITPKC, inositol 1,4,5-triphosphate kinase-C; TGF, transforming growth factor.

macrophages, basophils, eosinophils and endothelial cells. Elevated expression of CD40L during acute-phase Kawasaki disease and significantly higher expression in Kawasaki disease patients with coronary arterial lesions (CALs) have been reported [21]. Thus, it is likely that CD40L–CD40 signaling plays a facilitating role in Kawasaki disease progression and might be an effective target in molecular therapies.

Genome-wide association studies in Kawasaki disease patients of European descent identified a nonsynonymous SNP in the FCGR2A gene as influencing susceptibility [16]. FCG2A is expressed on the surface of monocytes/macrophages, dendritic cells and neutrophils, and transduces activating signals into the cells via an ITAM in the cytoplasmic domain when ligated with immune complexes.

Transforming growth factor (TGF)-β2, TGF-βR2 and SMAD3 are members of the TGF-β signaling pathway. SNPs in those genes influence Kawasaki disease susceptibility and coronary artery aneurysm formation [17].

In summary, genetic studies have generated much new information on the background of Kawasaki disease patients. Genetic studies of Kawasaki disease aim not only to predict disease susceptibility but also to improve our understanding of the pathogenesis of Kawasaki disease, and lead to new, improved therapies. In fact, genetic discovery by Onouchi *et al.* [12] that a SNP within the inositol 1,4,5-triphosphate kinase-C (ITPKC) gene confers both susceptibility to Kawasaki disease and risk for CALs led to use of calcineurin inhibitors to block this activation pathway in Kawasaki disease patients who are resistant to intravenous immunoglobulin (IVIG) treatment [22,23].

DERANGEMENT OF THE IMMUNE SYSTEM IN ACUTE-PHASE KAWASAKI DISEASE

As noted above, genetic studies are making important contributions to our understanding of the pathogenesis of Kawasaki disease. On the contrary, analysis of data for Kawasaki disease shows that abnormal immune responses to infectious agents play key roles in initiation of Kawasaki disease.

Many studies of cytokine networks in Kawasaki disease have focused on inflammatory cytokines such as TNF-α, IL-1, IL-2, IL-6, IL-8 and chemokines such as monocyte chemotactic protein-1 (MCP-1). These inflammatory cytokines and chemokines are elevated during the acute phase of Kawasaki disease. However, the mechanisms resulting in aberrant immune function or overexpression of inflammatory cytokines are not clear. Recently, Wang *et al.* [24▪] analyzed the serum Th1 and Th2 cytokine levels in patients with Kawasaki disease. They reported that the levels of IL-6, IL-20, TNF-α and interferon (IFN)-γ were significantly increased before IVIG treatment, and that the levels of IL-6, IL-10 and IFN-γ decreased rapidly after the treatment. The level of TNF-α decreased significantly after IVIG treatment of Kawasaki disease patients without CALs, but increased in IVIG-treated Kawasaki disease patients with CALs and in IVIG-resistant Kawasaki disease patients. They compared the cytokine levels before and after IVIG treatment and in Kawasaki disease patients with and without CALs, and concluded that determination of the serum Th1/Th2 cytokine profiles may help in predicting the prognosis and designing treatment strategies in Kawasaki disease patients.

It is known that the serum level of IL-17 and IL-17-induced cytokinesis increases in patients with acute-phase Kawasaki disease [17]. Jia *et al.* [25] suggested the existence of a T helper 17 (Th17) / regulatory T (Treg) cell imbalance in patients with Kawasaki disease. They demonstrated that the Th17 cell proportion and the expression levels of cytokines (IL-17, IL-6 and IL-23) and transcription factors (IL-17A/F, RAR-related orphan receptor γt (RORγt)) were significantly upregulated, whereas the Treg proportions and expression levels of Treg

transcription factors (FoxP3) were significantly downregulated in patients with acute Kawasaki disease. In addition, the Th17 proportions were significantly upregulated during the acute phase in the IVIG-resistant group compared with the IVIG-responding group. Therefore, Th17 expansion and Treg depletion can be said to be characteristics of acute Kawasaki disease.

It is well known that TNF-α is a key inflammatory cytokine that is initially produced by T lymphocytes, followed by a secondary TNF-α release from monocytes/macrophages. TNF-α mediates endothelial cell activation through increased expression of adhesion molecules and also upregulates expression of chemokines that are important in the orchestration of leukocyte–endothelial cell interactions. Th17 cells play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 [26]. IL-17A and IL-17F produced by Th17 cells have proinflammatory properties and act on a broad range of cell types to induce expression of such cytokines as IL-6, TNF-α, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as chemokines and metalloproteinases, and they also perpetuate tissue inflammation [27,28]. Thus, aberrant production of inflammatory cytokines such as TNF-α and IL-6 induced by an imbalance between Th17 and Treg cells may play an important role in the initiation stage of Kawasaki disease.

LESSONS FROM MURINE MODEL OF KAWASAKI DISEASE VASCULITIS

We established a murine model of systemic arteritis by injecting a *Candida albicans* cell wall polysaccharide (CAWS, CADS). The arteritis that develops in the model shows a predilection for manifesting in the coronary artery and aortic root. Histologically, the inflammation consists primarily of monocytes/macrophages and neutrophils. With that distribution of lesions and histological characteristics, the model resembles Kawasaki disease vasculitis, and for that reason it is considered to be a useful Kawasaki disease arteritis model [29]. Using that murine model, our research group demonstrated a relationship between development of vasculitis and overexpression of such proinflammatory cytokines such as TNF-α and IL-6 [30,31]. We administered anti-TNF-α drugs, which have been used as an alternative therapy for patients with IVIG-resistant Kawasaki disease, to treat the murine Kawasaki disease vasculitis. We investigated the relationship of TNF-α to the vasculitis on the basis of the histological findings. The anti-TNF-α drugs showed potent inhibitory effects on the vasculitis, suggesting that TNF-α plays an important role in the development of vasculitis [32▪]. Thus, there is a strong possibility that elucidation of the role of TNF-α in the process of development of vasculitis in the CAWS-induced murine Kawasaki disease vasculitis model will lead to development of new therapeutic strategies for Kawasaki disease. Moreover, recent study revealed that the development of vasculitis caused by CAWS administration was significantly suppressed in chemokine receptor (CCR)2 knockout mice compared with wild-type mice. The activation of CCR2-dependent macrophages might be involved in the pathogenesis of CAWS-induced vasculitis [33].

It was recently shown that Dectin-2, a member of the C-type lectin family, is the functional receptor for α-mannans, which comprises the fungal cell wall and is essential for host defense against *C. albicans* infection. CAWS contains α-mannans and can also bind to Dectin-2 as pathogen-associated molecular patterns (PAMPs) [34–36]. Dectin-2 recognizes the α-mannans in CAWS and transmits a signal inside dendritic cells and macrophages via the T cell receptor γ (TcRγ) chain. Dendritic cells and macrophages then produce such cytokines as IL-1β, IL-6 and IL-23, which promote the cells' differentiation from naive T cells to Th17 cells. IL-17A produced by Th17 cells recruits neutrophils to the inflammatory sites and activates T cells, B cells and macrophages by producing cytokines and chemokines, directly or indirectly. It is then likely that vascular inflammation develops (Fig. 1). According to the recent studies, IL-17 is produced not only by T lymphocytes but also by neutrophils [37] and myofibroblasts in the tunica intima of coronary arteritis of Kawasaki disease [38]. Thus, our conception of the interrelating effects between Th17 cells and IL-17 is becoming more complex.

What does seem sure is that CAWS derived from cell wall components of *C. albicans* induces systemic vasculitis in mice and CAWS-induced vasculitis has many similarities to Kawasaki disease, not only histologically but also in the immune response. If infection serves as the initial trigger for Kawasaki disease development, the likely route of invasion is via the respiratory or gastrointestinal tract. When hypothesizing *C. albicans* as a cause of Kawasaki disease, its origin is assumed to be as a part of the intestinal flora. It is known that the gut microbiota play critical roles in the development of lymphoid tissues and in immune system function. In addition, systemic autoimmune diseases have been suggested to be related to infections. Recent studies [39▪] in animal models have strengthened the idea that commensal microbiota contribute to systemic autoimmune disease even at sites distal to the intestinal mucosa. Animal models of autoimmunity are clearly

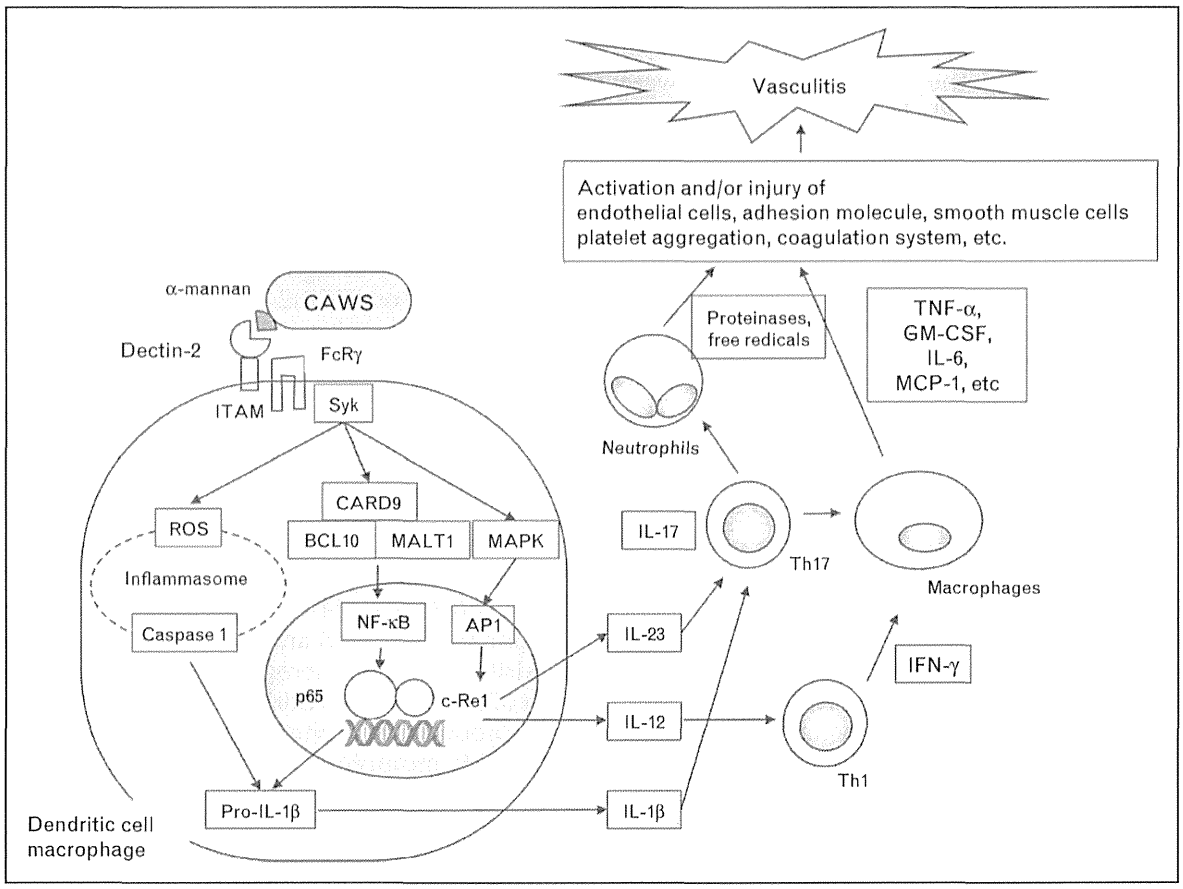

FIGURE 1. Hypothesized mechanism for the development of vasculitis induced by CAWS. Upon α-mannan binding to Dectin-2, Syk is recruited to the ITAM of the FcRγ chain and activates the CBM complex downstream. The CBM complex activates NF-kB to activate cytokine genes including pro-IL-1β, IL-23 and IL-12. IL-23 and IL-1β promote differentiation of Th17 cells. IL-17A produced by the Th17 cells recruits neutrophils and macrophages to the inflammatory sites and stimulates production of various pro-inflammatory cytokines/chemokines by the infiltrating cells. Endothelial cells and vascular smooth muscle cells are activated, and then inflammatory cells adhere to the endothelium and migrate within the vascular wall. It is the initiation of vasculitis. (The figure was cited from ref. [35] and modified slightly for inclusion in this review.) CAWS, *Candida albicans* cell wall polysaccharide; ITAM, immunoreceptor tyrosine-based inhibitory motif ; CBM, CARD9-BCL10-MALT1; NF-kB, nuclear factor-kappa B.

dependent on the colonization status. For example, germ-free mice have marked attenuation of disease in models of arthritis and experimental autoimmune encephalitis (EAE). Furthermore, in models of Th17 cell-dependent arthritis and EAE, monoassociation with segmented filamentous bacteria is sufficient to induce disease [39▪,40,41]. Induction of Th17 cells in the intestine strongly influences systemic disease in these models. It is obvious that the immune system plays a critical role in shaping the composition of the microbiota. However, we still lack even fundamental knowledge regarding fungi in the gut microbiota, especially in children, who are most susceptible to Kawasaki disease. At present, we are investigating the status of *C. albicans* in the gut microbiota of infants for comparison within Kawasaki disease patients.

CONCLUSION

Although microorganisms such as bacteria, superantigens derived from bacteria and viruses have been proposed as causes of Kawasaki disease, the cause of this disease remains unclear. High-dose IVIG administration has become the standard therapy for Kawasaki disease, but that treatment fails to provide sufficient efficacy in about 10% of Kawasaki disease patients. Today, with the cause still unclear, accurate understanding of Kawasaki disease patients' genetic background and immunoregulatory abnormality are very important for being able to administer effective therapy. Also, the intestinal microbiota play important roles in the development of the immune system in children, and imbalance in the gut microbiota may be involved in the onset of Kawasaki disease. Further studies are needed to

clarify the relationship between the Th17 cell-mediated immune response and the gut microbiota in Kawasaki disease.

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Conflicts of interest

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ORIGINAL ARTICLE

The role of TNF-α in a murine model of Kawasaki disease arteritis induced with a *Candida albicans* cell wall polysaccharide

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Abstract
Objectives Various inflammatory cytokines, including tumor necrosis factor-α (TNF-α), have been reported to play roles in Kawasaki disease (KD). Recently, anti-TNF-α therapy was reported to show efficacy in patients who do not respond to high-dose intravenous immunoglobulin therapy. However, there are many gaps in our understanding of the role that TNF-α plays in the development of KD arteritis as well as whether anti-TNF-α therapy causes any histological changes in the arteritis. Accordingly, the present histopathological study was carried out to elucidate the inhibitory effect of anti-TNF-α therapy on vasculitis as well as the role of TNF-α in the development of vasculitis in a murine model of KD vasculitis.

Methods We used two anti-TNF-α drugs (etanercept and infliximab) to treat a *Candida albicans*-induced murine model of KD vasculitis. We investigated the histopathological changes in terms of the incidence of vasculitis, the scope of lesions and the degree of inflammation.
Results Administration of etanercept to the mice reduced not only the incidence of vasculitis but also the scope of lesions and the degree of inflammation.
Conclusion Based on the histological findings, TNF-α is deeply involved in the development of vasculitis.

Keywords Arteritis · Arteritis model · *Candida albicans* · Kawasaki disease · Tumor necrosis factor-α

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Introduction

Kawasaki disease (KD) is an acute febrile disease of children that falls in the category of systemic vasculitis syndromes [1]. Ischemic heart disease originating in coronary arteritis has a major impact on the prognosis of child patients [2]. Intimate relationships between pro-inflammatory cytokines such as TNF-α and vasculitis have been reported in KD [3–8]. High-dose intravenous immunoglobulin therapy (IVIG therapy) is the first-choice treatment option for KD [9], and its efficacy is widely recognized. However, a recent nationwide survey in Japan revealed that 16.6 % of KD patients do not respond well to initial IVIG therapy [10]. A major issue today is how to effectively treat such non-responders. Most pediatricians have administered additional courses of IVIG, or a steroid, etc. However, recent years have seen the use of infliximab (IFX), an anti-TNF-α drug, as an additional treatment option for KD [11]. IFX is an anti TNF-α antibody which was originally developed in mice as a mouse monoclonal

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antibody. IFX is generally called a chimeric antibody because it contains both murine- and human-origin components. The efficacy of IFX has been well documented [12, 13]. On the other hand, there have also been reports pointing out the limitations of IFX therapy [14] and questioning its inflammation-suppressing efficacy in regard to cellular infiltration of the vessel wall [15]. In addition, it was recently reported that another anti-TNF-α drug, etanercept (ETA), also showed efficacy in treatment of KD [16]. Etanercept is a fusion protein produced by recombinant DNA, one part of which codes the human gene for soluble TNF receptor 2. However, none of those reports shed any light on the histological modifications that occur in vasculitis as a result of administration of the anti-TNF-α drugs. One reason for that is probably the difficulty of obtaining tissue samples from the coronary arteries, a frequent site involving vasculitis in KD. Studies that use human samples are often subject to limitations, making it necessary to perform analyses that use animal models.

In 1979, Murata [17] established a murine model of systemic arteritis by injection of a *Candida* cell wall polysaccharide. The arteritis that develops in that model shows a predilection for manifesting in the coronary artery and aortic root. Histologically, the inflammation consists primarily of histiocytes and neutrophils. With that distribution of lesions and histological characteristics, the model resembles KD vasculitis, and for that reason it is considered to be a KD arteritis model [18]. To date, our research group has used this murine model and has reported the relationship between vasculitis and pro-inflammatory cytokines [19, 20] and the feasibility of application of the model to therapeutic studies [21, 22].

The objectives of the present study were — by using the *C. albicans*-induced murine model of vasculitis — to generate histopathological evidence of the efficacy of anti-TNF-α drugs in inhibiting the development of vasculitis and to elucidate the role of TNF-α in the development of vasculitis.

Materials and methods

Animals

Four-week-old male C57BL/6 N mice were purchased from Japan SLC Inc. and used in experiments after 1 week of acclimatization. The animal room was maintained at 23 ± 2 °C and 60–70 % RH. The animals were fed a commercial diet (CLEA Rodent Diet CE-2, CLEA Japan Inc.) and had free access to tap water. The animals were raised in accordance with the relevant laws and regulations and Toho University's Animal Ethics Committee rules. The study design had been approved by the Committee (Approval No. 09-33-94).

Vasculitis-inducing substance

As reported previously [20, 23], *Candida albicans* (NRBC1385) was cultured in a complete synthetic medium, and a cell wall polysaccharide [mannoprotein-β-glucan complex; CAWS (*Candida albicans* water-soluble fractions)] extracted from the culture supernatant was used to induce vasculitis.

Induction of vasculitis

CAWS, 4 mg, was dissolved in 0.2 ml of PBS and injected into the peritoneal cavity of mice on 5 consecutive days. The mice were euthanized with carbon dioxide gas 28 days after the final injection, and various organs were subjected to histopathological studies (Fig. 1).

Anti-TNF-α drugs

Two anti-TNF-α drugs were employed, etanercept (ETA; Takeda Pharmaceutical Co., Ltd.) and infliximab (IFX; Mitsubishi Tanabe Pharma Corp.). ETA was administered subcutaneously (sc), while IFX was administered intraperitoneally (ip). Both drugs were administered twice weekly, for a total of 8 times. The drugs were diluted with distilled water immediately before administration (Fig. 1).

Experimental groups

The following anti-TNF-α drug and dosage groups were employed: E-1, ETA 5 mg/kg ($n = 10$); E-2, ETA 10 mg/kg ($n = 9$); E-3, ETA 20 mg/kg ($n = 25$); I-1, IFX 5 mg/kg ($n = 10$); I-2, IFX 10 mg/kg ($n = 10$); and I-3, IFX 50 mg/kg ($n = 25$). A control group was administered only CAWS, with no administration of either anti-TNF-α drug ($n = 51$).

Histopathological assessments

Tissue sections of various organs of the experimental animals were stained by the hematoxylin-eosin, Elastica van Gieson and Azan-Mallory staining methods. The stained specimens were carefully examined for inflammatory lesions of the blood vessels under a light microscope.

In particular, we carried out detailed histopathological studies of the heart, which is a frequent target organ for vasculitis. That is, with the objective of detecting even minute and mild inflammatory lesions that did not reach the extent of panvasculitis, we prepared serial sections of the coronary artery and aortic root, in accordance with the previously described methods [21]. The same site was anatomically divided into 5 segments, i.e., left coronary artery, right coronary artery, non-coronary sinus, left

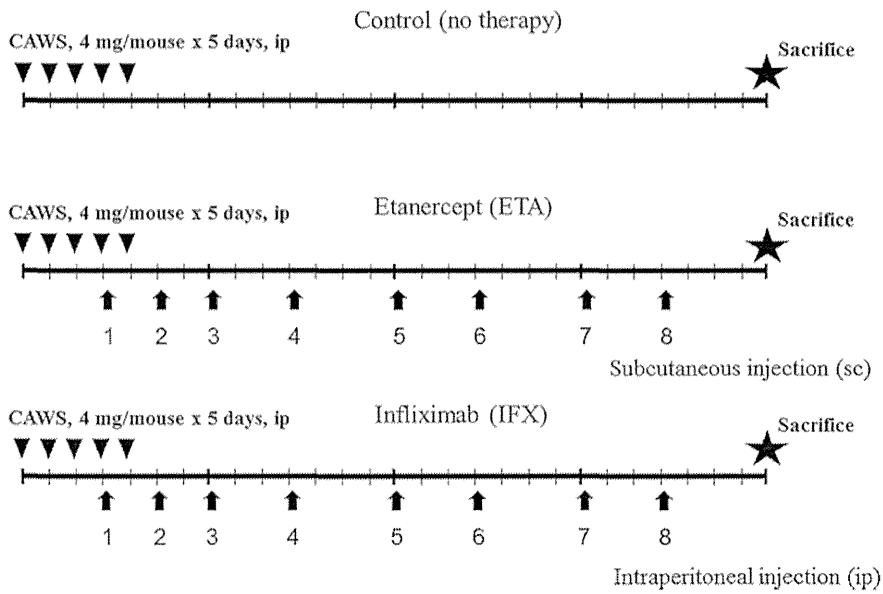

Fig. 1 Schedules for induction of vasculitis and administration of anti-TNF-α drugs. CAWS, 4 mg, was dissolved in 0.2 ml of PBS and injected into the peritoneal cavity of mice on 5 consecutive days to induce vasculitis. The mice were euthanized 28 days after the final injection. Either ETA (sc) or IFX (ip) was injected a total of 8 times, at the time-points indicated by the *arrows*

coronary sinus and right coronary sinus. The degree of inflammation in each segment was assessed using four scores: 0 = no inflammation, 1 = inflammation in the intima (i.e., endoarteritis), 2 = inflammation in the intima and adventitia, and 3 = inflammation in all layers of the vascular wall (i.e., panvasculitis) (Fig. 2). Panvasculitis was defined as a positive finding for vasculitis. Comparative investigation was performed regarding the incidence of vasculitis at the base of the heart (a frequent site of development of vasculitis) and the incidences of vasculitis in all organs. The total number of segments with lesions of score 1 or greater was defined as the extent of lesions, while the total score for all 5 segments was defined as the inflammation score for one mouse. These data were compared between the control and each drug-treated group.

We also compared the percentages of each score in each of the experimental groups with the objective of elucidating the histopathological changes caused by administration of each of the anti-TNF-α drugs.

Serum cytokine assay

Blood was drawn from the heart at the time of sacrifice of the mice (i.e., day 28 after CAWS injection), and the serum was separated. The murine TNF-α concentration in each serum was assayed using an ELISA kit (Invitrogen). The detection sensitivity limit of the ELISA kit was 3 pg/ml.

Study of binding affinities of ETA and IFX for mouse and human TNF-α

Dilution series of human TNF-α (Wako Pure Chemicals) and mouse TNF-α (Wako Pure Chemicals) were prepared and coated onto 96-well ELISA plates (Iwaki Glass) overnight at 4 °C. Then 100 μl of IFX or ETA was added to each well and reacted for 1 h at room temperature. Next, peroxidase-labeled anti-human IgG antibody (Sigma-Aldrich) was added to each well, followed by reaction for 1 h at room temperature. Finally, ethylbenzidine (Wako Pure Chemicals) was added as a chromogen, the reaction was allowed to proceed until there was sufficient color development, and then the absorbance at 450 nm was measured using a plate reader (Bio-Rad Model 550).

Statistical analyses

The data for the incidences of vasculitis and the percentages of each inflammation score were statistically analyzed for differences between the control group and each treatment group using the χ^2 test for goodness-of-fit. The extent of lesions and the inflammation score were analyzed using the Mann–Whitney test. A p value of <0.05 was defined as representing a statistically significant difference.

Results

Histopathological characteristics of vasculitis

The control and IFX groups showed high incidences of panvasculitis (score 3), defined as inflammation in all layers of the vascular wall of the coronary artery and aortic root (Fig. 3a–d). Histologically, inflammatory cell infiltration consisting mainly of histiocytes and neutrophils was observed in the lesion. The internal and external elastic lamina and the media were markedly damaged due to

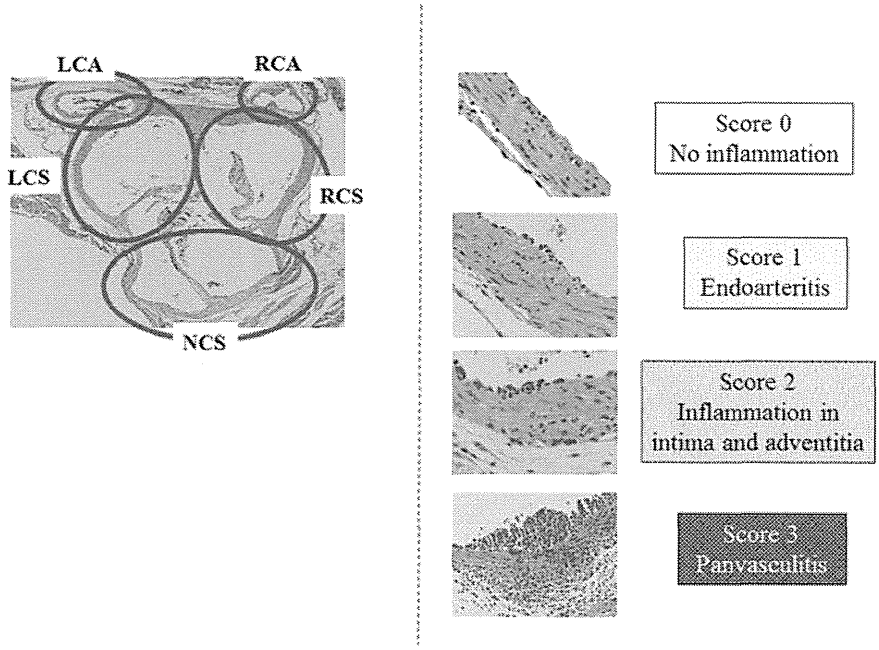

Fig. 2 Method for assessing the inflammatory lesions at the base of the heart. The base of the heart, including the coronary bifurcation, was anatomically divided into 5 segments, i.e., left and right coronary arteries (LCA and RCA), non-coronary sinus (NCS), and left and right coronary sinuses (LCS and RCS). The degree of inflammation in each segment was assessed using the four scores shown at the *right*

severe inflammation, and the vessel lumen was dilated because of destruction of the normal structure of the vessel wall due to the inflammation. Examination of the serial sections of the heart revealed, even in the sites not severe enough to qualify as panvasculitis, minute lesions with mild inflammation, such as adhesion of histiocytes and neutrophils to endothelial cells (i.e., endoarteritis, score 1), and endoarteritis together with inflammatory cell infiltration of the adventitia (score 2). In addition to the heart, although the incidences were low, panvasculitis had developed in medium ~ large vessels, including the renal artery, common iliac artery, intercostal artery, abdominal aorta, etc. The histological images of vasculitis did not differ as a function of the site of infiltration.

Even the ETA group showed similar images of panvasculitis, but they were low in incidence and small in size (Fig. 3e, f). Minute lesions, such as endoarteritis, were also observed.

Incidence of panvasculitis

The incidences of panvasculitis in each of the animal groups are shown in the Table 1. The incidences of panvasculitis in all organs, and also in the heart were significantly lower in E-2 and E-3 than in the control ($p < 0.05$, $p < 0.01$). However, the incidences were not significantly reduced in group E-1 or any of the IFX groups.

Extent of lesions and inflammation scores

The extent of the lesion (i.e., the total number of segments with lesions of score 1 or greater) was compared among the experimental groups, and the results were as follows: control group, 1.2 ± 1.2; E-1, 0.3 ± 0.5; E-2, 0.3 ± 0.6; E-3, 0.3 ± 0.6; I-1, 0.9 ± 1.2; I-2, 1.2 ± 1.5; and I-3, 1.0 ± 1.0. The extent of the lesion was significantly reduced in each of the ETA-administered groups ($p < 0.01$), whereas it was not reduced in the IFX-administered groups. The inflammation scores in the groups were as follows: control, 2.2 ± 2.7; E-1, 0.5 ± 1.0; E-2, 0.3 ± 0.7; E-3, 0.4 ± 0.9; I-1, 1.6 ± 2.3; I-2, 2.7 ± 4.0; and I-3, 1.5 ± 1.9. The inflammation score was significantly reduced in each of the ETA-administered groups ($p < 0.01$), whereas it was not clearly reduced in the IFX-administered groups (Fig. 4).

Changes in percentage of each inflammation score due to treatment

In group E-3, the percentage of each inflammation score was significantly changed compared with control group ($p < 0.01$). Lesions with a score of 1 or greater were reduced, whereas score 0 increased. The E-1 and E-2 groups showed nearly the same tendencies as seen in group E-3, although the differences were not statistically significant. In contrast, the percentages of each inflammation score in all three IFX groups (I-1, I-2 and I-3) were similar to those in the control group (Fig. 5).

Serum TNF-α assay

The levels were below the limit of detection in all experimental groups, regardless of the presence or absence of vasculitis.

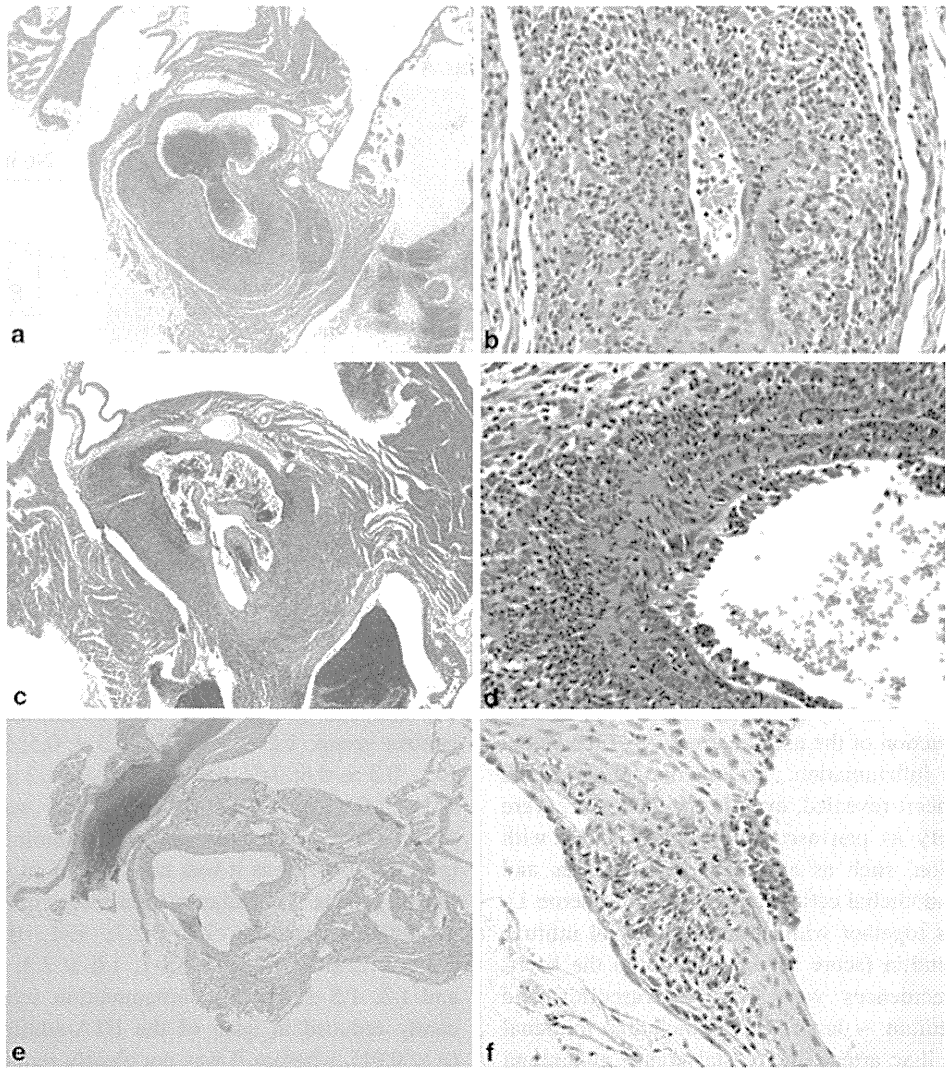

Fig. 3 Representative histopathological images of panvasculitis at the coronary artery and aortic root. **a** Panvasculitis in the control group (H&E ×40). The coronary artery and aortic root show involvement of severe inflammation. **b** Coronary arteritis in the control group (H&E ×400). Inflammatory cells consisting of neutrophils and macrophages are seen in all layers of the coronary artery. **c** Panvasculitis at the aortic root and coronary bifurcation in the IFX group (H&E ×40). **d** Coronary arteritis in the IFX group (H&E ×400). Extensive, severe panvasculitis is seen in the IFX group, similar to that in the control group. **e** Panvasculitis in the ETA group (H&E ×40). In the ETA group, even if panvasculitis develops, the lesion is smaller than in the control group. **f** High-power view of panvasculitis in the ETA group (H&E ×400). Macrophages and neutrophils are seen in all layers of the aortic root, but the inflammatory cell infiltration is milder than in the control group

Binding affinities of ETA and IFX for mouse and human TNF-α

ETA bound to both human and mouse TNF–α in a concentration-dependent manner. Conversely, IFX bound to human TNF–α in a concentration-dependent manner, but did not bind to mouse TNF-α, and the results were the same even if the concentration of TNF-α was increased (Fig. 6).

Discussion

The murine model of vasculitis induced using a *C. albicans* cell wall polysaccharide [17, 23] and a model produced using *Lactobacillus casei* (*L. casei*) [24] are representative animal models of Kawasaki disease (KD) arteritis. These models use physiologically active substances derived from microorganisms to induce vasculitis that is histopathologically similar to KD. The causes of KD remain unclear, but

Table 1 Incidence of panvasculitis

	n	All organs	Coronary artery and aortic root (heart)	Other than heart
Control	51	25 (49.0 %)	19 (37.3 %)	9 (17.6 %)
E-1	10	2 (20 %)	1 (10 %)	1 (10 %)
E-2	9	0 (0 %)*	0 (0 %)*	0 (0 %)
E-3	25	2 (8.0 %)**	1 (4 %)**	1 (4 %)
I-1	10	3 (30.0 %)	3 (30 %)	1 (10 %)
I-2	10	4 (40.0 %)	4 (40 %)	1 (10 %)
I-3	25	7 (28.0 %)	5 (20 %)	3 (12 %)

Incidence of panvasculitis in all organs and in the base of the heart. In E-2 and E-3, the incidence of panvasculitis was significantly reduced compared with the control

* $p < 0.05$

** $p < 0.01$

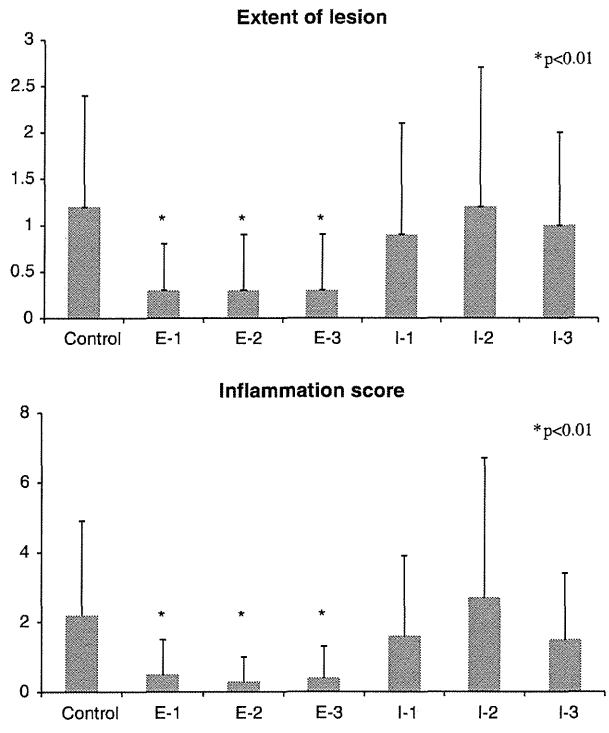

Fig. 4 The scope of lesions and inflammation scores. In each of the E-1, E-2 and E-3 groups, the scope of lesions and the inflammation scores were statistically significantly reduced compared with the control groups

infectious agents are suspected of being involved, and the present model is said to be very useful for investigating the causes and pathophysiology of KD. In recent years, we have used this *C. albicans*-induced murine vasculitis model and reported on the relationship between vasculitis and inflammatory cytokines [19, 20] and the feasibility of its application to therapeutic studies [21, 22]. On the other hand, studies using the *L. casei*-induced vasculitis model indicated a relationship between TNF-α and vasculitis [25]. However, to date, studies using those models have not carried out detailed histopathological experiments aimed at elucidating exactly how TNF-α is related to the establishment of vasculitis. In our present study, we treated the vasculitis in the *C. albicans*-induced model by administering two anti-TNF-α drugs and performed histopathological experiments regarding the relationship between TNF-α and vasculitis.

Those histopathological experiments revealed that administration of ETA not only reduced the incidence of vasculitis but also shrunk the extent of the lesions and markedly reduced the degree of inflammation. In therapeutic studies, it is important to investigate whether or not any therapeutic effect is dose-dependent. However, in our present experiments, all three of the administered dosages of ETA showed very potent lesion-shrinking and inflammation-alleviating effects, making it difficult to address the question of whether or not ETA exhibited dose-dependent efficacy in regard to those effects. It will be necessary to investigate the effects of a lower ETA dosage to prove that ETA's inhibitory effect on vasculitis is dose-dependent. Earlier, we reported on the efficacy of IVIG therapy in the present murine model of KD vasculitis [21]. Based on the results of our present experiments, we think that ETA is in no way inferior to IVIG therapy in terms of its vasculitis-inhibiting efficacy. The fact that the administered anti-TNF-α drugs showed potent vasculitis-inhibiting effects in this murine model of KD vasculitis means that TNF-α is a key cytokine playing important roles in both the onset and development of vasculitis in this animal model.

However, TNF-α in sera prepared from blood collected at the time of animal sacrifice was always below the limit of detection by ELISA, regardless of the presence or absence of vasculitis. A more sensitive assay than ELISA will be necessary to prove that TNF-α was not involved in vasculitis at 28 days after injection of CAWS.

We then investigated the percentages of each inflammation score with the objective of elucidating the mechanism by which ETA inhibits development of panvasculitis.

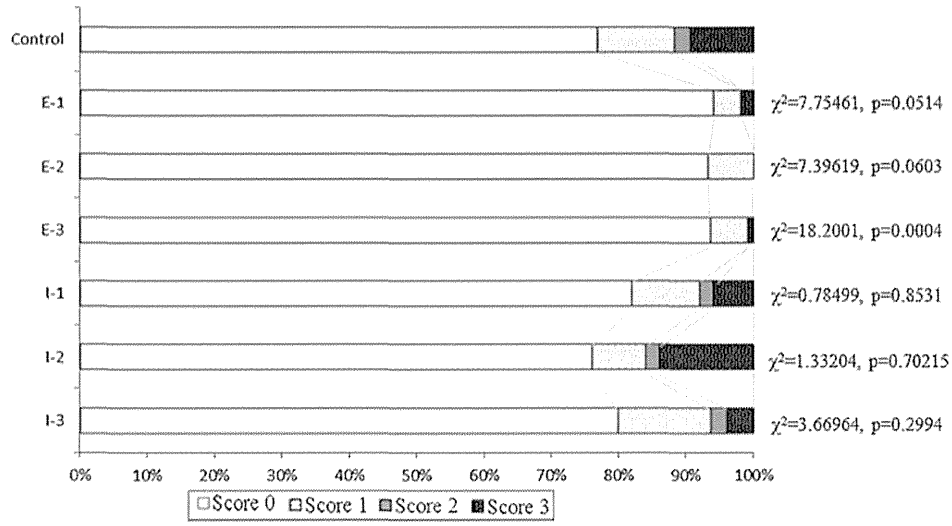

Fig. 5 Changes in the percentages of each inflammation score due to administration of the anti-TNF-α drugs. For each experimental group, the percentages of the total number of segments (i.e., the number of mice ×5 segments) having each score (i.e., 0, 1, 2 or 3) were calculated. Those percentages were then compared between the experimental groups. In group E-3, the percentage of each score differed significantly from the control group. Lesions assessed as score 1 or greater were decreased in percentage, while lesions scored as 0 were increased

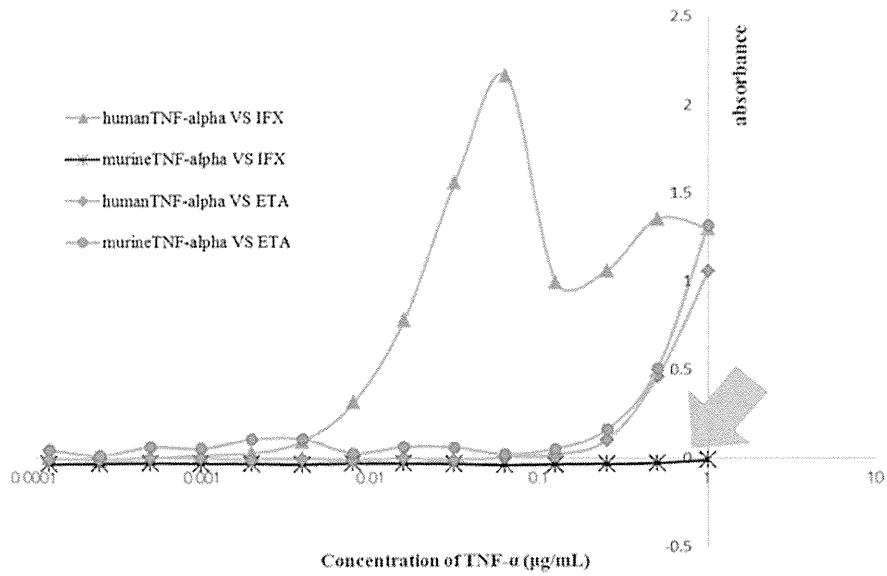

Fig. 6 Binding affinities of IFX and ETA for human and murine TNF-α. ETA bound to both human and murine TNF-α. IFX bound to human TNF-α in a concentration-dependent manner, but it did not bind to murine TNF-α (*arrow*)

The results showed that the incidences of lesions with an inflammation score of 1 or greater were reduced, while the incidence of lesions with a score of 0 was increased. It can be surmised that the process of progression of lesions from score 0 to score 1 was inhibited by ETA, and that ETA inhibits development of endoarteritis. This suggests that TNF–α is intimately involved in the onset and progression of endoarteritis in the early stage of the process of establishment of vasculitis. It is known that TNF-α directly and indirectly promotes adhesion between endothelial cells and inflammatory cells, especially neutrophils [26], findings that can be thought to support our present observations. However, our present experiments are unable to clarify whether the involvement of TNF-α is limited to just the onset and progression of endoarteritis, or extends even to the process of establishment of panvasculitis following endoarteritis. It is necessary to conduct time-course studies of the effects of anti-TNF-α agents on the histological changes that lead to the establishment of panvasculitis, as well as elucidate whether there are differences in the vasculitis-inhibiting effects of those agents as a function of when they are administered. Also, although cutaneous leukocytoclastic vasculitis was reported as an adverse reaction of ETA [27], we found no evidence of that adverse reaction in the ETA-administered animal groups in this study.

There are some reports regarding the efficacy of IFX in murine models of human diseases, such as asthma, muscular dystrophy and transplant atherosclerosis [28–30]. However, IFX, which is considered to be effective in the treatment of KD, was ineffective in our present murine model of KD vasculitis. This might be explained by the

fact that our binding affinity study found that IFX did not bind to murine TNF-α. IFX is a monoclonal antibody specific for human TNF-α, and, due to its characteristics as an antibody preparation, it was suggested that IFX binds only to specific target molecules on the surface of human TNF-α [31]. It can thus be thought that the difference in the structures of murine TNF-α and human TNF-α accounts for the difference in the therapeutic effects between ETA and IFX seen in the present murine model study. That is, the differences in the vasculitis-inhibiting effects of ETA and IFX in this study are due to the species differences between humans and mice. Accordingly, the results of this study are in no way to be construed as demonstrating that ETA is superior to IFX in treatment of KD.

Conclusion

We administered anti-TNF-α drugs to treat a murine model of KD vasculitis and investigated the relationship of TNF-α to vasculitis on the basis of histopathological observations. The results revealed that ETA shows potent inhibitory effects on the vasculitis in that model, and that TNF-α plays an important role in the development of vasculitis. It is highly likely that elucidation of the role of TNF-α in the process of development of vasculitis in the *C. albicans*-induced murine KD vasculitis model, which histopathologically resembles KD vasculitis, will help in clarifying the pathophysiology of KD vasculitis.

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Conflict of interest None.

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