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Circulating Myeloid Dendritic Cells is Decreased in the Acute Phase of Kawasaki Disease

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Abstract

Background: Kawasaki disease is the most prevalent vasculitis of children in the developed countries that affects middle-sized arteries. Though T-cells are known to be activated with ample production of cytokines in acute phase of Kawasaki disease, there is a paucity of data concerning dendritic cells (DCs), the most potent antigen presenting cells that initiates T-cell activation. This study examined change in circulating DCs in acute phase of Kawasaki disease.

Methods: Using multi-color flow cytometry, we determined circulating myeloid DC (mDC), $Lin^-HLA\text{-}DR^+CD11c^+$ cell, and plasmacytoid DC (pDC), $Lin^-HLA\text{-}DR^+CD123^+$ cell in 33 patients with acute phase of Kawasaki disease (aKD), 24 febrile controls (FC), and 13 healthy controls (HC). Blood chemistry data including cytokines were determined at the same time. Numbers of DCs were compared among 3 groups and before and after immunoglobulin treatment in aKD. Correlation between numbers of circulating DCs and blood chemistry data were determined.

Results: Number of circulating mDC was significantly lower in aKD on admission than in FC and HC [median (lower, upper quartile)=7260 (2463, 11550) vs. 12210 (9500, 22050) and 18600 (11520, 23460) cells/ml, $p < 0.001$]. This number of circulating DCs significantly correlated with disease severity represented by serum albumin (mDC, $r=0.56$, $p < 0.0001$; pDC, $r=0.39$, $p < 0.02$, respectively), C reactive protein (mDC, $r=-0.42$, $p < 0.005$), and interleukin-6 (mDC, $r=-0.55$, $p < 0.007$). Immunoglobulin treatment quickly restored number of mDC [7260 (2463, 11550) vs. 15200 (10840, 30965) after IVIG and 18600 (12950, 25510) cells/ml at convalescence, $p < 0.001$] in aKD.

Conclusions: This study indicates that number of circulating mDCs is decreased in acute Kawasaki disease, and may be involved in the pathophysiology.

Keywords: Kawasaki disease; Dendritic cell; Pathophysiology; Myeloid

Introduction

Kawasaki disease is the most prevalent systemic vasculitis, and affects middle-sized arteries including coronary arteries, of children in developed countries [1,2]. In acute phase of Kawasaki disease, ample cytokines are known to increase, but the precise pathophysiology of Kawasaki disease remains unknown [3-5]. In a recent report, a functional single nucleotide polymorphism (itpkc_3) in the inositol 1,4,5-trisphosphate 3-kinase C (ITPKC) gene, a negative regulator of T-cell activation, was significantly associated with Kawasaki disease susceptibility and also with an increased risk of coronary artery lesions in both Japanese and United States children [6]. However, little is known about the role of dendritic cells (DC), highly specialized antigen presenting cells initiating and regulating T cell response [7,8], in the pathophysiology of acute Kawasaki disease.

In the human circulation, two major subsets of DCs, myeloid DC (mDC) and plasmacytoid DC (pDC), have been identified [9,10]. Myeloid DC express CD11c, leukocyte integrin alpha subunit, and polarize naïve T cells toward the T-helper 1 (Th1) phenotype, whereas pDC express CD123, interleukin-3 receptor alpha chain, and polarize T cells toward the Th2 phenotype. These DC subsets recognize different microbial pathogens through specific receptors, which in turn induce different types of innate and adaptive immune responses [10]. Both DCs originate in the bone marrow and circulate briefly in peripheral blood as precursor DCs before migrating to the peripheral tissues. Immature DCs are activated after the capture of antigens in circulation or affected tissues, and then the activated DCs migrate through lymphatic vessels to lymphoid organs where they present processed antigens to naïve T cells [7-10]. Abnormalities of DC homeostasis have been involved in the pathophysiology of various human diseases, including autoimmune diseases [11-15], ischemic heart disease [16], and viral infection [17-19].

Especially, in patients with ischemic heart disease, the number of circulating DCs decreases and DC has been found to accumulate with activated T cells in atherosclerotic plaques [16]. Similarly, Yilmiz et al. reported that activated mDCs accumulate and co-localize with T cells in coronary artery lesions both in human patients with Kawasaki disease [20] and in a murine model of coronary arteritis mimicking Kawasaki disease [21]. In addition, Khor et al. recently identified susceptible alleles in ATP-binding cassette, subfamily C, member 4 (ABCC4) for Kawasaki disease from genome-wide linkage and association mapping

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[22]. ABCC4 is a multifunctional cyclic nucleotide transporter that known to stimulate the migratory capacity of dendritic cells.

Therefore we hypothesized that DCs are involved in the pathophysiology of acute phase of Kawasaki disease. The specific aim of this study was to determine the number of circulating DC in acute phase of Kawasaki disease compared with controls, its correlation with laboratory data including cytokines, and its change with immunoglobulin treatment.

Methods

Subjects were 33 patients with acute phase of KD who fulfilled the diagnostic criteria [23] of Kawasaki disease (aKD), 24 febrile controls (FC), and 13 healthy controls (HC) with comparable age (Table 1). All patients in aKD were treated with 2 g/kg of intravenous immunoglobulin treatment (IVIG) in a single infusion over 24 hours, and with 30 mg/kg of aspirin initially and 5 mg/kg of aspirin after resolution of fever either at Kurume University Hospital or St. Mary's Hospital, Kurume city, Japan. Initial IVIG was given at 5.6 ± 1.7 days of illness and 2 patients (5.7%) required additional IVIG because of resistance to IVIG. Also 1 patient (1.9%) required cardiotonic drugs and diuretics because of impaired cardiac contractility and moderate mitral valve regurgitation. In addition, 12 patients (34 %) showed transient dilation of coronary artery with more than + 2.5 standard deviation of population normal in diameter, but no patient left with coronary artery aneurysms at convalescence.

The primary diagnosis of FC included bacterial infection with unspecified focus in 15, pneumonia in 5, colitis in 1, lymphadenitis in 1, meningitis in 1, urinary tract infection in 1. All patients in FC were successfully treated with appropriate antibiotics.

In each group, blood samples were obtained on admission or recent visit and, in aKD, additionally at 48 hours after IVIG and at convalescence around 1 month after the onset. We determined numbers of circulating mDC and pDC using multi-color flow-cytometry, complete blood count including white blood cell count, platelet count, and hematocrit, and blood chemistry including C-reactive protein (CRP), serum sodium, and albumin.

We compared demographic data, numbers of mDC and pDC, laboratory data between groups and determined correlation between these variables and numbers of mDC as well as pDC.

Additionally, we determined serum cytokines in 20 of aKD, 10 of FC, and 9 HC at presentation and compared them among 3 groups. In addition, we determined correlation between numbers of mDC or pDC and serum cytokines in pooled data of aKD and HC.

Three-color flow cytometry

The numbers of DCs were determined using three-color flow cytometry as previously described elsewhere [24]. In brief, whole peripheral blood samples obtained from the subjects were incubated with phycoerythrin (PE)-conjugated anti–IL-3 receptor chain (CD123), PE-conjugated anti-CD11c, peridinin chlorophyll protein (PerCP)-conjugated anti–HLA-DR monoclonal antibody (mAb), and fluorescein isothiocyanate (FITC)-conjugated lineage cocktail 1 (Lin 1) for 20 minutes at room temperature. The Lin 1 contains mAb clones against CD3 (T cells), CD14 (monocytes / macrophages), CD16 (natural killer cells), CD19 (B cells), CD20 (B cells), and CD56 (natural killer cells). The erythrocytes were lysed with FACS lysing solution (Becton Dickinson, Franklin Lakes, NJ, USA). After washing with phosphate buffer saline, the stained cells were analyzed with a FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Dendritic cells were defined as the cells positive for PerCP-conjugated anti-HLA-DR mAb but negative for FITC-conjugated Lin1 (Figure 1a and b). Anti-CD11c and anti-CD123 mAb conjugated with PE were used for further identification of the mDC and pDC subsets (Figure 1c and d). Cells labeled with isotype control antibodies were included to determine background fluorescence. Three-color analysis was performed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the BD CellQuest™ software. The number of total WBCs in the samples was determined using an automated cell counter. The absolute number of mDCs and pDCs was calculated from the WBC count multiplied by the proportion of each subset within WBC, as determined by flow cytometric analysis.

Determination of serum inflammatory cytokines using cytometric beads array

One milliliter of peripheral blood was sampled, and serum levels of cytokines were quantified with the cytometric beads array (CBA) kits and BD CBA software (Becton Dickinson, Franklin Lakes, NJ, USA). Measured serum cytokines included Interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor-α (TNF-α). In general, each cytokine is categorized by its main action; IL-1β, IL-6, and TNF-α are thought to be pro-inflammatory cytokines, IL-8 to be a chemokine, IL-10 to be an immunosuppressive cytokine, and IL-12p70 to be regulator of DC-T cell interactions. These assay kits contained a mixture of five types of microbeads with distinct fluorescent intensities (FL-3) and were precoated with capture antibodies specific for each cytokine. Fifty microliters of serum, cytokine standard was added to the premixed micro beads in Falcon tubes (BD Biosciences, Carlsbad, CA). After the addition of 50 uL of a mixture of phycoerythrin (PE)-conjugated antibodies against the cytokines, the mixture was incubated for 3 hours in the dark at room temperature. This mixture was washed and centrifuged at 500*g* for 5 minutes and the pellet resuspended in 300 μL of wash buffer. A FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was calibrated with setup beads, and 3000 events were acquired for each sample. Individual cytokine concentration was determined by measuring individual fluorescence intensities (Fl-2) and computing with the standard reference curve of the software (BD CellQuest™ and CBA software).

This study protocol was approved by ethics committee of Kurume University School of Medicine and written informed consent to participate in this study was obtained from all parents or guardians of the subjects. Of note, these measurements including the number of

	Acute phase of Kawasaki disesae	Febrile Control	Healthy Control
number of Patients	33	24	13
Sex (Male / Female)	18 / 15	13 / 11	8 / 5
Age (years)	2.6 ± 2.1	2.2 ± 1.5	3.0 ± 2.5
Days of Illness (days)	5.6 ± 1.6	4.6 ± 2.2	-
C-reactive protein (mg/l)	86 ± 48*	75 ± 54*	1 ± 1
White blood cell (/μl)	13820 ± 5330*	15390 ± 7490*	8420 ± 2320
Hematocrit (%)	32.3 ± 2.8*	32.6 ± 3.1*	37.3 ± 2.8
Platelet count (x10^4/μl)	35.7 ± 10.4	34.8 ± 10.9	32.5 ± 4.7
Sodium (mmol/l)	134 ± 2*	135 ± 2*	138 ± 2
Albumin (g/l)	33 ± 4*,#	38 ± 4	41 ± 2

*, p <0.05 vs. Healthy Control
#, p<0.05 vs. Febrile Control

Table 1: Patients' Profile.

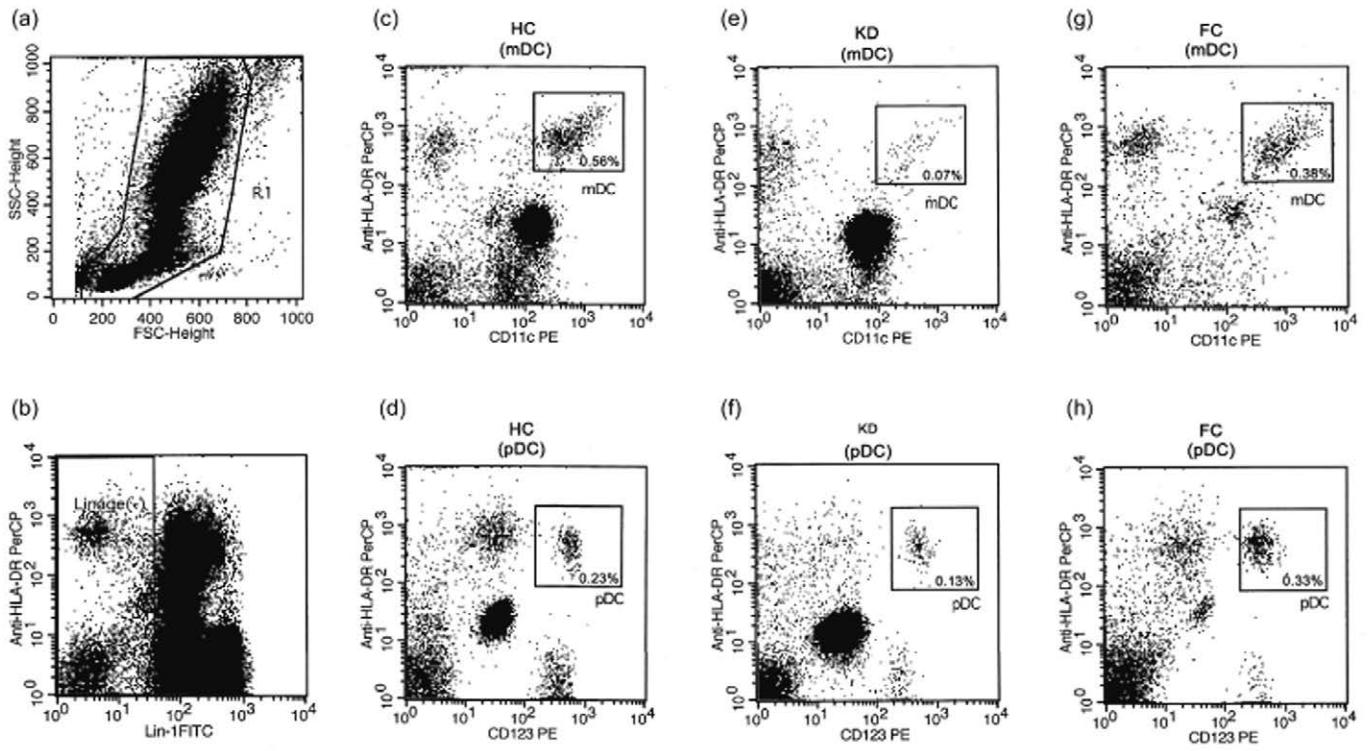

Figure 1: Identification of mDCs and pDCs in the circulation by flow-cytometry. Whole peripheral leukocytes were gated based on their forward and side scatter (R1 region) (a) and lineage-negative (Lin^-) cells were selected in R2 region (b). The cells in R1 and R2 were further analyzed for the expression of CD11c (c, e, g) and CD123 (d, f, h). Myeloid DCs and pDCs were defined as $Lin^-HLA\text{-}DR^+CD11c^+$ and $Lin^-HLA\text{-}DR^+CD123^+$cells, respectively. Representative profiles of the circulating DCs subsets of healthy control (c, d), patients with Kawasaki disease (e, f), and febrile control (g, h) are shown. Note the significantly decreased number of circulating mDCs in patients with Kawasaki disease.

DCs and inflammatory cytokines were made by 2 investigators (T.T. and H.O.) that were blinded to the clinical profile of each study subject.

Statistical analyses

Variables were expressed as mean and standard deviations or median and range as appropriate. We compared number of DCs, demographic data, and blood chemistry data among the 3 groups using the Kruskal-Wallis test and determined differences in consecutive changes of number of DCs among 3 time points using the Kruskal-Wallis test with post-hoc analysis using Dunn's method. In addition, we determined correlation between number of mDCs and blood chemistry data such as CRP, albumin, inflammatory cytokines, and chemokines in AKD using Spearman's rank correlation coefficient. Level of statistical difference was set at $p < 0.05$. All data analysis was performed by a commercially available statistical analysis software package (Statview 5.0, SAS Institute Inc, Cary, NC, USA, StatMateIV, Atms, Tokyo, Japan, or PASW Statistics 17.0, SPSS Inc, Chicago, IL, USA).

Results

There was no significant difference in age or sex distribution among 3 groups and number of febrile days at study between aKD and FC. As expected, aKD and FC showed significantly higher white blood cell count (13820 ± 5330 and15390 ± 7490 vs. 8420 ± 2320/μl) and CRP (86 ± 48 and 75 ± 54 vs. 1 ± 1 mg/l) but significantly lower hematocrit (32.2 ± 2.8 and 32.8 ± 3.0 vs. 37.3 ± 2.8 %) and sodium (134 ± 2 and 135 ± 3 vs. 138 ± 2 mEq/l) than HC (Table 1). There was no significant difference between aKD and FC in white blood cell counts, CRP, hematocrit, and sodium. However, aKD showed significantly lower serum albumin (33 ± 4 vs. 38 ± 4 and 41 ± 2 g/l) than FC or HC, characterizing blood chemistry profile in aKD.

Change in circulating DCs

Before IVIG, aKD showed significantly fewer circulating mDC [median (lower, upper quartile)=7260 (2463, 11550 vs. 12210 (9500, 22050) and 18600 (11520, 23460) cells/ml, $p < 0.001$] than FC and HC, but did not show any significant difference in circulating pDC compared with FC or HC [8100 (2553, 13090) vs. 8275 (5125, 13545) and 8690 (6060, 19600) cells/ml, p=0.43 (Figure 1c - h, 2A). In aKD, the number of mDC quickly restored [7260 (2463, 11550) vs. 15200 (10840, 30965) after IVIG and 18600 (12950, 25510) cells/ml at convalescence, $p < 0.001$], but the number of pDC did not change significantly [8100 (2553, 13090) vs. 10700 (6465, 14265) after IVIG and 13625 (11040, 16045) cells/ml at convalescence, NS] after IVIG (Figure 2B).

Of note, the number of mDC as well as pDC significantly positively correlated with serum albumin (mDC, $r=0.56$, $p < 0.0001$, Figure 3A, and pDC, $r=0.39$, $p < 0.02$, respectively) and the number of mDCs significantly negatively correlated with CRP ($r=-0.42$, $p < 0.005$, Figure 3B), but pDC did not. Either mDC or pDC did not correlate with serum sodium level.

Serum cytokines and its correlation with circulating DCs (Table 2)

aKD showed significantly higher IL-6 ($p < 0.0001$), IL-8 (p=0.0024), and IL-10 (p=0.0025), but significantly lower IL12-p70 (p=0.0035) than

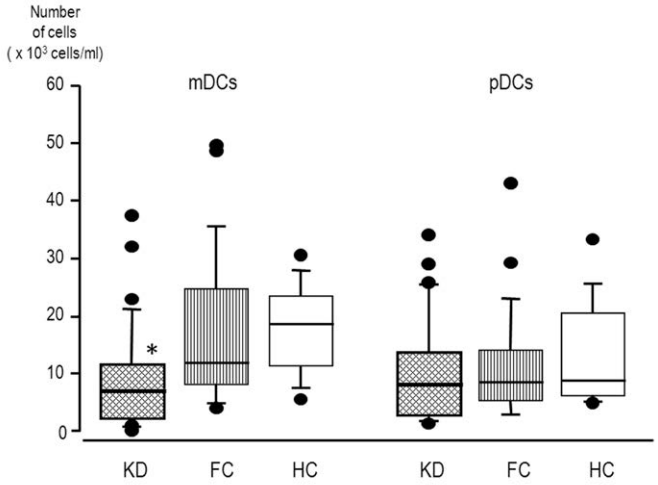

Figure 2A: Comparison of the number of circulating dendritic cells among 3 groups. Before immunoglobulin treatment, patients with Kawasaki disease showed significantly fewer circulating myeloid dendritic cells ($p < .01$) than febrile or healthy controls, but did not show any significant difference in circulating plasmacytoid dendritic cells compared with febrile or healthy controls ($p=0.43$).

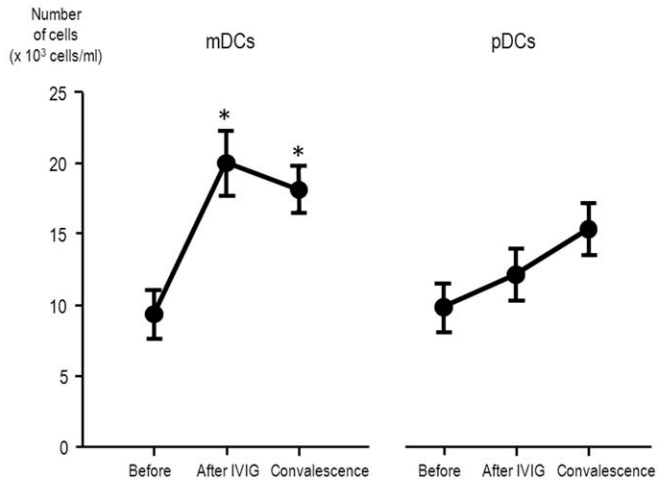

Figure 2B: Change in the number of circulating dendritic cells in patient with acute Kawasaki disease. After immunoglobulin treatment, the number of myeloid dendritic cells promptly normalized ($p <.001$) but the number of plasmacytoid dendritic cells increased gradually with time.

FC and HC (Figure 4). Among these elevated cytokines, IL-6 ($r=-0.50$, $p < 0.02$) and IL-10 ($r=-0.44$, $p < 0.04$), significantly inversely correlated with circulating DCs. There was no significant difference in IL-1β and tumor necrosis factor-α (TNF-α) among the groups.

Discussions

This study indicates that circulating mDCs might be involved in the pathophysiology of acute phase of Kawasaki disease. We demonstrated that aKD exhibited a specific and significant decrease in the absolute number of circulating mDC when compared to patients with other febrile disease or healthy controls. In addition, the number of circulating mDCs decreases with an inverse correlation with disease severity of Kawasaki disease and the number of circulating mDC is restored quickly by IVIG.

This association between reduced number of circulating DCs and diseases activity is reported in many types of autoimmune diseases such as Sjögren's syndrome [11,12], systemic lupus erythematosis [13], sarcoidosis [14], and graft versus host disease [15]. In patients with graft-versus-host disease [15], the decrease in circulating DCs is associated with the clinical manifestations of disease in temporal profile. When patients showed active skin or liver disease, the number of circulating DCs reduced. In these diseases, mature DCs were reported to be found in active lesion of the disease, such as in labial

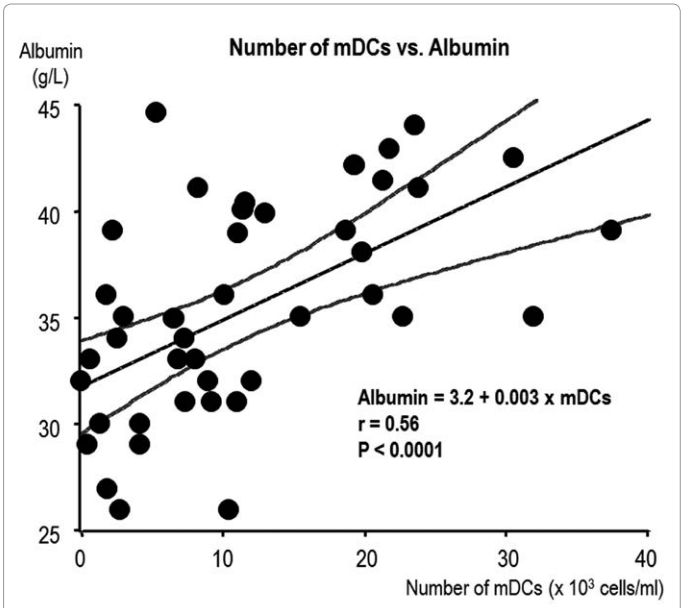

Figure 3A: Correlation between the number of myeloid dendritic cells and serum albumin. In pooled data of Kawasaki disease and healthy control, the number of myeloid dendritic cells significantly positively correlated with serum albumin ($r = 0.56$, $p < .0001$).

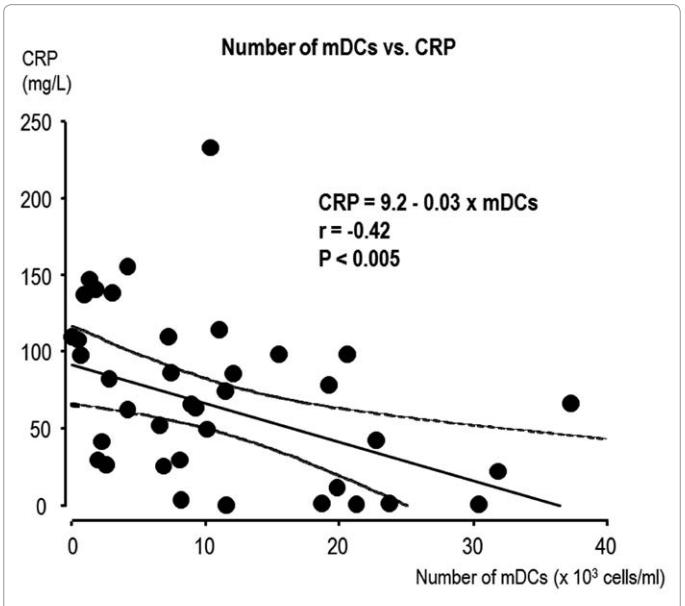

Figure 3B: Correlation between the number of myeloid dendritic cells and C-reactive protein. In pooled data of Kawasaki disease and healthy control, the number of myeloid dendritic cells significantly negatively correlated with C-reactive protein ($r = -0.42$, $p < .005$).

	All DCs	Myeloid DC	Plasmacytoid DC
IL-1β	Ns.	Ns.	Ns.
IL-6	R=-0.50, p=0.01	R = -0.46, p=0.003	R = -0.36, p=0.03
IL-8	Ns.	R = -0.34, p=0.03	Ns.
IL-10	R=-0.44, p=0.03	R = -0.41, p=0.01	R = -0.34, p=0.03
IL-12p70	Ns.	Ns.	Ns.
TNF-α	Ns.	Ns.	Ns.

Abbreviations
DC: Dendritic cell; IL: Interleukin; Ns: No significant difference; TNF-α: Tumor necrosis factor-α

Table 2: Correlation between cytokines and number of myeloid DC as well as plasmacytoidDC in acute phase of Kawasaki disease.

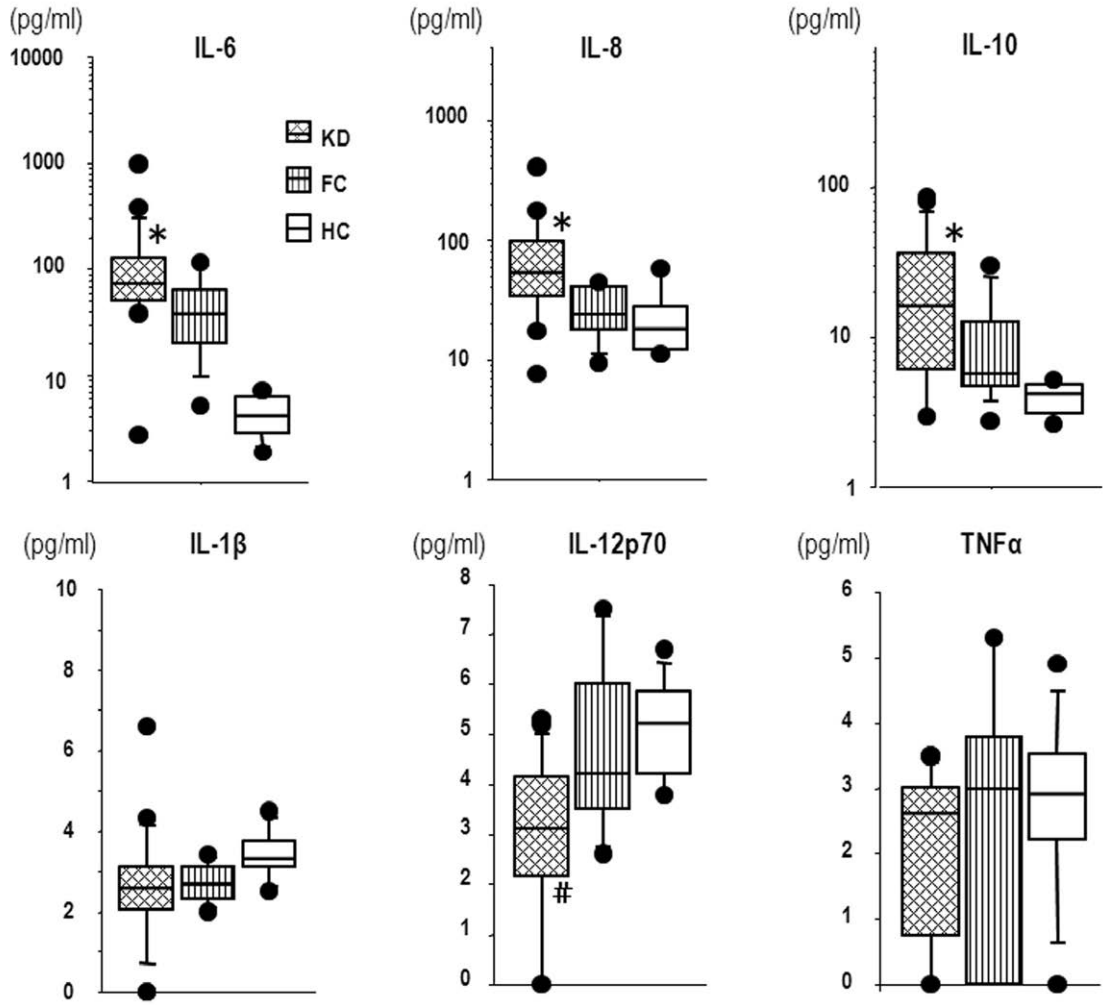

Figure 4: Comparison of inflammatory cytokines among 3 groups. Patients with Kawasaki disease showed significantly higher IL-6 (p<0.0001), IL-8 (p=0.0024), and IL-10 (p=0.0025), but significantly lower IL12-p70 (p=0.0035) than febrile and healthy controls. There was no significant difference in IL-1β and tumor necrosis factor-α among the groups.

salivary glands in Sjögren syndrome [11,12], in granulomas together with T cells in sarcoidosis [14], and in skin biopsy specimen of graft-versus-host disease [15]. In this study, the number of circulating DCs was associated with laboratory maker of disease severity of Kawasaki disease such as serum level of albumin; the characteristic change in KD [25,26] and levels of inflammatory pro-cytokines, CRP and IL-6.

Various possible mechanisms could be involved in decreased peripheral DCs. One hypothesis is that these cells are more susceptible to apoptosis and may undergo peripheral destruction [27]. However, it is possible that the reduced number of circulating DC could be due to their enhanced recirculation from blood to affected tissues. In Sjögren syndrome, a negative correlation was observed between the number of circulating mDCs and the frequency of tissue-infiltrated DCs [12]. Therefore, selective trafficking of DCs into target tissues and the resultant decrease of the circulating pool of DCs might be one of the mechanisms. Interestingly, IL-6 is reported to regulate the selective influx of DC subsets into an inflamed lymph node [28]. In mouse model, IL-6 has an important role in the accumulation of CD11b+DCs

and CD8+ DCs in response to the injection of bacterial peptidoglycan, as well as a role in regulating changes in overall lymph node cellularity [28]. Increased IL-6 in Kawasaki disease might not act as just one of the pro-inflammatory cytokines but also take some role in the recirculation of DCs to affected tissues.

On the other hand, the fact that number of circulating DCs is reduced at the initial presentation of Kawasaki disease should draw attention in terms of the etiological aspect because the initial trigger of this disease is still unknown. Number of circulating DCs is reported to decrease in infection of certain viruses such as influenza H1N1 virus [17], cytomegalovirus [18], and dengue virus [19]. Because clinical picture of Kawasaki disease, including hematological and blood chemistry data, is not compatible with these known viral infections, these viruses should not take etiological role. However, it may be possible that unknown virus infection [29] may induce an initial process and decrease circulating DCs.

Furthermore, the fact that immunoglobulin treatment is the most effective treatment in Kawasaki disease may strengthen the possible involvement of DCs. Regular immunoglobulin treatment is reported to be effective in experimental model of graft-versus-host disease [30] in which pathophysiological involvement of DCs is postulated and the reduced number of DCs is documented.

Cytokine profile in this study was compatible with previous reports except for TNF-α. Though many cytokines, such as IL-6, IL-8, and IL-10 in this study, were reported to increase in Kawasaki disease [31,32], IL-12p70 was not reported to increase [33]. Indeed, IL-12p70 in aKD was significantly lower than those in FC and HC in this study. Because production of IL-12p70 by DCs may depend on DC subset [34], this decreased level of IL-12p70 may be associated with decreased number of circulating mDCs. On the other hand, we could not show typical increase of TNF-α, because we measured TNF-α, unstable short half-life cytokine, and we should have measured soluble form receptor of TNF-α, which should reflect the true biological activity of TNF-α [35].

Study Limitation

This study has several limitations. Because we did not have methods to look for localization of DCs in live human subjects or the approval to examine affected tissue, such as lymph nodes, in patients with Kawasaki disease, the real mechanisms involved in the significant decrease of DCs are not clear. Also, it was difficult for us to examine functional status of DCs including co-stimulatory molecules because the severer the disease, the less DCs circulate and small subjects' body size did not allow us to obtain enough blood samples.

Conclusions

This study indicates that circulating mDC is decreased in acute phase of Kawasaki disease and may play a significant role in the pathophysiology of this disease.

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Part of this study was presented at the annual meeting of American Heart Association 2011, Orland, FL, USA.

Declaration

We declare that there is no conflict of interest in this study.

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