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IgG Epitopes Processed and Presented by IgG$^+$ B Cells Induce Suppression by Human Thymic-Derived Regulatory T Cells

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We described a human regulatory T cell (Treg) population activated by IgG$^+$ B cells presenting peptides of the heavy C region (Fc) via processing of the surface IgG underlying a model for B cell–Treg cooperation in the human immune regulation. Functionally, Treg inhibited the polarization of naive T cells toward a proinflammatory phenotype in both a cognate and a noncognate fashion. Their fine specificities were similar in healthy donors and patients with rheumatoid arthritis, a systemic autoimmune disease. Four immunodominant Fc peptides bound multiple HLA class II alleles and were recognized by most subjects in the two cohorts. The presentation of Fc peptides that stimulate Treg through the processing of IgG by dendritic cells (DC) occurred in myeloid DC classical DC 1 and classical DC 2. Different routes of Ag processing of the IgG impacted Treg expansion in rheumatoid arthritis patients. ***The Journal of Immunology*****, 2021, 206: 1194–1203.**

The immune regulation system limits inflammation through a variety of networks that involve innate and adaptive immune cells (1). Regulatory T cells (Treg) are key in controlling autoreactive and pathogenic immune responses (2, 3). Treg represent a heterogeneous population that include at least three lineages: thymic-derived natural Treg (nTreg) that recognize "self-antigens," inducible Treg (iTreg) or type 1 Treg that recognize foreign Ags, and follicular Treg that regulate follicular Th cells (3, 4).

Little is known about the self–fine specificities of human thymic-derived Treg. Single-cell gene expression studies have suggested great heterogeneity and TCR diversity in the recognition of self-antigens (5). Our group has shown that the heavy C region (Fc) of IgG1 directly activates and expands Treg in a classical HLA-restricted fashion and downregulates inflammation in Kawasaki disease (KD), an acute pediatric vasculitis of the coronary arteries (6, 7). The absence of Fc-specific Treg in circulation correlated with the development of coronary artery aneurysms in children with KD, suggesting the importance of these T cells in mediating immune homeostasis.

In the current study, we showed that IgG$^+$ B cells activated thymic-derived nTreg by presenting immunodominant Fc peptides via the Ag processing of the surface Ig. The Ag processing of surface IgG on B cells has been previously documented (8). However, the sequences of the immunodominant 15 mers reported in this study differed from the two "tregitopes," previously reported as immunogenic for iTreg in healthy adult donors (9).

We also studied in tandem the requirements for a productive Fc-specific Treg activation and expansion via the exogenous internalization of the IgG molecules by innate immune cells. Treg fine specificities were defined in healthy donors and in subjects with rheumatoid arthritis (RA), the most common inflammatory autoimmune arthropathy chosen as an autoimmune disease model (10).

Materials and Methods

Subjects details

The experiments using human cells were performed in accordance with relevant guidelines and regulations; institutional review board numbers are included with each study population. Informed consent was obtained from all adult subjects enrolled in the study. No children were enrolled. All the experimental protocols were approved by the Institutional Review Board committee at the University of California San Diego. All methods used in the study were carried out in accordance with relevant guidelines and regulations.

Forty healthy adult donors (19 males, 21 females, aged 24–66) were enrolled to study their Treg responses to the whole Fc and to define Treg fine specificities (University of California San Diego [UCSD] number 101213X). Eleven additional healthy donors (six males, five females, aged 24–63) were enrolled to study the Ag processing and presentation of the surface Ig by IgG$^+$ B cells and the exogenous whole Fc by PBMC. Two additional healthy donors (two males, aged 32 and 48) were enrolled to study naive T cell differentiation in Treg-naive T cells cocultures.

Twenty-five RA subjects, two males and 23 females, aged 31–77 y with mixed ethnicity were enrolled at the UCSD Rheumatology Clinic

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L.-E.H., postdoctoral fellow, executed the experiments under the supervision of A.F.; J.S. and A.S. studied the HLA class II binding affinity of the Fc peptides to 27 HLA class II alleles; J.C.B. provided financial support and edited the manuscript; Y.A. provided technical support for flow cytometry; and G.S.F. and D.L.B. provided rheumatoid arthritis samples for the study. A.F. designed, supported, and directed the study and wrote the manuscript.

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Abbreviations used in this article: ApoB, apolipoprotein B; cDC, classical dendritic cell; DC, dendritic cell; iTreg, inducible Treg; IVIG, IVIG therapy; KD, Kawasaki disease; nTreg, natural Treg; RA, rheumatoid arthritis; Treg, regulatory T cell; UCSD, University of California San Diego.

following written informed consent to study their Treg responses to the whole Fc and to define Treg fine specificities (UCSD number 140175). Four additional RA subjects were enrolled to study the Ag processing and presentation of the surface Ig by IgG^+ B cells and the Ag processing and presentation of the exogenous Fc by PBMC. Details of the study population are provided in Supplemental Table I.

Peptide sequences and synthesis

Sixteen Fc peptides, 15 aa in length (Table I), were synthesized by 9-fluorenylmethoxycarbonyl chemistry using a multiplex peptide synthesizer (Symphony X; Protein Technologies, 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. A control peptide, apolipoprotein B (ApoB) 3036–3050 that stimulated Treg in atherosclerosis (11), was used as a control in experiments involving IgG^+ B cell presentation to FACS-sorted, peptide-specific Treg.

Definition of peptide immunodominance in healthy adult donors and RA subjects

PBMC were separated from heparinized whole blood by Ficoll Histopaque density gradient. A total of 4×10^5 cells/well were stimulated in 96-well, flat-bottom plates (Falcon) either with individual Fc peptides (20 μg/ml) or the whole Fc protein (100 μg/ml) (purity ≥97%; Meridian Life Science) in the absence of exogenous IL-2. Treg expansion was assessed at 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. For the measurement of IL-10 in culture supernatants by Ag-capture ELISA, 4 μg/ml anti-human IL-10 primary Ab (BD Biosciences) and 2 μg/ml biotin-labeled secondary Ab (BD Biosciences) were used according to manufacturer instructions. Anti-human CD4 PerCp/Cy5.5 (clone RPA-T4) mouse IgG1κ (eBioscience) and anti-human CD25 BV421 (clone M-A251) mouse IgG1κ (BD Biosciences) Abs were used to enumerate $CD4^+$ $CD25^{high}$ T cells by standard flow cytometry methodologies. Each donor was tested once.

Phenotypical characterization of Fc peptide-specific Treg

The surface phenotype and FACS sorting of Fc peptide-specific $CD4^+$ $CD25^{high}$ Treg was obtained by using specific mAbs: anti-human CD4 PerCp/Cy5.5 (clone RPA-T4), mouse IgG1κ (eBioscience), and anti-human CD25 BV421 (clone M-A251) mouse IgG1κ (BD Biosciences). Expression of surface chemokine receptors on Treg, CCR7, and CXCR5, were measured with anti-human CCR7 PE/Cy7 (clone 3D12) rat IgG2aκ (eBioscience) and anti-human CXCR5 PE (clone J252D4) mouse IgG1κ (BioLegend). CTLA-4 was measured by anti-human CTLA-4 allophycocyanin (clone BNI3) mouse IgG2aκ (BioLegend); PD-1 was measured by anti-human PD-1 PE (clone EH12.2H7) mouse IgG1κ (BioLegend); ICOS was measured by anti-human ICOS PE/Cy7 (clone ISA-3) mouse IgG1κ (eBioscience). $CD4^+$ $CD25^{high}$ cells secreting IL-10 in PBMC cultures stimulated by peptides were 100% $CD3^+$ (anti-human CD3 AF700 [clone OKT3] mouse IgG2aκ [BioLegend]). $CD14^+$ monocytes (anti-human CD14 PE/Cy7 [clone M5E2] mouse IgG2aκ [BD Biosciences]) expressing CD4 and CD25 were also studied to rule out the unique contribution of the Treg as a source of IL-10 secretion in response to Fc peptides. To study the intracellular expression of FOXP3 and the production of IL-10, Treg lines were permeabilized and labeled with anti-human FOXP3 PE (clone 259D) IgG1κ (BioLegend) and anti-human IL-10 BV421 (clone JES3-JD70 rat IgG1κ (BioLegend). Data were analyzed with FlowJo software version 10 (Tree Star). The experiments were performed on two Treg lines that recognized Fc 181–195 and Fc 306–320.

HLA class II restriction of Fc peptide-specific Treg

A total of 5×10^3 FACS-sorted Treg lines from two donors were restimulated with 1×10^5 irradiated, autologous PBMC and 10 μg/ml Fc peptides in the presence of 10 μg/ml anti-human HLA class II framework Ab (clone Tu39) mouse IgG2aκ (BD Biosciences) to assess their restriction. Cell preparations were studied in duplicate and used for two assays: 1) measurement of intracellular IL-10 by flow cytometry and 2) measurement of IL-10 by ELISA (BD Biosciences) in culture supernatants 4 d after stimulation. An Ab, anti–HLA class I (clone W6/32) mouse IgG2aκ (BioLegend), was used as control in these experiments.

Suppression of naive T cell differentiation by Treg

Autologous $CD4^+$ $CD45RA^+$ naive T cells were obtained by FACS sorting with anti-human CD4 PerCp/Cy5.5 (clone RPA-T4) mouse IgG1κ (eBioscience) and anti-human CD45RA allophycocyanin (clone HI100) mouse IgG2bκ (eBioscience) and cocultured with FACS-sorted Treg lines or Treg supernatants prior to stimulation with a dose range of agonistic anti-CD3 (clone HIT3a) mouse IgG2aκ (BioLegend) and 1 μg/ml agonistic anti-CD28 (clone CD28.2) mouse IgG1κ (BD Biosciences) at a 1:1 ratio (5×10^3). IFN-γ secretion in culture supernatants was measured by ELISA (BD Biosciences) 72 h after stimulation, according to manufacturer's instructions. The experiments were performed with Treg from two healthy donors and one RA subject.

HLA binding assay

Binding affinities of Fc peptides to 27 different HLA class II alleles, including 15 HLA-DR, six HLA-DQ, and six HLA-DP, were measured using competition assays based on the inhibition of binding of a high-affinity, radiolabeled peptide to purified MHC molecules. MHC molecules were purified from cell lines, and assays were performed as detailed elsewhere (12). Briefly, 0.1–1 nM of radiolabeled peptide was coincubated at room temperature or 37°C with purified MHC in the presence of a mixture of protease inhibitors. Following a 2- to 4-d incubation, MHC bound radioactivity was determined by capturing MHC/peptide complexes on MHC locus-specific mAb-coated LUMITRAC 600 plates (Greiner Bio-One, Frickenhausen, Germany) and measuring bound cpm using the TopCount (Packard Instrument, Meriden, CT) microscintillation counter. The concentration of peptide yielding IC_{50} of binding of the radiolabeled peptide was calculated. Under the conditions used, where [label]<[MHC] and $IC_{50} \geq$[MHC], measured IC_{50} values are reasonable approximations of true K_D (13, 14). Each competitor peptide was tested at six different concentrations, covering a 100,000-fold range, and in three or more independent experiments. As a positive control, the unlabeled version of the radiolabeled probe was also tested in each experiment.

Ag processing and presentation of Fc peptides to FACS-sorted Treg lines

To establish peptide-specific Treg lines for functional assays, PBMC were stimulated with peptides at 20 μg/ml for 4 d prior to FACS sorting and screened for peptide-specific TCR recognition by measuring IL-10 in the culture supernatant by ELISA. To test the Ag processing of the surface IgG and the presentation of immunodominant peptides by autologous IgG^+ B cells, autologous IgG^+ and IgG^- B cells were FACS sorted from PBMC using anti-human CD19 allophycocyanin/Cy7 (clone SJ25C1) mouse IgG1κ (BD Biosciences) and anti-human IgG PE (clone G18-145) mouse IgG1κ (BD Biosciences). Treg were FACS sorted using anti-human CD4 PerCp/Cy5.5 (clone RPA-T4) mouse IgG1κ (eBioscience) and anti-human CD25 BV421 (clone M-A251) mouse IgG1κ (BD Biosciences).

IgG^+ B cells (and IgG^- B cells control) were cocultured with autologous, FACS-sorted, $CD4^+$ $CD25^{high}$ Treg lines at a ratio of 5:1 or 10:1 depending on the efficiency of the FACS sorting (1×10^4 or 2×10^4 B cells). Culture supernatants were collected after 72 h to measure IL-10 by ELISA (BD Biosciences). To address the role of ICOS as a costimulatory molecule for Treg activation by IgG^+ B cells, we cocultured B cells and peptide-specific Treg with an antagonistic Ab to human ICOS-L (clone MIH12) mouse IgG1κ (eBioscience) at the optimal concentration of 10 μg/ml. These experiments were performed with Treg from eight healthy donors and three RA subjects. Only seven healthy donors were presented in the paper. One of the donors showed a very minor secretion of IL-10 by IgG^+ B cells and has been excluded.

To address the processing of the exogenous Fc and presentation of the immunodominant peptides, 1×10^5 irradiated, autologous PBMC were cocultured with 2×10^3 FACS-sorted $CD4^+$ $CD25^{high}$ Treg lines in the presence of Fc protein (100 μg/ml) or peptide controls (20 μg/ml) for 72 h. IL-10 secretion in the culture supernatants was measured by ELISA (BD Biosciences). These experiments were performed with Treg derived from five healthy donors and three RA subjects. One of the healthy donors showed IL-10 secretion by irradiated PBMC alone and has been excluded.

Exogenous Fc internalization by different innate cells

The uptake of Fc fragments by different innate cells was studied by measuring the internalization of an IgG1-FITC (Sigma-Aldrich), or purified FITC-labeled Fc fragments (The Jackson Laboratory) at different time points in classical dendritic cell (cDC)1 and cDC2 myeloid DC (defined by $CD11c^+$ $CD11b^{-\text{ or low}}$ and $CD11c^+$ $CD11b^+$, respectively), $CD11c^-$ $CD11b^+$ $CD14^+$ macrophages, and $CD11c^-$ $CD11b^-$ $CD14^+$ monocytes derived from PBMC. These markers are distilled from studies that defined the linear differentiation of myeloid cells (15–17). The assay was carried out by pulsing 1×10^6 PBMC with 100 μg/ml IgG1-FITC or Fc-FITC for 0, 15, 30, or 60 min. After pulsing, cell preparations were washed and fixed with Cytofix Fixation Buffer (containing 4.2% paraformaldehyde; BD

Biosciences). Cell types were defined by surface staining using anti-human CD11c allophycocyanin (clone B-ly6) mouse IgG1κ, anti-human CD11b PE (clone ICRF44) mouse IgG1κ, and anti-human CD14 PE/Cy7 (clone M5E2) mouse IgG2aκ from BD Biosciences. PBMC from 10 healthy donors and one RA subject were studied in these experiments.

The expression of CD86, FcγRI, FcγRII, and FcγRIII in myeloid populations was defined by surface staining using anti-human CD11c allophycocyanin (clone B-ly6) mouse IgG1κ (BD Biosciences), anti-human CD11b PE (clone ICRF44) mouse IgG1κ (BD Biosciences), anti-human CD14 PE/Cy7 (clone M5E2) mouse IgG2aκ (BD Biosciences), anti-human CD86 BV605 (clone FUN-1) mouse IgG1κ (BD Biosciences), 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).

We also evaluated the downregulation of IL-10 secretion by Treg in Fc-stimulated (100 μg/ml) PBMC cultures in the presence of 10 μg/ml anti-human FcγRI (clone 10.1) mouse IgG1κ (BioLegend), anti-human FcγRII (clone IV.3) mouse IgG1κ (Bio X Cell), or anti-human FcγRIII (3G8) mouse IgG1κ (BioLegend). PBMC cultures stimulated with Fc protein without FcγR blockades served as controls. PBMC cultures from one healthy donor were set up in duplicate for two read outs: 1) measurement of intracellular IL-10 by flow cytometry 24 h after stimulation and 2) enumeration of $CD4^+$ $CD25^{high}$ T cells in PBMC 72 h after Fc stimulation.

Statistical analysis

Data were analyzed using Prism software version 7.0 (GraphPad Software, San Diego, CA). IL-10 secretion by Treg lines cocultured with IgG^- and IgG^+ B cells and Treg lines cocultured with PBMC in the presence of Fc protein and Fc peptide were assessed by two-tailed paired *t* test. The comparison of the percentage increase in Treg expansion in response to stimulation with the purified Fc protein between 26 healthy donors and 25 RA subjects was assessed by two-tailed nonparametric Mann–Whitney *U* test. A p value ≤ 0.05 was considered statistically significant.

Results

Characterization of immunodominant Fc peptides recognized by Treg that modulate naive T cell differentiation in healthy donors and subjects with RA

Our previous work in children with KD who received IVIG therapy (IVIG) and healthy adults identified 16 Fc peptides recognized by thymic-derived nTreg by screening a set of 64 peptides (15 aa in length) overlapping by 10 aas and spanning the whole Fc protein (7). In this study, we defined Fc peptide immunodominance and potency for Treg recognition, the function of Fc-specific Treg in regulating naive T cell differentiation, and the HLA binding affinity of the most immunogenic peptides to 27 HLA class II alleles, including DR, DP, and DQ.

The study population included 40 healthy donors and 25 RA subjects (Supplemental Table I). RA was chosen as an autoimmune disease model to address possible differences in the nTreg repertoire in comparison with KD, a self-reactive, systemic inflammatory condition (6). Sixteen IgG1 Fc peptide sequences (Table I), 15 aa in length, were used to determine Treg fine specificities in the RA patients and healthy controls. Treg responses in these experiments were evaluated by 1) IL-10 secretion in culture supernatants by ELISA and 2) $CD4^+$ $CD25^{high}$ T cell enumeration by flow cytometry 4 d after culture initiation.

Overall, 37 of 40 (92.5%) PBMC in healthy donors responded to at least one peptide, but a majority of the donors responded to between three and 16 peptides, suggesting that epitope spreading is pronounced in this antigenic model (Fig. 1A, Supplemental Fig. 1A). Eight peptides were recognized by most healthy subjects: Fc 306–320, which ranked highest, was recognized by 20 of 40 (50. 0%) donors; Fc 181–195, which ranked second, was recognized by 19 of 40 (47.5%) donors; Fc 271–285 and Fc 21–35, which ranked third, were recognized by 16 of 40 (40%) donors; Fc 186–200 and 301–315, which ranked fourth, were recognized by 14 of 40 (35.0%) donors; and Fc 65–75 and Fc 51–65, which ranked sixth and seventh, were recognized by 11 and 10 of 40 donors, respectively (27.5%, 25.0%). Three donors (numbers 8, 28, and 35) did not respond to any of the 16 peptides tested but may have responded to other Fc sequences not tested (7). Treg in PBMC responded to peptides as shown by IL-10 secretion by $CD4^+$ $CD25^{high}$ T cells 24 h after peptide stimulation measured by intracellular cytokine staining (Supplemental Fig. 1B). $CD4^+$ $CD25^{high}$ cells were confirmed to be $CD3^+$. Monocytes expressing CD4 and CD25 were not relevant in the IL-10 response in PBMC stimulated with Fc peptides (Supplemental Fig. 1B, 1C). Moreover, an antagonistic Ab that recognizes the common framework of HLA-DR, DP, and DQ inhibited IL-10 secretion by PBMC stimulated with a representative pan-HLA Fc peptide (181–195), thus confirming the HLA class II restriction of the Treg response (Supplemental Fig. 1D).

Table I. 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	SVFLFPPKPKDTLMI
126–140	PPKPKDTLMISRTPE
181–195	TYRVVSVLTVLHQDW
186–200	SVLTVLHQDWLNGKE
271–285	NNYKTTPPVLDSDGS
276–290	TPPVLDSDGSFFLYS
301–315	QGNVFSCSVMHEALH
306–320	SCSVMHEALHNHYTQ

When Fc peptides were used to stimulate Treg derived from RA subjects, we found that Treg from 19 of 25 (76%) patients responded to at least one Fc peptide, with a pattern of recognition comparable to healthy donors (Fig. 1A, Supplemental Fig. 2). Fc 21–35 and Fc 306–320 ranked highest and were recognized by 10 of 25 (40%) RA subjects; Fc 181–195, which ranked second, was recognized by 9 of 25 (36%) RA subjects; Fc 186–200 and Fc 301–315, which ranked third, were recognized by 7 of 25 (28%) RA subjects; Fc 61–75, which ranked fourth were recognized by 6 of 25 (24%) RA subjects; Fc 51–65 ranked fifth and was recognized by 5 of 25 RA subjects (20%); and Fc 271–285 ranked sixth and was recognized by 4 of 25 (16%) RA subjects. Subject number 16 showed a very high IL-10 secretion in the unstimulated controls, and although responsive to three peptides, we did not include the results in the heat map.

Phenotypically Treg expressed intracellular FOXP3 and surface CTLA-4 and PD-1 4 d after stimulation (Fig. 1B). Their functional phenotype was further validated by IL-10 secretion by Treg lines FACS sorted 4 d after peptide stimulation in response to anti-CD3 anti-CD28 (Fig. 1C).

To address the regulatory function of Treg specific for immunodominant Fc epitopes in modulating the differentiation of $CD4^+$ naive T cells toward a Th1 proinflammatory phenotype, we FACS sorted autologous $CD4^+$ $CD45RA^+$ naive T cells from healthy donors and one RA patient and cocultured them with autologous Treg lines following 4 d of in vitro stimulation with Fc peptides. Dose-dependent secretion of IFN-γ in the presence of agonistic CD3/CD28 stimulation and its regulation in naive T cell–Treg cocultures served as a readout in these experiments. IL-10–containing cell culture supernatants from Treg (day 4 after peptide stimulation in vitro) were also collected and cultured with naive T cells to determine a possible noncognate regulation of naive

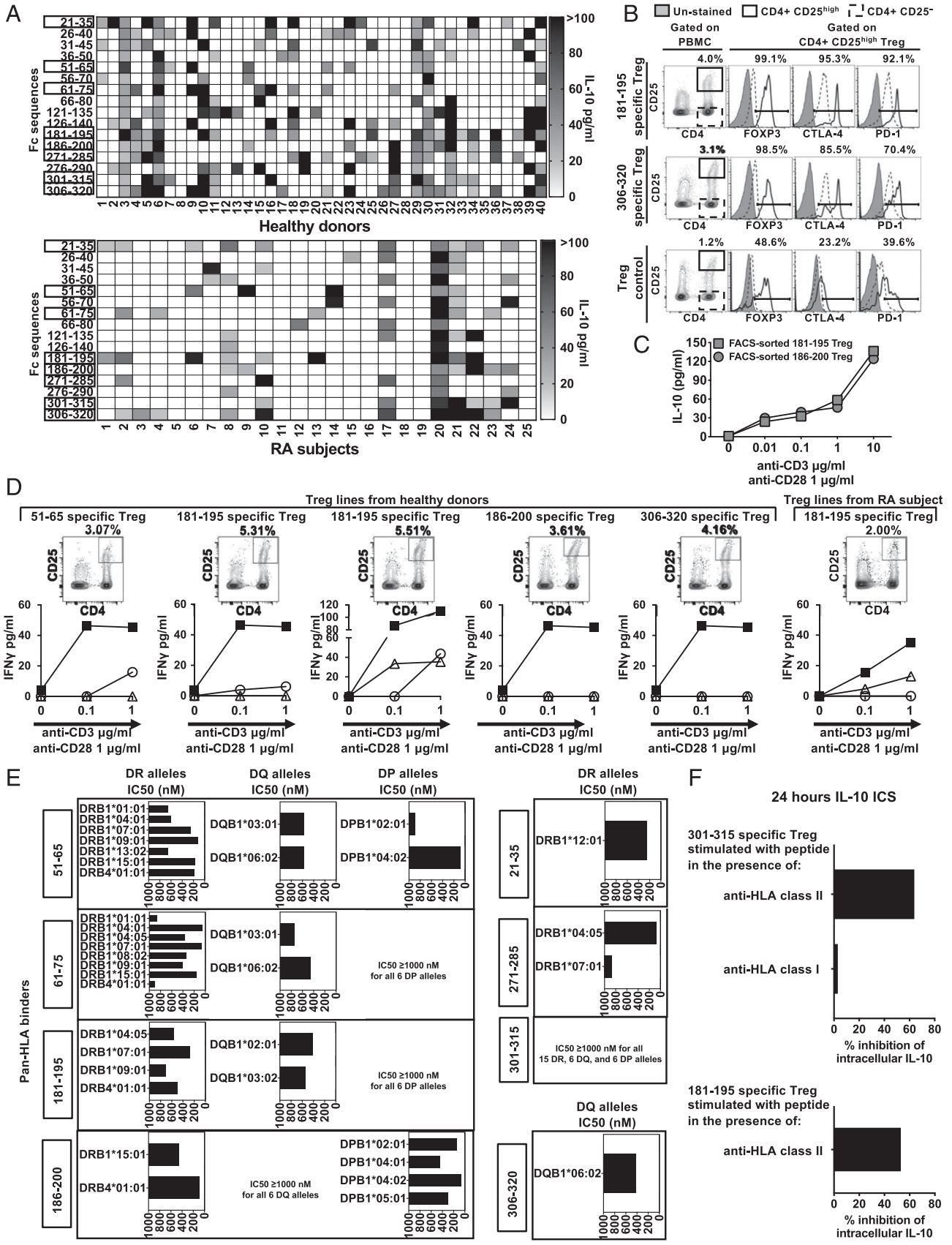

FIGURE 1. Treg fine specificity, phenotype, function, HLA class II restriction, and binding affinity of the eight immunodominant Fc peptides. (**A**) The heat maps show IL-10 secretion by PBMC cultures in response to 20 μg/ml ≥97% pure Fc peptides from 40 healthy donors and 25 RA subjects. IL-10 responses were below the level of detection in 3 out of 40 healthy donors (numbers 8, 28, and 45) and 5 out of 25 RA subjects (numbers 5, 11, 18, 19, and 25). One healthy donor (28) and one RA subject (11) showed a high base level of IL-10 in the unstimulated control. The other subjects possibly responded to Fc peptides not tested. (**B**) Phenotype of two representative Treg lines that recognize Fc 181–195 and Fc 306–320 and a *(Figure legend continues)*

T cells by Treg. Treg lines were equally effective in downregulating IFN-γ production by naive T cells regardless of their specificity, suggesting that different Fc sequences are equally potent in stimulating Treg effector regulatory functions. Of note, IFN-γ production by anti-CD3/anti-CD28–stimulated naive T cells was dramatically reduced not only in Treg-naive T cell cocultures, but also in naive T cell cultures that contained Treg culture supernatants (Fig. 1D). These results demonstrated that cognate interactions between Treg and naive T cells are not required to downregulate the development of proinflammatory Th1 cells from the naive T cell repertoire.

Next, the binding affinities of the eight immunodominant peptides were tested using a panel of 27 different HLA class II molecules. This panel was representative of the most common allelic variants found worldwide in mixed ethnicities (corresponding to our donor cohort) and encompassed all four HLA class II loci (DRB1, DRB3/4/5, DQ, and DP) (18). The data indicated that among the eight immunodominant Fc sequences, four peptides (Fc 51–65, Fc 61–75, Fc 181–195, Fc 186–200) bound multiple HLA class II alleles (Fig. 1E, left panels), one peptide (Fc 271–285) bound two DR alleles, two peptides (Fc 21–35 and Fc 306–320) showed monogamous restriction, and one peptide (Fc 301–315) bound poorly all the alleles tested (Fig. 1E, right panels). Despite the poor binding affinity, the HLA class II restriction of 301–315 was formally proven with a FACS-sorted Treg line restimulated with the peptide in the presence of an antagonistic Ab to human HLA class II. A Treg line specific for the pan-HLA binder peptide 181–195 served as control in this experiment, for which the readout was IL-10 intracellular staining 24 h after stimulation (Fig. 1F). In summary, several peptides bound different HLA alleles, explaining the broad Treg responses in the two cohorts studied that included subjects of diverse ethnicities (Supplemental Table I).

Ag processing of surface IgG by IgG+ B cells

The next question was how Fc-specific Treg recognize the Fc epitopes. We started by addressing the Ag-presenting role of mature IgG+ B cells that internalize, process, and present peptides from the surface IgG (cartoon model in Fig. 2A). Surface Ig is internalized very rapidly on B cells (8), and its Ag processing is rapid and efficient, with fast recycling of the IgG molecules. We also explored the Treg homing receptors for the lymph nodes and the germinal centers CCR7 and CXCR5, in which IgG+ B cells reside, and ICOS, which binds ICOS-L, a costimulatory molecule mostly expressed on B cells (19). The experimental design involved the following: 1) generation of Treg lines from healthy donors and RA subjects specific for the peptides that ranked high within the immunodominance, 2) FACS sorting of peptide-specific Treg 4 d after stimulation, 3) FACS sorting of autologous IgG+ and IgG− B cells, and 4) establishment of B cell–Treg cocultures in the absence of exogenous Fc and peptides. IL-10 secretion in the cocultures was the functional readout in these experiments. IgG+ B cell–Treg cocultures (5:1 and 10:1 ratios) were established with Treg lines that recognized Fc peptides 51–65, 181–195, 186–200, 271–285, and 306–320, representing different ranks in immunodominance and HLA binding in healthy donors and in RA subjects. Autologous IgG− B cells were used as controls to rule out autoreactive Treg responses to self-HLA molecules. The gating strategies for FACS sorting of Treg and IgG+ B cells and the lack of contaminating innate cells in the IgG+ B cell preparations is shown in Supplemental Fig. 3. The results of the B cell–Treg cocultures showed that IgG+ B cells presented all five Fc peptides tested to Treg derived from both healthy donors and RA subjects, regardless of their ranking (Fig. 2B). IL-10 in the culture supernatants was significantly increased when Treg cocultured with IgG+ B cells were compared with the cocultures with IgG B cells (Treg from healthy donors: $p = 0.0004$; Treg from RA subjects: $p = 0.0102$) (Fig. 2C). B cell purity after FACS sorting is shown in Supplemental Fig. 3B. IgG+ and IgG− B cells cultured alone did not secrete IL-10 (Fig. 2B).

To determine the unique presentation of Fc peptides by IgG+ B cells to Ag-specific Treg, we generated two Treg lines from the same healthy donor that recognized the ApoB peptide 3036–3050 and Fc 181–195 and tested them in coculture with FACS-sorted autologous IgG+ B cells. The results confirmed that only Fc epitopes were presented by IgG+ B cells (Fig. 2D). These results, together with the chemokine receptor expression, suggested Treg homing to the germinal centers.

Next, to address the role of ICOS–ICOS-L costimulation as a secondary signal for Fc-specific Treg activation and expansion, we tested IgG+ B cell presentation to two Treg lines that recognized Fc 181–195 and one Treg line that recognized Fc 306–320 in the presence or absence of an antagonistic Ab to ICOS-L. ICOS-L blockade strongly inhibited IL-10 secretion by Treg, suggesting that ICOS–ICOS-L interactions are essential costimulatory molecules mediating a productive TCR signaling for the activation of this Treg population (Fig. 2E).

Fc processing and peptide presentation by DC to Treg

An open question was how Fc-specific Treg expand after initial activation by IgG+ B cells. One possibility is that the exogenous Fc (circulating Igs) is processed and presented by classical APC, such as DC, that restimulate and expand Treg, explaining their high precursor frequency in circulation. To address this question, we studied

control Treg line nonspecific for the peptides. Percentage of $CD4^+$ $CD25^{high}$ Treg in PBMC stimulated with peptides and characterization of their expression of intracellular FOXP3 and surface CTLA-4 and PD-1. Solid lines represent $CD4^+$ $CD25^{high}$ Treg; dotted lines represent $CD4^+$ $CD25^-$ T cells; gray histograms represent unstained controls. (**C**) IL-10 secretion by two Treg lines restimulated with scalar doses of anti-CD3 (0.01, 0.1, 1, and 10 μg/ml) and 1 μg/ml anti-CD28 4 days after priming with Fc 181–195 and Fc 186–200 from healthy donor 29. (**D**) Treg suppression was explored by measuring the downregulation of the polarization of autologous $CD4^+$ naive T cells toward a Th1 proinflammatory phenotype. Upper panels, FACS-sorting strategy for $CD4^+$ $CD25^{high}$ Treg lines that recognize Fc 51–65, 181–195, 186–200, and 306–320 derived from two healthy donors and one RA subject 4 d after in vitro priming in the absence of exogenous lymphokines. Bottom panels, Cocultures of FACS-sorted, autologous $CD4^+$ $CD45RA^+$ naive T cells with FACS-sorted Treg lines (1:1 ratio, 5×10^3 cells per well) (open circle) or with Treg culture supernatants (open triangle) stimulated with anti-CD3 and anti-CD28 agonistic Abs. Naive T cells alone (solid square) served as controls in these experiments. IFN-γ secretion in culture supernatants was measured by ELISA after 72 h in culture. (**E**) The binding affinities of the eight immunodominant Fc peptides to 15 HLA-DR alleles, six HLA-DQ alleles, and six HLA-DP alleles were measured using a competition assay based on the inhibition of the binding of a high-affinity radiolabeled peptide to purified HLA molecules expressed as IC_{50} with an inverted *x*-axis. (**F**) HLA class II restriction of Fc 301–315 that bonded HLA class II alleles with low affinity. FACS-sorted Treg lines specific for Fc 301–315 were restimulated with irradiated, autologous PBMC and Fc peptide (20 μg/ml) in the presence of anti–HLA class II framework antagonistic Ab (10 μg/ml) for 24 h. Anti–HLA class I antagonistic Ab served as control. A Treg line specific for the pan-HLA class II binder 181–195 was tested side by side in this experiment. Intracellular IL-10 in $CD4^+$ $CD25^{high}$ Treg served as a readout. Data are presented as the percentage inhibition of IL-10 in Treg cultures restimulated with peptides in the presence of HLA class II blockade.

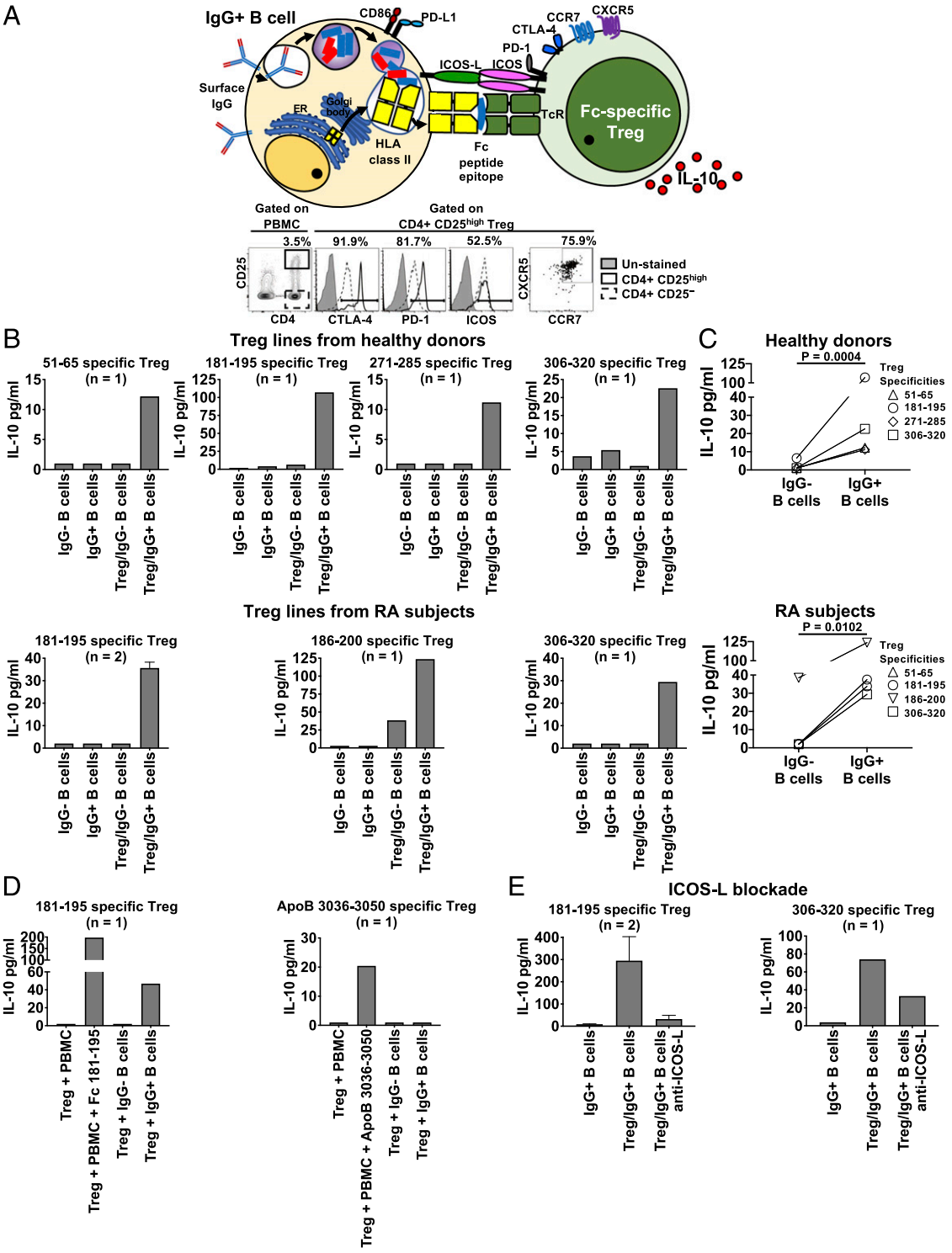

FIGURE 2. Processing and presentation of Fc peptides by autologous IgG$^+$ B cells to FACS-sorted, Fc peptide-specific Treg lines. (**A**) Cartoon model for IgG$^+$ B cell presentation to Treg phenotype that shows the double expression of the chemokine receptors CCR7 and CXCR5 and the costimulatory molecule ICOS on a representative Fc 181–195–specific Treg line. Percentage of CD4$^+$ CD25^{high} Treg in total PBMC is shown in the far left panel with their expression of CTLA-4, PD-1, CCR7, ICOS, CCR7, and CXCR5. Solid lines represent CD4$^+$ CD25^{high} Treg; dotted lines represent CD4$^+$ CD25$^-$ T cells; gray histograms represent unstained controls. (**B**) FACS-sorted, Fc peptide-specific Treg lines were cocultured with autologous IgG$^+$ B cells in the absence of exogenous Fc protein or peptides at a Treg/B cell ratio of 1:5 (Fc 51–65– and Fc 306–320–specific Treg lines from healthy donors; Fc 181–195– and Fc 306–320–specific Treg lines from RA patients) or 1:10 (Fc 181–195– and Fc 271–285–specific Treg lines from healthy donors; Fc 186–200–specific Treg line from an RA patient), depending upon the number of IgG$^+$ B cells obtained after FACS sorting. Treg cocultured with IgG$^-$ B cells served as a control for autoreactive Treg responses to self-HLA molecules in these experiments. IgG$^-$ and IgG$^+$ B cells cultured alone served as controls for the IL-10 secretions by B cells. (**C**) Dot plots show the IL-10 secretions from four Treg lines from healthy donors (upper panel) and four Treg lines from RA subjects (lower panel) cocultured with IgG$^-$ and IgG$^+$ B cells. The differences in IL-10 secretion from Treg lines in response to IgG$^-$ and IgG$^+$ B cells were assessed by two-tailed paired *t* tests. A *p* value <0.05 was considered to be statistically significant. (**D**) Treg lines specific for Fc 181–195 or ApoB 3036–3050 from an additional healthy donor were FACS sorted and cocultured with FACS-sorted autologous IgG$^+$ B cells (Treg/B cell ratio of 1:10). Treg cocultured with IgG$^-$ B cells or with irradiated, autologous PBMC in the presence of peptide served as controls. IL-10 secretion was measured *(Figure legend continues)*

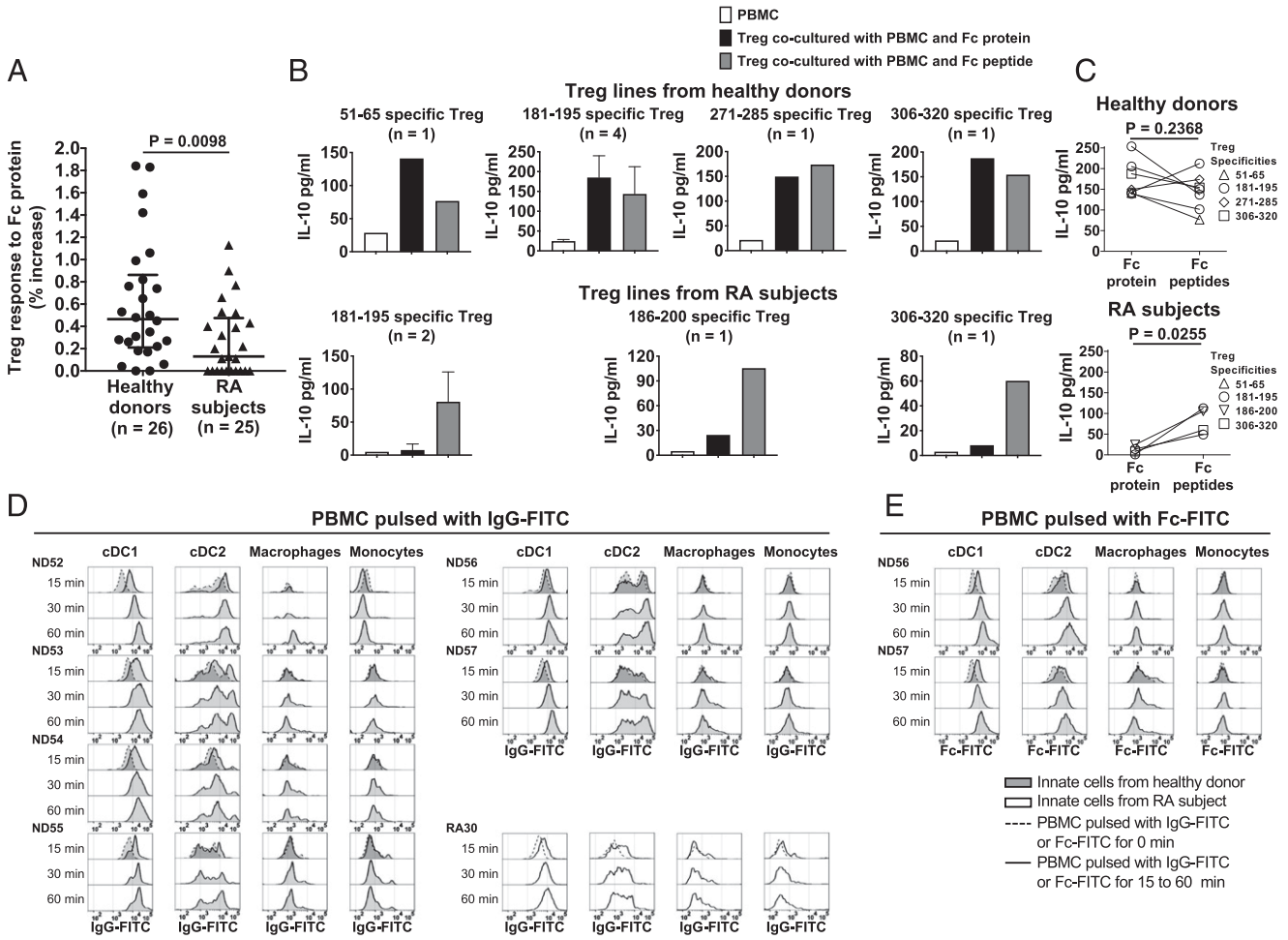

FIGURE 3. Treg expansion in response to the exogenous Fc protein presented by DC. (**A**) Percentage increase of $CD4^+$ $CD25^{high}$ T cells in PBMC cultures in response to 100 μg/ml Fc protein versus unstimulated controls. Twenty-six healthy donors (numbers 1–26) and 25 RA subjects (numbers 1–25) were studied (Supplemental Table I). (**B**) FACS-sorted Treg lines were cocultured with irradiated, autologous PBMC pulsed with the whole Fc protein (black bars) or Fc peptides (gray bars). Irradiated PBMC (white bars) served as controls in these experiments. IL-10 secretion was measured in culture supernatants by ELISA 72 h after coculture. Treg lines from different subjects specific for the same peptides are shown in the same graphs, and the mean ± SD are indicated. (**C**) Dot plots show the IL-10 secretions from the seven Treg lines from healthy donors (upper panel) and four Treg lines from RA subjects (bottom panel) cocultured with PBMC in the presence of Fc protein and Fc peptides. The differences of IL-10 secretion from Treg lines in response to Fc protein and Fc peptides were assessed by two-tailed paired *t* tests. A p value <0.05 was considered to be statistically significant. (**D**) Internalization of IgG1-FITC in cDC1, cDC2, macrophages, and monocytes from six healthy donors and one RA subject. PBMC were pulsed at 37°C, 5% CO_2 with IgG1-FITC in a time curve (0, 15, 30, and 60 min), washed, and fixed with paraformaldehyde prior to surface staining with mAbs to identify innate cells by surface markers. The histograms show the uptake of IgG1-FITC in a time course by different innate cells from six healthy donors (filled histograms) and one RA subject (empty histograms). The histograms with dotted line indicate the baseline of IgG-FITC at the time point 0. (**E**) The internalization of the Fc portion by innate cells from two out of six healthy donors was tested with a FITC-labeled Fc rather than the intact Ig, leading to comparable results.

the Treg expansion in response to purified Fc fragments in PBMC derived from healthy donors and RA subjects previously studied for peptide recognition. $CD4^+$ $CD25^{high}$ enumeration by flow cytometry on day 4 after culture served as the readout in these experiments.

In RA, $CD4^+$ $CD25^{high}$ Treg were less numerous in Fc-stimulated PBMC cultures than in healthy donors, suggesting reduced expansion, (Fig. 3A, Supplemental Fig. 4), although Treg from RA responded to minimal peptide epitopes, which do not require Ag processing, and to IgG^+ B cells.

To explain these results and to better understand the presentation of Fc peptides to Treg from the exogenous processing of Igs, we studied the presentation of purified Fc fragments by irradiated PBMC (that include DC, macrophages, and monocytes) to Treg lines specific for the same peptides naturally processed by IgG^+ B cells. Treg lines derived from four healthy donors that were specific for Fc 51–75, Fc 181–195, Fc 271–285, and Fc 306–320 were FACS sorted from PBMC stimulated for 4 d in vitro with peptides and restimulated in cocultures with autologous irradiated PBMC pulsed with purified Fc protein (100 μg/ml) or Fc peptides

in culture supernatants by ELISA 3 d after stimulation. (**E**) The requirements for ICOS–ICOS-L costimulation for a productive TCR signaling is shown with three representative Treg lines: two that recognized 181–195 and one that recognized 306–320 derived from two healthy donors. FACS-sorted Treg were cocultured with autologous IgG^+ B cells in the presence of an antagonistic Ab to ICOS-L. IL-10 secretion was measured in culture supernatants at day 3 from stimulation by ELISA. IgG^+ B cells cultured alone served as a control for the IL-10 secretions by B cells. Treg lines with the same specificities generated from different subjects are shown in the same graphs, and the mean ± SD are indicated.

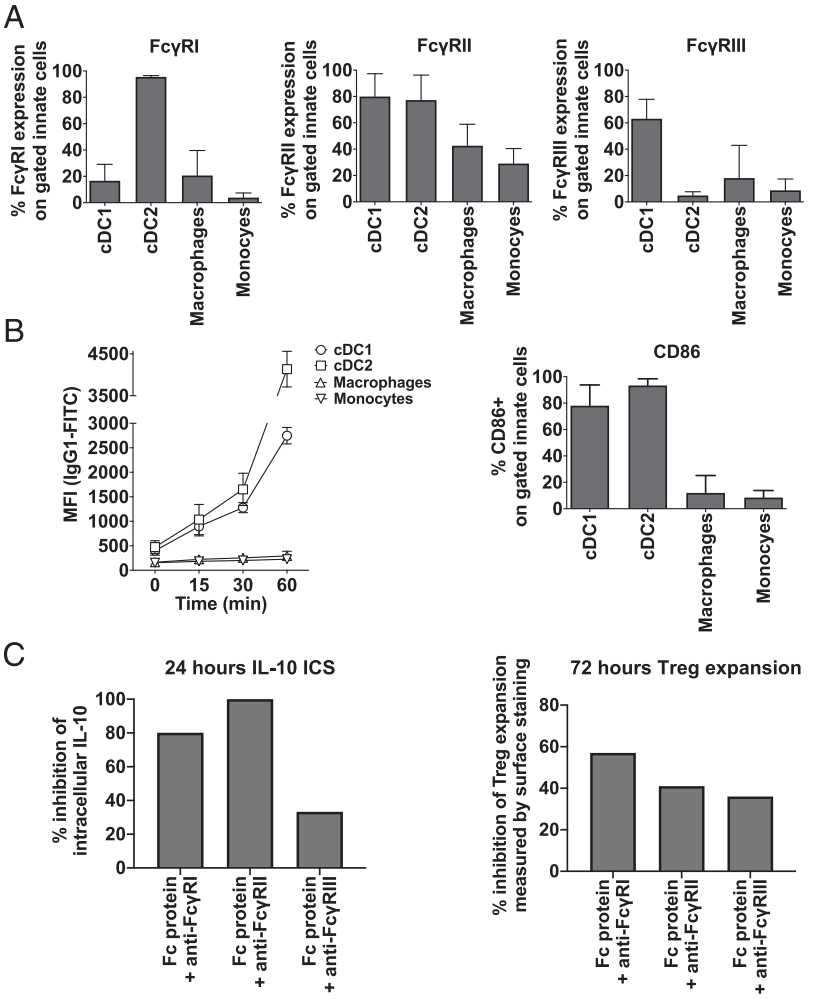

FIGURE 4. FcγRs expression on different innate cells and their role in expanding Fc-specific Treg. We studied the FcγRs expression, maturation stage, and internalization of IgG-FITC in different innate cell types from four additional healthy donors (**A**) Expression of FcγRI, FcγRII, and FcγRIII by cDC1, cDC2, macrophages, and monocytes. (**B**) IgG internalization at different time points by innate cells and their CD86 expression. Data are presented as mean fluorescence intensity (MFI), and the mean ± SD are indicated. (**C**) Inhibition of Treg expansion by functional blockades of FcγRI, FcγRII, or FcγRIII. PBMC were stimulated with Fc protein (100 μg/ml) with or without antagonistic Abs specific for FcγRI, FcγRII, or FcγRIII (10 μg/ml). The percentage inhibition of intracellular IL-10 in $CD4^+$ $CD25^{high}$ T cells in the presence of FcγRs blockades 24 h after simulation is shown in the left panel. The percentage inhibition of $CD4^+$ $CD25^{high}$ Treg 72 h after cultures is shown in the right panel.

(20 μg/ml). The IL-10 response was measured by ELISA in the culture supernatants 72 h later. All seven Treg lines from healthy donors responded with high IL-10 secretion to the exogenous soluble Fc (Fig. 3B, upper panel). Furthermore, Treg lines showed similar IL-10 responses to both Fc protein and Fc peptides ($p = 0.2368$) (Fig. 3C, upper panel).

Different results were obtained with the response to the Fc presented by PBMC to Treg lines from three RA subjects that recognized Fc 181–195, Fc186-200, and Fc 306–320 (Fig. 3B, bottom panel). All the Treg lines tested showed a significantly lower IL-10 response to the Fc protein presented by innate cells within PBMC as compared with Fc peptides ($p = 0.0255$) (Fig. 3C, bottom panel). These results are in sharp contrast to the Treg response to IgG^+ B cells via processing of the surface Ig of the same peptide epitopes that we previously showed.

To better understand whether the amount of Fc internalized by innate cells could play a role in a lesser peptide presentation to Treg, we studied the uptake of IgG in myeloid DC (cDC1 and cDC2), macrophages, and monocytes at different time points (15, 30, and 60 min) after incubation with a purified FITC-labeled IgG1 and an Fc-FITC. The results suggested a different uptake of the IgG and Fc depending upon timing and cell types; cDC1 ($CD11c^+$ $CD11b^{-\text{ or low}}$), and cDC2 ($CD11c^+$ $CD11b^+$) were very efficient at internalizing the IgG1-FITC in contrast to macrophages ($CD11c^-$ $CD11b^+$ $CD14^+$) and monocytes ($CD11c^-$ $CD11b^-$ $CD14^+$) (Fig. 3D).

In RA, cDC1 and cDC2 internalized the IgG but did so less efficiently and in a longer time, with the main differences at 30 and 60 min (Fig. 3D). These results suggested that the amounts of peptides processed and presented over time by cDC1 and cDC2 may not be sufficient to properly stimulate Treg.

IgG are known to be taken up by DC in the form of immune complexes. To exclude this possibility in our system, we repeated the experiments by enrolling two additional healthy donors and used Fc fragments rather than the intact Ig to study internalization by different APC types. The results obtained with the Fc alone were similar to the uptake of the IgG (Fig. 3E).

We then investigated the FcγRs expression in cDC1, cDC2, macrophages, and monocytes from four healthy donors and their ability to internalize IgG. The specific role of FcγRs in inhibiting Fc internalization and in turn Treg expansion was also explored in blockade experiments with antagonistic Abs. FcγRI was high on cDC2, FcγRII was high on cDC1 and cDC2, and FcγRIII was mainly on cDC1 (Fig. 4A).

cDC1 and cDC2 efficiently internalized IgG and expressed the activation/maturation marker CD86 while internalizing the IgG (Fig. 4B). Of interest, $CD14^+$ monocytes did not play an important role in internalizing Igs, and $FcγRIII^+$ monocytes, reported as proinflammatory, were scarce (Fig. 4B). Our experimental design could not exclude the role of surface binding of the IgG to FcγRs over time when we measured IgG uptake and internalization. However, macrophages were much less efficient than cDC1 and cDC2 in internalizing the Fc despite their expression of FcγRI, FcγRII, and FcγRIII (Fig. 4A), suggesting that the Fc protein is indeed internalized. All three FcγRs appeared to be relevant for Fc uptake and processing for Treg presentation. However, the

blockade of FcγRII led to an inhibition of 100% of intracellular IL-10 by Treg, indicating that FcγRII is the predominant FcγR involved in the Fc internalization for Ag processing. The other two FcγRs were also involved with the Fc Ag processing for Treg presentation; the blockade of FcγRI leaded to a substantial inhibition of intracellular IL-10 by Treg (79.5%). FcγRIII was also found somehow involved in the Fc Ag processing for Treg recognition (Fig. 4C). In conclusion, the functional blockade of all three receptors in Fc-stimulated PBMC, with a predominant role for FcγRII, resulted in a profound inhibition of IL-10 secretion by CD4$^+$ CD25^{high} T cells measured 24 h after stimulation and reduced CD4$^+$ CD25^{high} Treg expansion measured by surface staining (Fig. 4C).

Discussion

In this study, we characterized an important set of peptide epitopes derived from the heavy C region of IgG1 and their HLA affinity for presentation to human thymic-derived nTreg. Functionally, Treg suppressed CD4$^+$ naive T cell differentiation toward a Th1 proinflammatory phenotype in a cognate and noncognate fashion. These experiments showed a differential processing of the Fc by IgG$^+$ B cells that recycle the Ig compared with the exogenous processing of the Fc by specific DC populations.

There is great therapeutic potential in the harnessing the ability of Treg to control inflammation. Academic and industrial efforts are undergoing to treat a variety of inflammatory conditions via Treg transfer. In the absence of known human thymic-derived Treg fine specificities and the limited numbers of Treg in circulation, different approaches are under development, including FOXP3 transfection in bulk CD4$^+$ T cells, to expand iTreg. Treg transfer is certainly less efficient and much more expensive than the direct stimulation of the relevant Treg populations with the appropriate peptide sequences, as demonstrated by our work.

The Fc regions that we defined as immunodominant have been reported in the Immune Epitope Database and Analysis Resource after elution from HLA-DQ molecules in B cell lines from celiac disease patients (20), from B cell lines derived from subjects with DRB1*08:01-DQA1*04:01/DQB1*04:02 (DR8-DQ4) haplotypes (21), from chronic lymphocytic leukemia cell lines (22), and membranal and blood-soluble HLA (23). Therefore, the Fc peptides that we defined as immunodominant for Treg recognition are naturally processed and presented to T cells.

The internalization of the exogenous IgG occurred mostly in cDC1 and cDC2 CD86^{high} through all the FcγRs. In RA, FcγRII and FcγRIII have several isoforms that are linked to disease susceptibility (24, 25). Future genetic and phenotypic studies should address a possible link between FcγRII genetic variants and their correlation with Fc-Treg expansion in this patient population.

The important role of IgG$^+$ B cells in the activation of Fc-specific Treg confirmed previous data on the rapid internalization of the surface IgG on B cells (8). However, in children with KD, Fc-specific Treg are not detectable prior to IVIG. After IVIG, Fc-specific Treg expanded but not in the cohort that developed aneurysms (6). In the KD model, the expansion of Treg correlated with lower numbers of activated CD4$^+$ and CD8$^+$ T cells in circulation, suggesting that experienced T cells can be regulated by Treg in addition to naive T cells (6). IVIG is also successful in treating the new condition in children exposed to the SARS-CoV-2 virus, the Multisystem Inflammatory Syndrome in Children (26–28). Fc-specific Treg may be key in downregulating inflammation in these patients, and this is currently being addressed by our laboratory. The characterization of the immunomodulatory Fc peptides may explain the therapeutic effect of IVIG in neurologic diseases for which autoreactive T cells are pathogenic (29–31). In support of this hypothesis, the most immunogenic Fc peptide 306–320 in our study was restricted to DQB1*06:02, an HLA allele strongly associated with narcolepsy and Alzheimer disease, conditions for which IVIG has benefit (32, 33).

The fine specificity of the Treg indicated that conserved, immunodominant peptides derived from several regions of the Fc of the IgG1 were highly immunogenic, leading to a rapid T cell expansion in vitro. The shared recognition of these Fc epitopes was likely due to the fact that four of the eight immunodominant peptides bound multiple HLA alleles covering the HLA diversity of the general population. This has important implications for the therapeutic potential of these peptides for inflammatory conditions.

It is well known that the Ag processing of the surface Igs with the $(Fab)_2$ bound to cognate Ags and their presentation by B cells activates pathogen-specific T cells (34, 35). However, a role for IgG$^+$ B cells in downregulating inflammation by expanding Treg via the processing of the heavy C region and presentation of its peptides has not been previously described. The costimulatory pair ICOS/ICOS-L was essential for Fc-specific Treg expansion, suggesting that agonistic Abs to ICOS may improve therapeutic efforts involving in vivo Treg expansion.

Major efforts have been made to improve Ab isotypes for cancer and antiviral therapy by Fc glycan engineering (36). In cancer settings, the effort is directed toward enhancing proinflammatory responses to the cancer cell or pathogen. Our results suggested that it may also be important to consider the engineering of Fc depleted of the sequences that are immunogenic for Treg responses.

Treg regulate T cell responses via different mechanisms. Most recently, it has been suggested that naive T cells are suppressed by Treg that deplete MHC class II and the cognate Ag from DC, thus reducing Ag presentation to naive T cells (37). In our human model, cognate interactions were not required for the Fc-specific Treg to downregulate IFN-γ production by naive T cells. However, IL-10 downregulates MHC class II αβ peptide complexes at the plasma membrane of APC by affecting the Ag presentation (38). Also, the reduced IFN-γ secretion by effector cells mediated by Treg in lymph nodes could potentially decrease MHC class II molecule expression on monocytes and DC and thus further jeopardize the activation and polarization of naive T cells.

We recognize some limitations of our work. Our studies were hampered by the lack of access to human tissues and manipulation of the immune system to directly demonstrate the ontogeny of Fc-specific Treg.

In summary, we present, in this study, a model for mature IgG$^+$ B cells in controlling inflammation by expanding thymic-derived nTreg via a unique Ag processing that shapes adaptive regulation through the presentation of Fc peptides.

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Disclosures

The authors have no financial conflicts of interest.

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