Immune-monitoring in Kawasaki disease patients treated with infliximab and intravenous immunoglobulin

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Immune-monitoring in Kawasaki disease patients treated with infliximab and intravenous immunoglobulin

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Summary

The expansion of regulatory T cells (T_{reg}) controls inflammation in children with acute Kawasaki disease (KD). Blockade of tumour necrosis factor (TNF)-α is an emerging therapy for KD patients with refractory inflammation, but there is concern that this therapy could impede the host immune regulation. To define the effect of TNF-α blockade, we conducted ex-vivo immune-monitoring in KD subjects who participated in a randomized, double-blind, placebo-controlled clinical trial of the addition of infliximab to standard intravenous immunoglobulin (IVIG) therapy. We enumerated circulating myeloid and plasmacytoid dendritic cells (DC), regulatory T cells (T_{reg}) and memory T cells (T_{mem}) in 14 consecutive, unselected KD patients (seven treated with IVIG, seven with IVIG + infliximab) at three time-points: (i) acute phase prior to treatment, (ii) subacute phase and (iii) convalescent phase. Myeloid DC (mDC), but not plasmacytoid DC (pDC), were numerous in the peripheral blood in acute KD subjects and decreased in the subacute phase in both IVIG- and IVIG + infliximab-treated groups. The co-stimulatory molecule for antigen presentation to T cells and CD86 decreased in mDC from acute to subacute time-points in both treatment groups, but not in the single patient who developed coronary artery aneurysms. We also defined tolerogenic mDC that expand in the subacute phase of KD not impaired by infliximab treatment. T_{reg} and T_{mem} expanded after treatment with no significant differences between the two groups. Treatment of KD patients with infliximab does not adversely affect generation of tolerogenic mDC or the development of T cell regulation and memory.

Keywords: dendritic cells, infliximab, Kawasaki disease, T cells, vascular inflammation

Introduction

Kawasaki disease (KD) is an acute, T cell-mediated, self-limited vasculitis of the coronary arteries that is the most common cause of acquired heart disease in children [1]. Expansion of the regulatory T cell (T_{reg}) population with secretion of interleukin (IL)-10 is critical to recovery [2] (and Franco et al. submitted). The acute inflammation responds rapidly to intravenous immunoglobulin (IVIG) infusion, which may exert its beneficial effect through a number of different pathways including expansion of a heavy chain constant region (Fc)-specific T_{reg} population (Franco et al. submitted). IVIG resistance occurs in 10–15% of patients, and tumour necrosis factor (TNF)-α blockade has emerged as an effective strategy to limit inflammation in these patients [3]. Data are conflicting on the effect of TNF-α on myeloid dendritic cells (mDC) and T_{reg} maturation. Recent studies suggest that TNF-α inhibits phosphorylation of forkhead box protein 3 (FoxP3) by stimulating protein phosphatase 1, which leads to dysfunctional T_{reg} in patients with rheumatoid arthritis [4]. However, in contrast, in-vitro studies suggest that TNF-α is required for the terminal maturation and activation of DC, the most relevant antigen-presenting cells (APC) for T cell priming [5]. This raised concern that TNF-α blockade could impede the natural host immune mechanisms that lead to down-regulation of inflammation in KD patients. A Phase III, randomized, double-blinded, placebo-controlled
trial of the addition of infliximab (5 mg/kg) to standard IVIG therapy afforded an opportunity to study the effects of infliximab treatment on the emergence of tolerogenic mDC and T cell regulation over time. We defined the role of TNF-α-blockade on the activation and terminal differentiation of different DC populations and the expansion of Treg and memory T cells (Tmem) that participate in recovery from acute KD.

Materials and methods

Study population

Patients < 18 years of age who met American Heart Association criteria and presented within the first 10 days of fever were assigned randomly to receive infliximab or placebo prior to IVIG infusion as part of a Food and Drug Administration (FDA)-sponsored clinical trial (clinicaltrials.gov NCT#00760435). Patients were randomized according to a block randomization scheme stratified by site, age < 1 year or ≥ 1 year, and sex with balance achieved after every fourth patient. KD patients and paediatric patients with other acute inflammatory conditions were enrolled at Rady Children’s Hospital, San Diego, following written parental informed consent and patient assent as appropriate. The protocol was approved by the Institutional Review Board at UCSD. All KD patients were evaluated by echocardiography during the acute admission and at 2 and 6 weeks following diagnosis. All studies were evaluated by a single echocardiographer blinded to treatment assignment. The internal diameter of the right and left anterior descending coronary arteries was measured and expressed as normal coronary artery dimensions.

Study population

The CD4+ T cell phenotype was determined by staining with specific monoclonal antibodies: anti-CD4 PerCP-Cy5.5, mouse IgG1k, clone RPA-T4 and anti-CD25 PE and mouse IgG1k, clone BC96 from eBioscience. BD FACSCanto was used for data acquisition; data were analysed with FACSDiva (BD Biosciences) or FlowJo software (Tree Star, Inc., Ashland, OR, USA).

DC characterization

Myeloid and plasmacytoid DC were defined by a combination of the following markers detected by cell surface staining: anti-human CD11b-APC-cyanin 7 (Cy7), mouse immunoglobulin (Ig)G1k, clone ICRF44, anti-human CD11c-APC, mouse IgG1k, clone B-Ly6, anti-human CD14-phycoerythrin (PE) Cy7, mouse IgG2ak, clone M5E2, anti-human CD86-fluorescein isothiocyanate (FITC), mouse IgG1k, clone 2331(FuN-1), anti-human leucocyte antigen (HLA)-G, PE, clone 87G, mouse IgG2ak, anti-human CD85d [Ig-like transcript 4 (ILT-4)] peridinin chlorophyll (PerCP)-eFluor 710, rat IgG2ak, clone 42D1, anti-human CD1c-PE [blood dendritic cell antigen 1-phycocerythrin (BDCA-1-PE)], mouse IgG2a, clone AD5-8E7, anti-human HLA-DR-FITC, clone G41-6, mouse IgG2ak from BD Science and anti-IL3r (CD123), mouse IgG2ak, clone AC145 from magnetic affinity cell sorting (MACS) (Miltenyi Biotech, San Diego, CA, USA). In some experiments, mDC were FACS-sorted and stimulated in vitro with purified Fc fragments (Life Meridian Science).
Memory CD4+ and CD8+ T cell characterization

T cell memory subsets were defined by cell surface staining with the following antibodies: anti-human CD4, PerCP-Cy5.5, clone RPA-T4, mouse IgG1κ and anti-human CD8α, APC, clone RPA-T8 and mouse IgG1κ from eBioscience, in combination with anti-human IL-15Rα, FITC, clone eBioJM7A4, mouse IgG2bκ and anti-human CD197 (CCR7)-PE, clone 3D12 and rat IgG2aκ from BD Bioscience.

Results

Patient characteristics

Unselected KD subjects enrolled in a randomized clinical trial were studied consecutively to ensure an equal number of IVIG plus infliximab and IVIG plus placebo-treated subjects, as investigators were blinded to the treatment assignment. A total of seven infliximab-treated subjects (mean age 3.0 years, four males, median CRP 7.3 mg/dl) and seven placebo-treated subjects (median age 3.1 years, one male, median CRP 8.3 mg/dl) were studied (Table 1). All KD subjects received with IVIG (2 g/kg) and aspirin. One subject developed coronary artery aneurysms (placebo plus IVIG group) and three had transient dilation of one or more coronary arteries (two infliximab plus IVIG group, one placebo plus IVIG group) (Table 1).

Characterization of DC

mDC (defined as CD11c+CD11b+) were enumerated from PBMC from 14 acute KD patients prior to treatment and four acute febrile control children (Fig. 1). mDC represented 9.7–77.0% and 6.3–51.9% of the total PBMC in acute KD and febrile control patients, respectively. In most KD subjects, there was a decrease in the percentage of both total and activated (CD86+) mDC following treatment, regardless of the treatment arm (Fig. 2a,b). Of interest, the one patient who developed coronary artery aneurysms was in the IVIG plus placebo arm and had no decrease in either total or activated mDC between the acute and subacute phases (acute mDC 30.6%, subacute mDC 32.4%) (Fig. 2c,d). There was a striking correlation between the change in percentage of mDC and activated mDC (CD86+) and the change in left anterior descending (LAD) and right coronary artery (RCA) Z-scores between the acute and subacute time-points only for the subjects treated with infliximab plus IVIG (Fig. 2e,f). Three patients (43%) in each treatment group carried at least one copy of the risk allele for ITPKC (Table 1). There was no association between the percentage of mDC in the acute phase and ITPKC genotype (data not shown).

Plasmacytoid DC (pDC), defined as CD123+, CD11b− and CD14−, were detected at low levels during the acute phase (0.02–0.33% of PBMC) and circulating levels rose minimally at the subacute time-point (0.17–1.97% of PBMC) (Supporting information, Fig. S1).

Table 1. Demographic and clinical characteristics of patient populations.

<table>
<thead>
<tr>
<th></th>
<th>Kawasaki disease</th>
<th>IVIG + infliximab</th>
<th>Febrile controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 7</td>
<td>n = 7</td>
<td>n = 4</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>3.1 (1.4–5.2)</td>
<td>3.0 (0.3–9.4)</td>
<td>4.1 (1.1–10.5)</td>
</tr>
<tr>
<td>Males (%)</td>
<td>1 (14)</td>
<td>4 (57)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Median illness day (range)</td>
<td>6 (3–10)</td>
<td>7 (5–8)</td>
<td>5 (3–6)</td>
</tr>
<tr>
<td>CA status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneurysm</td>
<td>1</td>
<td>0</td>
<td>n.a.</td>
</tr>
<tr>
<td>Dilated</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Median Zmax (range)</td>
<td>1.2 (0–5.5)</td>
<td>1.2 (0–5–2.9)</td>
<td>n.a.</td>
</tr>
<tr>
<td>WBC (×10^3/μl)</td>
<td>12 (9.1–22.9)</td>
<td>14.7 (8.1–21.8)</td>
<td>9.9 (3.4–15.1)</td>
</tr>
<tr>
<td>ANC (μl)</td>
<td>8400 (4802–18837)</td>
<td>11256 (5913–16568)</td>
<td>5738 (2091–8758)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>61 (31–140)</td>
<td>67 (34–93)</td>
<td>36 (7–60)</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>8.3 (5.4–34.1)</td>
<td>7.3 (4.3–33.4)</td>
<td>2.7 (0.7–16.8)</td>
</tr>
<tr>
<td>ITPKC genotype: GG</td>
<td>4</td>
<td>4</td>
<td>n.a.</td>
</tr>
<tr>
<td>GC</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

ANC: absolute neutrophil count; CA: coronary artery; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ITPKC genotype: inositol-trisphosphate 3-kinase C, rs28493229; IVIG: intravenous immunoglobulin; lymph: lymphocytes; mono: monocytes; n.a.: not applicable; poly: polymorphonuclear leucocytes; WBC: white blood cell count; Zmax: maximal Z-score (internal diameter of the right and left anterior descending coronary arteries expressed as standard deviation units from the mean normalized for body surface area) during the first 6 weeks after diagnosis.
Tolerogenic CD14⁺ mDC circulate in acute KD and increase after therapy in the subacute phase

Within the mDC population in acute KD subjects, we detected a small percentage of CD14⁺ mDC (1.4–12.4% of PBMC). This population expanded during the subacute phase with a greater expansion in subjects who received infliximab, although the difference was not statistically significant with the small sample size studied (Fig. 3a). The majority of these cells also expressed CD86 (Fig. 3a). In two
additional acute KD subjects who were not part of the clinical trial, mDC were studied with an expanded panel of markers (Fig. 3b). It was necessary to study additional subjects for these markers, as there were insufficient cells from the subjects in the clinical trial who were undergoing immune monitoring. These mDC co-expressed ILT-4, HLA-G and low levels of BDCA-1, markers that in humans define IL-10-producing, tolerogenic mDC [7–9].

Patients with KD respond to IVIG infusion with a dramatic decrease in fever and inflammatory mediators. We used PBMC from a normal adult donor in order to have sufficient cells to address the role of IVIG in inducing IL-10 secretion by CD14+ mDC via Fcγ receptor stimulation. mDC were sorted by flow cytometry and the CD14+ subpopulation was stimulated in vitro with purified Fc fragments. Secretion of IL-10 was documented in response to Fc stimulation (Fig. 3c). This highlights a new mechanism of the anti-inflammatory action of IVIG in acute KD patients.

Treg

Consistent with previously published data from our group, Treg, defined as CD4+CD25high, circulated in the acute phase of KD and increased in number 2–4 weeks after treatment in 10 of 14 KD patients (71.4%), regardless of treatment arm (Fig. 4). These results suggest that TNF-α-blockade does not interfere with the development of Treg. It should be
noted that the expansion of the tolerogenic DC population coincided with rising numbers of circulating T_{reg} after therapy (Fig. 3a). There was no correlation between the degree of T_{reg} expansion and the ITPKC C allele.

Effector and central memory CD4⁺ and CD8⁺ T cells

Human memory T cells (T_{mem}) are defined by the expression of the IL-15 receptor and can be divided into an effector population that responds rapidly to antigen exposure within hours, and a central memory population that sustains the recall responses. The expression of the chemokine receptor CCR7, which is required for immune cells to home to secondary lymphoid organs, defines central memory T cells [10]. Both CD4⁺ and CD8⁺ memory T cells were detected in the acute phase and the circulating population expanded over time (Fig. 5). The development of T cell memory does not appear to be affected by TNF-α blockade (Fig. 5).

Discussion

We report here the immune-monitoring of infants and young children enrolled in a Phase III, randomized, double-blinded, placebo-controlled clinical trial of the addition of infliximab to standard IVIG therapy. KD subjects and febrile control children had abundant circulating mDC prior to the initiation of therapy. Overall, this population of cells contracted during the subacute phase with no significant difference between the two treatment arms. However, there was a trend towards a greater decrease in activated mDC and a greater expansion in tolerogenic mDC in subjects in the infliximab arm. In addition, the reduction in both total and activated mDC correlated strongly with the reduction in LAD Z-score only for subjects treated with infliximab. To the extent that the change in Z-score reflects a reduction in arterial wall inflammation, these data can be interpreted as showing a beneficial effect of infliximab treatment that parallels the fall in activated mDC and the expansion of tolerogenic mDC and T_{reg}. Further, we established that TNF-α blockade does not interfere with the expansion of regulatory and memory T cell populations.

There are few data regarding circulating numbers of mDC in both healthy and inflamed paediatric subjects [11,12]. In our study, we found higher numbers of circulating mDC in both our KD subjects and febrile controls than has been reported previously. In a study of healthy children across the paediatric age range, mDC and pDC were found in approximately equal numbers and the populations did not change over the first 18 years of life. However, there was a significantly lower percentage of mDC in females across the age spectrum [12]. Despite the fact that there was a skewed distribution with more females in the placebo plus IVIG group, the mean acute percentage of mDC between treatment arms was not different. In contrast to children with a chronic autoimmune disease, juvenile idiopathic
arthritides, both our acute KD and viral febrile control subjects had much higher circulating mDC. For KD, a disease of unknown aetiology, this suggests that the immune response parallels that of a canonical response to an acute infection.

A new mechanism of action of IVIG in KD has been identified recently by our group (Franco et al. submitted). A population of Treg that expand in response to the heavy chain constant region (Fc) of the immunoglobulin molecule secretes IL-10 and is key to the down-regulation of inflammation in KD. In a further extension of this observation, we report here the expansion of tolerogenic mDC following IVIG administration that correlates with Treg expansion in the subacute phase of KD. We documented IL-10 secretion in response to Fc stimulation in vitro by tolerogenic mDC sorted from a normal adult donor, and propose this as an additional mechanism of immune regulation mediated by IVIG in KD.

In this small cohort, we found no relationship between mDC or T cell subsets and the C allele of ITPKC that results in increased signalling through the calcineurin–nuclear factor of activated T cells (NFAT) pathway. Although it has been proposed that the presence of the C allele may alter gene expression in immune cells leading to a proinflammatory phenotype, we were unable to document an effect through the immune monitoring reported here [6].

Subjects treated with infliximab plus IVIG in the recently completed clinical trial had a more rapid fall in inflammatory markers, fewer days of fever and a greater decrease in LAD Z-score compared to subjects who received IVIG alone (Tremoulet et al. submitted). Here, we demonstrate an association between the decrease in activated mDC and the change in LAD Z-score only for subjects in the infliximab arm. If we use the change in LAD Z-score as a proxy for inflammation at the tissue level, then these results suggest that infliximab has benefit in these patients. The study of larger numbers of subjects with and without coronary artery dilation will be necessary to consolidate these preliminary findings. Further, the development of T cell regulation and memory was not affected adversely by TNF-α blockade, thus removing one of the theoretical concerns for the use of infliximab in a T cell-mediated disease.

We recognize several strengths and weaknesses in this study. This is the first extended immune monitoring of an acute, self-limited inflammatory illness in infants and children. Working with 1–5 ml of whole blood, we were able to perform extensive cell surface characterization of DC and T cell subsets. However, the limited blood volume obtained from these anaemic and lymphopenic ill children restricted the extent of cell characterization that could be performed. Specifically, we were unable to perform suppression assays to prove the functionality of the Treg. However, in the subacute and convalescent phase, we found decreased numbers of CD4+ and CD8+DR+ T cells as an indirect indication of Treg-mediated suppression. In addition, our small sample size makes this study hypothesis-generating, rather than definitive. The small number of subjects with coronary artery aneurysms precludes any definitive conclusions: a larger cohort of patients with vascular abnormalities will be studied to consolidate this observation.

Future studies comparing immune monitoring of KD patients and cohorts of children with well-characterized infections and autoimmune diseases will contribute to our understanding of KD pathogenesis. Immune monitoring such as the study presented here should be incorporated into future KD clinical trials to provide a more comprehensive picture of pharmacological action.

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Disclosure
The authors have no financial conflicts of interest.

Author contributions
Alessandra Franco designed and directed the immune-monitoring and all the experiments here reported. Jane C. Burns, Director of the Kawasaki disease Center at Rady Children’s hospital enrolled and treated Kawasaki disease patients with Adriana H. Tremoulet. Alessandra Franco wrote the paper with Jane C. Burns. Yali Song and Matthew Bujold technically executed the experiments described. Chisato Shimizu helped with the statistical analysis. Jhon Kanegaye provided the febrile controls for the study.

References


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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher’s website:

Fig. S1. Plasmacytoid dendritic cells (DC) expand in subacute Kawasaki disease (KD) patients after treatment. CD123+ plasmacytoid DC have been enumerated in placebo + intravenous immunoglobulin (IVIG) (red lines) and IVIG + infliximab-treated KD patients.