Expression of CD40 Ligand on CD4+ T-Cells and Platelets Correlated to the Coronary Artery Lesion and Disease Progress in Kawasaki Disease

Chih-Lu Wang, MD*; Yu-Tsun Wu, MD‡; Chieh-An Liu, MD*; Mei-Wei Lin, BS*; Chia-Jung Lee, BS*; Li-Tung Huang, MD*; and Kuender D. Yang, MD, PhD*

ABSTRACT. Objective. Kawasaki disease (KD) is an acute febrile vasculitic syndrome in children. CD40 ligand (CD40L) has been implicated in certain types of vasculitis. We proposed that CD40L expression might be correlated with coronary artery lesions in KD.

Methods. Blood samples were collected from 43 patients with KD before intravenous immunoglobulin (IVIG) treatment and 3 days afterward. Forty-three age-matched febrile children with various diseases were studied in parallel as controls. CD40L expression on T-cells and platelets were detected by flow cytometry, and soluble CD40L (sCD40L) levels were measured by enzyme-linked immunosorbent assay.

Results. We found that CD40L expression on CD4+ T-cells was significantly higher in patients with KD than in the febrile control (FC) group (28.69 ± 1.17% vs 4.37 ± 0.36%). CD40L expression decreased significantly 3 days after IVIG administration (28.69 ± 1.17% vs 13.53 ± 0.55%). CD40L expression on platelets from patients with KD was also significantly higher than in the FC group (8.20 ± 0.41% vs 1.26 ± 0.12%) and decreased after IVIG therapy. sCD40L levels were also significantly higher in KD patients with those of FC (9.69 ± 0.45 ng/mL vs 2.25 ± 0.19 ng/mL) but were not affected by IVIG treatment 3 days afterward (9.69 ± 0.45 ng/mL vs 9.03 ± 0.32 ng/mL). More interesting, we found that in KD patients, CD40L expression on CD4+ T-cells and platelets but not on CD8+ T-cells or sCD40L was correlated with the occurrence of coronary artery lesions.

Conclusions. CD40L might play a role in the immunopathogenesis of KD. IVIG therapy might downregulate CD40L expression, resulting in decrease of CD40L-mediated vascular damage in KD. This implicates that modulation of CD40L expression may benefit to treat KD vasculitis. Pediatrics 2003;111:140–147. URL: http://www.pediatrics.org/cgi/content/full/111/2/e140; Kawasaki disease, intravenous immunoglobulin, CD40 ligand, soluble CD40L.

ABBR EV IAT IONS. KD, Kawasaki disease; CD40L, CD40 ligand; IgG, immunoglobulin G; sCD40L, soluble CD40L; FC, febrile controls; IVIG, intravenous immunoglobulin; CAL, coronary artery lesions; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; IL, interleukin; TSST-1, toxic shock syndrome toxin-1.

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were commenced within 2 hours. Cells were acquired and analyzed by CellQuest software (Becton Dickinson). All experiments were performed in a total of 10,000 cells were acquired and analyzed by CellQuest software (Becton Dickinson). Isotype-matched FITC and PE-conjugated mouse IgG1 (Pharmigen) were used as negative controls. After incubation with FITC- and PE-conjugated mouse IgG1 (Pharmigen, San Diego, CA) and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson), CD61 and CD8 fluorescence was checked by microscopy to confirm that platelet purity exceeded 99%. Platelets were dual stained with FITC-conjugated anti-CD61 (Pharmingen), anti-CD4 and anti-CD8 fluorescein isothiocyanate monoclonal antibody conjugates for 30 minutes (4°C in darkness). A total of 10,000 cells/mm³ were acquired and analyzed by a FAScan. A total of 10,000 cells/mm³ were acquired and analyzed by CellQuest software (Becton Dickinson). Isotype-matched FITC and PE-conjugated mouse IgG1 (Pharmigen, San Diego, CA) and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson) were used as negative controls. After incubation with FITC- and PE-conjugated mouse IgG1 (Pharmigen) were used as negative controls. After incubation with FITC- and PE-conjugated mouse IgG1 (Pharmigen) and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson). Isotype-matched FITC and PE-conjugated mouse IgG1 (Pharmigen, San Diego, CA) and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson) were used as negative controls. After incubation with FITC- and PE-conjugated mouse IgG1 (Pharmigen) were used as negative controls. After incubation with FITC- and PE-conjugated mouse IgG1 (Pharmigen) and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson).

### METHODS

#### Patients and Samples Studied

Children who were admitted to Chang Gung Children's Hospital at Kaohsiung with the diagnosis of KD were enrolled in this study after informed consent was obtained. The treatment protocol was followed with the recommendation of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. Forty-three patients with KD and 43 age-matched febrile controls (FCs) were studied in parallel. Blood samples from FC and from KD patients before and 3 days after intravenous immunoglobulin (IVIG) therapy were collected. Serum samples were stored at −80°C until analysis. Blood leucocytes and platelets were subject to flow cytometric analysis. All of the KD and FC leukocytes were studied within 6 hours in parallel in each patient's assay. All patients with KD received 2-dimensional echocardiogram examinations by a pediatric cardiologist. Coronary artery lesion (CAL) was defined by internal diameter of coronary artery >3 mm. Detection of CD40L Expression on CD4+ and CD8+ T-Cells

Peripheral venous blood was drawn into sterile tubes containing heparin (Becton Dickinson, Heidelberg, Germany). Within 1 hour, 200 µL of whole blood was mixed with 20 µL of appropriate monoclonal antibody conjugates for 30 minutes (4°C in darkness). The following antibodies were used for staining: anti-CD3 peridin chlorophyll protein, anti-CD4 and anti-CD8 fluorescent isothiocyanate (FITC) (all from Becton Dickinson), and CD40L phycoerythrin (PE) (AnCell Croup, Bayport, MN). Isotype-matched FITC- and PE-conjugated mouse IgG1 (Pharmigen, San Diego, CA) were used as negative controls. We used a protein kinase C activator phorbol myristate acetate (32 nM) and calcium ionophore (A23187; 1 µg/mL) to stimulate CD40L expression on CD4+ and CD8+ T-cells. After incubation for 4 hours, each sample was harvested by red blood cell lysing buffer (Becton Dickinson), washed twice with cold phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde, and analyzed by FAScan. A total of 10,000 cells were acquired and analyzed by CellQuest software (Becton Dickinson). All experiments were performed in triplicate.

#### Detection of Membrane-Bound CD40L on Platelets

Peripheral venous blood was drawn into sterile tubes containing 3.8% sodium citrate (9:1). Platelet counts were measured as usual by clinical laboratory, Platelet-rich plasma (PRP) was obtained by centrifugation (1200 rpm for 15 minutes). The upper half of PRP was collected by Pasteur pipette to avoid contamination with red blood cells and leucocytes; the PRP was then checked by microscopy to confirm that platelet purity exceeded 99%. Platelets were dual stained with FITC-conjugated anti-CD61 and PE-conjugated anti-CD40L (TRAP1, IgG1; all from Becton Dickinson). Isotype-matched FITC and PE-conjugated mouse IgG1 (Pharmigen) were used as negative controls. After incubation with and/or without thrombin (0.5 U/mL) for 15 minutes, the PRP was then washed twice with cold PBS, resuspended in PBS with 1% paraformaldehyde, and analyzed by FAScan. A total of 10,000 cells were acquired and analyzed by CellQuest software (Becton Dickinson). All experiments were conducted in triplicate. Studies were commenced within 2 hours.

#### Measurement of sCD40L Levels

Levels of sCD40L were determined by enzyme-linked immunosorbent assay (detection limit: 0.095 ng/mL; Chemicon Corp, Temecula, CA) according to the manufacturer's instructions.

#### Statistical Analysis

Results were expressed as mean ± standard error of the mean. When 3 groups of individuals were compared, repeated measurement by analysis of variance was used as the test for statistical significance. The calculations were performed using the Statistical Package for Social Science (SPSS, version 8; SPSS Inc, Chicago, IL) software package. Statistical significance was achieved at P < .05. We used Window Multiple Document Interface for Flow cytometry (version 2.8; Scripps Research Institute, La Jolla, CA) to overlay histograms of different stages in patients with KD as well as FC to better present difference of the CD40L expression on T-cells before and after treatment.

### RESULTS

#### Demographic Data of the Patients Studied

As shown in Table 1, 43 patients with KD and 43 age-matched FCs were simultaneously studied. FCs include lobar pneumonia (n = 8), urinary tract infection (n = 6), acute gastroenteritis (n = 8), hand-foot-mouth disease (n = 6), anaphylactoid purpura (Heoch-Schoenlein purpura, n = 5), and those with simple upper respiratory tract infections (n = 10). There was no difference of demographic data between the 2 groups including age, duration of fever, leucocyte counts, C-reactive protein, and platelet counts.

#### Detection of CD40L Expression on CD4+ and CD8+ T-Cells From Patients With KD and FC

As shown in Fig 1, cell-surface CD40L expression on CD4+ T-cells was significantly higher in patients with KD than in FC (28.69 ± 1.17% vs 4.37 ± 0.36%; P < .001, n = 43) and decreased significantly 3 days after IVIG administration (19.89 ± 0.55%; P < .001, n = 43). As shown in Fig 2, cell-surface CD40L expression on CD8+ T-cells was also significantly higher in patients with KD than in FC (19.89 ± 0.9% vs 2.97 ± 0.23%; P < .001, n = 43), although the percentage and intensity was lower than that of CD4+ T-cells. CD40L expression also decreased significantly from the increased levels 3 days after IVIG administration (19.89 ± 0.9% vs 8.89 ± 0.55%; P < .001, n = 43). After in vitro stimulation by phorbol myristate acetate (32 nM) and A23187 (1 µg/mL) for 4 hours, patients with KD displayed increased CD40L expression on CD4+ T-cells and CD8+ T-cells, by comparison with the FC, as shown in Table 2.

#### TABLE 1. Demographic Data of FC and Patients With KD Before IVIG Therapy

<table>
<thead>
<tr>
<th></th>
<th>KD (n = 43)</th>
<th>FC (n = 43)*</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mo)</td>
<td>17.5 ± 1.8</td>
<td>21.7 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of fever (d)</td>
<td>6.7 ± 0.9</td>
<td>5.2 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Leukocyte counts (×10³ cells/mm³)</td>
<td>17.4 ± 1.3</td>
<td>15.5 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>76.3 ± 5.9</td>
<td>81.3 ± 6.5</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet counts (×10³ cells/mm³)</td>
<td>34.3 ± 3.6</td>
<td>30.5 ± 3.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant.

* The 43 FC consisted of lobar pneumonia (n = 8), urinary tract infection (n = 6), acute gastroenteritis (n = 8), hand-foot-mouth disease (n = 6), anaphylactoid purpura (n = 5), and simple upper respiratory tract infections (n = 10).
Fig 1. CD40L expression on CD4⁺ T-cells. A, A representative overlay histogram of CD40L expression on CD4⁺ T-cells in patients with KD and FC. Fluorescence intensity of the CD40L expression detected by PE-conjugated antibody is shown in the x axis. Numbers of CD4⁺ T-cells detected by FITC-conjugated antibody are shown in the y axis. B, CD40L expression on CD4⁺ T-cells was significantly higher in patients with KD than in FC (28.69 ± 1.17% vs 4.37 ± 0.36%; P < .001, n = 43) and decreased significantly 3 days after IVIG administration (28.69 ± 1.17% vs 13.53 ± 0.55%; P < .001, n = 43).
Fig 2. CD40L expression on CD8⁺ T-cells. A, A representative overlay histogram of CD40L expression on CD8⁺ T-cells in patients with KD and FC. Fluorescence intensity of the CD40L expression detected by PE-conjugated antibody is shown in the x-axis. Numbers of CD8⁺ T-cells detected by FITC-conjugated antibody are shown in the y-axis. B, CD40L expression on CD8⁺ T-cells was significantly higher in patients with KD than in FC (19.89 ± 0.97% vs 2.97 ± 0.23%; P < .001, n = 43) and decreased significantly 3 days after IVIG administration (19.89 ± 0.97% vs 8.89 ± 0.55%; P < .001, n = 43).
As shown in Table 3, additional analysis disclosed that KD patients in the acute stage with CAL showed significantly higher CD40L expression on CD4+ T-cells than those without CAL (37.26 ± 1.60% vs 25.37 ± 1.00%; P < .001). However, there was no difference in CD40L expression on CD8+ T-cells between KD patients with or without CAL (20.02 ± 1.70% vs 19.84 ± 1.19%; P = .934).

Detection of CD40L Expression on Platelets From Patients With KD and FC

CD40L expression on platelets from patients and FC was also studied by flow cytometric analysis. As shown in Fig 3, ex vivo expression of CD40L on platelets was significantly higher in KD than in FC (8.20 ± 0.41% vs 1.26 ± 0.12%; P < .001, n = 43) and decreased significantly after IVIG treatment 3 days afterward (8.20 ± 0.41% vs 3.88 ± 0.22%; P < .001, n = 43). After in vitro stimulation by thrombin (0.5 U/mL) for 15 minutes, there was no difference between the KD group and FC group (Table 2).

As shown in Table 3, KD patients with CAL also expressed significantly higher CD40L on platelets in the acute stage than those without CAL (9.48 ± 0.67% vs 7.70 ± 0.49%; P = .043).

Measurement of sCD40L Levels of Serum From Patients With KD and FC

As shown in Fig 4, sCD40L levels were significantly higher in KD patients than in FC (9.69 ± 0.45 ng/mL vs 2.25 ± 0.19 ng/mL; P < .001, n = 43) but were not affected by IVIG treatment 3 days afterward (9.69 ± 0.45 ng/mL vs 9.03 ± 0.32 ng/mL; P = .18, n = 43). As shown in Table 3, there were no differences in sCD40L levels between KD patients with or without CAL before IVIG therapy (9.77 ± 1.05 ng/mL vs 9.65 ± 0.49 ng/mL; P = .924).

**DISCUSSION**

KD is characterized by overactivation of the immune system targeting on vascular endothelium, resulting in systemic vasculitis or even coronary artery aneurysm. The CD40L–CD40 system is a crucial communication pathway for interaction among bone marrow–derived cells of the immune system. More recently, the interaction of CD40L with its receptor CD40 has been implicated in the modulation of immune and inflammatory responses, which are critical for the activation of tissue structure cells, including endothelial cells, smooth muscle cells, and epithelial cells as well as fibroblast, inducing production of a whole series of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor-α, along with induction of adhesion molecule expression on endothelial cells (eg, E-selectin, vascular cell adhesion molecule-1) and RANTES, in macrophages and vascular endothelial cells.

Consequently, CD40 signaling has been associated with the pathogenic processes of chronic inflammatory diseases, including autoimmune diseases, neurodegenerative disorders, graft-versus-host disease, cancer, and atherosclerosis. In patients with systemic lupus erythematosus, baseline expression of CD40L and CD40L regulation was recently shown to be abnormal. Most impressive, evidence increasingly shows that CD40L expression on T-lymphocytes, on platelets, or in soluble form plays an important role in the acute coronary syndrome, the most disastrous complication of KD.

Therefore, we explored CD40L expression in KD. To our knowledge, the present study is the first time abnormal CD40L expression in patients with KD has been demonstrated. We found that CD40L expression on CD4+ T-cells was significantly higher in KD than in FC, decreasing significantly 3 days after IVIG administration. Similarly, there was also significantly higher CD40L expression on CD8+ T-cells from patients with KD than in FC. CD40L expression on platelets from patients with KD was significantly higher than on platelets from FC. The increased CD40L expression on platelets in patients with KD...
decreased dramatically 3 days after IVIG treatment. In contrast, sCD40L levels were not affected by IVIG treatment 3 days afterward. It is interesting that CD40L expression on CD4+ T-cells and platelets but not on CD8+ T-cells is significantly correlated with the occurrence of coronary artery dilation, whereas sCD40L is not. This suggests that CD40L may play an important role in the immunopathogenesis of CAL in KD.

In patients with KD, overexpression of CD40L may...
activate CD40-positive target cells, either hematopoietic or structure cells, with the potential to activate further the immune system and elicit inflammatory reactions, resulting in vascular endothelial damage. Endothelial cells are able to express cell surface CD40 that, when ligated, signals augmented expression of vascular cell adhesion molecules and cytokines and secretion of tissue factors. Kotowicz et al. showed that ligation of CD40L with CD40 on human endothelial cells can lead to endothelial cell activation, induced expression of vascular cell adhesion molecule-1, intracellular adhesion molecule-1, and E-selectin with recruitment and activation of T-cells and neutrophils at sites of inflammation. Mach et al. demonstrated that activated human T-cells mediate contact-dependent expression of matrix metalloproteinases in vascular endothelial cells through CD40/CD40L signaling. They concluded that ligation of CD40 on endothelial cells can mediate aspects of vascular remodeling and neovessel formation during chronic immune reactions or even atherogenesis. We believe that the increase of CD40L expression on T-cells and platelets associated with elevated shedding of sCD40L in patients with KD may not only trigger immune activation but also attribute to pathogenic process of vascular inflammation.

In the past 30 years, identification of any infectious agent that may cause KD has been difficult. However, certain intracellular pathogens and superantigens from bacteria have been implicated in the immunopathogenesis of KD. By using a 3-phase screening procedure, Takahashi and colleagues recently identified a novel lymphotropic virus from peripheral blood mononuclear cells in patients with KD. This novel virus has an extensive but low-level homology (25%–33% identities and 28%–45% positives) with the African swine fever virus, which replicates in monocyte-macrophage lineage cells. Whether this lymphotropic virus contributes to the CD40L expression of CD4 T-cells and platelets deserves additional study. In contrast to viral pathogenesis, expansion of T-cells expressing TCRBV2 and TCRBV6 chains has been reported to be stimulated by streptococcal and staphylococcal superantigens, such as toxic shock syndrome toxin-1 (TSST-1), which likely contributes to immunopathogenesis of KD in a number of ways. Jabara et al. showed that TSST-1 promotes CD40L expression on T-cells. CD40L was preferentially expressed in the Vβ2 subset of T-cells expanded by TSST-1. Kum et al. also revealed that ligation of Vβ2-TCR by TSST-1 induced a rapid surface expression of CD40L on CD4 T-cells, leading to sequential T-cell proliferation and monocyte activation.

KD now has become the leading cause of acquired heart disease among children. There is evidence of persistent abnormal vascular wall morphology and vascular dysfunction in patients with previous KD at the site of regressed coronary aneurysms, including premature atherosclerosis. The arterial lesions continue to undergo active remodeling processes several years after the onset of the disease, even into adulthood. Takahashi et al. revealed evidence of atherosclerotic lesions by pathologic examination from autopsy patients older than 15 years of age with previous KD. Multiple lines of evidence also support the view of atherosclerosis as a chronic inflammatory disease and implicate components of immune activation in atherogenesis. Recent work has documented overexpression of CD40L in experimental and human atherosclerotic lesions. Notably, interruption of CD40/CD40L interaction not only di-
minated the formation and progression of atheroma but also fostered change in lesion biology and structure. On the basis of the findings, we propose that patients with KD, especially those patients associated with CAL, should be counseled to avoid potential risk factors for atherosclerosis, with long-term follow-up into adulthood. Furthermore, advanced understanding of the basic mechanisms of the relationship between CD40L interaction and long-term coronary arterial remodeling, as well as atherogenesis, may lead us to more effective and innovative treatments for patients with KD.

CONCLUSION

CD40L might play an important role in the immunopathogenesis of KD. IVIG treatment decreased cell-surface CD40L expression on CD4+ T-cells, CD8+ T-cells, and platelets but not on sCD40L, suggesting that downregulation of CD40L expression rather than shedding of sCD40L is involved in the decrease of CD40L-mediated vascular damage in KD. The precise mechanisms deserve additional investigation. On the basis of the results of this study, therapeutic modalities that are able to downregulate CD40L–CD40 interactions may represent a new therapeutic approach for KD vasculitis.

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