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ORIGINAL ARTICLE

Fine specificities of natural regulatory T cells after IVIG therapy in patients with Kawasaki disease

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Abstract

The activation of natural regulatory T cells (nTreg) recognizing the heavy constant region (Fc) of IgG is an important mechanism of action of intravenous immunoglobulin (IVIG) therapy in Kawasaki disease (KD). Lack of circulating Fc-specific nTreg in the sub-acute phase of KD is correlated with the development of coronary artery abnormalities (CAA). Here, we characterize the fine specificity of nTreg in sub-acute (2- to 8-week post-IVIG) and convalescent (1- to 10-year post-IVIG) KD subjects by testing the immunogenicity of 64 peptides, 15 amino acids in length with a 10 amino acid-overlap spanning the entire Fc protein. About 12 Fc peptides (6 pools of 2 consecutive peptides) were recognized by nTreg in the cohorts studied, including two patients with CAA. To test whether IVIG expands the same nTreg populations that maintain vascular homeostasis in healthy subjects, we compared these results with results obtained in healthy adult controls. Similar nTreg fine specificities were observed in KD patients after IVIG and in healthy donors. These results suggest that T cell fitness rather than T cell clonal deletion or anergy is responsible for the lack of Fc-specific nTreg in KD patients who develop CAA. Furthermore, we found that adolescents and adults who had KD during childhood without developing CAA did not respond to the Fc protein *in vitro*, suggesting that the nTreg response induced by IVIG in KD patients is short-lived. Our results support the concept that peptide epitopes may be a viable therapeutic approach to expand Fc-specific nTreg and more effectively prevent CAA in KD patients.

Keywords

Immunotherapy, immune-regulation, IVIG, Kawasaki disease, natural Treg

History

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Introduction

Natural regulatory T cells (nTreg) are selected in the thymus during fetal life and early childhood, recognize *self* antigens and play a central role in maintaining immunological tolerance [1,2]. We recently demonstrated that nTreg that recognize the heavy constant region of immunoglobulins (Fc) G (IgG) regulate vascular inflammation in Kawasaki disease (KD), a self-limited pediatric vasculitis of the coronary arteries [3]. KD is treated with high dose of intravenous immunoglobulin (IVIG), which leads to the rapid cessation of fever and inflammation in the majority of patients treated within 10 days of fever onset. However, even with timely IVIG treatment, 20–30% of patients will develop coronary artery abnormalities (CAA) including transient dilation and aneurysms [4]. We previously showed that activation and expansion of Fc-specific nTreg after IVIG was associated with positive clinical outcomes and absence of detectable CAA in KD children. Our studies further demonstrated functional peripherally induced Treg (pTreg) and tolerogenic dendritic cells (DC) are detectable in KD patients, including those with CAA. These results suggest that alterations in either fine specificity or other qualitative aspects might be associated with the failure of down-regulation of inflammation in the coronary arteries [3,5–7]. In this study, we describe the fine specificity of Fc-specific nTreg by testing their response to overlapping peptides covering the entire Fc molecule. We also tested the nTreg response to the whole Fc protein of adolescents and adults with a history of KD in childhood to assess the durability of the nTreg response years after IVIG and we compare it with sex-matched healthy donors. These studies suggest that Fc-specific nTreg fine specificity is similar in KD and healthy donors, but these responses are short lived in KD patients. Since this defect can be overcome by administration of large doses of IVIG in most KD patients, our results suggest that the administration of Fc-derived peptide epitopes may be a viable therapeutic approach to expand Fc-specific nTreg and prevent CAA.

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Material and methods

Study population

Sub-acute and convalescent pediatric KD patients were enrolled at Rady Children's Hospital San Diego following

Table 1. Demographic and clinical status of pediatric KD study subjects.

Subject #	KD #	Age at onset (years)	Illness day at phlebotomy	Sex	Race/ethnicity	Coronary artery Z max*
Sub-acute						
1	3855	6.5	Day 20	Female	Hispanic	1.6
2	3856	4.8	Day 20	Male	Hispanic	1.3
3	3858	6.4	Day 19	Female	White	2
4	3863	1.0	Day 21	Male	Hispanic	2.4
5	3871	1.5	Day 21	Male	Asian	3.1 (dilated)
6	3869	3.3	Day 22	Female	White	3.4 (dilated)
7	3870	15.5	Day 18	Female	White/Hispanic/Asian	1.3
8	3873	2.1	Day 10	Male	White	2.4
9	3872	2.0	Day 19	Male	White	1.2
10	3868	2.5	Day 54	Female	Asian	1
KD subject #	KD #	Age at onset (years)	Interval from disease onset (years)	Sex	Race/ethnicity	Coronary artery Z max*
Convalescent						
11	3661	1.4	2.6	Male	Hispanic	0.8
12	3706	0.65	2	Male	Hispanic/Asian	1.4
13	3709	5.8	2	Female	Hispanic	1.1
14	3747	5.7	2	Male	Asian/White	1.5
15	3089	5.7	10	Male	Hispanic/White	1.3
16	3725	1.4	1	Male	White	1.5

*Z score defined as the internal diameter of the right and left anterior descending coronary arteries expressed SD unity from the mean normalized for body surface area; normal Z score <2.5; Z_{worst} defined as the highest Z score of either coronary artery measured during the first 6 weeks after fever onset.

Table 2. Demographic characteristics of adolescent and adult subjects with a history of KD in childhood.

KD subject #	Sex	Age at KD onset (years)	Age at testing (years)	Race/ethnicity	IVIG treatment
A1	M	7	19	White	Yes
A2	M	3	19	Hispanic	Yes
A3	F	1	12	African–American	Yes
A4	M	4	16	White	Yes
A5	F	5	28	White	Yes
A6	M	2	19	Mixed	No
A7	F	4	21	Asian	Unknown
A8	F	1	42	Asian	No

parental informed consent and patient assent as appropriate. All the KD subjects were treated with IVIG 2 g/kg and aspirin 80–100 mg/kg/day until afebrile, then 3–5 mg/kg/day until the platelet count had returned to normal. All the sub-acute subjects were taking low-dose aspirin at the time of phlebotomy. KD subjects (10 sub-acute subjects: 5 males, 5 females aged 2.0–15.5 years at time of study) and 6 convalescent subjects: 5 males, 1 female, aged 2.4–15.7 years at time of study) were evaluated by echocardiography during the acute admission and at 2 and 6 weeks and 1 year following diagnosis. The internal diameter of the right and left anterior descending coronary arteries was measured and expressed as a Z score (SD 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 6 weeks after fever onset. Two of the subacute patients developed CAA despite IVIG treatment (Table 1). Heparinized blood samples (1–4 ml) were obtained 10- to 54-day post-IVIG (sub-acute cohort, subjects #1–10) and 1- to 2-year post-IVIG for five subjects (#11–14, 16) and 10-year post-IVIG for one subject (#15) (convalescent cohort).

As a comparison group for the Fc epitope mapping, 6 normal healthy donors (4 males, 2 females aged 25–59 years) were recruited at the Scripps Research Institute, La Jolla CA following written informed consent (IRB# 101213X).

To test the durability of the Fc-specific nTreg response, eight subjects who had KD during childhood without developing CAA (four males and four females aged 12–42 years, Table 2) were tested for Fc-specific nTreg responses to the whole Fc protein *in vitro* and compared to eight additional sex-matched healthy donors.

Peptides

Peptides were synthesized by Fmoc chemistry using a multiplex peptide synthesizer (Symphony X, Protein Technologies Inc., Tucson, AZ). Peptides were cleaved automatically on the synthesizer using trifluoroacetic acid.

Table 3. Sequences of 15-mer peptides used for experiments.

Fc position	Sequence	Fc position	Sequence
1–15	STKGPSVFPLAPSSK	161–175	VDGVEVHNAKTKPRE
6–20	SVFPLAPSSKSTSGG	166–180	VHNAKTKPREEQYNS
11–25	APSSKSTSGGTAALG	171–185	TKPREEQYNSTYRVV
16–30	STSGGTAALGCLVKD	176–190	EQYNSTYRVVSVLTV
21–35	TAALGCLVKDYFPEP	181–195	TYRVVSVLTVLHQDW
26–40	CLVKDYFPEPVTVSW	186–200	SVLTVLHQDWLNGKE
31–45	YFPEPVTVSWNSGAL	191–205	LHQDWLNGKEYKCKV
36–50	VTVSWNSGALTSGVH	196–210	LNGKEYKCKVSNKAL
41–55	NSGALTSGVHTFPAV	201–215	YKCKVSNKALPAPIE
46–60	TSGVHTFPAVLQSSG	206–220	SNKALPAPIEKTISK
51–65	TFPAVLQSSGLYSLS	211–225	PAPIEKTISKAKGQP
56–70	LQSSGLYSLSSVVTV	216–230	KTISKAKGQPREPQV
61–75	LYSLSSVVTVPSSSL	221–235	AKGQPREPQVYTLPP
66–80	SVVTVPSSSLGTQTY	226–240	REPQVYTLPPSRDEL
71–85	PSSSLGTQTYICNVN	231–245	YTLPPSRDELTKNQV
76–90	GTQTYICNVNHKPSN	236–250	SRDELTKNQVSLTCL
81–95	ICNVNHKPSNTKVDK	241–255	TKNQVSLTCLVKGFY
86–100	HKPSNTKVDKKVEPK	246–260	SLTCLVKGFYPSDIA
91–105	TKVDKKVEPKSCDKT	251–265	VKGFYPSDIAVEWES
96–110	KVEPKSCDKTHTCPP	256–270	PSDIAVEWESNGQPE
101–115	SCDKTHTCPPCPAPE	261–275	VEWESNGQPENNYKT
106–120	HTCPPCPAPELLGGP	266–280	NGQPENNYKTTPPVL
111–125	CPAPELLGGPSVFLF	271–285	NNYKTTPPVLDSDGS
116–130	LLGGPSVFLFPPKPK	276–290	TPPVLDSDGSFFLYS
121–135	SVFLF PPKPKDTLMI	281–295	DSDGSFFLYSKLTVD
126–140	PPKPKDTLMISRTPE	286–300	FFLYSKLTVDKSRWQ
131–145	DTLMISRTPEVTCVV	291–305	KLTVDKSRWQQGNVF
136–150	SRTPEVTCVVVDVSH	296–310	KSRWQQGNVFSCSVM
141–155	VTCVVVDVSHEDPEV	301–315	QGNVFSCSVMHEALH
146–160	VDVSHEDPEVKFNWY	306–320	SCSVMHEALHNHYTQ
151–165	EDPEVKFNWYVDGVE	311–325	HEALHNHYTQKSLSL
156–170	KFNWYVDGVEVHNAK	316–329	NHYTQKSLSLSPGK

Peptides were ≥97% pure as assessed by C18 reverse phase HPLC, and the identity of the peptides was verified by mass spectrometry. A total of 64 peptides, each 15 amino acids in length with a 10 amino acid-overlap for each peptide, spanning the whole Fc molecule were used to define the fine specificity of Fc-specific nTreg. The amino acid sequences of the 15-mer overlapping peptides are shown in Table 3.

Characterization of peptide-specific nTreg responses

Heparinized blood samples were collected in sodium heparin green top tubes for isolation of peripheral blood mononuclear cells (PBMC) from healthy adult donors and KD subjects. To enumerate Fc-specific nTreg that expand after IVIG infusion, we previously developed a method to avoid non-specific expansion of peripherally-induced (p)Treg by tolerogenic dendritic cells (DC) or the expansion of effector T cells [3]. PBMC were plated with scalar doses of purified Fc (1, 10 and 100 μg/ml; Life Meridian Science) or pools containing two peptides each (20 μg/ml/peptide, Table 3) at a concentration of 2×10^5 cells/well in 96-well flat-bottomed plates (Falcon) for 4 days. Cell cultures did not receive any exogenous IL-2 prior to the assays to prevent the expansion of non-Fc-specific nTreg and/or the expansion of effector T cells stimulated via Fcγ receptors.

On culture day 4, supernatants were collected to measure IL-10 secretion by ELISA with primary and secondary antibodies from BD Bioscience on all the subjects as previously described [3]. A positive nTreg response was defined as IL-10 secretion that exceeded 20 pg/ml. For four KD subjects and all eight of the normal adult donors, cells from these cultures were harvested on day 4 for FACS analysis. 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. BD FACSCanto was used for data acquisition; data were analyzed with FACSDiva (BD Biosciences) or FlowJo software (Tree Star, Inc).

HLA class II binding prediction for Fc-derived peptides

As a preliminary analysis to determine the potential HLA class II binding capacity of Fc-derived peptides, we scanned the sequences using the suite of class II algorithms available through the NIH-funded and La Jolla Institute for Allergy and Immunology-administered website, Immune Epitope Database (IEDB). For HLA class II binding predictions, the entire IgG Fc sequence was parsed into the set of 15-mer peptides, overlapping by 10 residues (Table 3) that we functionally tested for immunogenicity in KD subjects after IVIG and healthy donors. The capacity of each peptide to bind a panel of 27 common HLA class II DR, DQ and DP molecules was predicted using the IEDB consensus algorithm (www.iedb.org <http://www.iedb.org>) [8]. A peptide was considered a binder to any class II if its corresponding consensus score was ≤20th percentile [8]. Binding to any

specific allele was defined as a corresponding consensus prediction score ≤20th percentile. The number of alleles bound was tabulated for each peptide. A promiscuous binding peptide was operationally defined as a peptide binding or predicted to bind to 50% or more of the 27 alleles. The total number of class II molecules predicted to be bound was tabulated and plotted.

Results

Fine specificity of Fc-specific nTreg determined with 15-mer overlapping peptides in KD subjects after IVIG

Discrete regions within the Fc sequence were immunogenic for nTreg in subacute KD subjects after IVIG stimulating IL-10 secretion in 9 of the 10 subjects and including CD4 + CD25^{high} T cell expansion in all four subjects assessed (subject 1 recognized a unique set of epitopes, data not shown). Peptides were ranked based on IL-10 secretion and peptides 121–135 and 126–140 (pool 13) were the most immunogenic being recognized by nTreg in 8 of 16 (50%) KD subjects (Figure 1). Other sequences also resulted in expansion of the nTreg populations: 7 of 16 (44%) KD subjects responded to amino acid residues 276–290 and 281–295 (pool 28); three different peptide pools corresponding amino acid residues 51–65 and 56–70 (pool 6), amino acid residues 181–195 and 186–200 (pool 19), amino acid residues 301–315 and 306–320 (pool 31) were recognized in 6 of 16 (37%) KD subjects. Finally, 5 out of 16 (31%) KD subjects recognized amino acid residues 61–75 and 66–80 (pool 7). Of interest, the two KD subjects with CAA (subjects # 5 and 6) showed a measurable nTreg response to pool 28 and pool 6, respectively (Figure 1), suggesting that the fine specificity of this KD subgroup was similar to the remainder of the KD subjects tested.

The strength of the response varied among subjects possibly reflecting their HLA type and the precursor frequency of peptide-specific nTreg after IVIG. We observed a rapid expansion of the CD4 + CD25^{high} T cell population in some KD patients in response to peptide epitopes after IVIG, suggesting a very high precursor frequency (Figure 2).

Fine specificity of Fc-specific nTreg determined with 15-mer overlapping peptides in healthy adult donors

To compare Fc-specific nTreg responses that arise in KD patients after IVIG to the specificities of nTreg in healthy donors, we performed Fc epitope mapping in six healthy adult subjects. As observed in KD patients after IVIG, discrete regions within the Fc sequence were found to be immunogenic for nTreg and stimulated the secretion of IL-10 and

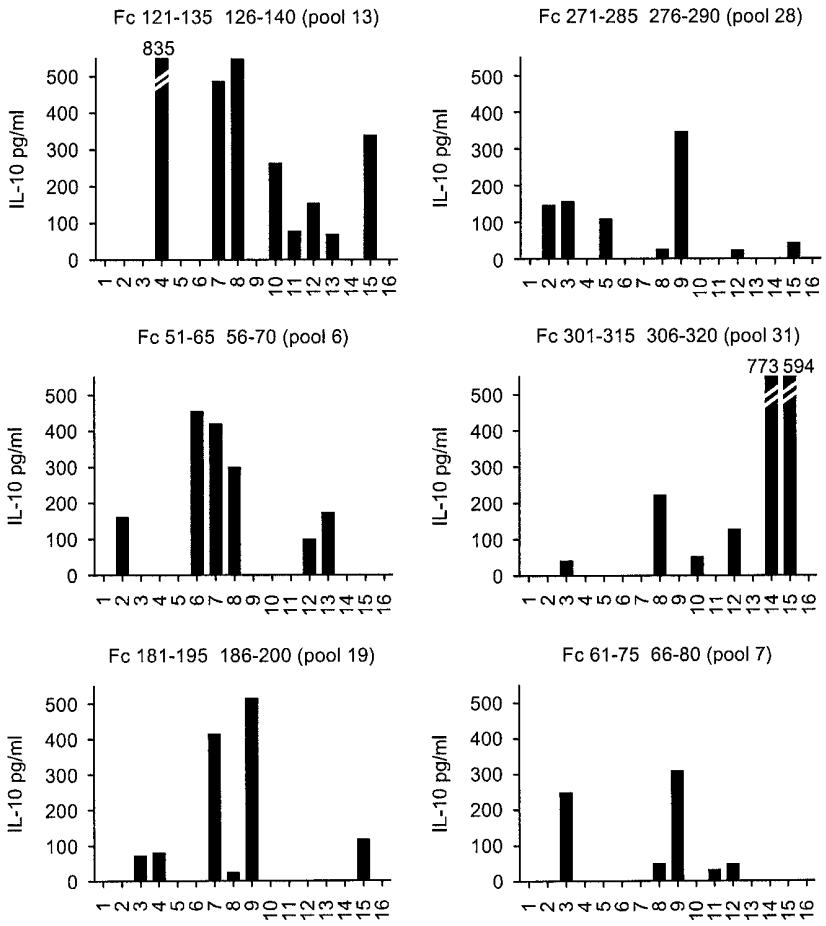

Figure 1. nTreg fine specificities in sub-acute and convalescent KD subjects. About 2 × 10^5 PBMC/well derived from 10 sub-acute and 6 convalescent KD subjects were cultured with pools of two Fc peptides (Table 3) for 4 days in the absence of exogenous lymphokines. IL-10 secretion in response to peptide stimulation was measured in culture supernatants by ELISA on day 4. Subjects 5 and 6 both developed CAA and IL-10 secretion was noted in their PBMC cultures incubated with peptides from Pools 28 and 6, respectively.

CD4 + CD25^{high} T cell expansion. The epitope ranking indicated that the immunodominant responses were similar in healthy controls compared to KD patients after IVIG. Peptides 121–135 and 126–140 (pool 13) were the most immunogenic and were recognized by nTreg in 5 of 6 (83%) donors (Figure 3). Several other pools defined as immunogenic in KD subjects also stimulated nTreg in healthy donors: 4 of 6 (67%) healthy donors responded to amino acid residues 276–290 and 281–295 (pool 28), and 301–315 and 306–320 (pool 31); 3 of 6 (50%) donors responded to amino acid residues 181–195 and 186–200 (pool 19); 2 of 6 (33%) donors responded to amino acid residues 51–65 and 56–70 (pool 6), and 61–75 and 66–80 (pool 7) (Figure 3). Responses to amino acid residues 21–35 and 26–40 (pool 3) and 31–45 and 36–50 (pool 4) were unique, as these pools were not immunogenic for the KD subjects (Figure 4).

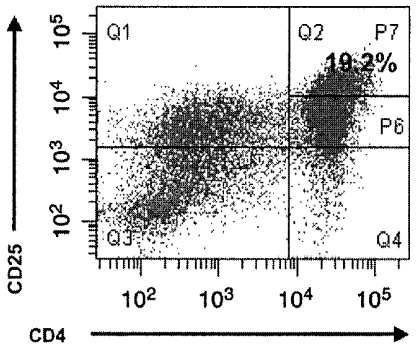

Figure 2. Response of nTreg to peptide pool 13. Enumeration of CD4 + CD25^{high} T cells from KD subject #10 in response to Fc 121–135 and 126–140 (pool 13), the most immunogenic sequences in this cohort of patients (P7, upper right).

nTreg from healthy adults who had KD in childhood without developing CAA fail to respond *in vitro* to whole Fc protein

We previously showed that acute KD subjects lack Fc-specific nTreg prior to IVIG [3]. IVIG therapy activates this nTreg repertoire in KD patients who do not develop CAA but it was unknown if the Fc-specific nTreg response to the whole Fc protein was long lasting or if it waned over time. Therefore, we tested CD4 + CD25^{high} nTreg expansion in response to Fc fragments in eight healthy adults who had KD during childhood without developing arterial complications (Table 2) and compared their response with eight healthy adult donors. nTreg derived from adolescents and adults years after IVIG treatment for KD did not expand in response to Fc fragments in sharp contrast to healthy adult controls (Figure 5).

HLA/Fc-derived peptide binding prediction analysis

It was previously shown that peptides predicted to bind 50% or more of the HLA alleles assessed at the 20% or better consensus prediction level correspond to the most dominant

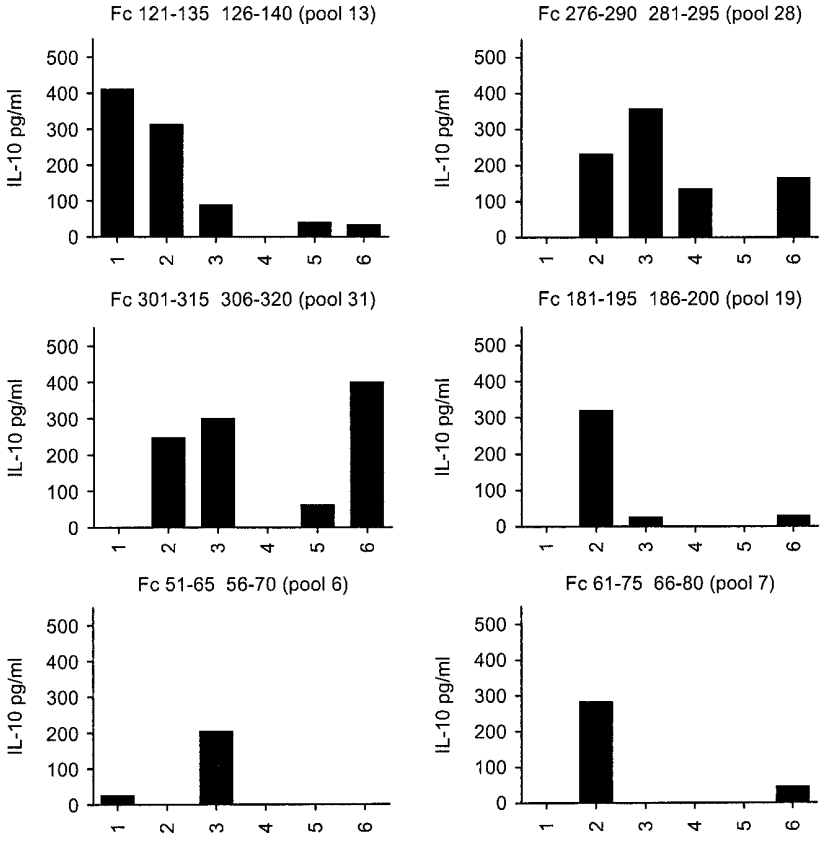

Figure 3. IL-10 secretion in PBMC cultures from healthy adult donors in response to peptide pools. About 2×10^5 PBMC/well derived from six healthy adult donors were cultured with pools of two Fc peptides (Table 3) for 4 days in the absence of exogenous lymphokines. IL-10 secretion by nTreg in response to peptide stimulation served as a read out in these experiments and was measured in culture supernatants by ELISA on day 4.

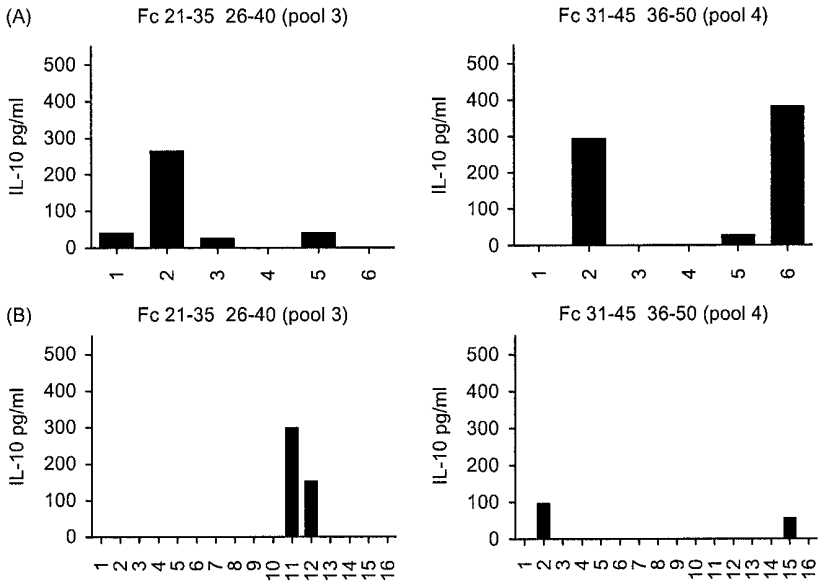

Figure 4. Pools 3 and 4 are more immunogenic in healthy donors than in KD patients. Panel A: IL-10 secretion in response to amino acid residues 21–35 and 26–40 (pool 3) and 31–45 and 36–50 (pool 4) in healthy donors. Panel B: nTreg responses to pools 3 and 4 in KD patients: only 2 of 12 KD subjects responded to these peptide pools.

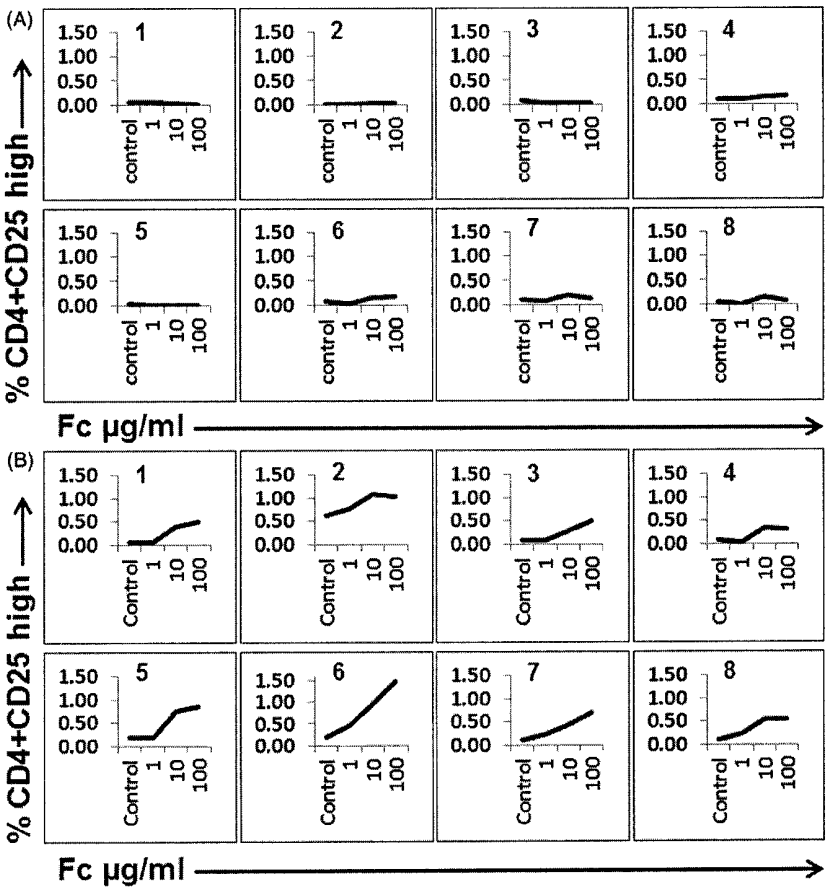

Figure 5. CD4 + CD25^{high} nTreg expansion in response to scalar doses of Fc. PBMC were cultured for 4 days with 0, 1, 10 or 100 µg/ml purified Fc fragments. Panel A: Fc-specific nTreg response in adult subjects who had KD in childhood. Panel B: Fc-specific nTreg response in healthy adult controls.

epitopes, and encompass a large fraction of the T cell responses to EPO [9], allergens such as timothy grass and mycobacteria tuberculosis antigens [10–12]. Since peptide:HLA binding is required, but not sufficient, to elicit an HLA class II restricted response, peptide:HLA binding predictions do not necessarily predict which peptides will be immunogenic as dominant epitopes tend to be promiscuous binders [10,13,14].

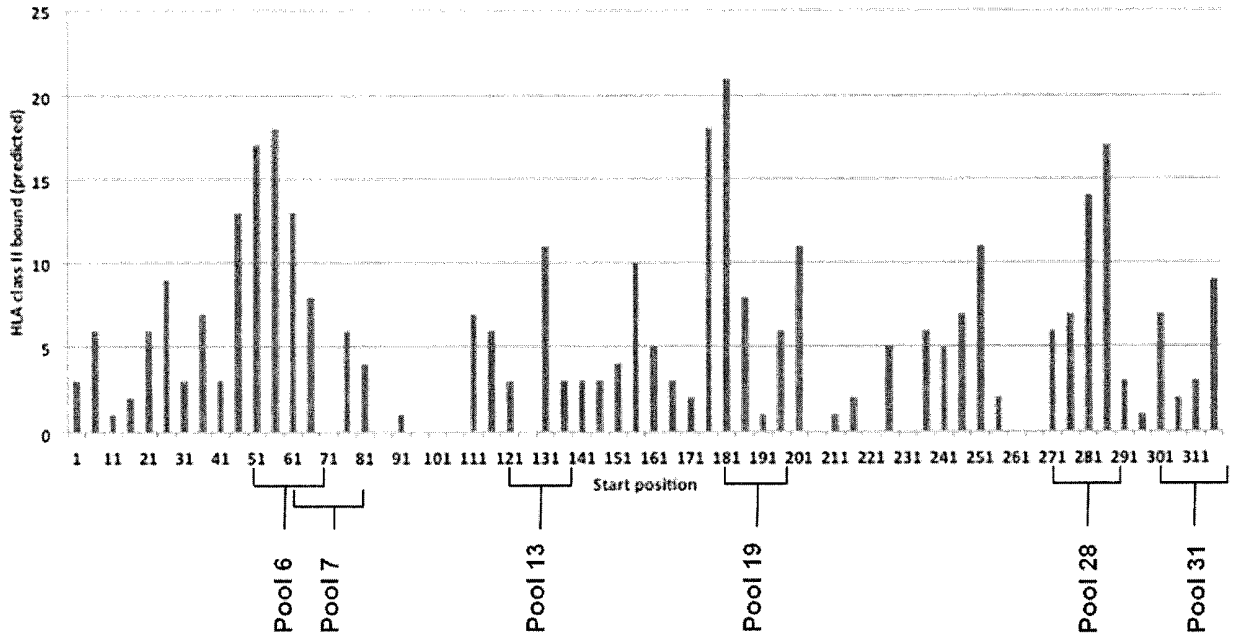

Figure 6. HLA binding predictions of peptides derived from the Fc sequence. IEDB consensus algorithm (www.iedb.org <http://www.iedb.org>) was used to predict HLA class II binding affinity of the Fc sequences described in Table 3. Immunogenic peptide pools are indicated.

Accordingly, the capacity of Fc peptides to bind a panel of 26 HLA class II alleles that are commonly expressed in representative ethnicities worldwide and in USA and for which epitope prediction tools are well established and publically available [8,15], were predicted using tools available through IEDB (www.iedb.org). Three major promiscuous binding regions were identified, corresponding to residues 181–185 51–56 and 56–70 (pool 6), 181–195 and 186–200 (pool 19), 271–285 and 276–290 (pool 28) (Figure 6).

These epitopes were compared with the HLA binding peptide patterns identified by the predicted binding analysis. Of the top four immunogenic pools, three corresponded to promiscuous binders (i.e. with predicted binding to $\geq$13 HLA alleles). Interestingly, pool 13 (peptides starting in positions 121 and 126), which was the most immunogenic, was not associated with predicted promiscuous binding. In fact, peptide 121 was predicted to bind only three alleles (HLA DRB1*0301, DRB1*1101 and DRB5*0101), and peptide 126 was not predicted to bind any. It is possible that this region might be associated with restriction by specific HLA alleles (monogamous restriction) that are present with high frequency in the donor cohort, and/or that were not present in the allele panel utilized for predictions. Future HLA typing studies will allow further elucidation of the pattern of response.

Discussion

In the present study, we mapped the Fc peptide regions recognized by nTreg from sub-acute and convalescent KD subjects after IVIG therapy, from adults with a history of KD in childhood and from healthy adult subjects. Using IL-10 secretion and CD4 + CD25^{high} T cell proliferation as a read-out, we defined discrete Fc regions that are immunogenic. Our previous work suggested that immune-regulation via Fc-specific nTreg influences the clinical fate of KD patients and identified this as one mechanism by which IVIG leads to clinical improvement in these patients [3]. However, 20–30% of KD patients go on to develop CAA with transient dilation or aneurysms despite timely treatment with IVIG and their nTreg do not respond to Fc stimulation *in vitro* [3,4,16].

By using Fc peptide epitopes as immunogens rather than the whole Fc protein, we demonstrated that nTreg from patients with CAA can expand *in vitro* (Figure 1). These results suggest that T cell fitness, rather than T cell anergy or T cell clonal deletion causes the lack of Fc-specific response in KD patients with CAA who do not appear to respond to the whole Fc protein [3]. We hypothesize that the immunodominant Fc sequences recognized in the cohorts could be candidates as therapeutic agents. These peptides would have significant advantages over whole IVIG in terms of safety, product reproducibility, and ease of definition and characterization.

The extension of these studies to a larger number of CAA patients will allow selection of the most suitable candidate epitopes. The predicted HLA binding analysis reported here supports the idea that most of the immunodominant Fc sequences recognized in the cohorts studied will bind multiple HLA alleles and would therefore be particularly attractive candidates as therapeutic peptides.

Among the Fc peptides identified as relevant for nTreg expansion after IVIG in KD patients, some include portions of the two long Fc sequences previously described as Tregitopes, pan-DR epitopes that were immunogenic in normal donors [17]. Our results confirm the relevance of these Fc peptides in expanding regulatory T cells and their pan-DR potential binding capacity, but also suggest that the Tregitopes are not the most immunogenic sequences of the Fc protein for nTreg

generation (Figures 1 and 3). Moreover, our data suggest that the Tregitopes are further trimmed for optimal HLA binding and TcR recognition since positions 51–65 and 56–60 (pool 6) contained within Tregitope 167 and positions 181–195 and 186–200 (pool 19) contained within Tregitope 289 were more immunogenic than other amino acid sequences within the two Tregitopes.

These studies suggest that Fc-specific nTreg responses in KD can be elicited by administration of large doses of IVIG in most patients. However, this regulatory T cell response is not long lasting and was undetectable in adult years after IVIG administration (Figure 5). We therefore postulate a focal immune regulatory defect in patients who develop KD that can be overcome in the majority of patients by administration of a large dose of IVIG.

Future studies of HLA typing of KD patients and measurement of the HLA binding affinity of the Fc peptides will highlight possible HLA alleles that bind poorly to stimulatory Fc sequences and may thereby explain the lack of nTreg responses to the whole Fc protein in KD patients who develop CAA. In those instances, amino acid substitutions within the relevant Fc epitopes may improve HLA binding and TcR affinity to generate super-agonistic peptides as a strategy to boost immune-regulation and down-regulate vascular inflammation.

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Declaration of interest

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