nature publishing group

Relationship between T-cell HLA-DR expression and intravenous immunoglobulin treatment response in Kawasaki disease

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BACKGROUND: Kawasaki disease (KD) is an acute febrile illness associated with the development of vasculitis. Administration of intravenous immunoglobulin (IVIG) is the standard treatment for KD. However, IVIG treatment is not effective in approximately 15% of children with KD. Some reports have presented evidence of immunological responses in IVIG-resistant KD patients. We assessed the possibility that T-cell activation is a contributing mechanism underlying this phenomenon.

METHODS: We analyzed human leukocyte antigen-DR (HLA-DR) expression on peripheral blood $CD4^+$ and $CD8^+$ T cells in 82 children with KD who were admitted to the hospital between October 2007 and February 2012. We compared the percentages of $HLA\text{-}DR^+$ T cells among the $CD4^+$ T-cell and $CD8^+$ T-cell populations for the IVIG-effective and IVIG-resistant groups.

RESULTS: Among the 82 subjects, 51 had IVIG-effective KD and 31 children had IVIG-resistant KD. The percentages of $HLA\text{-}DR^+$ T cells among the $CD4^+$ T-cell and $CD8^+$ T-cell populations in the IVIG-effective group were significantly lower than those in the IVIG-resistant group.

CONCLUSION: Our results suggest that increased T-cell HLA-DR expression is associated with IVIG resistance in KD patients, indicating that HLA-DR expression would be a useful tool for predicting IVIG responsiveness during KD pathogenesis.

Kawasaki disease (KD), known to be associated with immune-mediated damage of blood vessels, or vasculitis, is an acute febrile illness that primarily affects young children (1,2). The most serious complication of KD is the development of coronary arterial lesions (CALs), which, in some cases, can cause artery blockage, myocardial infarction, and death (3). Notably, high-dose intravenous immunoglobulin (IVIG) has been shown to reduce the incidence of CALs in the majority of KD patients, and has become the standard treatment option for this disease (4–6). However, IVIG treatment is not effective in 13–21% of cases (7). This varied response to treatment likely stems from our weak understanding of the underlying cause of KD. For example, although KD is known to involve the abnormal activation of the entire immune system, stimulating both innate and adaptive immunity (8), the details concerning the biological triggers of this response have not yet been characterized.

Activation of the immune system during KD appears to initially occur in the mucosal lymphoid tissues through the activation of T- and B-lymphocyte cells (8). This triggered activation, in turn, starts a systemic immune response leading to uncontrolled inflammation and vasculitis in genetically susceptible patients (9–11). Although a number of previous studies have sought to determine the cause of this disease, very few have addressed the role of the different peripheral T-cell populations in the acute phase of KD or in the effectiveness of IVIG treatment (12,13). Autopsy studies of children with KD who died during the acute phase clearly indicate that $CD4^+$ and $CD8^+$ T cells participate in the transmural infiltration of the coronary arterial wall (14). Notably, human leukocyte antigen-DR (HLA-DR), a cell-surface glycoprotein encoded by the HLA-DR region of the major histocompatibility complex (15), is a known marker of T-cell activation (16–18), but the function of this antigen on the different T-cell populations during KD is unknown. In this study, we have investigated the relationship between HLA-DR expression on peripheral $CD4^+$ and $CD8^+$ T cells, and thus the activation of these cells, and the response to IVIG treatment in children with KD.

RESULTS

Following IVIG treatment, we monitored the fever temperature for each patient over a 48-h time period. Group A, or the IVIG-effective patients with reduced fever after IVIG infusion, consisted of 51 patients (31 boys, 20 girls; aged 2.5 ± 2.3 y; median, 1.7 y), while Group B, or the IVIG-resistant patients with continued fever, consisted of 31 patients (23 boys, 8 girls; aged 3.8 ± 3.5 y; median, 3.1 y). **Tables 1** and **2** list the clinical features and general whole blood analysis, respectively, for the KD patients in both groups.

The results of our $CD4^+$ T cell and $HLA\text{-}DR^+CD4^+$ T cell analysis are shown in **Table 3** and **Figure 1**. The percentage of $HLA\text{-}DR^+CD4^+$ T cells among peripheral blood mononuclear

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Received 18 May 2014; accepted 19 October 2014; advance online publication 4 February 2015. doi:10.1038/pr.2015.12

Table 1. Clinical features of children with KD

	KD ($n = 82$)		*P* value
	IVIG effective ($n = 51$); Group A	IVIG resistant ($n = 31$); Group B	A vs. B
Median age (years (range))	1.7 (1.0–7.5)	3.1 (0.2–9.1)	0.257
Male (*n* (%))	31 (60.8)	23 (74.2)	0.240[a]
Pyrexia (*n* (%))	51 (100.0)	31 (100.0)	1.000[a]
CALs (*n* (%))	1 (2.0)	7 (22.6)	0.004**,[a]
Starting day of IVIG (day (range))	5 (3–9)	4 (3–11)	0.317

CALs, coronary arterial lesions; IVIG, intravenous immunoglobulin; KD, Kawasaki disease.
[a]Fisher exact test was used.
**$P < 0.01$.

Table 2. General analysis of whole blood isolated from children with KD

	KD ($n = 82$)		*P* value
	IVIG effective ($n = 51$); Group A	IVIG resistant ($n = 31$); Group B	A vs. B
White blood cell counts (/μl)	12,632 ± 4,537	15,441 ± 7,639	0.153
Hemoglobin concentrations (g/dl)	11.4 ± 1.1	10.6 ± 1.4	0.006**
Platelet counts ($\times 10^9$/l)	348 ± 76	444 ± 197	0.066
Albumin (g/dl)	3.7 ± 0.4	3.0 ± 0.8	<0.001†
Total bilirubin (mg/dl)	0.7 ± 0.8	1.1 ± 1.0	0.124
Aspartate aminotransferase (IU/l)	91.5 ± 154.8	155.1 ± 242.4	0.188
Alanine aminotransferase (IU/l)	85.3 ± 173.7	120.9 ± 158.2	0.056
C-reactive protein (mg/dl)	7.2 ± 4.5	11.1 ± 7.6	0.023*
Sodium (mmol/l)	135.1 ± 2.2	133.9 ± 1.6	0.005**
D-dimer (μg/ml)	2.5 ± 2.8	3.2 ± 2.0	0.011*

IVIG, intravenous immunoglobulin; KD, Kawasaki disease.
*$P < 0.05$; **$P < 0.01$; †$P < 0.001$.

Table 3. $CD4^+$ and $HLA\text{-}DR^+$ $CD4^+$ T cells in children with KD

	KD ($n = 82$)		P value
	IVIG effective ($n = 51$); Group A	IVIG resistant ($n = 31$); Group B	A vs. B
PBMCs (/μl)	4,318 ± 1,829	5,499 ± 4,570	0.339
$CD4^+$ T cells (/μl)	1,601 ± 957	1,876 ± 1,323	0.293
$CD4^+$ T cells among PBMCs (%)	35.6 ± 10.7	34.4 ± 7.3	0.916
$HLA\text{-}DR^+$ $CD4^+$ T cells (/μl)	70 ± 47	131 ± 110	0.004**
$HLA\text{-}DR^+$ $CD4^+$ T cells among PBMCs (%)	1.7 ± 1.1	2.3 ± 1.0	0.005**
$HLA\text{-}DR^+$ $CD4^+$ T cells among $CD4^+$ T cells (%)	5.1 ± 3.4	6.8 ± 2.8	0.003**

IVIG, intravenous immunoglobulin; KD, Kawasaki disease; PBMCs, peripheral blood mononuclear cells.
**$P < 0.01$.

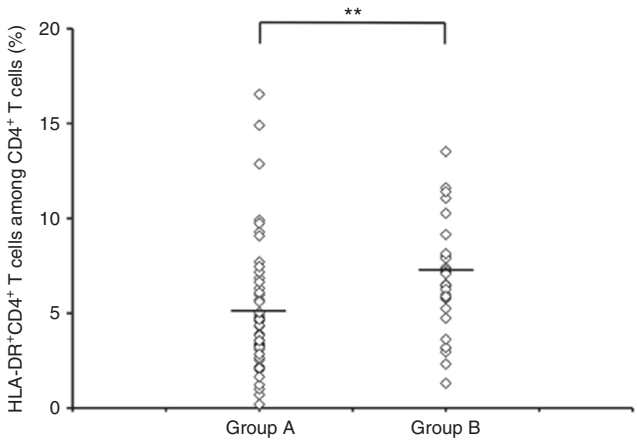

Figure 1. The relationship between intravenous immunoglobulin (IVIG) effectiveness and $HLA\text{-}DR^+CD4^+$ T cells. The percentages of peripheral blood $HLA\text{-}DR^+CD4^+$ T cells among $CD4^+$ T cells in patients with Kawasaki disease (KD) were analyzed by flow cytometry. Group A: IVIG-effective KD patients, Group B: IVIG-resistant KD patients. **$P < 0.01$. Horizontal lines indicate means.

cells was significantly lower in Group A compared to Group B ($P = 0.005$). The $HLA\text{-}DR^+CD4^+$ T-cell count and percentage among the $CD4^+$ T cells in Group A were also significantly lower than those observed for Group B ($P = 0.004$ and $P = 0.003$, respectively). Taken together, these data indicate a significant relationship between the level of HLA-DR expression, and thus $CD4^+$ T-cell activation, and the effectiveness of IVIG treatment.

The results of our $CD8^+$ T cell and $HLA\text{-}DR^+CD8^+$ T cell analysis are shown in **Table 4** and **Figure 2**. $CD8^+$ T cells and $HLA\text{-}DR^+CD8^+$ T cells exhibited very similar trends to those observed for $CD4^+$ T cells and $HLA\text{-}DR^+CD4^+$ T cells. The count of $HLA\text{-}DR^+CD8^+$ T cells in peripheral blood mononuclear cells was significantly lower in Group A compared to Group B ($P = 0.019$), while the percentage of $HLA\text{-}DR^+CD8^+$ T cells among the $CD8^+$ T cells in Group A was significantly less than that in Group B ($P = 0.046$). The relationship between the level of HLA-DR expression on the $CD8^+$ T cells and the effect of IVIG was similar to that found for the $CD4^+$ T cells, whereby increased HLA-DR expression is correlated to IVIG-resistance.

DISCUSSION

We have previously reported on the pathogenesis of KD (19–27); however, our understanding of what causes KD or KD-related complications, such as CALs, is limited. In terms of changes in peripheral immunocyte counts in KD patients, an increase in the number of white blood cells has been correlated with an increase in neutrophil count, but there was not a large variation in the number of mononuclear cells (19). Further, the number of $CD14^+$ monocytes/macrophages and $CD19^+$ B cells does appear to be increased among mononuclear cells, whereas the number of $CD4^+$ and $CD8^+$ T cells was shown to be slightly decreased (19,20). However, the increased or decreased expression of these cell surface receptors does not clearly indicate their activation level or function during KD. In this regard, we have utilized the expression of HLA-DR to act as a marker of

Table 4. $CD8^+$ and HLA-DR^+ $CD8^+$ T cells in children with KD

	KD ($n = 82$)		*P* value
	IVIG effective ($n = 51$); Group A	IVIG resistant ($n = 31$); Group B	A vs. B
PBMCs (/μl)	4,318 ± 1,829	5,499 ± 4,570	0.339
$CD8^+$ T cells (/μl)	1,000 ± 493	1,315 ± 1,116	0.202
$CD8^+$ T cells among PBMCs (%)	23.8 ± 7.8	23.8 ± 6.7	0.863
HLA-DR^+ $CD8^+$ T cells (/μl)	96 ± 81	186 ± 192	0.019*
HLA-DR^+ $CD8^+$ T cells among PBMCs (%)	2.5 ± 2.2	3.4 ± 2.9	0.071
HLA-DR+CD8+ T cells among $CD8^+$ T cells (%)	9.9 ± 7.4	13.7 ± 10.1	0.046*

IVIG, intravenous immunoglobulin; KD, Kawasaki disease; PBMCs, peripheral blood mononuclear cells.
*$P < 0.05$.

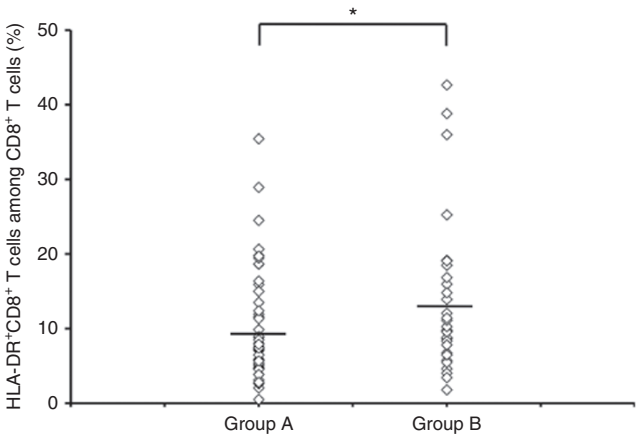

Figure 2. The relationship between intravenous immunoglobulin (IVIG) effectiveness and HLA-DR^+CD8^+ T cells. The percentages of peripheral blood HLA-DR^+CD8^+ T cells among $CD8^+$ T cells in patients with Kawasaki disease (KD) were analyzed by flow cytometry. Group A: IVIG-effective KD patients, Group B: IVIG-resistant KD patients. *$P < 0.05$. Horizontal lines indicate means.

T-cell activation (16–18), and have previously shown that its expression is low in $CD4^+$ and $CD8^+$ T cells during the acute phase of KD (20,28,29). This data was further corroborated by another research group, which observed that the number of HLA-DR^+CD8^+ T cells does not increase during the acute phase of KD (30). Further, in patients with systemic lupus erythematosus, another disease that affects the immune system, the percentage HLA-DR^+CD8^+ T cells was found to be higher among patients with active systemic lupus erythematosus compared to those with quiescent systemic lupus erythematosus (31).

Although the underlying cause of KD remains unknown, IVIG has become the standard treatment option, and has been shown to alter a number of immune-related factors. For example, it has been reported that nuclear factor-κB (NF-κB) is activated in the peripheral $CD14^+$ monocytes/macrophages of KD patients, and this activation can be reduced by IVIG treatment (21–23). Further, the increased number of peripheral monocytes/macrophages expressing both $CD14^+$ and $CD16^+$ (FcγRIII), a low-affinity IgG receptor, observed in KD patients is also strongly suppressed by treating with IVIG (24,25). However, while many studies have demonstrated the effects of IVIG treatment on KD, few of these have utilized flow cytometry (28,29). Flow cytometry allows for more accurate counting and measurement of cellular properties in comparison to other methods and is therefore a very useful clinical tool. Furthermore, even fewer studies have utilized this technique to determine the cause of IVIG-resistance.

It is likely that the aberrant immune activation occurring during KD involves a complex cascade of factors, involving both B and T cell secreted cytokines and other signaling molecules. Thus, considering the numerous mechanisms likely involved in KD pathogenesis, it is not surprising that the effectiveness of IVIG treatment can be somewhat variable. Suzuki *et al.*, (32) for example, have shown that the expression of soluble interleukin (IL)-2 receptor (another marker of T-cell activation) is elevated in IVIG-resistant KD patients. In this study, we investigated the relationship between HLA-DR expression on peripheral $CD4^+$ and $CD8^+$ T cells and IVIG responsiveness in patients with KD and found that HLA-DR expression on $CD4^+$ and $CD8^+$ T cells is significantly elevated in IVIG-resistant KD patients compared to IVIG-effective KD patients. When used in combination with our prior work demonstrating that the number and activation of $CD4^+$ and $CD8^+$ T cells is lower in the acute phase of KD patients, our data imply that KD patients with weak suppression of $CD4^+$ and $CD8^+$ T-cell activation during the acute phase may not respond to high-dose IVIG therapy. Taken together, these results indicate that HLA-DR expression on $CD4^+$ and $CD8^+$ T cells may be used as a predictive marker for the effectiveness of IVIG treatment in KD patients.

Notably, this study is not without its limitations. The size of the study is small and further studies should be conducted using a larger number of subjects in order to determine the broad implications for KD patients. Further, additional work is needed concerning the prevalence of IVIG-effective patients with high HLA-DR expression in the KD patient population to determine the full function of this cell surface glycoprotein during disease pathogenesis. In this study, we also did not investigate the proportion of HLA-DR^+CD3^+ T cells because of technical limitations. Detection of $CD3^+$ on T cells is necessary to distinguish $CD8^+$ T cells from natural killer (NK) cells, which are also involved in the immune response (33). The number of NK cells has also been shown to decrease during acute KD (34), similar to the number of $CD4^+$ and $CD8^+$ T cells, and HLA-DR is also expressed on a subset of NK cells during the initiation and amplification of inflammatory responses (35). Thus, while we do not explicitly distinguish between the NK and $CD8^+$ T cells, we believe that the change in HLA-DR expression in the general population of CD8 expressing cells (NK and $CD8^+$ T cells) is still the driving force behind the IVIG resistance observed in these patients. Moreover, this study should be complimented by a follow-up evaluation of HLA-DR-positive NK cell function during KD pathogenesis.

Finally, because of the complex nature of this disease, we suspect that the best way to determine IVIG effectiveness prior to treatment is to use a combination of markers, including HLA-DR, as highlighted here, as well as other factors that have been found to be highly expressed in IVIG-resistant KD patients. This would include markers such as polycythemia rubra vera 1 and granulocyte colony-stimulating factor (28) as well as the T helper type 17/regulatory T-cell balance and plasma concentrations of IL-17A and IL-6 (29). When used together, this combination of testing would utilize differences in gene expression, immune cell activation, and cytokine secretion to determine the probable result of IVIG treatment, thus providing us with a powerful clinical tool to determine the optimal treatment option for KD patients.

In conclusion, HLA-DR expression was significantly higher in both the $CD4^+$ and $CD8^+$ T-cell populations in IVIG-resistant KD patients compared to the IVIG-effective KD patients, suggesting that T-cell HLA-DR expression is associated with IVIG resistance. Thus, we suspect that assessing HLA-DR expression on T cells would be a useful tool for predicting IVIG responsiveness during the acute phase of KD, particularly when used in combination with other markers. Further studies should be performed to determine suitable clinical testing for HLA-DR, as well as combination testing, prior to IVIG treatment in order to determine the best course of treatment for the patient.

METHODS

Subjects

This study included 82 children with KD who were admitted to the Department of Pediatrics at Yamaguchi University Hospital between October 2007 and February 2012. There were 54 boys and 28 girls, aged 2.8 ± 2.6 y (median, 1.8 y). All the patients fulfilled the criteria described in the fifth revision of the Diagnostic Guidelines for KD (36). Only typical cases, in which the subject showed at least five of the six principal symptoms associated with KD or four of the principal symptoms associated with CALs, were included in this study. Further, patients that had been administered steroid or immunosuppressive therapy as adjunctive primary therapy with IVIG were excluded. To the 82 patients fulfilling these inclusion and exclusion criteria, we then administered IVIG (2 g/kg) and oral aspirin (30 mg/kg daily). This study was approved by the Institutional Review Board at Yamaguchi University Hospital, and the parents of the patients enrolled in this study all provided informed consent. We considered patients who showed reduced fever within 48 h after the end of IVIG infusion as "IVIG-effective" cases (Group A), and those of patients with continued fever as "IVIG-resistant" cases (Group B).

Flow Cytometry

We collected heparinized whole blood from children in the acute phase of KD that were enrolled in this study just before IVIG infusion. The blood samples were tested shortly after collection. All the reagents and equipment used for fluorescence-activated cell sorting (FACS) analysis were purchased from BD Biosciences (Franklin Lakes, NJ). Samples were labeled with peridinin chlorophyll protein (PerCP)-conjugated anti-HLA-DR along with FITC-conjugated mouse anti-human CD4 or FITC-conjugated mouse anti-human CD8. Samples were also labeled with FITC-conjugated mouse IgG1 and PerCP-conjugated mouse IgG2a as a negative control. Erythrocytes were lysed with FACS Lysing Solution. The cells were centrifuged and resuspended in FACS Flow. For each sample, 5,000 cells were analyzed by a FACScaliber flow cytometer equipped with CellQuest software (BD Biosciences).

Statistical Analysis

Differences between the two groups were analyzed using the Mann–Whitney *U*-test or the Fisher exact test, and *P* values less than 0.05 were considered statistically significant. These analyses and calculations were performed using the Statistical Package for Social Sciences (SPSS), version 12.0 (SPSS, Chicago, IL).

ACKNOWLEDGMENTS

We sincerely thank the volunteers for participating in these studies.

STATEMENT OF FINANCIAL SUPPORT

No financial assistance was received to support this study.

Disclosure: The authors have no conflicts of interest to declare.

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