**Original Article**

**Distinct expressions of immunoglobulin signal related molecules between human Kawasaki disease and BALB/c mice treated with Lactobacillus casei cell wall extract**

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**Abstract:** Background: Kawasaki disease (KD) is an acute febrile multi-system vasculitic syndrome occurring mostly in infants and children. It is also the most commonly acquired heart disease in children. The exact etiology and pathogenesis of KD remain unclear. Genome-wide association study identified FCGR2A, CD40 and B lymphoid tyrosine kinase (BLK) to be susceptibility locus for Kawasaki disease. Plasma clusterin level was also noted being able to predict the occurrence of CAL and IVIG resistance of KD. Single intraperitoneal injections of Lactobacillus casei cell wall extract (LCWE) inducing an acute inflammatory coronary arteritis in mice is reported resemble the coronary arteritis found in KD. This study was conducted to investigate whether these immunoglobulin-related significant molecules (clusterin, BLK, CD40/CD40L, and Fcγ receptor that observed in human KD are also play important roles in LCWE-treated mice. Material and methods: Wild-type male BALB/c mice were intraperitoneally injected with LCWE (1 mg/mL) to induce vasculitis. The target molecules in mice were examined post injections as indicated. Results: There was higher Fcγ receptor IIIA (CD16) expression on CD4+ splenocytes of LCWE treated mice than control group. However, LCWE treatment did not change the CD40, CD40L and Fγ receptor IIB expression of splenocytes. LCWE treatment also did not change BLK abundance of splenocytes. Plasma clusterin level did not alter after LCWE treatment at day 3, 7 or day 14. Conclusion: There are many inconsistent expressions between LCWE-treated mice and Kawasaki disease in human. Thus LCWE-treated mice cannot totally present human KD. We should be very carefully when apply the laboratory finding of LCWE-treated mice to human KD.

**Keywords:** Kawasaki disease, LCWE, B lymphoid tyrosine kinase, Fcγ receptors

**Introduction**

Kawasaki disease (KD) is an acute febrile multi-system vasculitic syndrome of unknown etiology occurring mostly in infants and children. It is characterized by prolonged fever, bilateral non exudative conjunctivitis, erythema of the lips and oral mucosa, changes in the extremities, skin rash, and cervical lymphadenopathy. The incidence of KD among children younger than 5 years is 66 per 100,000 per year in Taiwan. KD is the most commonly acquired heart disease in children worldwide. Among cardiovascular complications, coronary artery lesions (CAL) can particularly cause long term morbidity and mortality. Without proper treatment early, there will be a 15-25% chance of serious cardiovascular complications. Early introduction of intravenous immunoglobulin (IVIG) therapy has dramatically decreased the frequencies of CAL and mortality.

Because of more susceptible for specific races, early studies paid much attention on genetic
susceptibility. Previous researches on genetic susceptibility of KD showed human leukocyte antigen (HLA)-DR, A2, B44, B5, BW22, Bw51, Bw4415 and HLA-G being candidate hot spots [1-5]. With the progress of genome-wide association studies (GWAS), the most interesting candidates of KD-susceptibility genes are the B lymphocyte kinase (BLK), caspase-3 (CASP3) gene, Fc fragment of IgG low affinity IIa receptor (FCGR2A), inositol 1,4,5-triphosphate 3-kinase (ITPKC) gene, HLA and CD40 gene currently [6-11]. FCGR2A is expressed on many immune cells surface, such as monocytes, dendritic cells, macrophages, and neutrophils, and transduces activation signals into cell as ligated with immune complexes [12]. Evidences had shown higher serum circulating immune complex active immunoreaction through FCGR2A during the acute phase of KD [13, 14]. The expression of BLK in leukocytes was increased in patients in the acute stage of KD [10]. The CD40L expression on CD4(+) T-cells was significantly higher in patients with KD than in the febrile control group. CD40L expression decreased significantly 3 days after IVIG administration [15]. This implicates that modulation of CD40L expression may benefit to treat KD vasculitis. With proteomic approach, we had found clusterin increased in KD patients, whereas immunoglobulin free light chains were decreased, as compared with controls [16]. Plasma clusterin levels even could predict the occurrence of CAL and IVIG resistance of Kawasaki disease [17, 18].

Several animal model of KD had been developed with Candida albicans water soluble fraction (CAWS), Bacille Calmette Guérin (BCG) or Lactobacillus casei cell wall extract (LCWE) [19-22]. Single injection of LCWE through intraperitoneal route produces an acute inflammatory coronary arteritis in mice that mimics the coronary arteritis found in KD. The coronary arteritis is accompanied by pancarditis and coronary artery lesions in early and later stage, respectively. This model is important for it provide the opportunity to study the natural history of coronary artery lesions over a relatively short life span. It also provides a model for immunological and pharmacological interventions without increasing human life risk [23]. In spite of the similar vasculitis manifestation between KD in human and animal model, rare research address their distinctness. In this study, we try to investigate the expression of these immunoglobulin-related significant molecules (clusterin, BLK, CD40, CD40L, and Fcγ receptor) of KD in LCWE treated mice.

Material and methods

LCWE preparation

LCWE was prepared as previously described. In brief, the Lactobacillus casei (ATCC 11578) [24, 25] (Bioresource Collection and Research Center, Taiwan) were cultured in Lactobacillus MRS broth (Difco, Detroit, MI, USA) at 37°C. After harvested, the cells were treated with 4% SDS (Sigma-Aldrich, St. Louis, MO, USA) overnight, and then incubated with 250 μg/ml RNase, Dnase I, and trypsin (Sigma-Aldrich) to remove proteins, nucleic acids, and lipids. The pellet was then sonicated for 2 h at a pulse setting of 9 second pulse/5 second pause at 20 kHz frequency (VibraCell™. Sonics & Materials Inc., Newtown, CT) in a dry ice/ethanol bath. Following 1 h of centrifugation at 20,000×g, the supernatant concentration was determined on the basis of its rhamnose content by phenol-sulfuric acid colorimetric assay. The endotoxin concentration was determined by the Limulus amoebocyte lysate assay (Associates of Cape Cod Inc., East Falmouth, MA) and <1.5 pg/μg. The Lactobacillus casei cell wall extraction (LCWE) was then sterilized by irradiation and suspended in sterile PBS at a concentration of 1 mg/ml and stored frozen at -20°C for further study use.

Induction of KD in BALB/c mice

This study was performed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital Kaohsiung Medical Center. Wild-type male BALB/c mice (National Laboratory Animal Center, Taiwan) were used in this study as previous described [24, 25]. All animal experiments were performed in accordance with legislation on the protection of animals and are approved by the animal care committee of Chang Gung Memorial Hospital. To induce a mouse model of coronary arteritis, 4- to 5-wk-old WT mice were injected intraperitoneal with 1 ml of PBS containing 1 mg of LCWE. Other WT mice were injected intraperitoneal with 1 ml of PBS alone.
LCWE treated mice differ from Kawasaki disease

Table 1. Blood cell profile and white blood cell classification in LCWE treated mice or not at day 3. The results are presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>LCWE</th>
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<tr>
<td>Blood cell profile</td>
<td></td>
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</tr>
<tr>
<td>WBC (10^3/mL)</td>
<td>3.83±0.23</td>
<td>5.03±0.62</td>
</tr>
<tr>
<td>RBC (10^6/µL)</td>
<td>8.26±0.08</td>
<td>8.69±0.14</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>12.82±0.43</td>
<td>13.71±0.18</td>
</tr>
<tr>
<td>PLT (10^3/µL)</td>
<td>402.25±77.31</td>
<td>491.02±56.53</td>
</tr>
<tr>
<td>White blood cell classification (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>43.20±4.05</td>
<td>36.07±2.78</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>53.20±3.45</td>
<td>56.70±1.91</td>
</tr>
<tr>
<td>Monocyte (%)</td>
<td>1.25±0.18</td>
<td>4.30±0.82</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>1.90±0.35</td>
<td>1.53±0.17</td>
</tr>
<tr>
<td>Basophil (%)</td>
<td>0.40±0.07</td>
<td>1.10±0.65*</td>
</tr>
</tbody>
</table>

*p < 0.05 as compared with PBS group.

as a negative control. The mice were sacrificed at 3, 7, or 14 days post-injection. Mice was intramuscular injection with anesthetics containing ketamine (50 mg/Kg) (Reg. No. 003542, United Biomedical, Hsinchu, Taiwan) and rompun (50 mg/Kg) (Reg. No. 06713, Bayer, Taipei, Taiwan) at 1:1 mixture, and cardiac puncture, perfusion and then organ removed.

Peripheral blood analysis

The specimens of blood were collected in heparin rinsed tubes. Total blood cell counts and white blood cell differential counts were measured using a Sysmex XT-1800i system (Sysmex, Hyogo, Japan). For lymphocyte subset analysis, leukocytes were stained with PerCP-conjugated anti-rat CD4 (Catalog #FAB554C, R&D Systems, Inc., Minneapolis, MN, USA), and AlexaFluor 450-conjugated anti-rat CD8a (Catalog #FAB116V, R&D Systems, Inc.). Data were acquired using a FACS Aria I cytometer (Becton Dickinson, Franklin, NJ, USA) and analyzed using FlowJo software.

CD40/CD40L and Fcy receptors detection with flow-cytometry

Splenocytes were separated from whole spleens as previously described [26]. In brief: the spleen was washed with PBS and then pressed with a syringe plunger through a 30 µm nylon mesh (Preseparation Filter # 130-041-407, MiltenyiBiotec). After erythrocytes were lysed, the remaining splenocytes were washed and counted and 2×10^6 cells/ml will be plated in a 24 well plate in enriched medium (RPMI 1640 medium supplemented with 1% non-essential amino acids, 1% pyruvate, 10% heat-inactivated fetal bovine serum and antibiotics). Cultured cells are then stimulated with or without 5 µg/ml of Concanavalin A (ConA) for 6 hrs. The cells were washed and stained with anti-CD4-PerCP and anti-CD8a-AlexaFluor 450 (R&D Systems, Inc.) for 30 min. Then the cells were stained with anti-CD40 (Catalog #FAB440F, R&D Systems, Inc.), anti-CD40L (Catalog #FAB1163P, R&D Systems, Inc.), anti-FcyR IIB (Catalog # 17-0321, eBioscience, San Diego, CA), and anti-FcyR IIIA (Catalog # 19601F, R&D Systems, Inc.) for 30 min and then fixed with paraformaldehyde/PBS. The expressions of cell surface markers were analyzed determined using FACS Calibur flow cytometer (BD Biosciences).

Traditional western blotting

Protein samples (50 µg each) were boiled with gel loading buffer for 5 min, subjected to 12% SDS-PAGE. After transfer to a polyvinylidene fluoride membrane and block with PBS-Tween containing 5% dry milk, the membranes were then incubated for 2 h with the following anti-phospho-BLK (Catalog #ab192670, Abcam; Cambridge, UK), anti-BLK (Catalog #AF2679, R&D Systems, Inc.) or anti-GAPDH (Catalog #ab9484, Abcam; Cambridge, UK) antibody diluted 1:500 in TBS containing 1% skim milk. After five washes with 0.1% T-TBS, the membranes were incubated for 1 h with peroxidase-labeled secondary antibody diluted 1:1000 in T-TBS. After washes with T-TBS, the membranes were incubated for 1 h with peroxidase-labeled secondary antibody diluted 1:1000 in T-TBS. After washes with T-TBS, the membranes were developed using enhanced chemiluminescence (ECL) Plus kit. The signal was obtained by using Bio-Rad Molecular Imager ChemiDoc MP and quantified by Image Lab version 5.0 software (Bio-Rad).

Simple western experiments

Simple Western system (Simon™, Protein-Simple, San Jose, CA) was used to efficiently and quantitatively analyze protein expression according to manufacturer’s instructions. Briefly, cell pellets were washed and lysed in cold radioimmunoprecipitation assay (RIPA) lysis buffer (Cat no: R0278, Sigma) containing protease inhibitors (Complete MiniTM; Roche Diagnostics, Indianapolis, IN, USA). The supernatants were collected, aliquoted, and stored.

LCWE treated mice differ from Kawasaki disease

In -80°C prior to use. During denaturation, the protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) and freshly made 1 M DTT was used to reduce samples. A biotinylated ladder contains 6 proteins at molecular weights was used for molecular weight determination. Protein lysates (approximately 5 μg protein per lane) were loaded into capillary and electrophoresis together with fluorescent standards. Phospho-BLK (p-BLK) and GAPDH were identified using specific primary antibodies, probed with HRP-conjugated secondary antibodies, detected or quantitated by chemiluminescent signals. p-BLK protein abundance was normalized to GAPDH levels.

**Enzyme-linked immunosorbent assay**

Plasma clusterin levels were detected with enzyme-linked immunosorbent assay (ELISA) tests according to manufacturer’s instructions.

**Statistics**

For most parameters, statistical analysis was performed using Mann-Whitney U test. Data were expressed as the mean ± standard error of the mean. A p-value of less than 0.05 was defined to be statistically significant for all tests. All analyses statistical tests were performed using with SPSS 15.0 for Windows XP (SPSS, Inc., Chicago, IL, USA).

**Results**

*Peripheral blood leukocyte subsets exhibited no change by LCWE treatment*

At first, we evaluated the effect of LCWE treatment on the complete blood count and differential count. Since inflammatory cells infiltration could be observed in the adventitia of coronary arteries as early as day 3 post-LCWE injection [27]. We especially focused the changes of on
LCWE treated mice differ from Kawasaki disease

D3 [22]. As showed in Table 1, there was no significant difference between the LCWE treated group and PBS group in total leukocyte counts, leukocyte differential count, red blood cell counts and platelet counts at D3.

Unique expressions of immunoglobulin associated cascade molecules on splenocytes of LCWE treated mice

Splenocytes were then analyzed and compared for the expressions of CD4, CD8a, CD16 (FcyRIIA), CD32 (FcyRIIB), CD40 and CD40L. There was no significant difference on the expressions of CD4/CD8a ratio between LCWE group and PBS group (data not shown). There was also no significant difference on the CD16, CD32, CD40 or CD40L expressions of total splenocytes between LCWE group and PBS group at D3, D7 or D14 (data not shown). Then we investigated the expressions of CD16, CD32, CD40 and CD40L on helper T cells by gating the CD4+ cells. We found the CD4+ splenocytes of LCWE treated mice showing higher CD16 expression than PBS group at D3 with ConA stimulation (Figure 1; Table 2). However, the differential expression of CD16 on CD4+ splenocytes between LCWE and PBS group was not appeared at D7 and D14.

Table 2. Expressions of indicated molecules on LCWE treated mice CD4+ splenocytes that corresponding to significant markers in human Kawasaki disease

<table>
<thead>
<tr>
<th>% Gated</th>
<th>3 days</th>
<th></th>
<th>7 days</th>
<th></th>
<th>14 days</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>LCWE</td>
<td>PBS</td>
<td>LCWE</td>
<td>PBS</td>
<td>LCWE</td>
</tr>
<tr>
<td>CD16</td>
<td>11.12±6.05</td>
<td>24.55±15.15*</td>
<td>12.5±2.26</td>
<td>14.74±5.44</td>
<td>21.4±6.01</td>
<td>25.76±12.15</td>
</tr>
<tr>
<td>CD32</td>
<td>11.04±1.45</td>
<td>15.93±5.48</td>
<td>17.60±1.84</td>
<td>18.89±2.42</td>
<td>11.69±1.56</td>
<td>11.31±1.61</td>
</tr>
<tr>
<td>CD40</td>
<td>9.24±4.13</td>
<td>15.93±3.32</td>
<td>9.97±5.84</td>
<td>6.65±5.63</td>
<td>17.29±5.47</td>
<td>21.27±13.91</td>
</tr>
</tbody>
</table>

*P=0.038.

Figure 2. Western blot of total B lymphoid tyrosine kinase (BLK) and phosphor-BLK (p-BLK) of splenocytes with/out LCWE treated mice. A. Representative traditional Western blot showing the abundance of total BLK abundance for PBS and LCWE treated mice splenocytes. B. Quantification of total BLK (relative to GAPDH) was shown with mean ± SE. C. Representative simple Western measurement showing the abundance of p-BLK abundance for PBS and LCWE treated mice splenocytes. D. Quantification of p-BLK (relative to GAPDH) was shown with mean ± SE. Data presented were calculated from six replicated experiments.
LCWE treated mice differ from Kawasaki disease

Spleen phospho-BLK (p-BLK)/total BLK displayed no significant change by LCWE treatment

Next, we studied the abundances of p-BLK and total BLK protein in splenocytes of LCWE or PBS treated mice. At first, we analyzed the abundances of p-BLK and total BLK protein with traditional Western blot method. LCWE treated mice did not change the abundance of total BLK of splenocytes (Figure 2A and 2B). However, the amount of p-BLK protein in splenocytes was too rare to be presented with traditional Western blot method. We used Simple Western system to detect the p-BLK. As showed in Figure 2C and 2D, there was also no significant difference on the p-BLK expression of splenocytes between LCWE group and PBS group at D3.

LCWE treatment did not alter plasma clusterin levels of mice

Finally, we tried to determine the effect of LCWE treatment on clusterin concentration in mice plasma with ELISA kit. The clusterin concentration in mice plasma was about 40 to 60 μg/ml. LCWE treatment did not change plasma clusterin levels of mice no matter at D3, D7 or D14 (Figure 3).

Discussion

Evidences had shown higher serum circulating immune complex active immunereaction through FCGR2A during the acute phase of KD [13, 14]. The expression of BLK in leukocytes were increased in patients in the acute stage of KD [10]. The CD40L expression on CD4(+) T-cells was significantly higher in patients with KD than in the febrile control group. CD40L expression decreased significantly 3 days after IVIG administration [15]. From this study, we found there was higher Fcy receptor IIIA (CD16) expression on CD4+ splenocytes of LCWE treated mice than control group. However, LCWE treatment did not change the CD40, CD40L and Fγ receptor IIB (CD32) expression of splenocytes. LCWE treatment also did not change BLK abundance of splenocytes. Plasma clusterin level did not alter after LCWE treatment at day 3, 7 or day 14. The result of this study demonstrated the distinct features of some important immunologic molecules in LCWE-treated mice as compared with KD patients. Candida albicans water soluble fraction (CAWS), Bacille Calmette-Guérin (BCG) or Lactobacillus casei cell wall extract (LCWE) have been tried to establish a disease model that mimic human KD [19-22]. Among them, BALB/c mice treated with LCWE is especially important because it provide a coronary arteritis model for immunological and pharmacological interventions [23]. LCWE treated mice indeed showed some immunologic alteration similar to KD in human [25]. Both human KD patients and LCWE-treated mice showed higher plasma IL-6, monocyte chemoattractant protein (MCP)-1, and TNF-α [25] in acute phase. Monocytes of human KD patients and LCWE-treated mice also presented TLR-2 activation in acute phase [25]. However, different immunologic manifestations existed between human KD patients and LCWE treated mice. For example, IL-6, MCP-1, and TNF-α were significantly decreased after IVIG treatment but remained statistically higher than those in controls. The CALs in KD patients often occurred before the systemic inflammation completely subsided. In contrast, spontaneous decline of IL-6, MCP-1, and TNF-α occurred in LCWE-treated mice.
LCWE treated mice differ from Kawasaki disease

mice, nearly reaching basal levels without any treatment at 7 days post injection, which was then followed by the development of CALs [24, 25]. In fact, even different host species react differently to LEWC treatment. LCWE treatment was reported to induce arthritis rather than cardioangitis in rats [28]. Thus LCWE-treated mice cannot totally present human KD. We should be very carefully when apply the laboratory finding of LCWE-treated mice to human KD.

Mouse and human Fc receptors for IgG (FcγRs) can be defined by their function and affinity for IgG. The human IgG receptor family consists of two high-affinity receptor hFcγRI, FcRn; and several low to medium affinity activating receptors (hFcγRIIA, hFcγRIIB, hFcγRIIC, hFcγRIIIA, and hFcγRIIIIB) [29-31]. Among these receptors, hFcγRI, hFcγRIIA, hFcγRIIC, and hFcγRIIIA have activating function. Considering the signals triggered by FcR crosslinking, the hFcγRIIB own inhibitory function, which contains an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytoplasmic domain [29, 32]. Although these inhibitory FcγRIIB receptors are expressed mainly on monocytes, macrophages, their presence cannot explain the immunomodulatory capacity of IVIG. Direct IVIG effects at the level of the activating FcγRs worth of our attention for IVIG-related immunomodulation [33]. In contrast to human, mouse has 3 high affinity FcγRs: FcγRI, FcγRIIb, FcγRn for mouse IgG1, IgG2a, IgG2b or IgG3 as indicated [29, 34, 35]. There are two low affinity FcγRs: FcγRIIb and FcγRIII in mice [29]. The human FcγRs are not totally identical to mouse FcγRs. Major differences on expression patterns between human FcγR and their mouse homolog exist: (1) the expression of mouse FcγRI is restricted to monocyte-derived DCs but not of human; (2) the expression of FcγRIIb of human is mainly restricted to B cells and basophils but not of mouse; (3) the expression of human FcγRIIIA is restricted to NK cells and monocytes/macrophages, but not of mouse; (4) FcγRIV exist in mice rather than in humans [36, 37]; and (5) FcγRIIA, FcγRIIC, and FcγRIIIIB exist in humans rather than in mice [29, 38]. Recent studies also suggested 3 major differences between human and mouse FcγRs binding abilities: (1) all human activating FcγRs bind the major human IgG subclass IgG1, whereas only mouse activating FcγRII binds mouse IgG1; (2) human inhibitory hFcγRIIB has a lower affinity for IgG1, IgG2, and IgG3 than all other hFcγRs, which is not the case in mice for IgG1 and IgG2b; and (3) no human FcγR binds human IgE, however mouse FcγRIIB, FcγRIII, and FcγRIIV bind mouse IgE [29, 34, 35]. Studies using FcγRIIIA−/− mice have reported that FcγRIIIA triggers Arthus reaction, passive cutaneous anaphylaxis (PCA), IgG1-inducedPSA, K/BxN arthritis, and collagen-induced arthritis [39-42]. Recent reports also have revealed that activating mice FcγRII mediates the anti-inflammatory effects of intravenous immunoglobulin in experimental thrombocytopenia when expressed on dendritic cells or on macrophages [43, 44]. Thus in our study, the increased expression of FcγRII A on CD4+ splenocytes in LCWE treated mice also provide its anti-inflammatory role.

In spite of the similar vasculitis manifestation between KD in human and animal model, rare research address their distinctness. In this study, we investigated the expression of some important molecules (clusterin, BLK, CD40L, and Fcγ receptor) of KD in LCWE treated mice. LCWE-treated mice cannot totally present human Kawasaki disease. For better disease approach, other models including in vitro study are still needed for human KD.

Acknowledgements

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Disclosure of conflict of interest

None.

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LCWE treated mice differ from Kawasaki disease


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LCWE treated mice differ from Kawasaki disease


