

Inhibition of differentiation, amplification, and function of human T_H17 cells by intravenous immunoglobulin

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Background: T_H17 cells play a critical role in the pathogenesis of several autoimmune and allergic diseases. Intravenous immunoglobulin (IVIg), a therapeutic preparation of polyclonal IgG that is increasingly used in the treatment of diverse autoimmune and allergic diseases, might target T_H17 cells to exert therapeutic effects.

Objective: We sought to examine whether IVIg interferes with the development and function of human T_H17 cells.

Methods: T_H17 cells were differentiated from naive human CD4⁺ T cells in the presence of TGF- β and IL-21. T_H17 cells were amplified by stimulating memory CD4⁺ T cells in the presence of IL-1 β and IL-6. The effect of IVIg was examined on the differentiation and amplification of T_H17 cells, expression of the T_H17 lineage-specific transcription factor retinoic-acid-related orphan receptor C, secretion of T_H17 effector cytokines, and phosphorylation of signal transducer and activator of transcription 3, a transcription factor that plays an important role in T_H17 cell development and function.

Results: IVIg inhibits the differentiation and amplification of human T_H17 cells, as well as the production of their effector cytokines IL-17A, IL-17F, IL-21, and CCL20. The inhibitory effects of IVIg on T_H17 cells are F(ab')₂ dependent and involve interference with the expression of retinoic-acid-related orphan receptor C and activation of signal transducer and activator of transcription 3. Also, IVIg significantly enhanced forkhead box protein 3–positive regulatory T cells among the memory CD4⁺ T cells.

Conclusion: These results reveal a novel mechanism of action of IVIg in achieving a therapeutic effect in autoimmune and

allergic diseases, in which T_H17 cells play a key modulatory role in sustaining the chronic inflammatory response. Our results also suggest a reciprocal regulation of T_H17 and regulatory T-cell populations by IVIg. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Intravenous immunoglobulin, allergy, autoimmunity, inflammation, T_H17 cells, IL-17, regulatory T cells, retinoic-acid-related orphan receptor C, forkhead box protein 3

CD4⁺ T_H cells are the major components of the adaptive immune system that play a prime role in the sustenance and regulation of the antigen-specific immune response. On activation, under the influence of the cytokine milieu, naive T_H cells differentiate into various subsets with distinct functions. These subsets include T_H1, T_H2 and induced regulatory T (Treg) cells. Recently, T_H17 cells, a novel subset of T_H cells that express the lineage-specific transcription factor retinoic-acid-related orphan receptor C (RORC) (ROR γ t in mice) and secrete the effector cytokines IL-17A and IL-17F, have been identified. T_H17 cells require signal transducer and activator of transcription 3 (STAT3) signaling for their differentiation and amplification.^{1,2}

In addition to their role in protection against extracellular pathogens, such as fungi, T_H17 cells play a critical role in the pathogenesis of several autoimmune, allergic, and inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, psoriasis, allergic rhinitis, asthma, allergic contact dermatitis, systemic lupus erythematosus, anti-neutrophil cytoplasmic antibody–associated vasculitis, dermatomyositis, and pemphigus.^{3–9} T_H17 cells produce IL-17A, IL-17F, IL-21, IL-22, and CCL20, which induce massive tissue reactions by promoting the recruitment of inflammatory cells.³ These effector cytokines, in addition to immune cells, also target nonimmune cells, such as fibroblasts, endothelial cells, and epithelial cells, to induce the production of the proinflammatory mediators IL-6, TNF- α , IL-1 β , prostaglandin E₂, nitric oxide, matrix metalloproteinases, and chemokines (CXCL1, CXCL8, and CCL2).^{1–10} Furthermore, the development of T_H17 cells is dependent on the inflammatory cytokines IL-6, IL-21, IL-1 β , and IL-23, thus sustaining the vicious inflammatory circle.¹¹

Both experimental and clinical studies have clearly demonstrated the role of T_H17 cell–derived cytokines in the pathogenesis of several autoimmune, allergic, and inflammatory diseases.^{3,11,12} Thus new therapeutic strategies that can efficiently interfere with the development and function of T_H17 cells are being explored.^{13–17} In this context intravenous immunoglobulin (IVIg) represents one of the promising approaches that might target T_H17 cells.

IVIg, a therapeutic preparation of polyclonal IgG obtained from pooled plasma of several thousand healthy donors, is being

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Abbreviations used

APC: Antigen-presenting cell
 Foxp3: Forkhead box protein 3
 HSA: Human serum albumin
 IVIg: Intravenous immunoglobulin
 pSTAT3: Phosphorylated STAT3
 RORC: Retinoic-acid-related orphan receptor C
 STAT3: Signal transducer and activator of transcription 3
 Treg: Regulatory T

increasingly used in the treatment of diverse autoimmune, allergic, and inflammatory diseases.¹⁸⁻²⁶ IVIg is believed to exert beneficial effects in patients through several mutually non-exclusive mechanisms.^{18,27-30} Furthermore, IVIg can directly interact with T cells to exert immunomodulatory effects.^{18,31} Interestingly, a therapeutic effect of IVIg has also been demonstrated in autoimmune diseases in which T_H17 cells have been implicated in the pathogenesis.^{3,18,20,22} Therefore we hypothesized that IVIg modulates human T_H17 cell differentiation, propagation, and function to exert beneficial effects in autoimmune patients.

METHODS**Cell-culture reagents and antibodies**

The mAbs anti-CD3 (clone UCHT1) and anti-CD28 (clone 37407) and the cytokines TGF- β , IL-1 β , and IL-6 were from R&D Systems (Lille, France). IL-21 and plasma-derived human serum albumin (HSA) were from ImmunoTools (Friesoythe, Germany) and Laboratoire Française de Biotechnologies (Les Ulis, France), respectively.

Therapeutic IVIg

Octagam (Octapharma, Vienna, Austria) was used throughout the study. Octagam (100 mg/mL; 0.6 mmol/L) was dialyzed against a large volume of PBS followed by RPMI-1640 medium at 4°C for 18 hours to remove the stabilizing agents.³² F(ab')₂ fragments of IVIg were prepared by means of pepsin digestion (2% wt/wt; Sigma, St Louis, Mo) followed by chromatography on a protein G Sepharose column (Pharmacia, Uppsala, Sweden). F(ab')₂ fragments were free of intact IgG and Fc fragments, as assessed by means of SDS-PAGE and ELISA. Octagam contained a negligible amount of T-cell cytokines (0-5 pg/mL/100 mg) and tested negative for endotoxins (ToxinSensor Chromogenic LAL Endotoxin Assay Kit; GenScript, Piscataway, NJ).

Cell purification

PBMCs were purified from buffy coats of healthy donors. Untouched total CD4⁺ T cells were isolated by means of negative selection with the CD4⁺ T-cell isolation kit II (Miltenyi Biotech, Paris, France). Furthermore, naive (CD45RO⁻) and memory (CD45RO⁺) CD4⁺ T cells were separated with CD45RO microbeads (Miltenyi Biotech). Finally, CD4⁺CD25⁻CD45RO⁻ naive T cells were obtained by depleting CD25⁺ cells with CD25 microbeads (Miltenyi Biotech) to remove the recently activated cells among the naive CD4⁺ T cells. The purity of cells was greater than 95% in all the subpopulations.

T-cell stimulation and culture

Ninety-six-well U-bottom plates were coated with 1.5 μ g/mL anti-CD3 mAb. Plates were then rinsed once with serum-free RPMI-1640 medium. CD4⁺ T cells (5×10^4 per well/200 μ L) were stimulated in serum-free X-VIVO 15 medium with soluble anti-CD28 mAb (1.0 μ g/mL) in the presence of cytokines that included TGF- β (5 ng/mL) and IL-21 (25 ng/mL) for naive T cells and IL-1 β (12.5 ng/mL) and IL-6 (25 ng/mL) for memory T cells. Equimolar concentrations (0.15 mmol/L) of IVIg (25 mg/mL), F(ab')₂ fragments (16 mg/mL), or HSA (10 mg/mL) were added to the cells after 12 hours of

initiation of culture. The stimulated T cells were cultured for 5 to 6 days at 37°C in 5% CO₂. Supernatants were collected either daily or at the end of culture for the analysis of cytokines, and cells were used for intracellular staining. In addition, dose-response and time-response curves of IVIg were also performed.

Intracellular staining and cytokine assays

For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Sigma, Saint Quentin Fallavier, France) and ionomycin (500 ng/mL, Sigma) at 37°C for 5 hours with GolgiStop (BD Biosciences, Le Pont de Claix, France) for the last 3 hours. Surface staining was done with fluorescence-conjugated CD4 mAb (BD Biosciences). Cells were fixed, permeabilized (Fix/Perm; eBioscience, Paris, France), and incubated at room temperature with fluorescence-conjugated mAbs to RORC (eBioscience), IFN- γ (BD Biosciences), IL-17A (eBioscience), and forkhead box protein 3 (Foxp3; eBioscience). Ten thousand cells were acquired for each sample, and the data were processed by using FACS DIVA software (BD Biosciences).

Intracellular phosphorylated STAT3 (pSTAT3) staining was performed on activated T cells at different time points. Cells were harvested and fixed in prewarmed BD Cytofix buffer by incubating for 10 minutes at 37°C. Fixed cells were washed twice with staining buffer (1% FCS/PBS) and then permeabilized with chilled BD Phosflow Perm Buffer III on ice for 30 minutes. Cells were then washed twice with staining buffer, and phycoerythrin-conjugated anti-STAT3 pY705 (BD Biosciences) was added to cells and incubated at room temperature for 45 to 60 minutes. Cells were washed and suspended in staining buffer before data acquisition.

Levels of IL-17A and CCL20 (DuoSet ELISA kits, R&D Systems), IL-17F (ELISA Ready-SET-Go, eBioscience), and IL-21 (ELISA MAX Deluxe Sets, BioLegend, San Diego, Calif) in cell-free culture supernatants were quantified by means of ELISA. Cutoff values were 15 pg/mL for IL-17A, 30 pg/mL for IL-17F, 16 pg/mL for IL-21, and 15 pg/mL for CCL20 (see Table E1 in this article's Online Repository at www.jacionline.org).

Statistical analysis

The paired Student *t* test was used to determine the statistical significance of the data. A *P* value of less than .05 was considered significant.

RESULTS**Inhibition of differentiation and function of human T_H17 cells by IVIg**

For the differentiation of human T_H17 cells, CD4⁺CD45RO⁻CD25⁻ naive T cells were cultured in serum-free medium in the presence of T-cell receptor stimulators and TGF- β and IL-21 for 6 days.³³ To closely mimic *in vivo* differentiating conditions, we did not use neutralizing antibodies against IFN- γ and IL-4, the cytokines that are known to inhibit the development of T_H17 cells.

IVIg was added to the T-cell culture 12 hours after initiation of stimulation to investigate the effect of IVIg on the differentiation of T_H17 cells. Compared with cells differentiated in the presence of TGF- β and IL-21 alone, IVIg significantly reduced the percentage of IL-17A⁺, RORC⁺, and IL-17A⁺/IFN- γ ⁺ cells (Fig 1). Equimolar concentrations of HSA, used as a protein control for IVIg, did not inhibit the differentiation of T_H17 cells (Fig 1). We excluded the possibility that the reduction of IL-17-producing cell numbers by IVIg is not due to apoptosis or the toxic effects of IVIg on cells (data not shown). Furthermore, IVIg did not alter the expression of Foxp3 (Treg cells) among the naive T_H cells cultured under T_H17-polarizing conditions (Fig 1, B). A prominent decrease in IFN- γ -producing cell numbers was

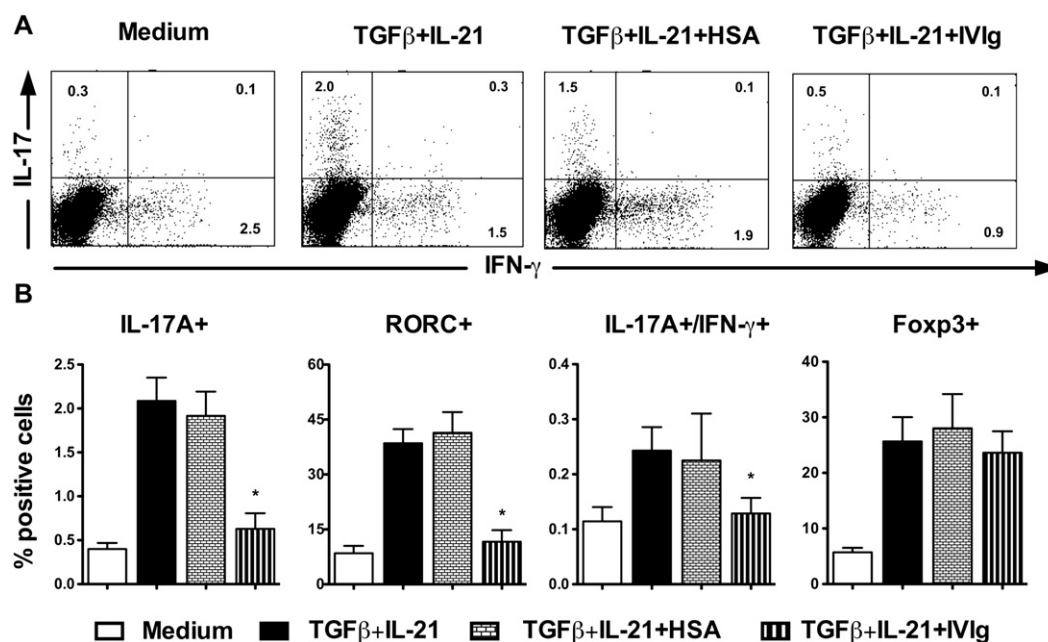


FIG 1. Inhibition of human T_H17 cell differentiation by IVIg. **A**, Flow cytometric analysis of intracellular IL-17 and IFN-γ in naive T_H17 cells after 6 days of culture for T_H17 cell differentiation. A representative dot plot of 8 independent experiments is shown. **B**, Percentage of IL-17A⁺, RORC⁺, IL-17A⁺/IFN-γ⁺, and Foxp3⁺ cells (means ± SEMs, n = 8 donors) during T_H17 cell differentiation. *P < .05, paired t test.

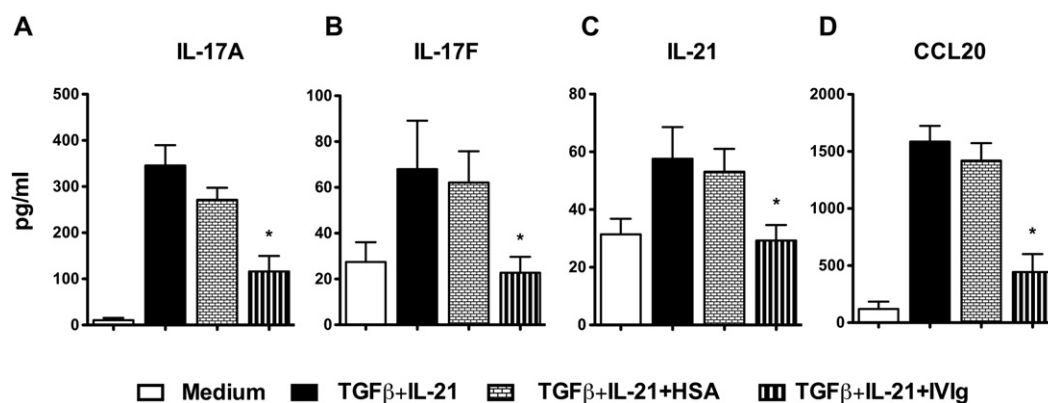


FIG 2. IVIg-mediated suppression of T_H17 cytokines and chemokine by the differentiating T_H17 cells. The figure depicts the amount (in picograms per milliliter) of secretion of IL-17A (**A**), IL-17F (**B**), IL-21 (**C**), and CCL20 (**D**) by the naive T_H17 cells after 6 days of culture for T_H17 cell differentiation. Results (means ± SEMs) are from 8 healthy donors. *P < .05, paired t test.

also observed in IVIg-treated cells (Fig 1, A), which is in consensus with the previous reports of the effect of IVIg on T_H1 cells.^{34,35}

Furthermore, IVIg significantly suppressed the amount of IL-17A secreted by differentiated T_H17 cells, whereas HSA had no significant effect (Fig 2, A). This effect of IVIg on IL-17A production was similar to that of other known inhibitors, such as IFN-β, 1,25-dihydroxyvitamin D₃, and dexamethasone (see Fig E1, A, in this article's Online Repository at www.jacionline.org). The kinetic study showed that IVIg inhibits the differentiation of IL-17-producing cells throughout the differentiation phase (see Fig E2, A, in this article's Online Repository at www.jacionline.org). The dose-response and time-response curves indicated that

IVIg is able to inhibit T_H17 cell differentiation at a dose of 20 mg/mL or greater and even when IVIg was added at 72 hours after initiation of T_H17 cell differentiation (see Figs E3, A, and E4, A, in this article's Online Repository at www.jacionline.org). Interestingly, the suppression of IL-17A production by IVIg was sustained even after removal of IVIg from the culture, thus indicating a long-lasting effect of IVIg on differentiation of T_H17 cells (see Fig E5, A, in this article's Online Repository at www.jacionline.org).

We further substantiated the IVIg-mediated inhibition of T_H17 cell differentiation by investigating other effector molecules that are produced by T_H17 cells. In addition to IL-17A, IVIg significantly suppressed the secretion of IL-17F, IL-21, and CCL20 by

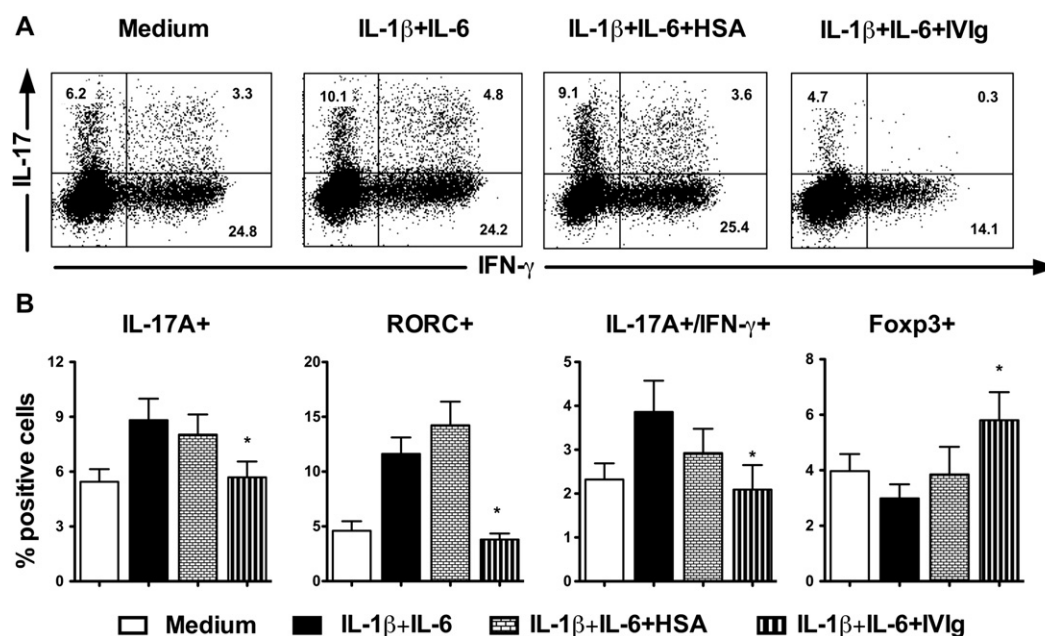


FIG 3. Inhibition of human T_H17 cell amplification by IVIg. **A**, Flow cytometric analysis of intracellular IL-17 and IFN- γ in the memory T_H cells after 6 days of culture for T_H17 amplification. Results are representative of 8 independent experiments. **B**, Percentage of IL-17A⁺, RORC⁺, IL-17A⁺/IFN- γ ⁺, and Foxp3⁺ cells (means $\pm$ SEMs, $n = 8$ donors) during T_H17 amplification. * $P < .05$, paired t test.

the differentiating T_H17 cells (Fig 2, B-D). HSA did not alter the production of these effector molecules. Taken together, our results demonstrate that IVIg interferes with the differentiation process of human T_H17 cells.

Inhibition of amplification of memory T_H17 cells by IVIg

Inflammatory cytokines have been shown to amplify memory T_H17 cells.^{1,33} Accordingly, CD4⁺CD45RO⁺ memory T cells were stimulated with IL-1 β and IL-6 in the presence of anti-CD3 and anti-CD28 antibodies for 6 days, and IVIg was added to the T-cell culture 12 hours after initiation of stimulation. Similar to the effect on the differentiation of T_H17 cells, IVIg significantly decreased the percentage of IL-17A⁺, RORC⁺, and IL-17A⁺/IFN- γ ⁺ cells compared with the culture conditions that are stimulated with IL-1 β and IL-6 alone (Fig 3). In contrast to naive T_H cells, IVIg was able to significantly enhance Foxp3⁺ Treg cells among the memory T_H cells (Fig 3, B).

The inhibition of T_H17 amplification by IVIg was also evident in the amount of secretion of IL-17, wherein a significant decrease in the production of IL-17A was observed (Fig 4, A). The effect of IVIg on IL-17A production by amplified memory T_H17 cells was similar to that of 1,25-dihydroxyvitamin D₃ (see Fig E1, B). The kinetic study indicated that IVIg inhibits the IL-17-producing cells throughout the amplification phase (see Fig E2, B). The dose-response and time-response curves indicated that IVIg is able to inhibit T_H17 cell amplification at a therapeutic dose of 25 mg/mL and when IVIg was added at 12 and 24 hours after initiation of T_H17 cell amplification (see Figs E3, B, and E4, B). However, in contrast to the effect on differentiating T_H17 cells, IVIg did not sustain its inhibitory effect on IL-17A production by memory T_H17 cells after removal of IVIg from the culture (see Fig E5, B).

IVIg significantly suppressed the production of IL-17F, IL-21, and CCL20 by the amplifying memory T_H17 cells (Fig 4, B-D). However, HSA did not alter the levels of these molecules. Thus in addition to differentiation, IVIg also significantly inhibits the amplification of memory T_H17 cells and the production of their effector molecules.

Inhibition of differentiation and amplification of T_H17 cells by IVIg is F(ab')₂ dependent

To further decipher the mechanisms of IVIg-mediated suppression of the differentiation and amplification of T_H17 cells, we investigated the involvement of F(ab')₂ fragments of IVIg in this process. Similar to previous reports,^{31,32} we observed that both intact IVIg and IVIg-derived F(ab')₂ recognize CD4⁺ T cells (see Fig E6 in this article's Online Repository at www.jacionline.org). In addition, we did not observe the expression of any of the Fc receptors (Fc γ R) on T cells (data not shown), indicating that in our experimental conditions, in which pure CD4⁺ T cells were used, the IVIg-mediated suppressive effect on T_H17 cells involves interaction of F(ab')₂ regions of IVIg with T cells.

Interestingly, F(ab')₂ fragments of IVIg at equimolar concentrations to IVIg added to the T-cell culture after 12 hours of initiation of stimulation significantly inhibited the secretion of IL-17A, IL-17F, and CCL20 from both differentiated and memory T_H17 cells (Fig 5). The extent of suppression by F(ab')₂ fragments was similar to that of intact IVIg. Although not significant, IL-21 was also downregulated by F(ab')₂ fragments of IVIg. The percentage of IL-17-producing cells was also significantly decreased in the F(ab')₂ fragment-treated cells (data not shown). These results thus demonstrate that suppression of the differentiation and amplification of T_H17 cells by IVIg is F(ab')₂ dependent.

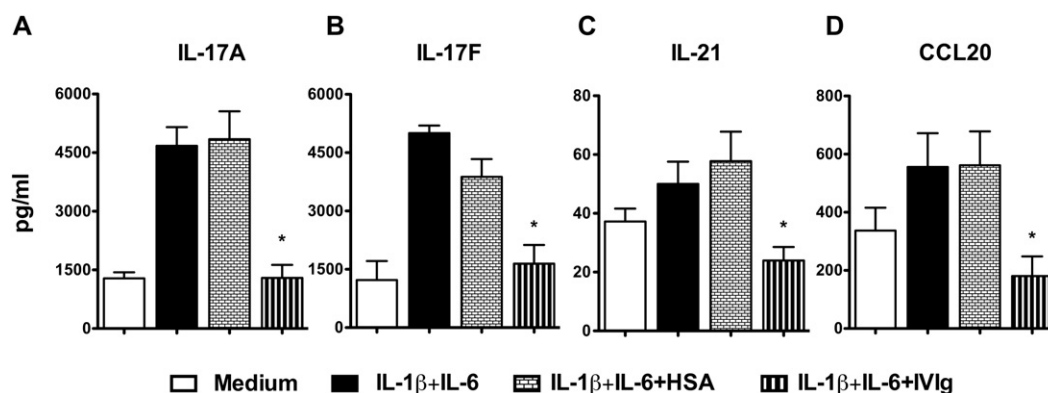


FIG 4. Suppression of T_H17 cytokines and chemokine in the amplifying memory T_H17 cells by IVIg. The figure shows the amount (in picograms per milliliter) of secretion of IL-17A (A), IL-17F (B), IL-21 (C), and CCL20 (D) by the memory T_H cells after 6 days of culture for T_H17 amplification. Results (means $\pm$ SEMs) are from 8 healthy donors. * $P < .05$, paired t test.

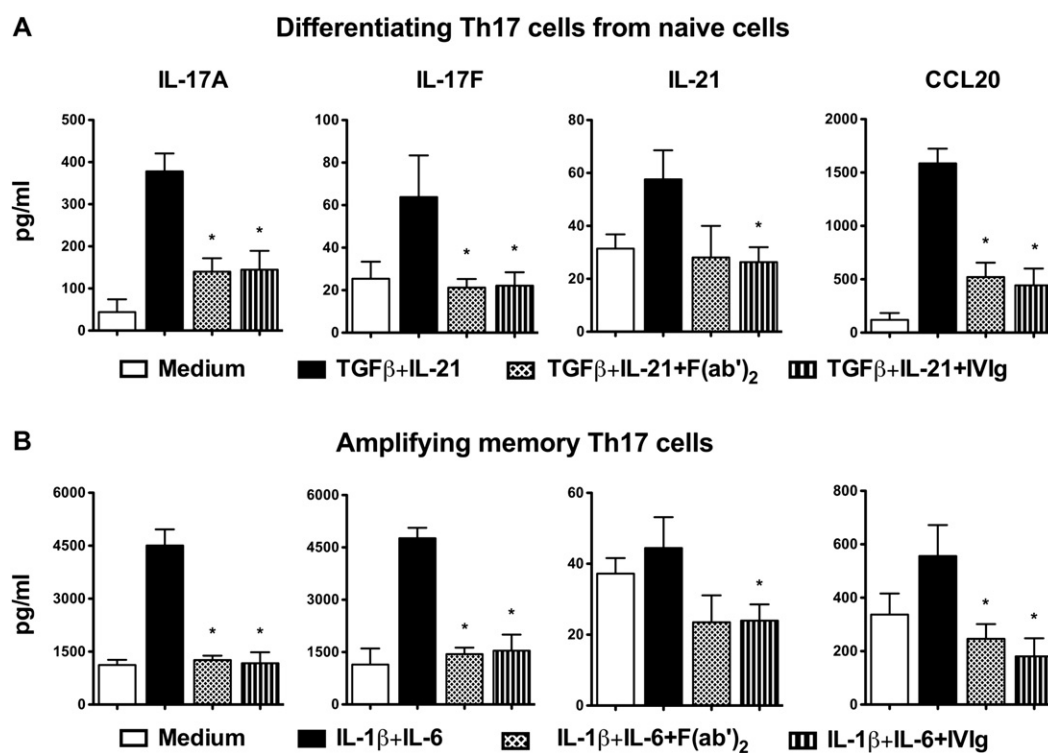


FIG 5. IVIg-mediated suppression of differentiation and amplification of T_H17 cells is $F(ab')_2$ dependent. The amount (in picograms per milliliter) of secretion of IL-17A, IL-17F, IL-21, and CCL20 by the naive $CD4^+$ T cells cultured for T_H17 cell differentiation (A) and the memory $CD4^+$ T cells cultured for T_H17 amplification (B) is depicted. Results (means $\pm$ SEMs) are from 5 donors. * $P < .05$, paired t test.

IVIg interferes with cytokine signaling to mediate the suppression of differentiation and amplification of T_H17 cells

STAT3 signaling plays a crucial role early in the development of T_H17 cells.¹¹ After binding of IL-21 and IL-6 to their respective receptors on cells, STAT3 undergoes activation by phosphorylation (at Y705) and facilitates the expression of RORC and the inhibition of Foxp3. Therefore we explored the probable modulation of sustained STAT3 phosphorylation (pSTAT3) by

IVIg as a mechanism of inhibition of T_H17 cell development and function.

A significant level of pSTAT3 was observed in both naive and memory T_H cells under T_H17 culture conditions after 18 hours of cytokine stimulation (Fig 6). Interestingly, IVIg reduced pSTAT3 levels in both populations of T_H cells (Fig 6). During the differentiation phase of T_H17 cells, IVIg inhibited pSTAT3 from the early time points, whereas in memory T_H17 cells the inhibition of pSTAT3 was evident from 24 hours of culture.

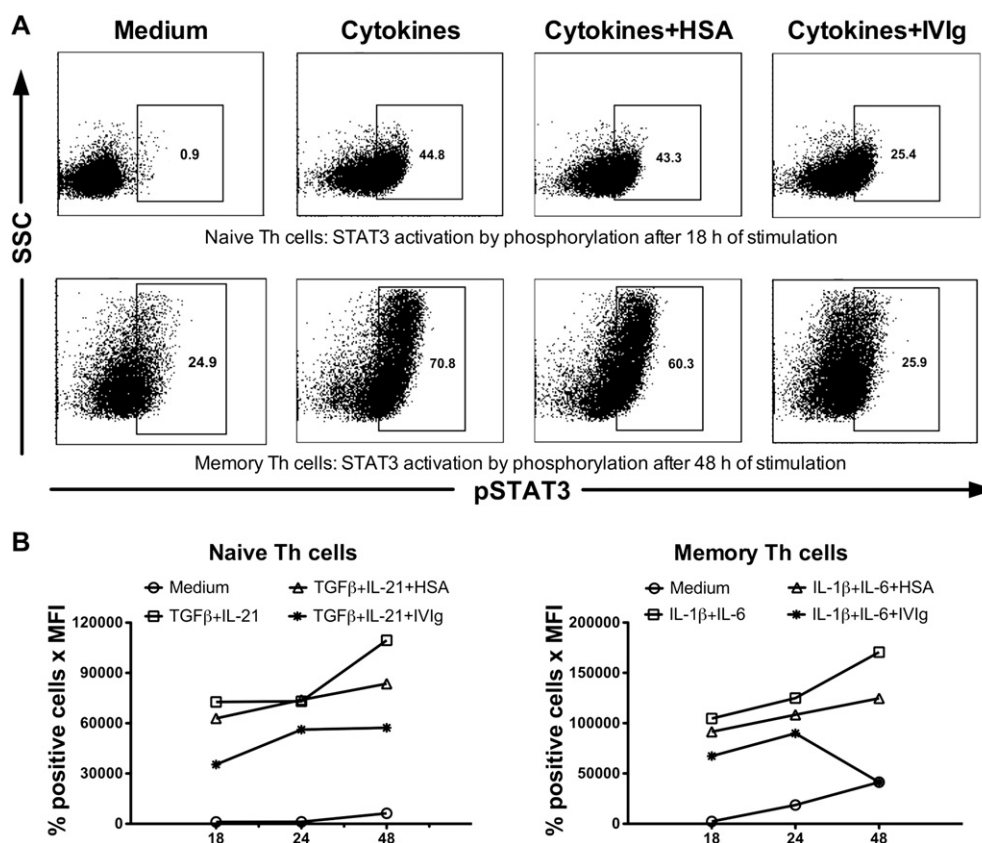


FIG 6. IVIg modulates the sustained phosphorylation of STAT3 to mediate inhibition of T_H17 cell differentiation and amplification. **A**, Phosphorylation status of STAT3 (pSTAT3) at indicated time points, as analyzed by means of flow cytometry. **B**, Mean expression of intracellular pSTAT3 levels (percentage of pSTAT3⁺ cells $\times$ pSTAT3 mean fluorescence intensity [MFI]) in naive T_H cells (left panel) and memory T_H cells (right panel). Results are representative of 3 independent experiments.

These results thus indicate that IVIg-mediated suppression of T_H17 cell differentiation and amplification involves inhibition of pSTAT3.

DISCUSSION

IVIg is increasingly used in the treatment of several autoimmune, inflammatory, and allergic diseases.¹⁸⁻²⁶ Numerous mutually non-exclusive mechanisms have been proposed to explain the beneficial effects of IVIg in patients with these immune-mediated diseases.^{18,28-30} Because IVIg therapy is beneficial in the treatment of diseases in which T_H17 cells have a critical role in pathogenesis, we hypothesized that in addition to other mechanisms, the therapeutic efficacy of IVIg in several immune disorders implicates interference with T_H17 cell differentiation, amplification, and function.

In line with this hypothesis, our results demonstrate that IVIg inhibits the differentiation and amplification of T_H17 cells and the production of the effector cytokines IL-17A, IL-17F, IL-21, and CCL20 by interfering with the cytokine signaling and expression of the lineage-specific transcription factor RORC. In addition, our preliminary results also indicate that IVIg suppresses IL-17A production *in vivo* in patients with autoimmune conditions, such as myositis (data not shown), thus confirming the therapeutic relevance of our observations. These effects are specific to IVIg because HSA used at equimolar concentrations did not influence the development and function of T_H17 cells. Thus our findings reveal a novel mechanism of action of IVIg in achieving a beneficial

therapeutic effect in patients with autoimmune diseases in which T_H17 cells play a key modulatory role in sustaining the chronic inflammatory response.

In our study, in which pure $CD4^+$ T cells were used in the absence of other accessory immune cells, IVIg-mediated suppression of T_H17 cell function was mediated through $F(ab')_2$ fragments, indicating that the antigen-binding variable regions of IVIg interact with surface receptors (including cytokine receptors), leading to interference in T_H17 development and function. In line with these observations, IVIg inhibited pSTAT3, a transcription factor that plays an important role in T_H17 cell differentiation, amplification, and stabilization. However, under *in vivo* conditions, we favor a cooperative effect of both $F(ab')_2$ and Fc-dependent mechanisms by which IVIg modulates T_H17 cells.^{18,28-30} We believe that IVIg can target T_H17 cells *in vivo* through several multipronged mechanisms, either by modulating antigen-presenting cells (APCs) or through expanding Treg cells or directly affecting T_H17 cells. In accordance with this, IVIg has been shown to modulate the APC-derived cytokines and APC-mediated stimulation of T cells.^{32,36} Furthermore, as shown in the present report and elsewhere, IVIg directly targets T cells by inhibiting T-cell proliferation and activation and secretion of various T-cell cytokines.^{18,28} The inhibitory effect of IVIg on T_H17 cells *in vivo* might also involve expansion of natural Treg cells.^{31,37,38} In fact, antigen-specific Treg cells are shown to regulate T_H17 -mediated inflammatory processes.^{39,40}

Interestingly, in our study IVIg-mediated inhibition of T_H17 cell differentiation from naive T_H cells was not associated with the enhancement of Foxp3⁺ Treg cells. Moreover, the naive T_H cells were depleted of CD25⁺ cells to remove the recently activated T_H cells and the naive Treg cells. This finding is in consensus with our previous observation, wherein IVIg did not induce *de novo* generation of Treg cells in mice.³¹ However, among the memory cells, suppression of T_H17 amplification and function by IVIg was associated with a significant increase in Foxp3⁺ Treg cell numbers. These results thus confirm the previous reports that IVIg expands the Treg cell population both *in vitro* and *in vivo*.^{31,37,41} Together, our results suggest a reciprocal regulation of the T_H17 and Treg cell population by IVIg.

We observed that in addition to inhibition of T_H17 cell differentiation and amplification, IVIg also suppressed IL-17⁺/IFN-γ⁺ and IFN-γ⁺ cells. This is in contrast to some of the molecules explored for T_H17 inhibition, such as halofuginone and pioglitazone, which selectively inhibit T_H17 cells without affecting T_H1 development.^{14,15} However, 1,25-dihydroxyvitamin D₃ and dexamethasone inhibit IFN-γ in addition to IL-17.¹⁷ Despite the prominent role of T_H17 cells in several inflammatory and autoimmune diseases, the importance of IFN-γ-producing T_H1 cells is not clearly ruled out in these diseases.^{1,2} Moreover, memory T_H17 cells can express IFN-γ after activation, as evidenced by the high percentage of IL-17⁺IFN-γ⁺ T cells in the memory CD4⁺ T-cell pool, thus suggesting the plasticity of T_H17 cells.² Given that both T_H1 and T_H17 cells mediate diverse autoimmune processes, therapies that have broad cellular targets, as in the case of IVIg, enable more efficient control of these pathologies.

Key messages

- IVIg inhibits T_H17 cell differentiation, propagation, and production of the effector cytokines IL-17A, IL-17E, IL-21, and CCL20.
- In addition to other mechanisms, the therapeutic efficacy of IVIg in patients with autoimmune and allergic diseases might implicate interference with T_H17 cell development and function.

REFERENCES

- Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature* 2008;453:1051-7.
- Zhou L, Chong MM, Littman DR. Plasticity of CD4⁺ T cell lineage differentiation. *Immunity* 2009;30:646-55.
- Miossec P, Korn T, Kuchroo VK. Interleukin-17 and type 17 helper T cells. *N Engl J Med* 2009;361:888-98.
- Louten J, Boniface K, de Waal Malefyt R. Development and function of TH17 cells in health and disease. *J Allergy Clin Immunol* 2009;123:1004-11.
- Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells: origins, features and possible roles in rheumatic disease. *Nat Rev Rheumatol* 2009;5:325-31.
- Hashimoto T, Akiyama K, Kobayashi N, Mori A. Comparison of IL-17 production by helper T cells among atopic and nonatopic asthmatics and control subjects. *Int Arch Allergy Immunol* 2005;137(suppl 1):51-4.
- van Beelen AJ, Teunissen MB, Kapsenberg ML, de Jong EC. Interleukin-17 in inflammatory skin disorders. *Curr Opin Allergy Clin Immunol* 2007;7:374-81.
- Larsen JM, Bonefeld CM, Poulsen SS, Geisler C, Skov L. IL-23 and T(H)17-mediated inflammation in human allergic contact dermatitis. *J Allergy Clin Immunol* 2009;123:486-92.
- Ciprandi G, Filaci G, Battaglia F, Fenoglio D. Peripheral Th-17 cells in allergic rhinitis: New evidence. *Int Immunopharmacol* 2010;10:226-9.
- Dong C. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat Rev Immunol* 2008;8:337-48.
- Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol* 2009;27:485-517.
- Wang YH, Liu YJ. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol* 2008;20:697-702.
- Zhang X, Jin J, Peng X, Ramgolam VS, Markovic-Plese S. Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and by inhibiting the expression of IL-17 transcription factor RORC in CD4⁺ lymphocytes. *J Immunol* 2008;180:6988-96.
- Sundrud MS, Koralov SB, Feuerer M, Calado DP, Kozhaya AE, Rhule-Smith A, et al. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science* 2009;324:1334-8.
- Klotz L, Burgdorf S, Dani I, Saijo K, Flossdorf J, Hucke S, et al. The nuclear receptor PPAR gamma selectively inhibits Th17 differentiation in a T cell-intrinsic fashion and suppresses CNS autoimmunity. *J Exp Med* 2009;206:2079-89.
- Ramgolam VS, Sha Y, Jin J, Zhang X, Markovic-Plese S. IFN-β inhibits human Th17 cell differentiation. *J Immunol* 2009;183:5418-27.
- Colin EM, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, et al. 1,25-Dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum* 2010;62:132-42.
- Kazatchkine MD, Kaveri SV. Immunomodulation of autoimmune and inflammatory diseases with intravenous immune globulin. *N Engl J Med* 2001;345:747-55.
- Orange JS, Hossny EM, Weiler CR, Ballow M, Berger M, Bonilla FA, et al. Use of intravenous immunoglobulin in human disease: a review of evidence by members of the Primary Immunodeficiency Committee of the American Academy of Allergy, Asthma and Immunology. *J Allergy Clin Immunol* 2006;117(suppl):S525-53.
- Gold R, Stangel M, Dalakas MC. Drug Insight: the use of intravenous immunoglobulin in neurology—therapeutic considerations and practical issues. *Nat Clin Pract Neurol* 2007;3:36-44.
- Mazer BD, Giclas PC, Gelfand EW. Immunomodulatory effects of intravenous immunoglobulin in severe steroid-dependent asthma. *Clin Immunol Immunopathol* 1989;53(suppl):S156-63.
- Mazer BD, Gelfand EW. An open-label study of high-dose intravenous immunoglobulin in severe childhood asthma. *J Allergy Clin Immunol* 1991;87:976-83.
- Leung DY. The immunoregulatory effects of IVIG in Kawasaki disease and other autoimmune diseases. *Clin Rev Allergy* 1992;10:93-104.
- Gelfand EW, Landwehr LP, Esterl B, Mazer B. Intravenous immune globulin: an alternative therapy in steroid-dependent allergic diseases. *Clin Exp Immunol* 1996;104(suppl 1):61-6.
- Rabinovitch N, Gelfand EW, Leung DY. The role of immunoglobulin therapy in allergic diseases. *Allergy* 1999;54:662-8.
- Prins C, Gelfand EW, French LE. Intravenous immunoglobulin: properties, mode of action and practical use in dermatology. *Acta Derm Venereol* 2007;87:206-18.
- Lemieux R, Bazin R, Neron S. Therapeutic intravenous immunoglobulins. *Mol Immunol* 2005;42:839-48.
- Tha-In T, Bayry J, Metselaar HJ, Kaveri SV, Kwekkeboom J. Modulation of the cellular immune system by intravenous immunoglobulin. *Trends Immunol* 2008;29:608-15.
- Nimmerjahn F, Ravetch JV. Anti-inflammatory actions of intravenous immunoglobulin. *Annu Rev Immunol* 2008;26:513-33.
- Seite JF, Shoenfeld Y, Youinou P, Hillion S. What is the contents of the magic draft IVIg? *Autoimmun Rev* 2008;7:435-9.
- Ephrem A, Chamat S, Miquel C, Fisson S, Mouthon L, Caligiuri G, et al. Expansion of CD4⁺CD25⁺ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis. *Blood* 2008;111:715-22.
- Ephrem A, Lacroix-Desmazes S, Carbonnel C, Misra N, Donkova V, Pashov A, et al. Inhibition of maturation and function of dendritic cells by intravenous immunoglobulin. *Blood* 2003;101:758-65.
- Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. IL-21 and TGF-β are required for differentiation of human T(H)17 cells. *Nature* 2008;454:350-2.
- Andersson J, Skansen-Saphir U, Sparrelid E, Andersson U. Intravenous immune globulin affects cytokine production in T lymphocytes and monocytes/macrophages. *Clin Exp Immunol* 1996;104(suppl 1):10-20.
- Tha-In T, Metselaar HJ, Tilanus HW, Boor PP, Mancham S, Kuipers EJ, et al. Superior immunomodulatory effects of intravenous immunoglobulins on human T-cells and dendritic cells: comparison to calcineurin inhibitors. *Transplantation* 2006;81:1725-34.

36. Aubin E, Lemieux R, Bazin R. Indirect inhibition of in vivo and in vitro T-cell responses by intravenous immunoglobulins due to impaired antigen presentation. *Blood* 2010;115:1727-34.
37. Kessel A, Ammuri H, Peri R, Pavlotzky ER, Blank M, Shoenfeld Y, et al. Intravenous immunoglobulin therapy affects T regulatory cells by increasing their suppressive function. *J Immunol* 2007;179:5571-5.
38. De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, et al. Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." *Blood* 2008;112:3303-11.
39. Fletcher JM, Loneragan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, et al. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* 2009;183:7602-10.
40. Crome SQ, Clive B, Wang AY, Kang CY, Chow V, Yu J, et al. Inflammatory effects of ex vivo human Th17 cells are suppressed by regulatory T cells. *J Immunol* 2010;185:3199-208.
41. Tha-In T, Metselaar HJ, Bushell AR, Kwekkeboom J, Wood KJ. Intravenous immunoglobulins promote skin allograft acceptance by triggering functional activation of CD4+Foxp3+ T cells. *Transplantation* 2010;89:1446-55.

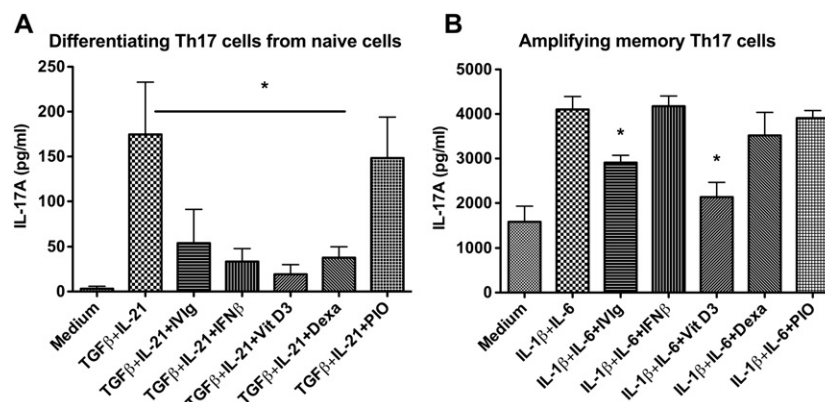


FIG E1. Comparison of the inhibitory effect of IVIg and other known inhibitors on human T_H17 cells. Amount of secretion of IL-17A (in picograms per milliliter, means $\pm$ SEMs, $n = 3$ donors) in differentiating (**A**) and amplifying (**B**) T_H17 cells under the influence of IFN- β (2,000 IU/mL), 1, 25-dihydroxyvitamin D3 (Vit D3; 100 nmol/L), dexamethasone (1,000 nmol/L; Dexa), pioglitazone (PIO; 10 μ mol/L), and IVIg (25 mg/mL) is shown. * $P < .05$, paired t test.

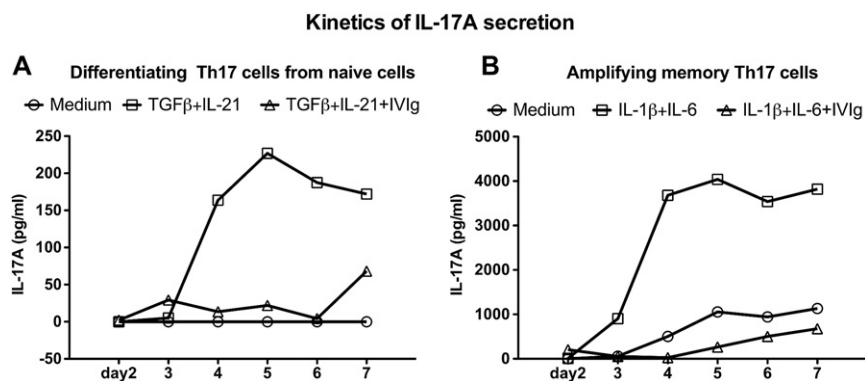


FIG E2. Kinetic study of the effect of IVIg (25 mg/mL) on T_H17 cell differentiation and amplification. Amount of secretion of IL-17A (in picograms per milliliter) during differentiation (**A**) and amplification (**B**) of T_H17 cells is shown. Results are representative of one of the 3 independent experiments.

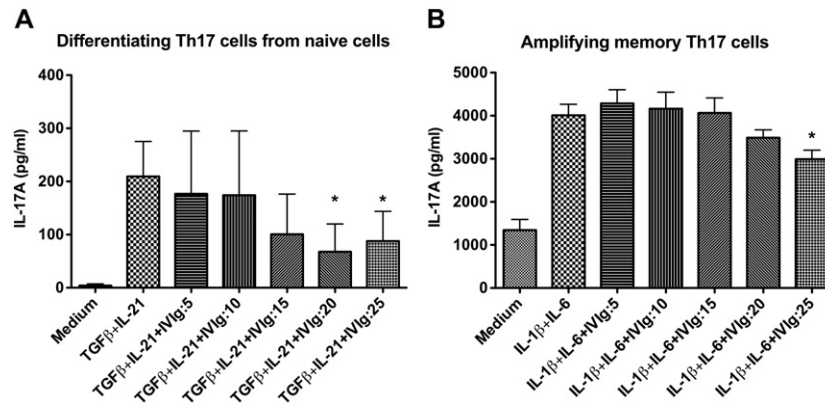


FIG E3. Dose-dependent effect of IVIg on human T_H17 cell differentiation and amplification. Amount of secretion of IL-17A (in picograms per milliliter, means $\pm$ SEMs, $n = 5$ donors) during differentiation (**A**) and amplification (**B**) of T_H17 cells is shown. IVIg was added at concentrations of 5, 10, 15, 20, and 25 mg/mL. * $P < .05$, paired t test.

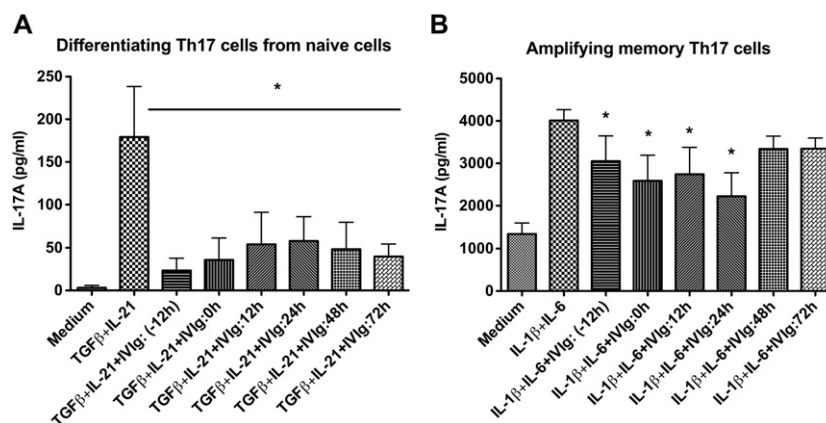


FIG E4. Time-dependent effect of IVIg on human T_H17 cell differentiation and amplification. Amount of secretion of IL-17A (in picograms per milliliter, means $\pm$ SEMs, $n = 5$ donors) during differentiation (**A**) and amplification (**B**) of T_H17 cells is shown. IVIg (25 mg/mL) was added to the cells at -12 , 0 , 12 , 24 , 48 , and 72 hours after cytokine stimulation. $*P < .05$, paired t test.

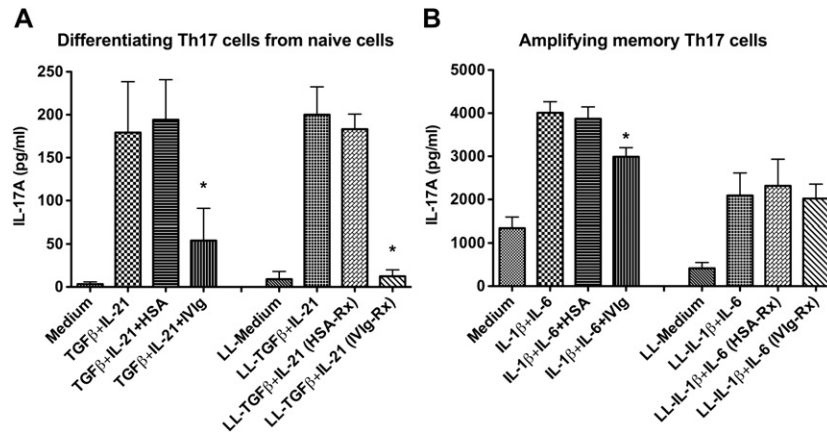


FIG E5. Long-lasting inhibitory effect of IVIg on human T_H17 cell differentiation and amplification. Secretion of IL-17A (in picograms per milliliter, means $\pm$ SEMs, $n = 3-5$ donors) during differentiation (**A**) and amplification (**B**) of T_H17 cells is shown. After the first round of treatment, cells were washed and restimulated (LL) only with anti-CD3 and anti-CD28 in the presence of cytokines. LL, Long-lasting. * $P < .05$, paired t test.

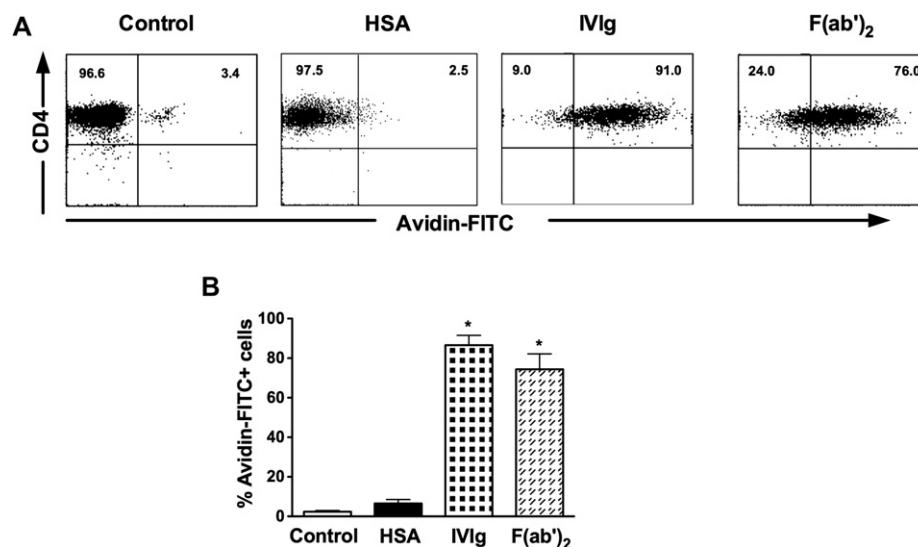


FIG E6. IVIg and F(ab')₂ fragments of IVIg bind to T_H cells. **A**, Flow cytometric analysis of binding of biotinylated IVIg, F(ab')₂ fragments, and HSA to CD4⁺ T cells. **B**, Percentage (means $\pm$ SEMs, $n = 3$) of IVIg-, F(ab')₂-, and HSA-bound T_H cells. FITC, Fluorescein isothiocyanate. * $P < .05$, paired t test.

TABLE E1. Assay limits and cutoff values for ELISA kits for cytokine estimation

Assay limit (pg/mL)	High	Low	Cutoff
IL-17A	1000	15.625	15
IL-17F	4000	30.25	30
IL-21	2000	16.00	16
CCL20	1000	15.625	15

A 7-point standard curve with 2-fold serial dilutions was obtained with the standards.