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Research Article

Intravenous immunoglobulin induces IgG internalization by tolerogenic myeloid dendritic cells that secrete IL-10 and expand Fc-specific regulatory T cells

Li-En Hsieh[1], Jaeyoon Song[1], Adriana H. Tremoulet[1,2], Jane C. Burns[1,2], and Alessandra Franco[*]

[1]University of California San Diego, School of Medicine, Department of Pediatrics, La Jolla, CA 92093-0641, USA
[2]Rady Children's Hospital, San Diego, CA 92123, USA
*Correspondence: Alessandra Franco, 9500 Gilman Drive, La Jolla, CA 92093-0641, USA. Email: alfranco@health.ucsd.edu

Abstract

Intravenous immunoglobulin (IVIG) is used as an immunomodulatory agent in many inflammatory conditions including Multisystem Inflammatory Syndrome-Children (MIS-C) and Kawasaki disease (KD). However, the exact mechanisms underlying its anti-inflammatory action are incompletely characterized. Here, we show that in KD, a pediatric acute vasculitis that affects the coronary arteries, IVIG induces a repertoire of natural Treg that recognize immunodominant peptides in the Fc heavy chain constant region. To address which antigen-presenting cell (APC) populations present Fc peptides to Treg, we studied the uptake of IgG by innate cells in subacute KD patients 2 weeks after IVIG and in children 1.6–14 years after KD. Healthy adults served as controls. IgG at high concentrations was internalized predominantly by two myeloid dendritic cell (DC) lineages, CD14+ cDC2 and ILT-4+ CD4+ tmDC mostly through Fcγ receptor (R) II and to a lesser extent FcγRIII. Following IgG internalization, these two DC lineages secreted IL-10 and presented processed Fc peptides to Treg. The validation of IVIG function in expanding Fc-specific Treg presented by CD14+ cDC2 and ILT-4+ CD4+ tmDC was addressed in a small cohort of patients with MIS-C. Taken together, these results suggest a novel immune regulatory function of IgG in activating tolerogenic innate cells and expanding Treg, which reveals an important anti-inflammatory mechanism of action of IVIG.

Graphical Abstract
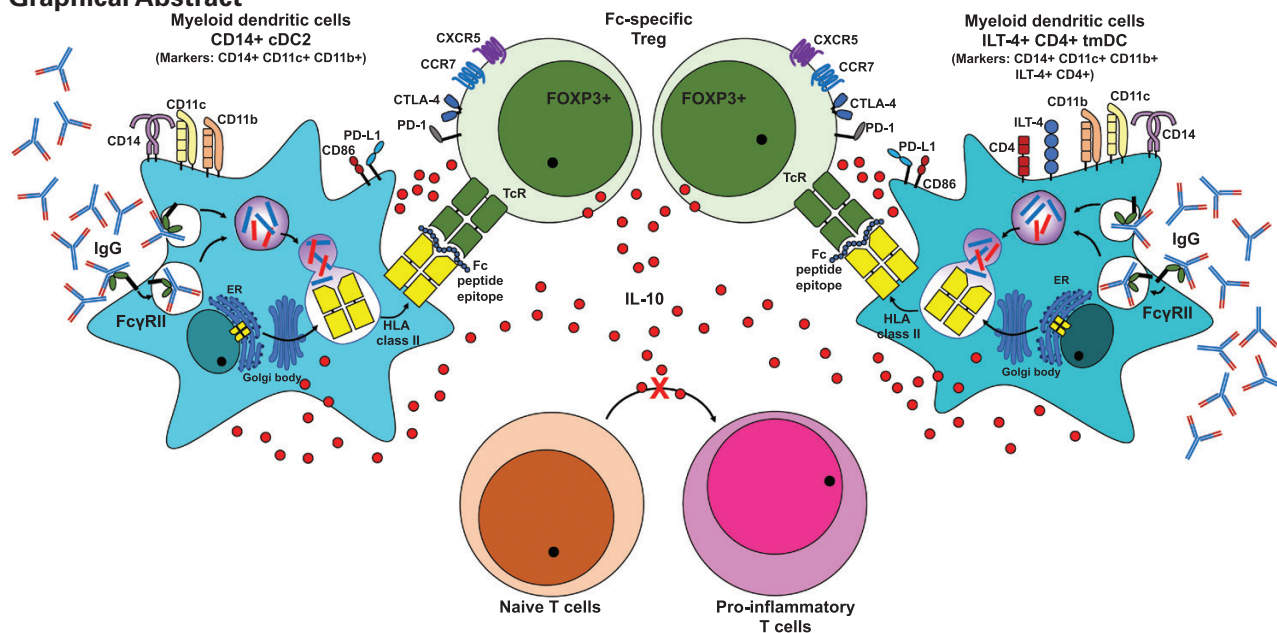
Keywords: IgG, tolerogenic myeloid dendritic cells, Fcγ receptors, natural regulatory T cells

Abbreviations: APC: antigen presenting cells; CAA: coronary artery aneurysms; cDC1: myeloid type 1 dendritic cells; cDC2: myeloid type 2 dendritic cells; DC: dendritic cells; FcγRs: Fcγ receptors; IVIG: Intravenous immunoglobulin therapy; KD: Kawasaki disease; MIS-C: Multisystem Inflammatory Syndrome in Children; PBMC: peripheral blood mononuclear cells; pDC: plasmacytoid dendritic cells; tmDC: tolerogenic myeloid dendritic cells; Treg: regulatory T cellse

Introduction

Immune regulation involves cells of the innate and adaptive immune systems that interact within a complex network that is still largely unexplored [1]. Our previous work suggested that the heavy chain constant region (Fc) of IgG has profound immune regulatory functions via two mechanisms: (a) the activation of a unique population of tolerogenic myeloid dendritic cells [2] and (b) the expansion of natural regulatory T cells (Treg) that recognize immunodominant Fc peptides in a classical HLA-restricted fashion [3]. Fc-specific Treg are activated by IgG⁺ B cells via a unique antigen processing pathway [4] and by the antigen processing of exogenous IgG internalized and presented by myeloid dendritic cells [3].

Intravenous immunoglobulin (IVIG) is used to treat a variety of inflammatory conditions including Kawasaki disease (KD), an acute pediatric vasculitis that affects the coronary arteries and is the leading cause of acquired heart disease in the pediatric age group, and Multisystem Inflammatory Syndrome-Children (MIS-C) an acute pediatric complication of SARS-CoV-2 infection. We previously reported an association between Fc-specific regulatory T cell (Treg) responses and clinical outcome [5], as well as a strong correlation between the severity of the acute clinical presentation and the numbers of circulating tolerogenic myeloid dendritic cells (tmDC) [2, 6].

Here we defined the immunodominant Fc peptides that expanded Treg in KD children studied 2 weeks after IVIG infusion and the persistence of this Treg repertoire in circulation in IVIG treated children that had KD years prior to the study. We also studied the Fcγ receptors (R) responsible for the uptake of IgG and explored the internalization of IgG by antigen-presenting cells to define the cells that best process and present Fc peptides to Treg.

Materials and methods

Study populations

Four cohorts were enrolled in the study, namely, subacute KD patients, healthy children with a remote history of KD, healthy adult controls, and subacute MIS-C patients. The experiments using human samples were performed in accordance with relevant guidelines and regulations, and the study protocols for KD subjects and healthy adult donors were approved by the Institutional Review Board at the University of California San Diego (IRB #140220 and #101213X, respectively). Pediatric subjects were enrolled at Rady Children's Hospital, San Diego, following written parental informed consents and patient assent as appropriate. Healthy adult donors were enrolled at the Scripps Research Institute Normal Blood Donor Services following written consents.

A total of 42 KD subjects were enrolled 2 weeks after receiving IVIG. Of these, 18 had coronary artery aneurysms (CAA) as defined by the American Heath Association criteria of the internal diameter of the right or left coronary arteries normalized for body surface area (Z score) ≥ 2.5. Nine children were enrolled 1.5–14 years after KD (Table 1). Peripheral blood mononuclear cells (PBMC) from 32 subacute KD subjects (KD #1 – #32, 21 males and 11 females, aged 0.26 to 13.6 years) were used to study the fine specificities of the Treg with 15 amino acid (aa)-long Fc peptides. Four subacute KD subjects (KD #33 – #36, three males and one female, aged 0.5–15 years) were enrolled to study the internalization

Table 1. KD subjects enrolled in this study

	Age at time of blood draw	Sex	Race/ethnicity	Zmax*
1	6.0	M	Hispanic	1.87
2	2.3	M	Hispanic	1.39
3	9.9	M	Hispanic	0.31
4	3.4	F	Caucasian	1.63
5	1.4	M	Hispanic	6.15
6	1.3	M	Mixed	2.88
7	10.8	M	Caucasian	1.10
8	5.8	F	Hispanic	1.54
9	4.8	M	Asian	3.30
10	5.5	F	Hispanic	2.47
11	3.17	M	Hispanic	1.41
12	9.97	F	Caucasian	1.03
13	3.13	F	Caucasian	1.77
14	1.09	M	Caucasian	1.98
15	0.59	M	Hispanic	3.04
16	0.5	M	Hispanic	2.24
17	1.9	F	Caucasian	0.90
18	8	F	Mixed	1.46
19	0.9	M	Hispanic	7.53
20	1.8	M	Hispanic	3.19
21	3.1	M	African American	1.72
22	3.3	M	Asian	2.25
23	5.6	M	Mixed	1.07
24	13.6	F	Mixed	0.19
25	0.6	M	Hispanic	2.52
26	4.1	F	Mixed	1.88
27	1.7	M	Mixed	2.71
28	6.2	M	Asian	1.41
29	0.26	F	Asian	6.9
30	5.8	M	Mixed	0.88
31	4.8	M	Caucasian	2.12
32	0.4	F	Asian	3.2
33	2.9	M	Hispanic	0.5
34	1.7	F	Hispanic	0.9
35	0.5	M	Hispanic	1.15
36	15	M	Hispanic	−1.1
37	3	M	Caucasian	0.7
38	0.9	M	Mixed	3.5
39	1.2	F	Hispanic	2
40	5	M	Hispanic	1
41	3	F	Mixed	1.8
42	2	M	Caucasian	1
43	3.8	M	Caucasian	1.41
44	4.1	F	Caucasian	1.05
45	7	M	Asian	9.16
46	15	M	Asian	6.65
47	15	M	Asian	4.86
48	6	F	Asian	35.85
49	4.5	M	Mixed	4.54
50	17	M	Hispanic	9.5
51	9.5	M	Caucasian	15.3

*Zmax = maximal internal diameter of the right and left anterior descending coronary arteries determined by echocardiography and normalized for body surface area and expressed as standard deviation units (Z score).

of IgG-FITC by different APC populations and the Treg response to the whole Fc protein. The Treg response to the Fc protein was studied in six additional subacute KD subjects (KD #37–#42, four males and two females, aged 0.9–5 years). PBMC from three children with a remote history of KD (KD #43–#45, three males, aged 7–15 years) were studied for IgG internalization by different APC and Fc-specific Treg recognition. Treg expansion in response to the Fc was also studied in six children with a remote KD history (KD #46–#51, five males and one female, aged 3–17 years). Ten healthy adult donors (ND #1–#10, eight males and two females, aged 20–70 years) were enrolled to study their Treg responses to the whole Fc protein, the internalization of IgG-FITC by different APC populations and their FcγRs expression. Five MIS-C patients treated with IVIG (#1–#5, four males and one female, aged 3.7–12.6 years), four studied in the subacute phase and one a year after IVIG, were enrolled to verify the expansion of Fc-specific Treg and antigen presentation by the same APC identified in KD and healthy controls.

Peptide sequences and synthesis

Sixteen 15 aa-long peptides derived from human IgG1 Fc sequences (Table 2) were synthesized by Fmoc chemistry using a multiplex peptide synthesizer (Symphony X, Protein Technologies Inc., Tucson, AZ). Synthesized peptides were automatically cleaved on the synthesizer using trifluoroacetic acid. The purity of the peptides was ≥97% as measured by C18 reverse phase-HPLC, and the identity of the peptides was verified by mass spectrometry.

Treg priming by Fc peptides and whole Fc protein to determine peptide immunodominance and to study Treg response to the whole Fc protein

PBMC were separated from heparinized whole blood by Ficoll Histopaque density gradient. 4×10^5 cells/well were stimulated in 96-well, flat-bottom plates (Falcon) either with individual Fc peptides (20 µg/ml) or scalar doses of the whole Fc protein (1, 10, and 100 µg/ml) (purity ≥ 97%, Meridian Life Science) in the absence of exogenous IL-2 for 4 days. Treg

Table 2. IgG1 Fc peptides tested for Treg recognition

Fc position	Sequence
21–35	TAALGCLVKDYFPEP
26–40	CLVKDYFPEPVTVSW
31–45	YFPEPVTVSWNSGAL
36–50	VTVSWNSGALTSGVH
51–65	TFPAVLQSSGLYSLS
56–70	LQSSGLYSLSSVVTV
61–75	LYSLSSVVTVPSSSL
66–80	SVVTVPSSSLGTQTY
121–135	SVFLF PPKPKDTLMI
126–140	PPKPKDTLMISRTPE
181–195	TYRVVSVLTVLHQDW
186–200	SVLTVLHQDWLNGKE
271–285	NNYKTTPPVLDSDGS
276–290	TPPVLDSDGSFFLYS
301–315	QGNVFSCSVMHEALH
306–320	SCSVMHEALHNHYTQ

expansion was assessed on Day 4 by two different methodologies: (1) measurement of IL-10 in culture supernatants by ELISA and (2) enumeration of CD4$^+$ CD25^{high} T cells by flow cytometry. IL-10 in the culture supernatants was measured by antigen-capture ELISA using 4 µg/ml of anti-human IL-10 primary antibody (BD Bioscience) and 2 µg/ml of biotin labeled secondary antibody (BD Bioscience) according to manufacturer instructions. CD4$^+$ CD25^{high} T cells were defined by anti-human CD4 PerCp/Cy5.5 (clone RPA-T4, mouse IgG1κ, eBioscience) and anti-human CD25 BV421 (clone M-A251, mouse IgG1κ, BD Bioscience) antibodies using standard flow cytometry methodologies. To study the intracellular FOXP3 expression in CD4$^+$ CD25^{high} Treg, cell preparations were fixed with 1X FOXP3 Fix/Perm buffer (BioLegend) followed by a permeabilization step using 1X FOXP3 Perm buffer (BioLegend) following the manufacturer's instructions and stained by anti-human FOXP3 PE (clone 259D, mouse IgG1κ, BioLegend). The data were acquired by using BD FACSCanto II (BD Bioscience) and analyzed with FlowJo software version 10 (Tree Star).

IgG internalization by antigen-presenting cells

The efficiency of different APC populations in the internalization of IgG was defined by measuring by flow cytometry the uptake of a purified IgG-FITC (Sigma) after pulsing at different time points and fixation of the cell surface. In PBMC cultures, monocytes were defined by CD14$^+$ CD11c$^-$ CD11b$^-$, macrophages were defined by CD14$^+$ CD11c$^-$ CD11b$^+$, myeloid cDC1 were defined by CD14$^-$ CD11c$^+$ CD11b$^-$, myeloid cDC2 were defined by CD14$^+$ CD11c$^+$ CD11b$^+$ or CD14$^-$ CD11c$^+$ CD11b$^+$, tolerogenic myeloid DC (tmDC) were defined by CD11c$^+$ CD11b$^+$ CD14$^+$ ILT-4$^+$ (CD85d) and CD4$^+$, plasmacytoid DC (pDC) were defined by CD14$^-$ CD11c$^-$ CD11b$^-$ CD123$^+$, and B cells were defined by the expression of CD19.

A total of 2.5×10^5 PBMC were pulsed with scalar doses of IgG-FITC (1, 10, and 100 µg/ml) for 15, 30, or 60 min, washed with cold 1X PBS, and fixed with BD Cytofix fixation buffer (containing 4.2% paraformaldehyde, BD Bioscience) at 4°C for 20 min. Non-stimulated PBMC served as control. Cell preparations were then stained with specific monoclonal antibodies to define cell populations as follows: anti-human CD11c allophycocyanin (clone B-ly6, mouse IgG1κ), anti-human CD11b BV650 (clone ICRF44, mouse IgG1κ), and anti-human CD14 PE/Cy7 (clone M5E2, mouse IgG2aκ), anti-human CD4 AF700 (clone RPA-T4, mouse IgG1κ), CD19 allophycocyanin/Cy7 (clone SJ25C1, mouse IgG κ), CD123 BV711 (clone 6H6, mouse IgG1κ) from BD Bioscience, and anti-human ILT-4 PerCp/eFlour710 (clone 42D1, rat IgG2aκ) eBioscience. To measure the expression of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) by different APCs, we used anti-human FcγRI BV510 (clone 10.1, mouse IgG1κ, BioLegend), anti-human FcγRII FITC (clone FLI8.26, mouse IgG2bκ, eBioscience), and anti-human FcγRIII BV421 (clone B73.1, mouse IgG1κ, BioLegend).

Characterization of the FcγRs involved in the IgG internalization by different APC

To address the role of FcγRI, FcγRII, and FcγRIII, in the uptake of IgG-FITC in different APC populations, PBMC were co-cultured with 10 µg/ml of antagonistic antibodies to FcγRs during pulsing with IgG-FITC. Anti-human FcγRI, clone

10.1, mouse IgG1κ, BioLegend, anti-human FcγRII, clone IV.3, mouse IgG1κ, Bio X Cell, and anti-human FcγRIII, 3G8, mouse IgG1κ, BioLegend were used alone or in combination prior to fixing and staining cell preparations. BD LSRFortessa (BD Bioscience) was used for the acquisition and the data were analyzed with FlowJo software version 10 (Tree Star).

Results

Characterization of immunodominant Fc peptides recognized by Treg after IVIG in subacute KD subjects

To determine the fine specificity of Fc-specific Treg known to expand after IVIG therapy in KD [5], we studied the Treg responses in PBMC from 32 KD subjects in the subacute phase (2–6 weeks after receiving IVIG) (Table 1). We synthetized 16 Fc peptides derived from human IgG1 sequences that were previously identified to be immunodominant in healthy adult donors and subjects with rheumatoid arthritis (RA) [3] (Table 2). PBMC were stimulated for 4 days *in vitro* with 16 individual peptide epitopes and the Treg recognition of the peptides in culture was determined by measuring IL-10 secretion in culture supernatants and the expansion of CD4+ CD25high T cells by flow cytometry. Overall, PBMC from 28/32 (87.5%) subacute KD subjects showed IL-10 responses to at least one peptide. Of these 28 subjects, 16 (57.1%) showed responses to multiple (≥3) peptides (Fig. 1). Treg expanded in response to eight peptides: Fc 306-320 and Fc 56-70 were recognized by 14 (43.8%) subjects, Fc 181-195 was recognized by 12 (37.5%) subjects, Fc 26-40 was recognized by 11 (34.4%) subjects, Fc 121-135 and Fc 126-140 were recognized by 10 (31.3%) subjects. Fc 21-35 and Fc 301-315 were recognized by 9 (28.1%) subjects (Fig. 1B). Two KD subjects with CAA (#6 and #32) and two KD subjects without CAA (#2 and #8) did not respond to any of the 16 peptides tested. These results suggested that the pattern of peptide immunodominance in KD after IVIG was similar to the natural Treg response previously described in healthy adult donors [3].

IgG at high concentrations is internalized by tolerogenic myeloid dendritic cells that stimulate Treg expansion

To define the timing and the APCs that best internalize IgG to process and present to Treg, we studied the up-take of scalar doses of IgG-FITC in monocytes, macrophages, myeloid DC (cDC1 and cDC2), ILT-4+ CD4+ tmDC, plasmacytoid DC (pDC), and B cells at different time points (15, 30, and 60 min) from two subacute KD subjects 2 weeks after IVIG therapy. We defined the APC that were best at presenting the Fc to activate Fc-specific Treg by using the fluorescence intensity (and mean fluorescence) of IgG-FITC in the cytoplasm of cell populations defined by the surface markers.

Surprisingly, high concentrations of IgG-FITC (100 μg/ml), and less efficiently lower concentrations, were internalized with different efficiencies depending upon APC cell type. Myeloid CD14+ cDC2 and ILT-4+ CD4+ tmDC were most efficient in internalizing the IgG-FITC in sharp contrast to monocytes, macrophages, cDC1, pDC and B cells (Fig. 2A). CD4+ CD25high Treg expanded in PBMC cultures from the same subjects stimulated with purified Fc fragments *in vitro* in a dose-dependent manner (Fig. 2B). In a subset of these subjects, FOXP3 up-regulation in response to the Fc was measured in CD4+ CD25high Treg by intracellular staining (Supplementary Fig. S1). High levels of IL-10 secretion by CD14+ cDC2, ILT-4 CD4+ tmDC, and Treg were measured in culture supernatants, consistent with our previous results on the role of the Fc in stimulating regulatory innate and adaptive immune cells [2, 3, 5].

IVIG-stimulated Fc-specific Treg are detected in circulation years after IVIG therapy

An important question was the persistence of Fc-specific Treg in circulation after IVIG. Prior to IVIG, acute KD subjects lack this important Treg repertoire that can be found in healthy donors [3, 5]. To address this question, we enrolled eight additional subacute KD and nine children with a remote history of KD treated with IVIG. The results suggested
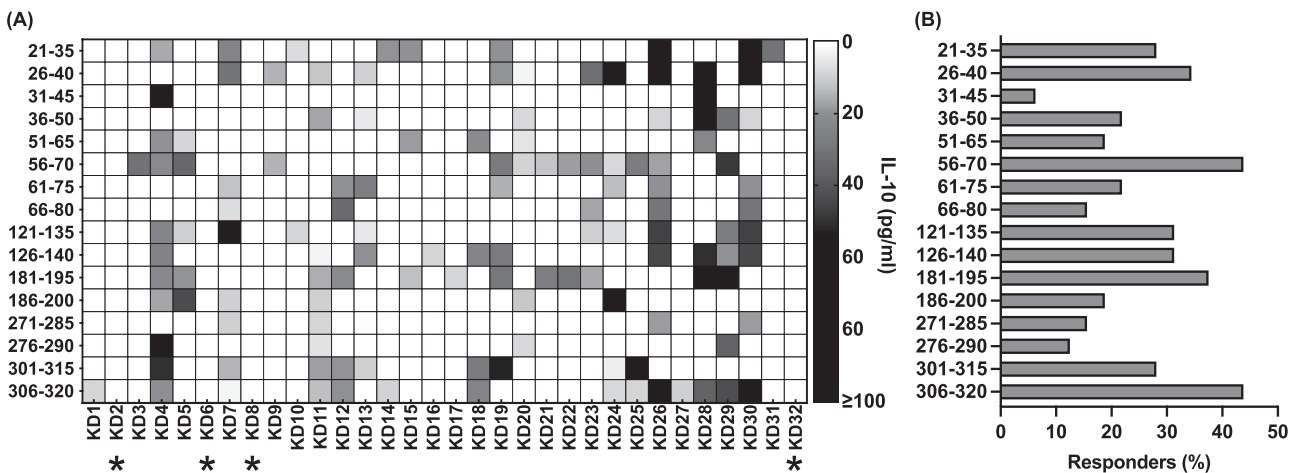

Figure 1: Fine specificities of Fc-specific Treg in subacute KD subjects after IVIG. PBMC were isolated from 32 subacute KD subjects 2–6 weeks after IVIG (21 males and 11 females, aged 0.26–13.6 years) and stimulated *in vitro* for 4 days in the absence of exogenous IL-2 with 16 individual Fc peptides (Table 2). IL-10 secretion in the culture supernatants served as a read-out to measure the Treg response. (A) IL-10 responses to the 16 Fc peptides from 32 subacute KD subjects. Shaded boxes indicate a positive IL-10 response (>20 pg/ml and above un-stimulated control) to a specific Fc peptide epitope. Gradients represent differences within the IL-10 secretion (right scale). The > 20 pg/ml cut off has been chosen based on the ELISA sensitivity and the difference with the IL-10 measurable in the unstimulated controls. *Four subjects (KD #2, 6, 8, and 32) had no detectable IL-10 response. (B) Percentage of the subjects that responded to each individual peptide tested.
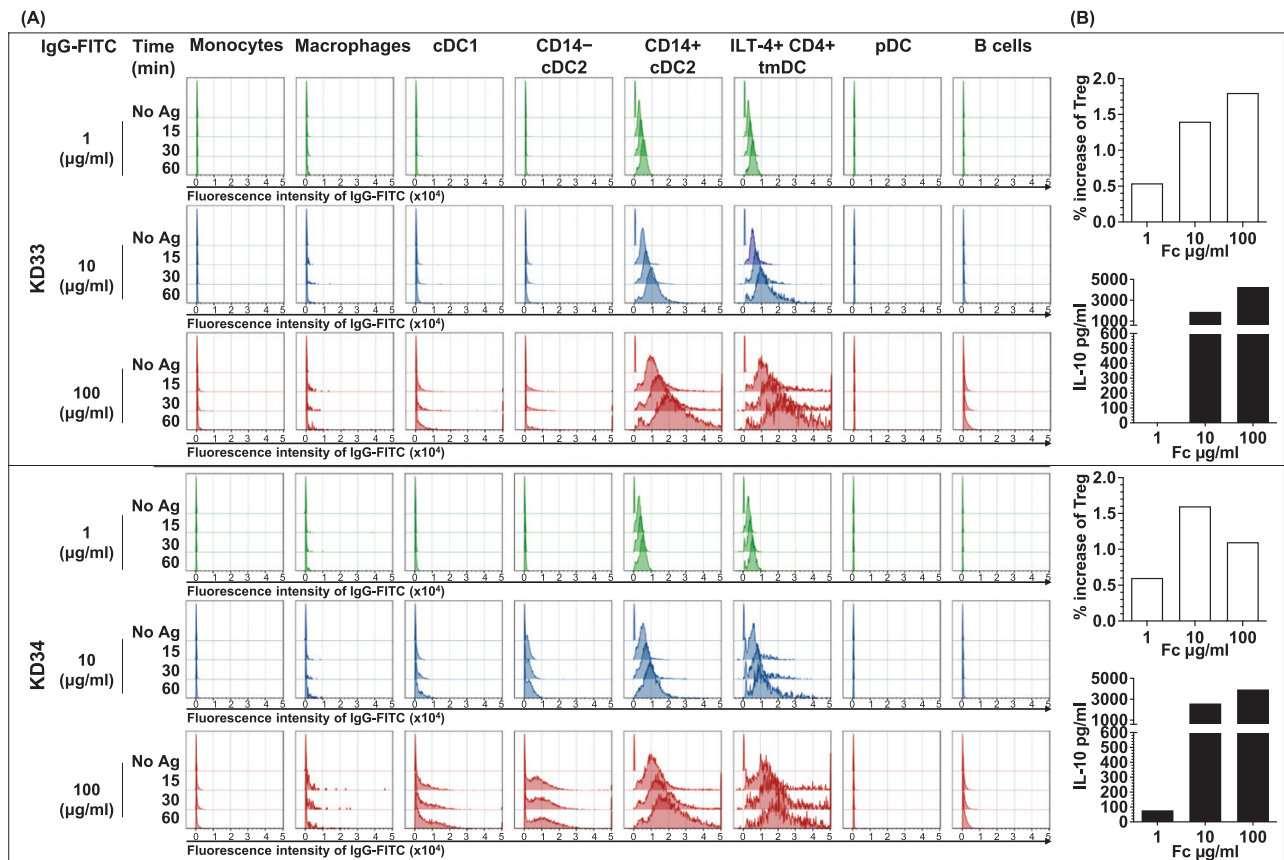
Figure 2: Internalization of IgG-FITC by different APC populations and expansion of Fc-specific Treg in subacute KD subjects after IVIG. Two subacute KD subjects (KD #33, a 2.9 year male, and #34, a 1.7 year female, Table 1) were enrolled to study the efficiency and dose-dependency of the internalization of an IgG-FITC by different APC populations in conjunction with the measurement of Treg expansion (% CD4+ CD25high T cells) in culture in response to Fc protein stimulation determined by flow cytometry. (A) Internalization of scalar doses of IgG-FITC (1, 10, and 100 µg/ml) by monocytes (CD11c− CD11b− CD14+), macrophages (CD11c− CD11b+ CD14+), cDC1 (CD11c+ CD11b− CD14−), CD14− cDC2 (CD11c+ CD11b+ CD14−), CD14+ cDC2 (CD11c+ CD11b+ CD14+), ILT-4+ CD4+ tmDC (CD14− cDC2 co-expressing ILT-4 and CD4), pDC (CD11c− CD11b− CD123+ CD14−), and B cells (CD19+). Cell preparations were pulsed with IgG-FITC for 15, 30, and 60 min, fixed, and stained with monoclonal antibodies to identify APC populations. Unstimulated PBMC served as baseline control. IgG-FITC internalization is shown as histograms with linear scales representing the internalization of 1 µg/ml (shown in green), 10 µg/ml (shown in blue), 100 µg/ml (shown in red) IgG-FITC in a time course. CD14+ cDC2 and ILT-4+ CD4+ tmDC were the only two APC populations that showed high levels of internalized IgG-FITC. Efficient internalization of IgG-FITC was only observed with 100 µg/ml of IgG-FITC. (B) Fc-specific, dose-dependent percent increase of the CD4+ CD25high Treg and IL-10 secretion in response to stimulation with 1, 10, and 100 µg/ml Fc protein.

that Fc-specific Treg expanded after IVIG exposure and that this repertoire was detectable several months after therapy, although with a lower precursor frequency than in the subacute phase (Fig. 3 right panels versus left panels). No differences were observed in IL-10 secretion between the two cohorts because of the contribution of CD14+ cDC2 and ILT-4+ CD4+ tmDC.

Characterization of the FcγRs involved in IgG internalization by tmDC

We then investigated the expression of different FcγRs and their relevance in uptaking IgG for antigen presentation in monocytes, macrophages, cDC1, CD14− cDC2, CD14+ cDC2, ILT-4+ CD4+ tmDC, pDC and B cells side-by-side with the IgG-FITC internalization. PBMC from four subacute KD and four healthy children with a remote KD history were investigated (Table 1). We also determined the specific role of FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) in the entry of IgG-FITC in blockade experiments with antagonistic antibodies.

We found a variable distribution of FcγRs depending upon APC type with no significant differences between subacute KD and children with remote KD (Fig. 4A). CD14+ cDC2 and ILT-4+ CD4+ tmDC expressed FcγRI and FcγRII (up to 100% of the populations) and, to a lesser extent, FcγRIII. These cells efficiently internalized the IgG-FITC with no differences between the two KD cohorts (Fig. 4A and B). Blocking the entry of IgG-FITC with anti-FcγRII led to a profound inhibition of IgG uptake, suggesting that FcγRII is the most efficient FcγR for internalization of IgG (Fig. 4C). FcγRIII was also found to play a role, especially in ILT-4+ CD4+ tmDC (Fig. 4C). Less relevant was FcγRI for IgG internalization, although this receptor was highly expressed by the CD14+ cDC2 and IL-T4+ CD4+ tmDC (Fig. 4C).

Treg from healthy donors respond to the Fc internalized processed and presented by tolerogenic mDC

We previously reported that the Fc-specific Treg repertoire in healthy donors has comparable fine specificities as Treg
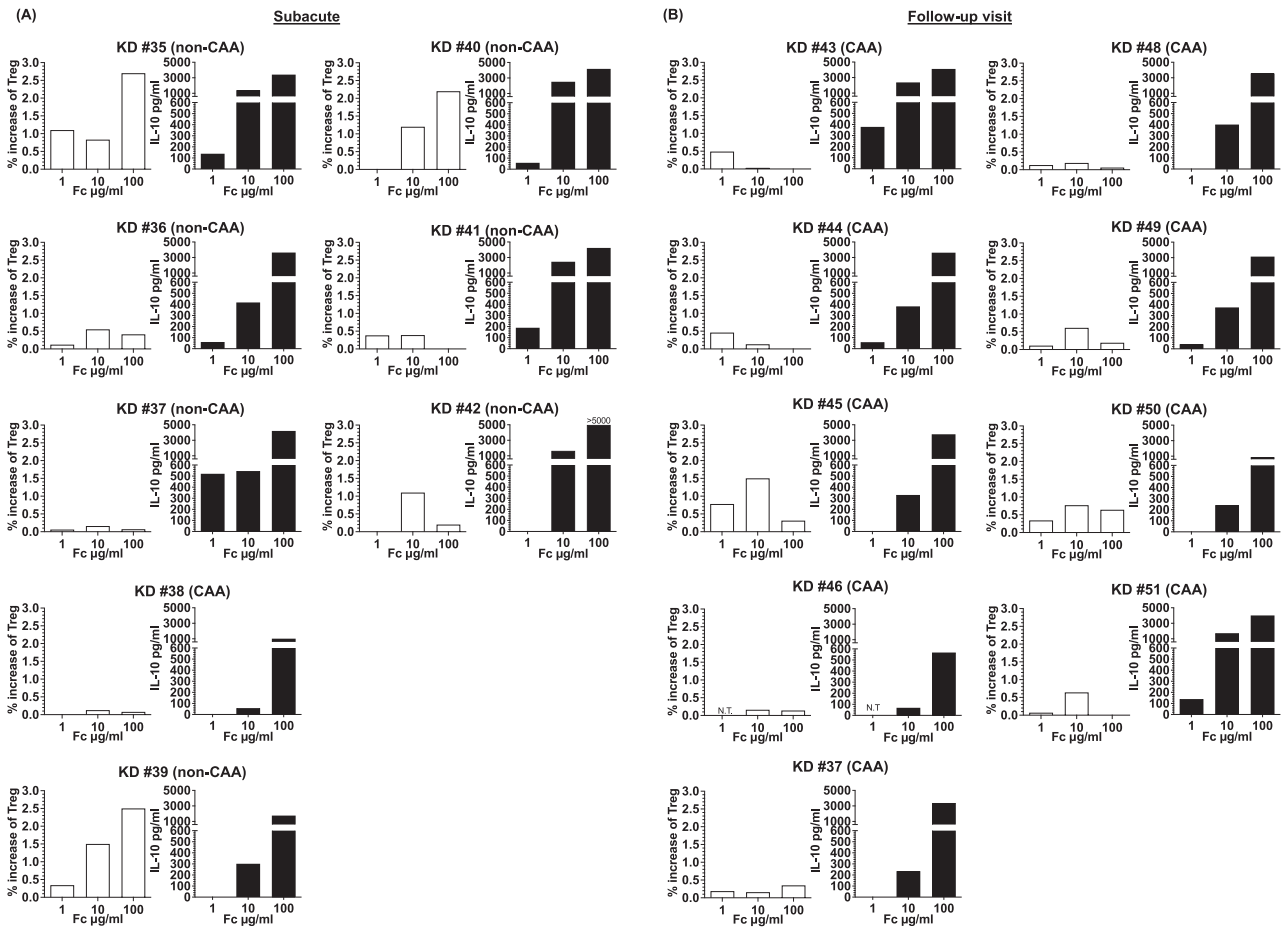
Figure 3: Treg expansion and IL-10 secretion in response to the Fc protein in subacute KD subjects after IVIG and children with a remote history of KD. CD4+ CD25high Treg expansion and IL-10 secretion in the PBMC cultures in response to a scalar doses of Fc protein were studied in two cohorts (A) eight subacute KD subjects (KD #35–42; 6 males and 2 females, aged 0.5–15 year, Table 1) and (B) nine subjects with a remote history of KD (KD #43–51; eight males and one female, aged 3–15 year, Table 1). CD4+ CD25high Fc-specific Treg were detected in both subacute KD subjects and children 1.6–14 years later suggesting that IVIG primes a Treg repertoire that endures. N.T.: not tested.

stimulated by IVIG in subacute KD subjects [3]. Here we addressed the APC compartment that best internalized the Fc to present processed peptides to Treg to validate the role of tmDC that we found uniquely involved in the Fc presentation to Treg in KD through FcγRII and FcγRIII. We enrolled 10 healthy adult donors and defined the expansion of CD4+ CD25high Treg in response to scalar doses of Fc in PBMC cultures, the IL-10 secretion in culture supernatants, and IgG-FITC uptake by APCs. We determined the expression of FcγRs and their role within the IgG-FITC internalization in blockade experiments. The results supported a similar level of Fc-specific Treg expansion in healthy donors compared to the subacute KD children who had received IVIG (Fig. 5A). When we measured the entry of IgG-FITC in different APCs, we found that high concentrations of IgG-FITC were internalized by suppressor myeloid DC, CD14+ cDC2, and ILT-4+ CD4+ tmDC. In adult controls, canonical myeloid DC, namely CD14− cDC2 internalized the IgG-FITC, although only at high concentrations (Fig. 5B). The expression of FcγRs on APC was similar to the KD subjects (Fig. 5C), as well as the prevalent efficiency of FcγRII over other FcγRs in the internalization of the IgG-FITC (Fig. 5D).

IVIG induces Fc-specific Treg presented by CD14+ cDC2 and ILT-4+ CD4+ tmDC in MIS-C

To validate the function of IVIG in immune regulation, we studied the Fc-specific Treg expansion and IgG-FITC internalization in PBMC cultures from a small cohort of MIS-C patients. KD and MIS-C have some clinical similarities but different pathogenesis and intensity of inflammation. Both diseases are commonly treated with IVIG.

For these experiments, four MIS-C were enrolled in the subacute phase (three males and one female aged 3.7–12.9 years) and one year after acute symptoms and IVIG (male, 10 years old).

The results supported a similar level of Fc-specific Treg expansion and Fc dose-dependent IL-10 secretion in MIS-C compared to KD children who had received IVIG and healthy controls (Fig. 6A). As observed in KD after IVIG and healthy donors, CD14+ cDC2 and ILT-4+ CD4+ tmDC were activated by Fc stimulation as shown by the expression of CD86 (Fig. 6B). Also consistent with previous results, the distribution of FcγRs on APCs in MIS-C showed high FcγRII expression and intermediate FcγRIII expression in CD14+ cDC2 and ILT-4+ CD4+ tmDC (Fig. 6C). When we measured the entry of IgG-FITC in different APCs, we
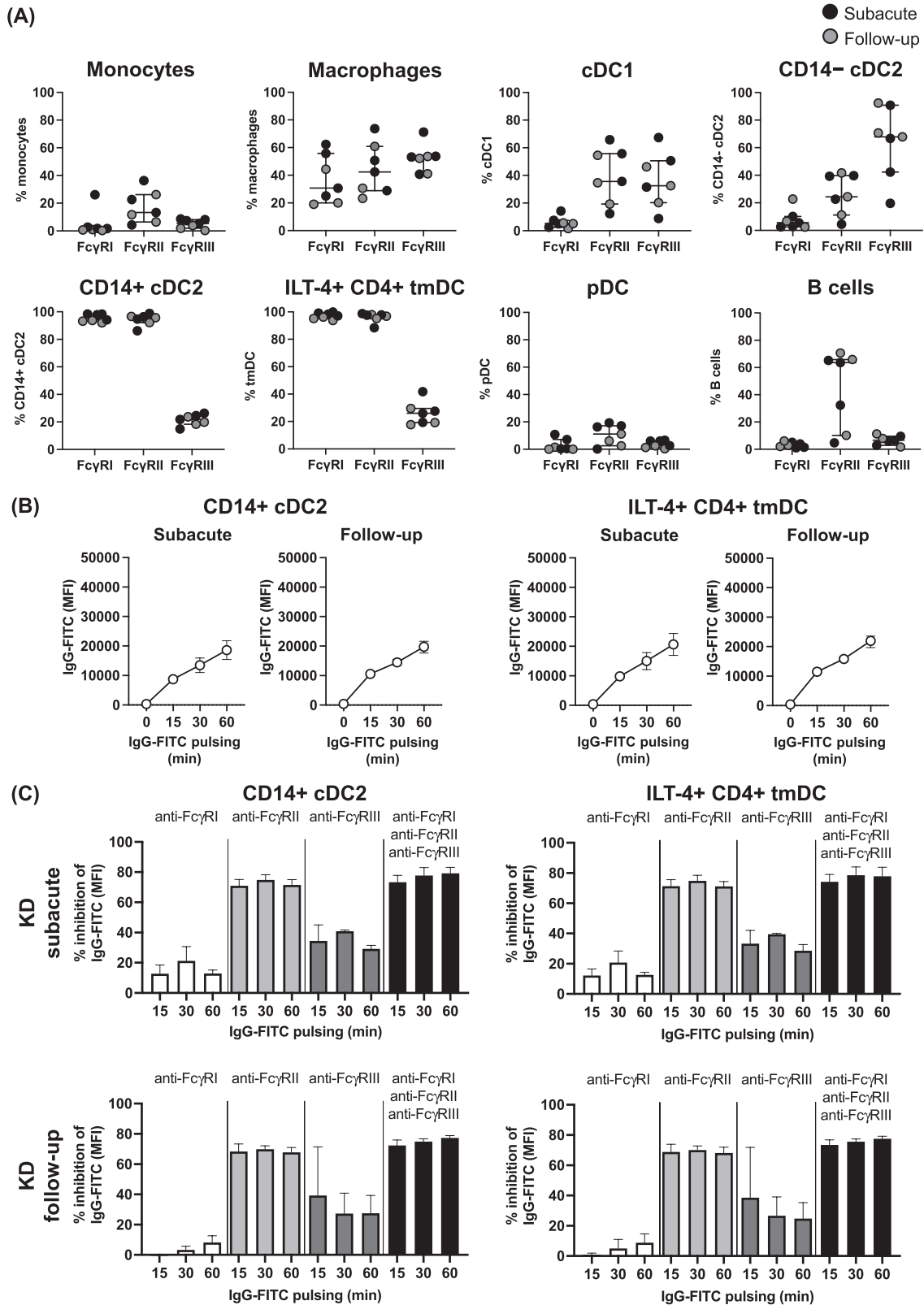
Figure 4: Expression of FcγRs on different APC populations and their role in the internalization of IgG-FTIC. To better understand the role of FcγRs in internalizing IgG-FITC, we studied the expression of FcγRI, FcγRII, and FcγRIII by flow cytometry and the effect of blocking monoclonal antibodies in monocytes, macrophages, cDC1, CD14⁻ cDC2, CD14⁺ cDC2, ILT-4⁺ CD4⁺ tmDC, pDC, and B cells. Four subacute KD subjects (KD #33–36; three males and one female, aged 0.5–15 yo, Table 1) and three subjects studied 3–14 years after KD (KD #43–45; three males, aged 7–15 year, Table 1). (A) FcγR expression on different APC: Black circles: subacute KD subjects after IVIG. Gray circles: subjects with remote KD. (B) IgG-FITC uptake by tolerogenic myeloid DC (CD14⁺ cDC2 and ILT-4⁺ CD4⁺ tmDC) were studied in two subacute KD subjects (KD #35, a 0.5 year male and 36, a 15 year male, Table 1) and three children studied 3–12 years after KD (KD #45–47, three males aged 7–15 yo, Table 1). The uptake of IgG-FITC was quantified by measuring the mean fluorescent intensity (MFI) of the FITC signal from the APC populations. The kinetics of IgG-FITC uptake by CD14⁺ cDC2 and ILT-4⁺ CD4⁺ tmDC were similar between the subacute and remote KD subjects. (C) Percent inhibition of IgG-FITC internalization by blocking monoclonal antibodies to FcγRI, FcγRII, and FcγRIII. A significant reduction in MFI of IgG-FITC in the presence of blockade of FcγRII, in both CD14⁺ cDC2 and ILT-4⁺ CD4⁺ tmDC was observed. To a lesser extent, FcγRIII blockade also reduced the IgG uptake.
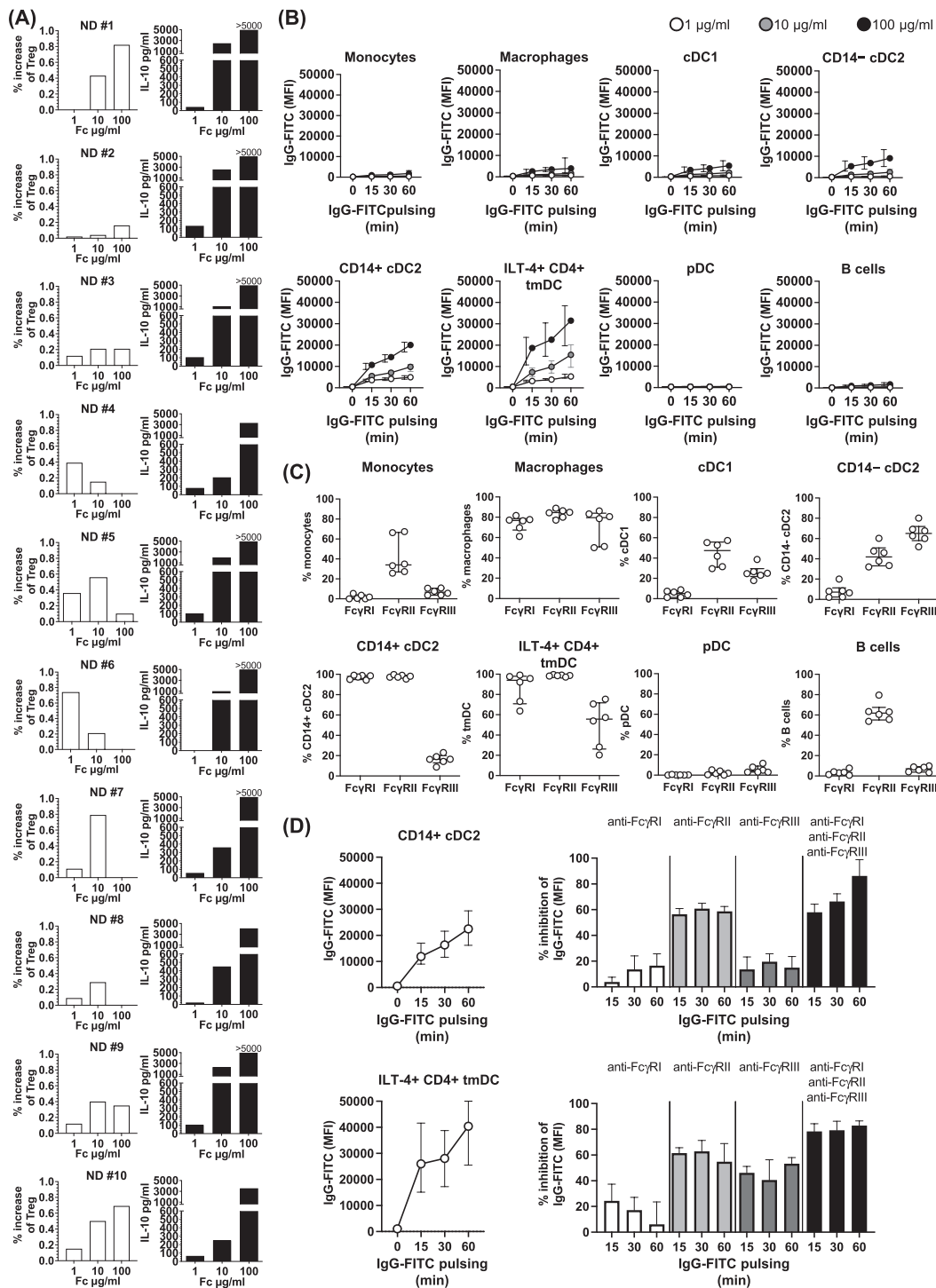
Figure 5: Treg response to the Fc protein and internalization of IgG-FITC by different APC populations in healthy adult donors. Ten healthy adult donors (eight males and two females, aged 20–70 year) were enrolled to study the Treg responses to the Fc protein, the dynamic of the entry of the IgG-FITC uptake by different APC type, the expression of FcγRs and their role in the inhibition in the IgG-FITC entry into the cytoplasm of APC. (A) Percentage increase in PBMC of CD4+ CD25high Treg (opened bars, left panels) and IL-10 secretion (closed bars, right panels) in response to the stimulation of 1, 10, and 100 μg/ml of Fc protein. Fc-specific Treg responses were detectable in all the healthy adult donors tested (that never received IVIG). IL-10 have been found abundantly secreted in response to Fc stimulation. (B) Internalization of IgG-FITC (1, 10, and 100 μg/ml) by different APC populations. White, gray, and black circles show pulsing with 1, 10, and 100 μg/ml of IgG-FITC respectively. Error bars indicate the mean ± range. Similar to subacute KD after IVIG therapy and children that had KD and far from IVIG therapy (follow-up visit), CD14+ cDC2 and ILT-4+ CD4+ tmDC were the two APC populations that showed the highest capability in internalizing the IgG-FITC. As in KD, a significant increase of the MFI of IgG-FITC was only observed when the PBMC were pulsed with a high concentration (100 μg/ml) of IgG-FITC. (C) Expression of FcγRI, FcγRII, and FcγRIII by different APC populations. The median and the IQR were indicated by the error bars. No significant differences of FcγR expression on APC populations was found between KD subjects and healthy adult donors. (D) Internalization of IgG-FITC (100 μg/ml) by CD14+ cDC2 and ILT-4+ CD4+ tmDC (left panels) and the percent inhibition of internalization of IgG-FITC in the presence of FcγR blockades (right panels) in the healthy adult donors. Error bars show the mean ± range. Like KD subjects, anti-FcγRII significantly decreased the internalization of IgG-FITC in both, CD14+ cDC2 and ILT-4+ CD4+ tmDC. Anti-FcγRIII showed a lesser effect in inhibiting the entry of the IgG-FITC and the role of FcγRI was minimal.
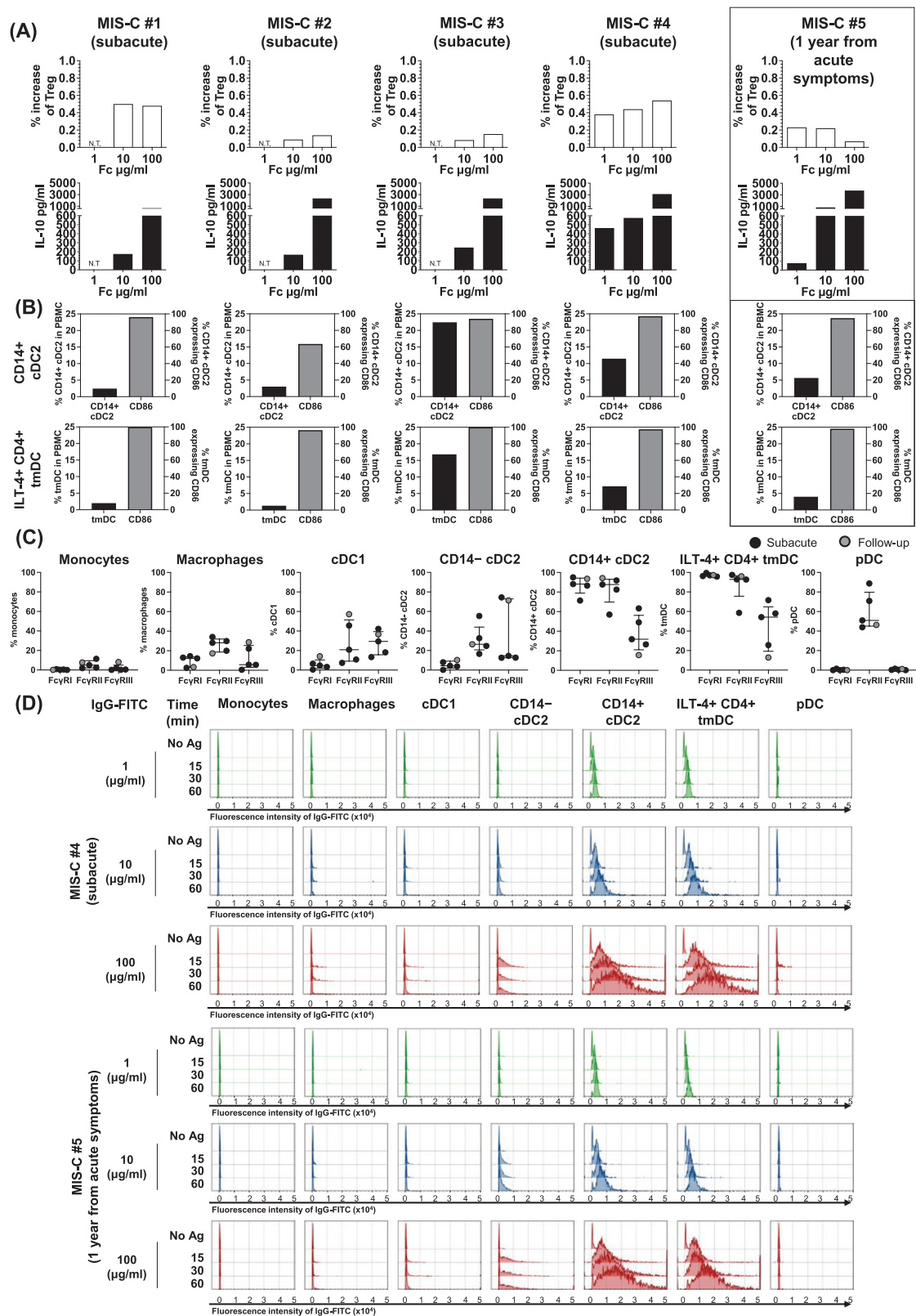
Figure 6: Treg response to the Fc protein and internalization of IgG-FITC by different APC populations in MIS-C. Four MIS-C were enrolled in the subacute phase (three males and one female aged 3.7–12.9 years) and one year after acute symptoms and IVIG (male, 10 years old) were enrolled to study the Treg responses to the Fc protein, the expression of FcγRs and the dynamic of the entry of the IgG-FITC uptake by different APC type. (A) Percentage increase in PBMC of CD4^{+} CD25^{high} Treg (opened bars, upper panels) and IL-10 secretion (closed bars, bottom panels) in response to stimulation by scalar doses of Fc protein. Cell numbers is a limitation in these lymphopenic children and the lowest part of the curve could not be tested in three of five subjects. Fc-specific Treg responses were detectable in all the MIS-C patients tested. IL-10 was secreted in response to Fc stimulation. (B) CD86 expression CD14^{+} cDC2 and ILT-4^{+} CD4^{+} tmDC. (C) Expression of FcγRI, FcγRII, and FcγRIII by different APC populations. Median and IQR are indicated by the error bars. (D) Internalization of IgG-FITC (1, 10, and 100 μg/ml) by different APC populations. CD14^{+} cDC2 and ILT-4^{+} CD4^{+} tmDC showed the highest capacity to internalize the IgG-FITC. Increased MFI of IgG-FITC was only observed when the PBMC were pulsed with a high concentration (100 μg/ml) of IgG-FITC.

found that high concentrations of IgG-FITC were internalized by suppressor myeloid DC, CD14+ cDC2 and ILT-4+ CD4+ tmDC in MIS-C, similar to the findings in KD patients and healthy controls (Fig. 6D).

Discussion

Treg is a plastic and heterogenous cell population and play a major role in controlling immune homeostasis in secondary lymphoid organs, thus preventing the adverse expansion of self-reactive T cells and B cells [7–9]. We previously reported that in the acute phase of KD and before IVIG therapy, Fc-specific Treg could not be found in circulation, in contrast with other acute pediatric febrile infectious controls [5]. Here we defined an important function of IVIG in expanding Treg that recognize a set of peptide epitopes derived from the heavy chain constant region of IgG in children recovering from KD who were studied 2–6 weeks after initial treatment and several years after treatment. The high dose of IVIG administered to these patients allowed the expansion of Treg whose T cell receptors (TcRs) recognize a small set of immunodominant peptides, including pan-HLA Fc epitopes that bind multiple HLA class II alleles [3]. Of interest, IVIG induces the expansion of Treg that recognize the same Fc regions that we previously found to be immunodominant in healthy donors [3]. Some of these peptides have been eluted and sequenced from HLA class II molecules suggesting their relevance in being processed and presented by APC [10–13]. Here we elucidate the antigen uptake of IgG reporting that only a subset of innate APCs, namely, CD14+ cDC2 and ILT-4+ CD4+ tmDC, efficiently internalize IgG. In turn, these cells secrete IL-10 and prime Treg by processing the Fc and presenting immunodominant peptides defined here.

Our results point to critical immune regulatory functions for IgG that is internalized by tolerogenic APC, but only at high concentrations. This observation supports the concept that high doses of IVIG are needed in inflammatory diseases like KD and MIS-C to achieve the desired stimulation of Treg and the production of IL-10 to control inflammation. Our data may help to explain the clinical observation made by Japanese investigators in the early days of IVIG as a treatment for KD that administration of the Fc portion of IVIG was more effective in controlling inflammation than infusing preparations enriched for the F(ab)$_2$ fragment [14, 15]. In KD, the F(ab)$_2$ portion of IgG recognizes and eliminates neutrophils [16] while our observations suggest that the Fc boosts immune regulation. IVIG is currently used to treat children with MIS-C triggered by SARS-CoV-2 infection [17–19] and the role of the Fc in innate and adaptive immune regulation here reported may contribute to the multifactorial mechanisms of action of IVIG in this hyperinflammatory state.

The observation that immunoglobulins need to remain in circulation and therefore are not internalized and processed by innate cells that carry FcγRs is novel. In fact, the function of FcγRs in the light of IgG internalization and antigen processing for Treg presentation is also different than the canonical signaling: preferentially FcγRII is involved in the internalization of IgG and only in a subset of tolerogenic dendritic cells, although FcγRII is expressed on multiple APCs.

A polymorphism in FcγRII has been reported to influence KD susceptibility with the A allele (coding for histidine) conferring elevated disease risk [20]. Substitution of A to G in FcγRII (rs1801274) changes the amino acid sequence at position 131 from arginine to histidine. It is possible that this polymorphism in the FcγRII influences receptor binding to IgG on cell surfaces compromising the internalization of IgG and, in turn, the expansion of Fc-specific Treg in KD. IVIG may favor the expansion of Fc-specific Treg via large doses of IgG that can by-pass the problem of a lower affinity receptor.

We recognize the limitations of our studies that are a consequence of working with pediatric patients with small blood volumes that can be obtained for research purposes. For example, the elution of Fc peptides from CD14+ cDC2 and ILT-4+ CD4+ tmDC was not feasible because of the limitation in primary cell numbers. We could not formally prove the Fc antigen processing but we used the presentation of Fc peptides to Treg as a read-out. Lack of access to secondary lymphoid organs did not permit the study of tissue-resident innate cells and their anatomical localization to prime Treg.

In conclusion, we highlight the important role of IVIG in activating the innate and adaptive immune regulation in KD and MIS-C. Our results, including the characterization of immunodominant immunomodulatory Fc peptides in KD, may also explain the therapeutic value of IVIG in other inflammatory conditions including neurological diseases where autoreactive T cells play a central role in the pathogenesis [21–24].

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Li-En Hsieh performed the experiments, analyzed the results, contributed to the experimental plan and contributed to the manuscript preparation. Jaeyoon Song performed the ELISA to measure IL-10 secretion in the culture supernatants. Adriana H. Tremoulet and Jane C. Burns diagnosed and treated KD and MIS-C children. Jane C. Burns also participated to the editing of the manuscript. Alessandra Franco designed, directed, supported the study and wrote the manuscript.

Ethical approval

The experiments using human samples were performed in accordance with relevant guidelines and regulations. The study protocols for KD subjects and healthy adult donors were approved by the Institutional Review Board at the University of California San Diego (IRB #140220 and #101213X, respectively).

Patient consent

Pediatric subjects were enrolled at Rady Children's Hospital, San Diego, following written parental informed consents and patient assent as appropriate. Healthy adult donors were enrolled at the Scripps Research Institute Normal Blood Donor Services following written consent.

Data availability

The data underlying this article are available within the article.

References

1. Rieckmann JC, Geiger R, Hornburg D, Wolf T, Kveler K, Jarrossay D, et al. Social network architecture of human immune cells unveiled by quantitative proteomics. *Nat Immunol* 2017, 18, 583–93. doi:10.1038/ni.3693.

2. Franco A, Kumar J, Lin G, Behnamfar N, Hsieh LE, Shimizu C, et al. Pediatric tolerogenic DCs expressing CD4 and immunoglobulin-like transcript receptor (ILT)-4 secrete IL-10 in response to Fc and adenosine. *Eur J Immunol* 2018, 48, 482–91. doi:10.1002/eji.201747139.

3. Hsieh L-E, Sidney J, Burns JC, Boyle DL, Firestein GS, Altman Y, et al. IgG epitopes processed and presented by IgG+ B cells induce suppression by human thymic-derived regulatory T cells. *J Immunol* 2020, 206, 1194–203.

4. Guagliardi LE, Koppelman B, Blum JS, Marks MS, Cresswell P, Brodsky FM. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature* 1990, 343, 133–9. doi:10.1038/343133a0.

5. Franco A, Touma R, Song Y, Shimizu C, Tremoulet AH, Kanegaye JT, et al. Specificity of regulatory T cells that modulates vascular inflammation. *Autoimmunity* 2014, 47, 95–104. doi:10.3109/08916934.2013.860524.

6. Burns JC, Hshieh L-E, Kumar J, Behnamfar N, Sivilay N, Tremoulet AH, et al. Characterization of circulating immune cells in acute Kawasaki disease suggests exposure to different antigens. *Clin Exp Immunol* 2020, 202, 263–72.

7. van der Veeken J, Gonzalez AJ, Cho H, Arvey A, Hemmers S, Leslie CS, et al. Memory of inflammation in regulatory T cells. *Cell* 2016, 166, 977–90.

8. Sakaguchi S, Ohkura N. Regulatory T cells: roles of T cell receptor for their development and function. *Semin Immunopathol* 2010, 32, 95–106.

9. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune diseases. *Nat Immunol* 2018, 19, 665–73. doi:10.1038/s41590-018-0120-4.

10. Bergseng E, Dørum S, Arntzen MO, Nielsen M, Nygård S, Buus S, et al. Different binding motifs of the celiac disease-associated HLA molecules DQ2.5, DQ2.2, and DQ7.5 revealed by relative quantitative proteomics of endogenous peptide repertoires. *Immunogenetics* 2015, 67, 73–84.

11. Muixí L, Gay M, Muñoz-Torres PM, et al. The peptide-binding motif of HLA-DR8 shares important structural features with other type 1 diabetes-associated allele. *Genes Immun* 2011, 12, 504–12. doi:10.1038/gene.2011.26.

12. Nelde A, Kowalewski DJ1, Backert L, Schuster H. HLA ligandome analysis of primary chronic lymphocytic leukemia (CLL) cells under lenalidomide treatment confirms the suitability of lenalidomide for combination with T-cell-based immunotherapy. *Oncoimmunology* 2018, 7, e1316438.

13. Ritz D, Sani E, Debiec H, Ronco P, Neri D, Fugmann T1. Membranal and blood-soluble HLA class II peptidome analyses using data-dependent and independent acquisition. *Proteomics* 2018, 18, e1700246.

14. Hsu CH, Chen MR, Hwang FY, Kao HA, Hung HY, Hsu CH. Efficacy of plasmin-treated intravenous gamma-globulin for therapy of Kawasaki syndrome. *Pediatr Infect Dis J* 1993, 6, 509–12.

15. Harada K. Intravenous gamma-globulin treatment in Kawasaki disease. *Acta Paediatr Jpn.* 1991, 33, 805–10. doi:10.1111/j.1442-200x.1991.tb02612.x.

16. Zhu YP, Shamie I, Lee JC, et al. Immune response to intravenous immunoglobulin in patients with Kawasaki disease and MIS-C. *J Clin Invest* 2021, 131, 1–15.

17. Feldstein LR, Rose EB, Horwitz SM, et al. Multisystem inflammatory syndrome in U.S. children and adolescents. *N Engl J Med* 2020, 383, 334–46. doi:10.1056/NEJMoa2021680.

18. Dufort EM, Koumans EK, Chow EJ, et al. Multisystem inflammatory syndrome in children in New York state. *N Engl J Med* 2020, 383, 347–58.

19. Diorio C, Henrickson SE, Vella LA, et al. Multisystem inflammatory syndrome in children and COVID-19 are distinct presentations of SARS-CoV-2. *J Clin Invest* 2020, 130, 5967–75. doi:10.1172/JCI140970.

20. Khor CC, Davila S, Breunis WB, Lee YC, Shimizu C, Wright VJ, et al. Genome wide association study identifies FCGR2A as a susceptibility locus for Kawasaki disease. *Nat Genet* 2011, 43, 1241–6.

21. Steele NZ, Carr JS, Bonham LW, Geier EG, Damotte V, Miller ZA, et al. Fine-mapping of the human leukocyte antigen locus as a risk factor for Alzheimer disease: a case-control study. *PLoS Med* 2017, 14, e1002272.

22. Dodel R, Neff F, Noelker C, Pul R, Du Y, Bacher M, et al. Intravenous immunoglobulins as a treatment for Alzheimer's disease: rationale and current evidence. *Drugs* 2010, 70, 513–28. doi:10.2165/11533070-000000000-00000.

23. Latorre D, Kallweit U, Armentani E, Foglierini M, Mele F, Cassotta A, et al. T cells in patients with narcolepsy target self-antigens of hypocretin neurons. *Nature* 2018, 562, 63–8. doi:10.1038/s41586-018-0540-1.

24. Lecendreux M, Berthier J, Corny J, Bourdon O, Dossier C, Delclaux C. Intravenous immunoglobulin therapy in pediatric narcolepsy: a nonrandomized, open-label, controlled, longitudinal observational study. *J Clin Sleep Med* 2017, 13, 441–53. doi:10.5664/jcsm.6500.