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RESEARCH ARTICLE

Specificity of regulatory T cells that modulate vascular inflammation

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Abstract

Intravenous immunoglobulin therapy (IVIG) is the treatment of choice for many immune-mediated diseases, yet its mechanisms of action are incompletely elucidated. We investigated the possibility that IVIG played a direct role in the expansion of regulatory T cells (Treg) that recognize the heavy chain constant region of immunoglobulin G (Fc) as a mechanism for the recovery of Kawasaki disease (KD), a T cell mediated pediatric vasculitis of the coronary arteries. We successfully generated Fc-specific Treg clones from sub-acute KD subjects that did not develop arterial complications after IVIG and defined an unusual functional phenotype: Fc-specific Treg secrete IL-10 and small amounts of IL-4 but not TGF-β. Antigen presentation studies demonstrated that these Treg clones can be activated by autologous B cells that express IgG on their cell surface in the absence of exogenous Fc. The IgG molecule has to be canonically processed and presented by autologous MHC molecules to be recognized by Treg. In support of the importance of this novel Treg population in downsizing vascular inflammation, KD patients with dilated coronary arteries or aneurysms despite IVIG treatment failed to expand Fc-specific Treg. Our results point to a specificity of a previously un-described Treg population for the clinical benefit provided by IVIG therapy in children.

Keywords

IVIG, immune-regulation, Kawasaki disease, vasculitis, Treg

History

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Introduction

Kawasaki disease (KD), an acute, self-limited pediatric vasculitis of the coronary arteries, responds to a single infusion of intravenous immunoglobulin (IVIG) with abrupt cessation of fever and reduction in acute phase reactants [1]. Treg are the most abundant T cell clonal population in the circulation in KD [2], suggesting that they play an important role in the self-limited nature of the disease.

As a result of IVIG therapy, Treg expand *in vivo*, as reported in human autoimmune diseases [3] and in KD [4]. Recently it has been reported that human Treg plays an important role in post-infarct cardiac healing [5] suggesting that immune-regulation is key to resolve vascular inflammation.

However, the mechanism for Treg expansion after IVIG in KD has not been fully elucidated. Recently our group defined a tolerogenic, IL-10-secreting, dendritic cell (DC) population that is stimulated by the heavy constant region of immunoglobulin (Fc). The expansion of tolerogenic DC correlates with the expansion of Treg after IVIG treatment [4]. The mechanism for this Treg expansion may be the up-regulation of prostaglandin E2 on DC [6]. However, Treg numbers are not the whole story. As an illustration of this, in KD patients who develop coronary arterial abnormalities after IVIG, populations of tolerogenic DC and Treg expand [4]. However, it is the Treg specificities, rather than numbers, that appear to determine the regulation of vascular inflammation after IVIG therapy in KD.

Treg limits inflammation and restore immune tolerance to self and not-self in humans, thus playing a critical role in allergy, autoimmunity and transplantation [7–9]. Their most relevant characteristic is the secretion of interleukin-10 (IL-10), a suppressive cytokine that down-regulates the effector functions of cells of the innate and adaptive immune system [10,11].

Two main Treg lineages were initially described [12]: (1) natural Treg (nTreg) are derived from the thymus during fetal life and recognize self peptides [13,14]; (2) peripherally-induced Treg recognize not-self, arise from naïve T cells under appropriate conditions (i.e. transforming grow factor (TGF)-β [15,16] and following repeated antigenic stimulation [17–19]. The maintenance and plasticity of Treg and an understanding of the epigenetic alterations that influence their development and functions are still under investigation [20]. Treg plasticity and stability largely depend on the specific inflammatory environment [21–23].

Here we describe a novel Treg population that specifically recognizes the Fc of IgG presented by autologous mature IgG+ B lymphocytes, that expands following IVIG infusion, and that is distinct from the previously described Treg that recognize exogenous pan-DR Fc epitopes in normal healthy

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donors [24]. Expansion of this Fc-specific Treg population is associated with resolution of vascular inflammation, while failure to expand is associated with progressive damage to the vascular wall and coronary artery aneurysm formation in infants and young children with KD.

Materials and methods

Study population

KD patients and pediatric patients with acute inflammatory conditions were enrolled at Rady Children's Hospital San Diego following parental informed consent. The protocol was approved by the Institutional Review Board at UCSD. All KD patients enrolled in this study (Table 1) were evaluated by echocardiography during the acute admission and at two and six weeks following diagnosis. The internal diameter of the right and left anterior descending coronary arteries was measured and expressed as *Z* scores (standard deviation units from the mean normalized for body surface area; normal *Z* score <2.5). Z_{worst} was defined as the highest *Z* score of either coronary artery measured during the first six weeks after fever onset. All KD subjects were treated with IVIG 2 g/kg and aspirin 80–100 mg/kg/d until afebrile, then 3–5 mg/kg day until the platelet count had returned to normal. All subjects were taking low dose aspirin at the time of the sub-acute phlebotomy. No subject in this series had IVIG resistance defined as persistent or recrudescent fever at least 36 h after completion of the initial IVIG infusion. Subjects 6, 16 and 20 received infliximab 5 mg/kg IV prior to IVIG infusion as part of a clinical trial of intensification of initial IVIG therapy (clinicaltrials.gov).

Heparinized blood samples (1–4 ml) were obtained prior to IVIG treatment at the time of diagnosis (acute) and two weeks after IVIG therapy (sub-acute).

Pediatric patients with acute inflammatory conditions studied as controls (acute viral infections, bacterial infections, systemic drug allergy, juvenile idiopathic arthritis and Henoch–Schoenlein purpura) had blood sampled only once and were aged 1.6–15.4 years (Table 2).

Fc-specific Treg detection and characterization of Treg phenotype

To enumerate Fc-specific Treg that activate after IVIG infusion, we developed a method to avoid non-specific

Table 2. Acute pediatric febrile controls.

Control subject #	Age, yrs	Sex	Race/Ethnicity	Illness day	Final diagnosis
1	2.3	F	Hispanic	14	Viral stomatitis
2	2.1	M	Hispanic	~3–5	Viral syndrome
3	1.6	F	Hispanic	5	Viral syndrome
4	6.6	F	Hispanic/ Caucasian	6	Viral
5	1.7	F	American Indian	4	Viral/her angina
6	5.8	F	Caucasian	6	Adenovirus
7	9.6	M	Hispanic	10	Viral syndrome
8	3.6	F	Caucasian	2	Abscess
9	2.2	M	Asian	5	Viral
10	15.4	F	Caucasian	2	Bactrim drug reaction
11	2.3	F	Caucasian	16	Juvenile idiopathic arthritis (JIA)
12	3.8	F	Hispanic	10	Juvenile idiopathic arthritis (JIA)
13	4.6	F	Hispanic	~21	Henoch–Schoenlein purpura (HSP)

Table 1. KD subjects enrolled in the study.

KD subject #	Age, yrs	Sex	Race/Ethnicity	Illness day[a]	Coronary artery *Z*-max[b]	Fc-specific Treg response
1	1.4	M	Hispanic	4	0.8	+
2	5.4	F	Caucasian/Asian	5	0.7	+
3	0.6	M	Hispanic/Asian	6	1.4	+
4	2.0	M	Caucasian/Hispanic/ Native American	8	1.7	+
5	5.8	F	Hispanic	5	1.1	+
6	5.3	M	Caucasian	5	1.2	+
7	0.3	M	Asian	3	1.2	+
8	3.7	M	Caucasian	8	0.9	+
9	6.9	M	Caucasian	6	1.0	+
10	2.9	M	Hispanic	7	1.6	+
11	1.9	M	Caucasian	4	3.3 (dilated)	−
12	7.9	M	Hispanic	4	2.8 (dilated)	−
13	2.0	F	Mixed	8	4.7 (aneurysm)	−
14	0.9	M	Hispanic	6	5.6 (aneurysm)	−
15	5.7	F	Asian	10	2.7 (dilated)	−
16	1.6	M	Caucasian	5	1.3	+
17	2.9	F	Hispanic	5	−0.1	+
18	6.4	F	Asian	6	1.0	+
19	1.9	M	Asian/Caucaisan	8	2.3	+
20	0.8	M	Hispanic	4	8.1 (aneurysm)	−
21	5.6	M	Asian/Caucasian	4	1.5	+

Subjects 6, 16, 20 were also treated with infliximab.
[a]Illness day 1 = onset of fever.
[b]*Z*-max scores = maximum internal diameter for the left anterior descending or right coronary artery expressed as standard deviation units from the mean (*Z*-score) normalized for body surface area.

expansion of Treg by tolerogenic DC or the expansion of effector T cells. Peripheral blood mononucleated cells (PBMCs) were plated with scalar doses of purified Fc (1, 10 and 100 μg/ml; Life Meridian Science, Memphis, TN) at a concentration of 4×10^5 cells/well in 96 flat-bottomed plates (Falcon) for 4 d. Cell cultures did not receive any exogenous IL-2 prior to the assay: withholding IL-2 feeding in Fc-stimulated PBMC prevented the expansion of non-Fc-specific Treg and/or the expansion of effector T cell stimulated via Fcγ receptors. On day 4, culture supernatants were collected to measure interleukin 10 (IL-10), interferon γ (IFNγ and IL-2 by enzyme-linked immunosorbent assay (ELISA) and to perform fluorescence-activated cell sorting (FACS) analysis as described below.

CD4+ $CD25^{high}$ T cell surface phenotype was determined by staining with specific monoclonal antibodies: anti-CD4 PerCP-Cy5.5, mouse IgG1κ, clone RPA-T4 and anti-CD25 PE, mouse IgG1κ, clone BC96 from eBioscience (San Diego, CA). BD FACSCanto was used for data acquisition; data were analyzed with FACSDiva (BD Biosciences, San Francisco, CA) or FlowJo software (Tree Star, Inc, Ashland, OR). Intracellular FOXP3+ was measured with a kit from eBioscience (anti-human FOXP3 PE, mouse IgG1κ, clone 259/D/C7, anti-CD4 FITC, mouse IgG1κ, clone RPA-T4 and anti-CD25 antigen presenting cell (APC), mouse IgG1κ, clone M-A251). Surface anti-IL-7 r was measured using anti-CD127 PE, mouse IgG1κ, clone HIL-7 R-M21; CD45RA by using anti-CD45RA-APC, mouse IgG2bκ, clone HI100 from BD Bioscience; IL-15r with anti-IL15r FITC, mouse IgG2b, clone JM74A; CCR7 with anti-CCR7 PE, rat IgG2aκ, clone 3D12; CCR6 with anti-CCR6 PE-Cy7, mouse IgG1κ, clone R6H1; CCR4 with anti-CCR4 PE, mouse IgG1κ, clone 1G1 from eBioscience. Levels of IL-10 and IL-4 produced by PBMC, Treg lines and Treg clones were measured by ELISA with primary and secondary antibodies from BD Bioscience. mRNA was extracted from Treg clones using TRIZOL according to manufacture's instructions. mRNA transcript abundance levels from cDNA derived from 25 ng of total RNA were measured using Taqman 5′-nuclease gene expression assay (Applied Biosystem, Foster City, CA) for IL-10, IL-4, TGF-β, IL-17 and CTLA-4. Results were normalized for the housekeeping gene TAF1b.

Activated and memory CD4+ and CD8+ T cell characterization

Activated T cells were detected by staining with anti-human HLA-DR, PE, clone LN3, mouse IgG2b,κ, in combination with anti-human CD4, PerCP-Cy5.5, clone RPA-T4, Mouse IgG1,κ and anti-human CD8α, APC, clone RPA-T8, Mouse IgG1,κ from eBioscience. Memory T cells were detected with anti-human IL-15Rα, FITC, clone eBioJM7A4 and Mouse IgG2b, from BD Bioscience.

Treg cloning and expansion

Fc-specific Treg lines were generated from PBMC stimulated with purified Fc (100 μg/ml). T cell lines were established by plating 4×10^5 cells/well in 96 flat-bottomed plates (Falcon). Each well was evaluated as an individual T cell line. On day 4 after stimulation *in vitro*, cell cultures were fed 100 U/ml of recombinant IL-2 (Peprotech, Rocky Hill, NJ), expanded for two days, and tested for specificity at day 6 from the first Fc stimulation *in vitro* [25]. The specificity of the T cell lines was determined by measuring IL-10 in T cell culture supernatants collected at 48 h following stimulation with one of the following: (1) irradiated autologous PBMC (negative control); (2) irradiated autologous PBMC pulsed with 20 μg/ml Fc; (3) irradiated allogeneic PBMC pulsed with 20 μg/ml Fc (MHC-restriction control). Treg lines that responded by producing IL-10 to autologous PBMC pulsed with Fc, but not to autologous PBMC alone or allogeneic PBMC pulsed with Fc, were cloned by limiting dilution (0.3–1 c/w) in the presence of 2×10^4 irradiated autologous *Epstein–Barr virus* (EBV)-transformed B cells per well pulsed with 20 μg/ml Fc as APC/antigen source. EBV-transformed B cell lines were obtained by infecting PBMC with supernatant from an EBV-producing marmoset-derived cell line B95-8 purchased from American Tissue Culture Collection (ATCC). T cell clones were stimulated weekly with irradiated autologous EBV lines pre-pulsed with Fc fragments and expanded with 100 U/ml of IL-2 every other day.

Cloning to obtain IgG− and IgG+ autologous and allogenic B cell lines as APC source

IgG+ autologous B cells to address the recognition of endogenous Fc by Treg (in addition to exogenous Fc) were obtained from EBV-transformed B cells derived from acute KD patients cloned early after transformation by limiting dilution. B cell clones were screened for surface IgG expression using anti-human CD19, APC-cy7, clone SJ25c1, mouse IgG1κ and anti-human IgG, PE, clone G18-145, Mouse IgG1κ, that recognizes the framework of all IgG isotypes from BD Bioscience.

Statistical analysis

Data analysis was conducted using Prism version 5.0 software (Graphpad Software, San Diego, CA). Statistical significance of the observed Fc-specific Treg expansion versus no antigen control was assessed by Wilcoxon matched-pairs signed rank test. Non-parametric Mann–Whitney test was used to compare the percent increase in Treg between groups of KD children. p Values less than 0.05 were considered significant.

Results

IL-10 increases in culture supernatants of PBMC from sub-acute KD patients stimulated with Fc fragments

To address a possible role of the Fc in stimulating Treg in an antigen-specific, MHC-restricted manner, we developed an assay, starting with a small number of specific precursors, to measure IL-10 secretion and CD4+ $CD25^{high}$ T cell expansion in response to scalar doses of purified Fc fragments (1, 10, 100 μg/ml) in bulk PBMC cultures.

After Ficoll-hypaque separation, PBMCs from 15 sub-acute KD subjects who had received IVIG 2 weeks earlier (Table 1) were plated with scalar doses of purified Fc fragments for 4 d. Culture supernatants were collected to measure IL-10, IFNγ and IL-2. The measurement of IFNγ and

IL-2 was included in these experiments to address a possible expansion of pro-inflammatory effector T cells by Fc stimulation.

IL-10 secretion by PBMC was documented in the culture supernatants of all 15 sub-acute KD subjects studied after IVIG therapy (subjects 1–14 and 20) (Figure 1), regardless of their coronary artery status. The highest concentration of Fc tested (100 μg/ml) was the most effective (Figure 1). The Fc fragments did not stimulate pro-inflammatory T cells with these experimental conditions as evidenced by the absence of IFNγ and IL-2 in these culture supernatants (Figure 1).

Fc-specific Treg expands only in KD patients with normal coronary arteries after IVIG but not in patients with arterial abnormalities despite the presence of IL-10

To address the importance of the Treg specificity for the Fc in influencing clinical outcome, we measured the Treg response to purified Fc fragments in the same cultures in which IL-10 was detected (Figure 1). Under these experimental conditions, only 4 d in culture in the absence of IL-2 *in vitro*, only Treg responding to the Fc via T cell receptor (TcR) recognition can survive and proliferate. Ten KD patients who did not develop arterial abnormalities after IVIG therapy (subjects 1–10, Figure 2) showed a brisk expansion of CD4+ $CD25^{high}$ T cells after 4 d in response to scalar doses of Fc. Notably, Treg from the five patients who developed coronary artery abnormalities failed to respond to Fc stimulation *in vitro* (subjects 11–14 and 20, Figure 2). Presumably, the source of the IL-10 in the subjects that developed arterial abnormalities was tolerogenic DC that we found abundant during the acute phase of KD and increase in numbers after IVIG in the sub-acute phase regardless from the clinical outcome [4].

When we compared the magnitude of the Fc-specific Treg response in PBMC from the acute and sub-acute KD subjects, marked Fc-specific Treg expansion after IVIG treatment was observed only in patients with normal arteries but not in patients who developed arterial abnormalities (Figure 3).

As shown in Figure 4, FOXP3 analysis by intracellular staining confirmed the lack of Treg expansion in Fc-stimulated cultures from a sub-acute KD patient who developed coronary arterial dilation, subject 15, in contrast to a sub-acute KD patient with normal coronary arteries after

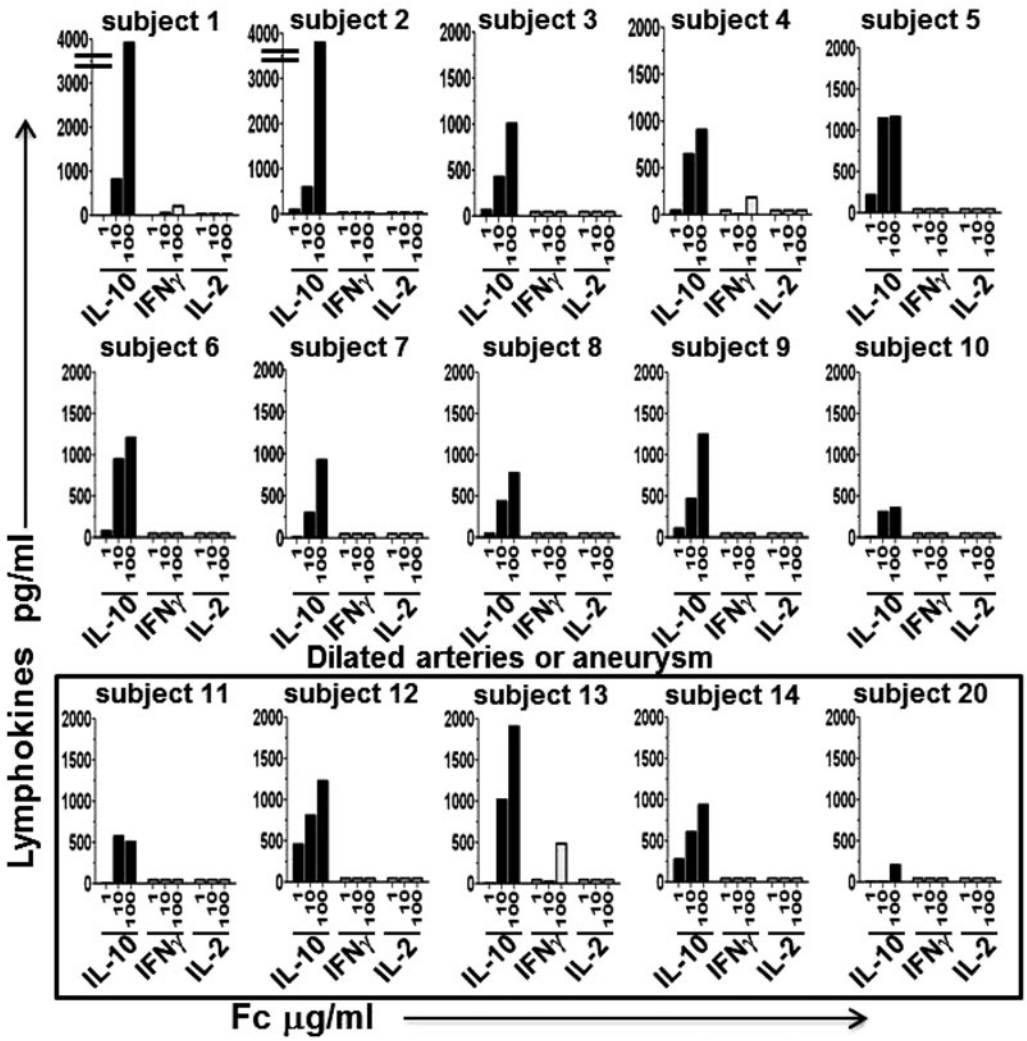


Figure 1. PBMC derived from sub-acute KD subjects secrete IL-10, but not IFNγ or IL-2 in response to scalar doses of Fc. PBMC from 15 KD subjects, 10 with normal arteries after IVIG therapy (subjects 1–10) and 5 with dilated arteries or aneurysm after IVIG therapy (subjects 11–14 and 20) were cultured with scalar doses of purified Fc fragments (Meridian Life Science, purity ≥97%) for 4 d in the absence of exogenous lymphokines. Supernatants were harvested and tested for IL-10, IFNγ and IL-2 secretion by ELISA.

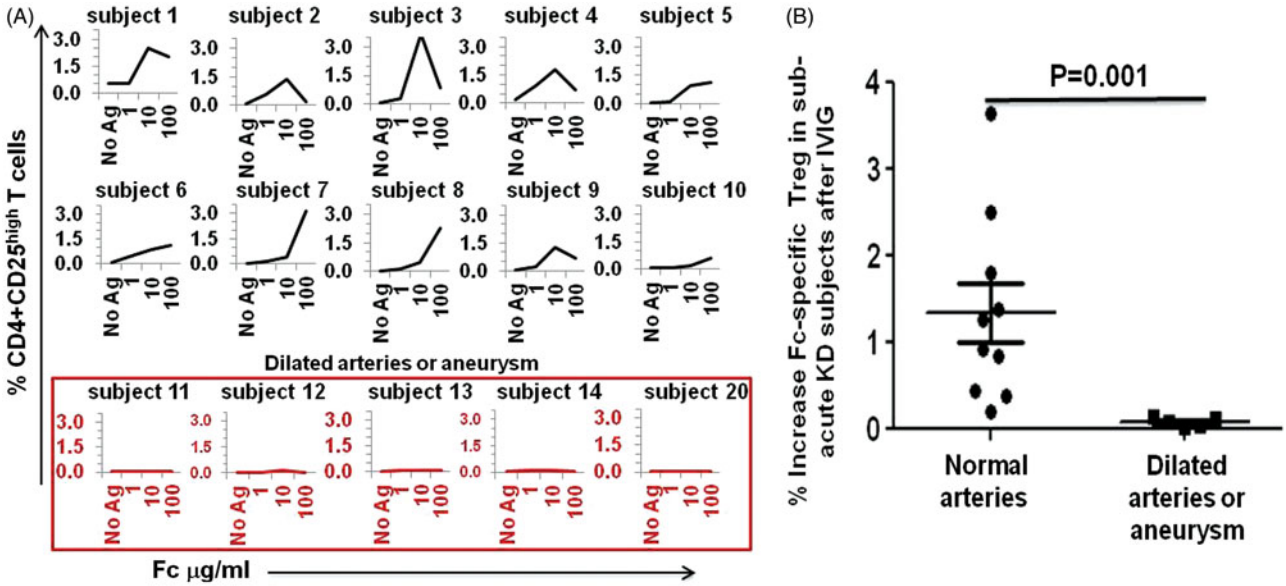


Figure 2. Enumeration of Fc-induced Treg in sub-acute KD patients with normal arteries after IVIG. Panel A: CD4+ CD25^{high} Treg expansion to scalar doses of Fc was analyzed by flow cytometry in PBMC cultures derived from 15 sub-acute KD subjects, 10 with normal arteries (subjects 1–10) and 5 with dilated arteries or aneurysm (subjects 11–14 and 20) previously studied for lymphokine production. PBMC were cultured for 4 d with 0 (no antigen), 1, 10 or 100 µg/ml of purified Fc fragments (Meridian Life Science, purity ≥97%). Panel B: Summary of the Treg response in 15 sub-acute KD subjects described in panel A. The median % increase in Treg expansion for the subjects with normal coronary arteries was calculated with an Fc dose of 10 µg/ml.

IVIG therapy (subject 16, Table 1). These results further support the concept that the expansion of Fc-specific Treg plays an important role in resolution of the acute vasculitis in KD patients.

In five Treg responders, 10 µg/ml Fc was more immunogenic than 100 µg/ml (subjects 1, 2, 3, 4, 9; Figure 2), in sharp contrast to the higher dose required to stimulate IL-10 secretion in the same patients (Figure 1). These results may reflect different MHC haplotypes that bind relevant Fc peptides recognized by Treg with different TcR affinities. The FACS images summarized in Figure 2 are provided in supplemental Figure 1A. As a control for antigenic specificity, we tested Treg expansion in response to increasing concentrations of F(ab)$_2$ fragments and documented no expansion (supplemental Figure 1B).

Although we were unable to perform classic suppression assays due to the limited number of cells and the very young age of the patients who did not yet complete vaccination protocols, we observed that T cells with a pro-inflammatory phenotype decreased as the Fc-specific Treg increased. Specifically, activated CD4+ and CD8+ DR+ T cells, but not IL-15 (receptor)r+ T cells, decreased in Fc-stimulated PBMC cultures in which the Treg expanded (subjects 18 and 19, Figure 5).

Characterization of Fc-specific Treg clones derived after IVIG from sub-acute KD subjects with normal coronary arteries

Treg clones were generated from Fc-specific Treg lines derived from IVIG-treated, sub-acute KD subjects with normal coronary artery internal diameters and were screened for specificity. B-cell lines derived from four KD subjects were cloned by limiting dilution and screened for IgG expression. We measured IL-10 production by Treg clones in response to irradiated, autologous, IgG-negative B cell lines alone (control) or the same B cell lines incubated with Fc fragments. We expanded and further characterized the phenotype and lymphokine profile of Treg clones that made IL-10 in response to Fc-pulsed autologous IgG-negative B cells but not to the same B cells without Fc. Treg clones that secreted IL-10 in response to autologous B cells in the absence of Fc stimulation were considered to be autoreactive for self-antigens other than Fc and were not studied further.

Fc-specific Treg clones produced high amounts of IL-10 and lower amounts of IL-4 but not TGF-β (Figure 6A). The production of IL-10 and IL-4, but not TGF-β were confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of RNA extracted from individual clones, which also demonstrated high transcript abundance for CTLA-4, a canonical marker for all Treg lineages, and no expression of IL-17 (Figure 6A). T cells were further defined by surface and intracellular markers: CD4+ CD25^{high}, IL-7r (CD127)^{low} and CD45RA^{low} FOXP3^{high} (Figure 6B). The memory marker IL-15r and CCR6, the homing receptor for vessels, were not expressed by Fc-specific Treg clones, which did express high levels of CCR7, an important homing receptor for lymphoid organs (Figure 6B). The low expression level of CD127 coupled with absent expression of CCR4 excluded the possibility of contamination by T helper (h) 2 cells in this T cell clonal effort [26].

Fc-specific Treg clones respond to autologous IgG+ B cells in the absence of exogenous Fc

We next addressed whether Fc-specific Treg clones respond to both: (1) exogenous Fc fragments presented by autologous IgG negative B cells and (2) endogenous Fc derived from membrane IgG on the cell surface of autologous IgG+ B

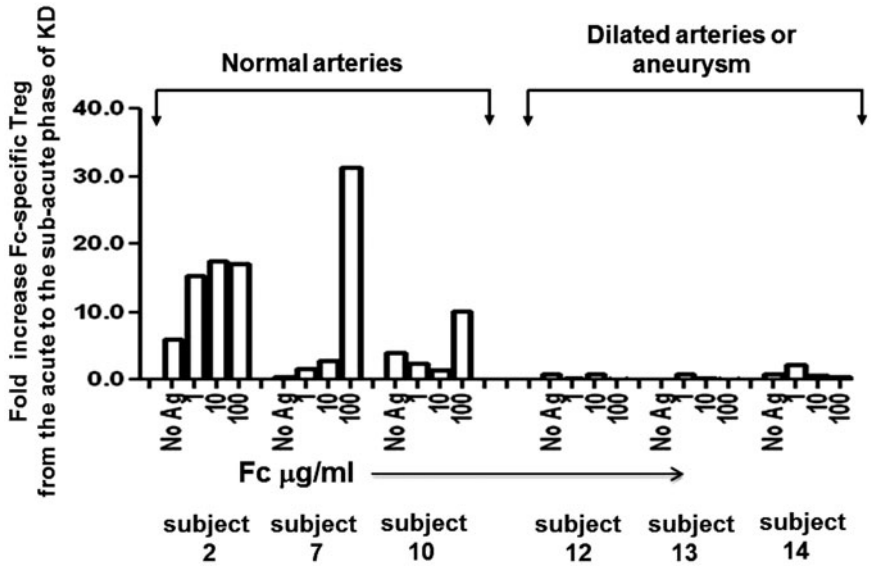


Figure 3. Differential expansion of Fc-specific Treg from the acute to the sub-acute phase of KD depending upon clinical outcome. We studied the fold-increase of the Fc-specific Treg repertoire from the acute to sub-acute phase in six KD subjects: three subjects with normal coronary arteries after IVIG therapy (subjects 2, 7, 10), three subjects with dilated arteries or aneurysm despite IVIG therapy (subjects 12, 13, 14).

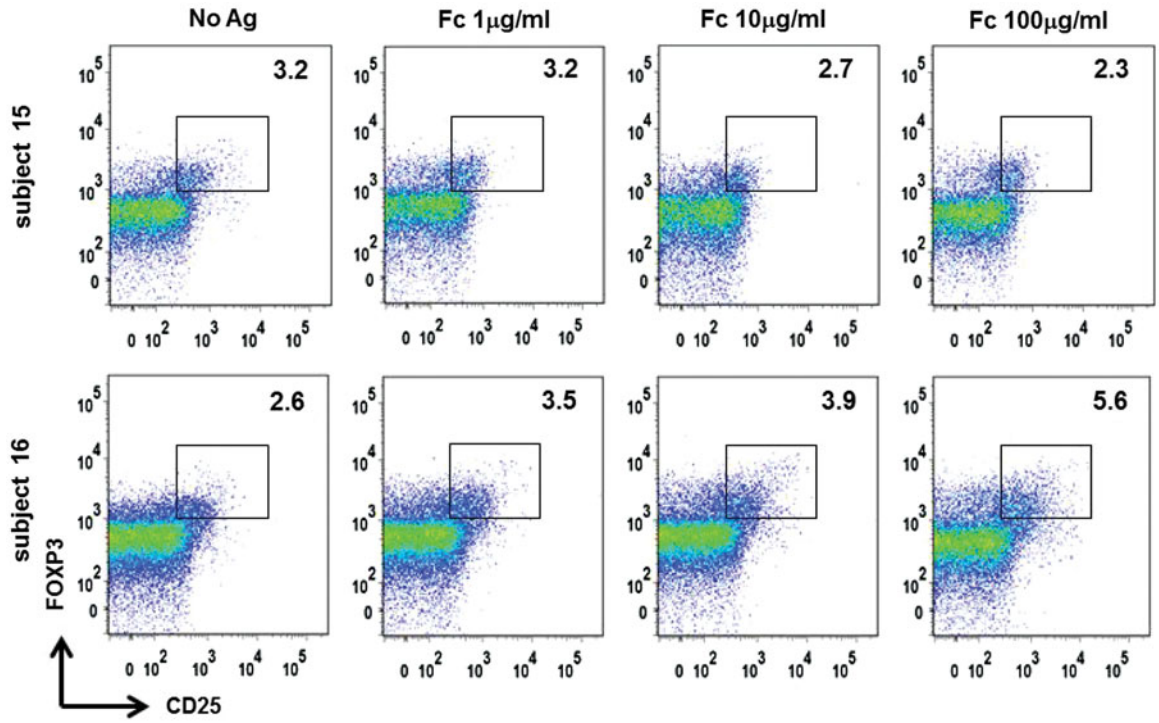


Figure 4. FOXP3+ T cells expansion in response to Fc. Intracellular staining for FOXP3^{high} expression in CD4+ T cells was assessed in Fc-stimulated PBMC cultures from two sub-acute patients after IVIG therapy. Subject 15 had dilated coronary arteries by echocardiogram and there was no expansion of the Fc-specific Treg population. Subject 16 had normal coronary arteries and showed expansion of the Treg population in response to 100 μg/ml Fc.

cells. When autologous, live, irradiated IgG+ B cells were used as APCs, Fc-specific Treg clones responded by secreting IL-10 and IL-4 in the absence of exogenous Fc (Figure 7A). To determine if conventional antigen processing of IgG molecules by B cells is required to stimulate Fc-specific Treg, we tested in parallel the antigen presenting capacity of live versus paraformaldehyde-fixed autologous IgG+ B cell lines (Figure 7A). MHC restriction was required for Fc presentation and Treg response because only autologous, but not allogeneic, IgG+ B-cells induced IL-10 and IL-4 secretion (Figure 7B). The requirement for TcR signaling rather than Fcy receptor stimulation on T cells was further confirmed by the dose-dependent IL-10 production by Treg clones in response to an anti-CD3 agonistic antibody (supplemental Figure 2).

Treg expand when stimulated *in vitro* by Fc in children with acute infections and drug reactions, but not in untreated children with autoimmune diseases

To address if this Fc-specific Treg population physiologically expand during other acute pediatric inflammatory conditions, we tested the magnitude of Treg expansion in Fc-stimulated

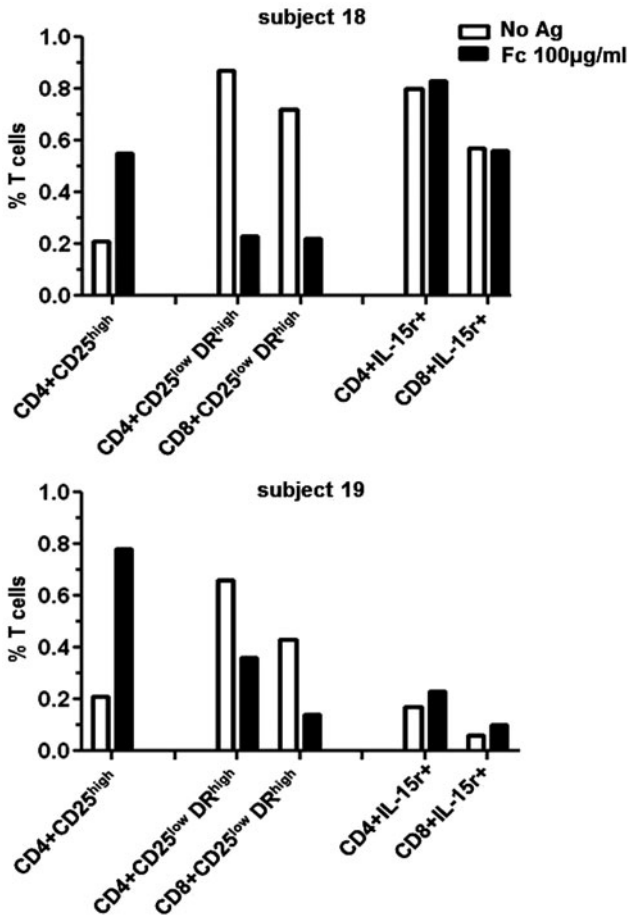


Figure 5. Activated CD4+ and CD8+ $CD25^{low}$ DR^{high} but not memory IL15r+ T cells decrease in PBMC cultured with Fc when Treg expand. PBMC from two sub-acute KD subjects with normal arteries after IVIG (subjects 18 and 19) were cultured with 100 µg/ml purified Fc fragments or media alone as control to address the fate of activated T cells and memory T cells in Fc-stimulated cultures in which Treg expand. Treg were defined as CD4+ $CD25^{high}$; activated CD4+ and CD8+ T cells as $CD25^{low}$ and DR^{high}; and memory T cells as IL-15r+. After 4 d in culture with Fc, Treg expanded but activated CD4+ and CD8+ T cell numbers decreased. IL-15r+ memory T cells remained unchanged.

PBMC in a variety of controls that included children with self-limited viral infections ($n = 7$), bacterial infections ($n = 2$), systemic drug reaction ($n = 1$) and three newly diagnosed, untreated autoimmune diseases (juvenile idiopathic arthritis, $n = 2$, Henoch–Schoenlein purpura, $n = 1$). In 10 of 13 pediatric subjects with acute viral or bacterial infection or systemic drug reactions, but not autoimmunity, Fc-induced Treg responses were detected (median Treg $\% = 0.28\%$, 95% CI 0.20–0.37 at 10 µg/ml of purified Fc) (Figure 8).

Discussion

There is a growing list of the immune modulatory functions of IVIG that includes expansion of IL-10-secreting DC stimulated by Fc fragments and the non-specific, IL-10 mediated expansion of Treg observed in humans and mice [3,4,6,27,28]. To this list, we can now add a newly discovered action of IVIG: expansion of a population of Treg that recognize the Fc region of IgG presented in a conventional MHC-restricted, TcR-mediated fashion by mature B cells. Following IVIG therapy, this Fc-specific Treg population expands in KD patients with normal coronary arteries but fails to expand in patients who develop dilated or aneurysmal coronary arteries despite IVIG treatment. Although IL-10 secretion in response to Fc stimulation of PBMC in sub-acute KD patients after IVIG is important, it is not sufficient to resolve the vasculitis, since in the absence of Fc-specific Treg expansion following *in-vivo* boost by IVIG therapy it did not prevent coronary arterial abnormalities. Our results suggest that the specificity of the Treg, as for any other adaptive immune response, is critical for the suppression *in vivo* of pathogenic T cells. This Treg population expands during acute inflammation in patients suffering from a variety of different infections, but not in children with new-onset, untreated autoimmune disease.

The recognition of endogenous IgG presented by mature B cells appears to maintain Fc-specific Treg that have been activated by IVIG *in vivo*. These Treg are fully functional in very young infants and children. B cell antigen presentation of endogenous Fc by mature B cells and circulating IgG presented by conventional APC (i.e. DC) keep stimulating Fc-specific Treg: the antigen (Fc) is always present under physiological conditions and IgG-expressing mature B cells reside in normal lymph nodes, tonsils, Peyer patches, bone marrow and other secondary lymphoid organs.

The phenotype of Fc-specific Treg clones that expand after IVIG in KD patients suggests that they down-regulate acute vasculitis by suppressing pro-inflammatory T cells not in the inflamed coronary arteries but in the lymph nodes and other secondary lymphoid organs as evidenced by the expression of CCR7 [19]. In fact, CCR6, which is a critical chemokine receptor for T cell homing to the vessels [29], was not expressed on any of the Treg clones studied. Lack of CCR6 expression on Fc-specific Treg clones further supports the idea that this T cell subset is different from auto-reactive, peripherally induced Treg previously reported in humans [19] or pathogen-induced human Th17 T cells that produce IL-10 regulated by IL-1β [30]. The Treg population described herein is also different from the Treg that respond to optimized, pan-DR-binding exogenous Fc peptides (Tregitopes), which have been previously described in adult healthy donors [24]. Those T cells were CCR7 negative and did not express high levels of CTLA-4, which we detected in all the Fc-specific Treg clones studied. Moreover, the Treg described here secrete low amounts of IL-4 that may be functionally important to sustain B cell survival and expansion. The results suggest in fact a new model of T-B cooperation.

An important role of Fc-specific Treg and mature B cell–Treg interactions in immune-regulation is further supported by the blunted expansion of this Treg specificity in children with newly diagnosed, untreated autoimmune diseases. Collectively, it appears as if a defect in the Treg-mediated suppression of the pro-inflammatory T cell response in KD patients with coronary artery abnormalities and in children with autoimmune disorders is associated with disease progression.

Finally, in light of these results, one may speculate on the importance of the Fc-specific Treg as an immunological

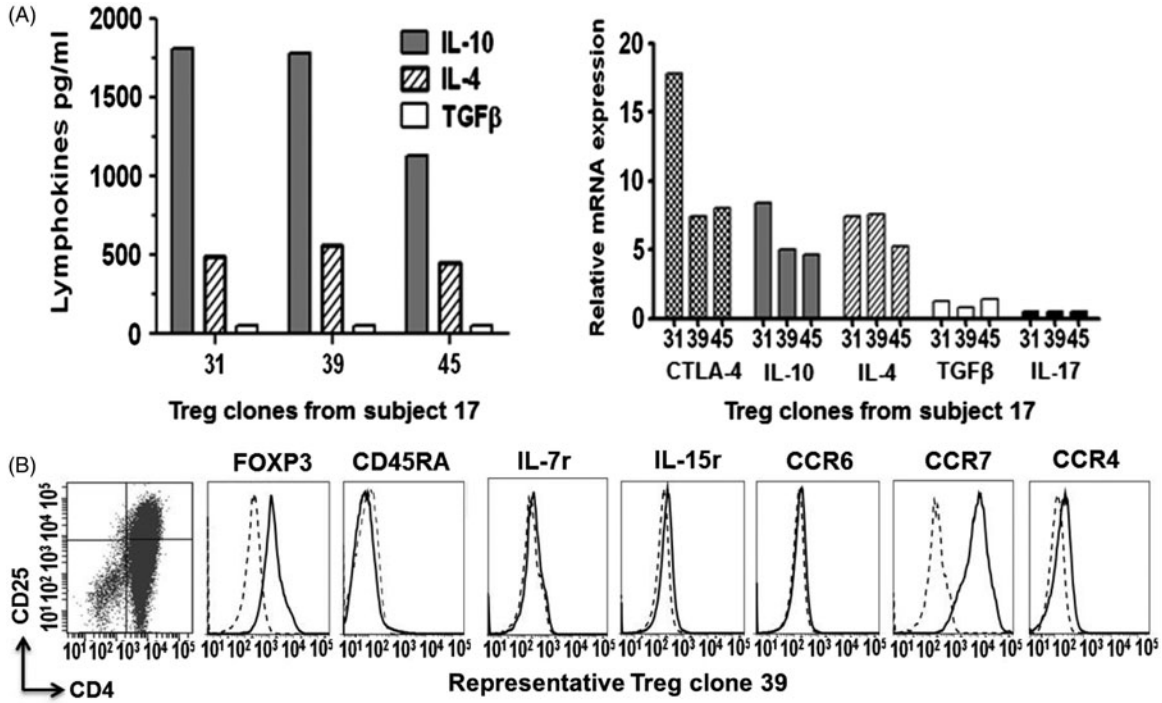
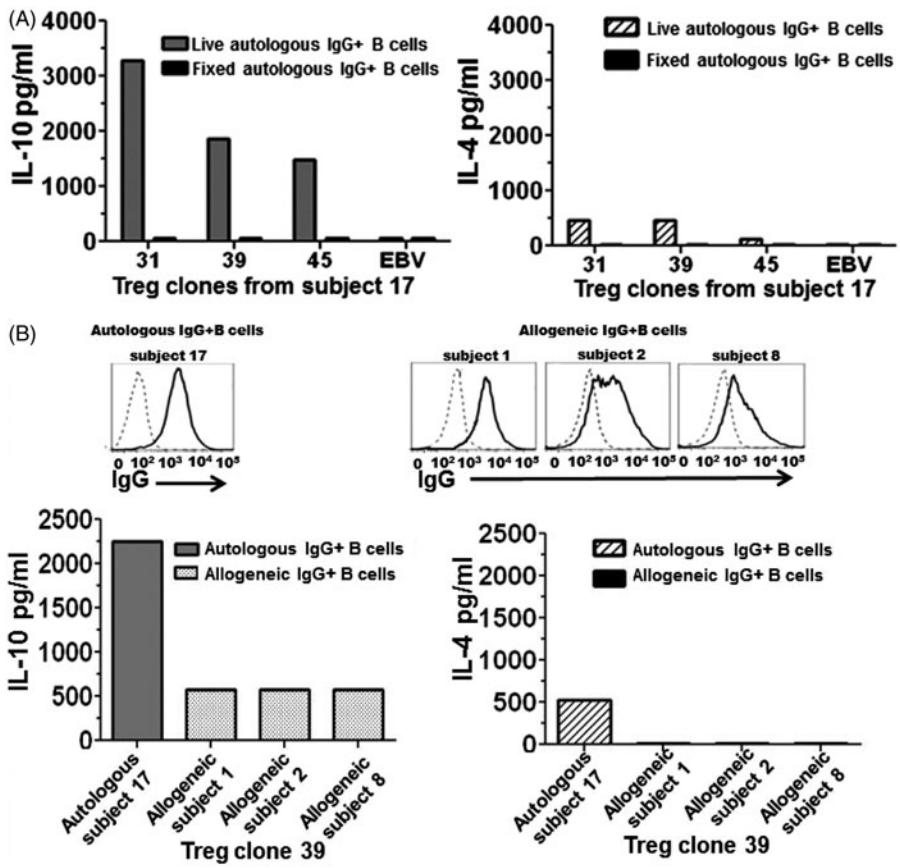


Figure 6. Characterization of Fc-specific Treg clones. Three representative Treg clones generated from KD subject 17 two weeks following IVIG treatment. Panel A: left: production of IL-10, IL-4 and TGF-β measured by ELISA 48 h after stimulation with autologous, irradiated B cells pulsed with 20 μg/ml Fc fragments; Right: qRT-PCR analysis of cell lysates from the same three Treg clones. Panel B: Phenotype of a representative Treg clone characterized as CD4+ CD25^{high}, intracellular FOXP3^{high}, CD45RA^{low}, IL-7r^{low}, IL15r−, CCR6-, CCR7^{high} and CCR4-.

Figure 7. Fc-specific Treg clones recognize endogenous processed IgG presented by autologous B cells in an MHC-restricted, TcR-mediated manner. Panel A: Live, but not paraformaldehyde-fixed, autologous EBV-transformed IgG+ B cells stimulate Treg clones to secrete IL-10 and IL-4 in the absence of exogenous Fc demonstrating that (A) IgG needs to be conventionally processed for Treg recognition; (B) the Treg response is not directed to self MHC molecules. Panel B: Only autologous, but not allogeneic IgG+ B cells activate a Fc-specific Treg clone, demonstrating that the Fc-specific Treg response is MHC-restricted.



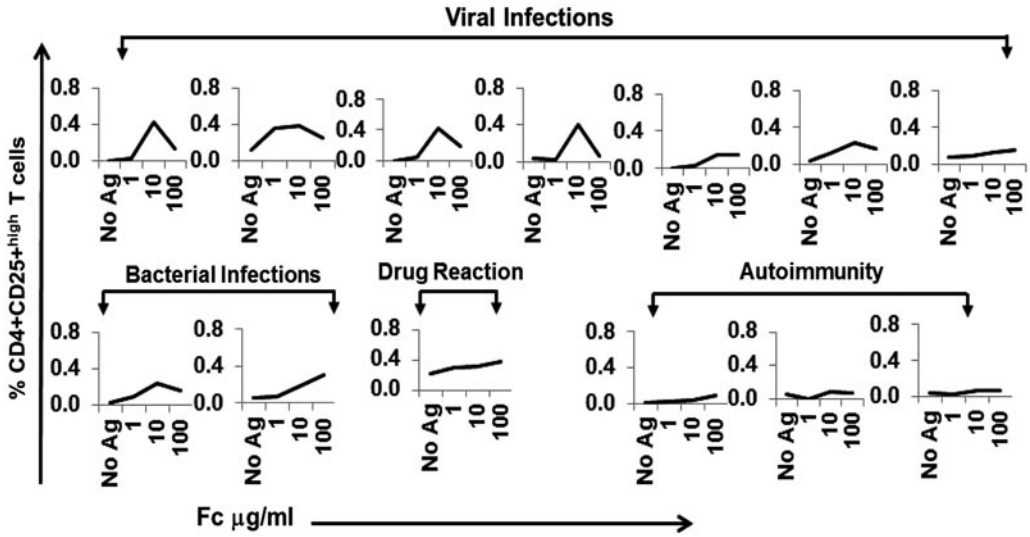


Figure 8. Treg expand in response to Fc in acute pediatric inflammatory conditions but not in subjects with acute autoimmune conditions. Fc-induced Treg expansion in cultured PBMC was detected after Fc stimulation in seven subjects with acute viral infections, two subjects with bacterial infections and one subject with a systemic drug reaction. Three patients with new-onset, untreated autoimmune conditions failed to show Treg expansion in response to Fc.

barrier in maintaining maternal–fetal tolerance during pregnancy. It is now accepted that the transfer of maternal IgG occurs during the third trimester through the MHC I-related receptor Fc receptor for IgG (FcRn) [31,32]. It has also been demonstrated that fetal liver B cells present Ig peptides to CD4+ T cells and generate Ig-specific T cell expansion that can be detected post-partum [33]. It is therefore possible that Fc-specific Treg are instructed in fetal lymphoid organs, fetal liver and thymus by maternal antibodies through TcR recognition of Fc peptides as described in the present report. In support of this hypothesis is the observation that the highest Treg response to Fc was observed in the youngest children in our study (subjects 3 and 7), aged 6 and 3 months old, respectively.

Our work opens the door to the development of novel therapeutic agents. The identification of immunodominant, pan-MHC binding naturally processed Fc peptide(s) could lead to a low cost and large scale alternative to IVIG in KD and a novel therapy for inflammatory diseases. After mapping the immunodominant peptides in the Fc, it may be possible to create highly immunogenic peptide(s) by amino acid substitutions at major TcR contacts that could be used to expand Treg in KD patients at increased genetic risk of arterial abnormalities and in patients with autoimmune disease who lack the Treg response to the natural Fc sequence.

We recognize several strengths and limitations to our work. We describe here a novel role of Tregs as powerful regulators of immunopathology in pediatric patients and point to a new model of B cell–T cell cooperation in generating Treg via TcR-mediated Fc-dependent stimulation. However, there are always challenges associated with immunologic research in pediatric populations. Limitations of cell numbers in these ill, anemic and lymphopenic patients did not permit conventional suppression assays *in vitro*. However, the temporal association between Fc-specific Treg expansion and the resolution of fever and circulating inflammatory markers may be viewed as an *in-vivo* suppression assay. The number of patients studied with coronary artery aneurysms and with new onset autoimmune disease was small, so these findings must be validated in the study of expanded cohorts.

In summary, we demonstrate the role of exogenous IgG and mature B cells in the control of an acute pediatric vasculitis of the coronary arteries via the expansion of Treg that recognize the Fc portion of IgG. Our results suggest a novel mechanism for the anti-inflammatory action and clinical benefit provided by IVIG therapy in patients with KD and, potentially, other vasculitides [34]. Measuring the expansion of Fc-specific Treg may be helpful for clinical risk assessment in the case of KD and potentially for monitoring other immune-mediated pathological conditions and response to IVIG therapy.

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The paper is dedicated to the loving memory of Dr. Eli Sercarz, father of immune-regulation.

Declaration of interest

The authors have no financial conflict of interest.

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Supplementary material available online

Supplementary Figures 1 and 2