

Intravenous Immunoglobulin Therapy Affects T Regulatory Cells by Increasing Their Suppressive Function

Aharon Kessel,* Hana Ammuri,* Regina Peri,* Elsa R. Pavlotzky,* Miri Blank,[†] Yehuda Shoenfeld,[†] and Elias Toubi^{1*}

Intravenous Ig therapy (IVIg) is reported to be a useful regimen in treating autoimmune diseases. In this study, we asked whether IVIg (in vitro) could increase the expression of TGF- β , IL-10, and the transcription factor FoxP3 in T regulatory (Treg) cells, and the idea that IVIg could enhance suppressive properties of these cells. CD4⁺ T cells from 12 healthy individuals were cultured in the presence or absence of IVIg vs human control IgG during 16, 24, and 36 h. Using FACS analysis and gating on CD4⁺CD25^{high} Treg cells, we assessed the expression of intracellular TGF- β , IL-10, and FoxP3. In addition, the production of TNF- α by stimulated CD4⁺ T cells alone or in culture with CD25⁺ by itself or together with IVIg was also assessed. The presence of IVIg with Treg cells in culture significantly increased the intracellular expression of TGF- β ($17.7 \pm 8.5\%$ vs $29.8 \pm 13\%$; $p = 0.02$), IL-10 ($20.7 \pm 4.7\%$ vs $34.2 \pm 5.2\%$; $p = 0.008$) and FoxP3 ($20.8 \pm 5.2\%$ vs $33.7 \pm 5.9\%$; $p = 0.0006$) when compared with cells cultured alone or with control human IgG. The suppressive effect of CD4⁺CD25⁺ T cells presented as the decrease of TNF- α production by stimulated CD4⁺CD25⁻ (effector T cells) was further increased by adding IVIg to cell culture. We hereby demonstrate an additional mechanism by which IVIg could maintain self-tolerance and decrease immune-mediated inflammation. *The Journal of Immunology*, 2007, 179: 5571–5575.

The usage of i.v. Ig (IVIg) has been proven to be beneficial in various autoimmune and immune-mediated inflammatory conditions such as idiopathic thrombocytopenic purpura (ITP),² Kawasaki disease, and dermatomyositis (1, 2). In addition, IVIg is widely used (empirically) in other autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome, and various vasculitides (3–5). The long list of reported mechanisms by which IVIg induces its immunomodulatory effects, points to the many pathways that are targeted by this unique therapy.

The presence of a wide range of anti-idiotypic Abs in IVIg was reported to be one of the main mechanisms for the beneficial effect of this therapy (6, 7). These Abs bind pathogenic autoantibodies and prevent them from targeting autoantigens and the formation of immune complexes. IVIg was shown to induce a reversible blockade of the Fc receptors on phagocytic cells by saturating or down-regulating the affinity of Fc receptors, disabling by that sensitized phagocytic cells to function in ITP (8). In addition IVIg is protective against complement-mediated tissue damage, when it inhibits the binding of C3; C4 to target structures, such as in dermatomyositis (9). The ability of IVIg to neutralize proinflammatory cytokines such as TNF- α , was also proven in some patients to be one

of the important mechanisms by which IVIg is beneficial in rheumatoid arthritis (RA) or Kawasaki disease (10).

Considering the effect of serum factors as IFN- α (IFN- α), on the activity of dendritic cells (DCs), the effects of IVIg on the differentiation of DCs mediated by serum from SLE patients was assessed (11). IVIg was found to abrogate DC differentiation induced by IFN- α present in serum from SLE patients by 36%. These findings indicate that IVIg down-regulate DCs by inhibiting the ingestion of nucleosomes by immature DCs. Inhibition of expression of HLA and CD80/CD86 on DCs offers a plausible explanation for the efficacy of IVIg in SLE and other immune-mediated inflammatory conditions.

Recently, IVIg was reported to induce apoptosis in human lymphocytes and monocytes via Fas-mediated pathway (12). It has also been shown to inhibit endothelial cell proliferation and mRNA expression of cytokines, adhesion molecules and chemokines in endothelial cells (13). In a most recent study, B cell-activating factor was reported to be a new target for IVIg treatment in autoimmune diseases (14).

Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs), are engaged in dominant control of self-reactive T cells, contributing to the maintenance of immunologic self-tolerance. Their repertoire of Ag specificities is as broad as that of naive T cells, and they are capable of recognizing both self and nonself Ags, thus enabling them to control various immune responses. In addition to Ag recognition, signals through various accessory molecules (TLRs) and via the up-regulation of FoxP3 (a specific T regulatory transcription factor) and or inhibitory cytokines such as IL-10 and TGF- β , control their activation, expansion, and tune their suppressive activity (15). Treg cells are characterized by their ability to suppress effector T cells of either TH1 or TH2 phenotype involved in mediating inflammation in a cell contact and Ag-specific manner. Many studies have already shown that the lower number of Treg cells as well as alterations in their suppressive function may lead to the development of autoimmune disease in otherwise normal animal and human models (16–18).

*Division of Clinical Immunology, Bnai-Zion Medical Centre, Rappaport Faculty of Medicine, Technion, Haifa, Israel; and [†]Department of Medicine B and Centre for Autoimmune Diseases, Chaim Sheba Medical Centre, Tel-Hashomer, Incumbent of the Laura Schwarz Kipp Chair for Research of Autoimmune Diseases, Sackler Faculty of Medicine, Tel-aviv, Israel

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¹ Address correspondence and reprint requests to Dr. Elias Toubi, Division of Clinical Immunology, Bnai-Zion Medical Centre, Technion, Haifa 31048, Israel. E-mail address: elias.toubi@b-zion.org.il

² Abbreviations used in this paper: ITP, idiopathic thrombocytopenic purpura; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; DC, dendritic cell.

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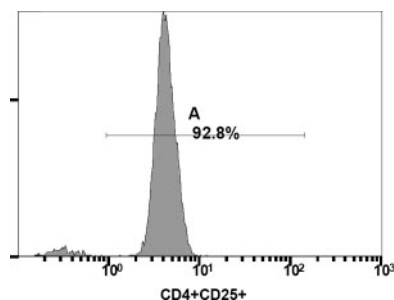


FIGURE 1. A representative figure of $CD4^+CD25^+$ lymphocyte isolation. PBMCs were isolated on Lymphoprep. Then, $CD4^+$ T cells were isolated by negative selection using the CD4 isolation kit for magnetic separation, and achieving >95% purity. For $CD4^+CD25^+$ lymphocyte isolation, $CD4^+$ T cells were incubated with mouse anti-human CD25 MicroBeads ($20 \mu\text{l}/10^7$ cells) and separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells on a positive selection column. A representative isolation of 92.8% Treg cells is shown by FACS analysis.

In previous studies we demonstrated the increase of Treg numbers as well as the restoration of their sensitivity to undergo spontaneous apoptosis following anti-TNF therapy in patients with active rheumatoid arthritis. This suggested that Treg cells are a potential target for the treatment of patients with autoimmune diseases.

The possible increase of Treg suppressive activity by IVIg therapy has yet to be investigated, and therefore we asked to analyze whether in vitro IVIg could possibly increase the expression of intracellular IL-10, TGF- β and the transcription factor FoxP3 in Treg cells. We also show that IVIg increase the suppressive abilities of Treg cells in decreasing the secretion of TNF- α - by effector $CD4^+$ T cells.

Materials and Methods

Study material

PBMCs, isolated $CD4^+$ T cells, and purified $CD4^+CD25^{\text{high}}$ Treg cells were obtained from 12 healthy individuals aged 24–36 years. Informed consent was obtained, and the study was approved by the local Helsinki committee at Bnai-Zion Medical Center (Haifa, Israel).

The source of IVIg was Omrigam (Omrix Pharmaceutical). C-IgG from one healthy individual (serving as a control for IVIg) was loaded on anti-human IgG Sepharose column (Sigma-Aldrich). The bound IgG was eluted by using 5M MgCl_2 and dialyzed against PBS (pH 7.4). Both affinities purified individual IgG and IVIg were dialyzed overnight against enriched RPMI 1640 at 4°C and adjusted to be used in cell culture at final concentration of 6 mg/ml.

Purification of T cell subsets

PBMCs were isolated on Lymphoprep (Axis-Shield). $CD4^+$ T cells were isolated by negative selection using the CD4 isolation kit for magnetic separation (MAC system; Miltenyi Biotec) according to the manufacturer's instructions, achieving >95% purity. For $CD4^+CD25^+$ lymphocyte isolation (Treg cells), $CD4^+$ T cells were incubated with mouse anti-human CD25 MicroBeads ($20 \mu\text{l}/10^7$ cells; Miltenyi Biotec) and separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells on a positive selection column (MS; Miltenyi Biotec). The resulted purity of Treg cells was between 90 and 94%, with a contamination of 6–10% of $CD4^+CD25^-$ cells (Fig. 1).

Flow cytometric analysis

To analyze the expression of intracellular IL-10, TGF- β and the transcription factor FoxP3, purified $CD4^+$ T lymphocytes were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 5 U/ml streptomycin, and recombinant human IL-2 at a concentration of 5 U/ml. Cells were cultured in 24-well plates (Cellstar, Greiner bio-one) at a final concentration of 1×10^6 cells/well in triplicates alone or supplemented with IVIg (6 mg/ml) or with human C-IgG (6 mg/ml) during 12 h. After washing the cells, staining with CD4-FITC and CD25-PC5 (Iotest; Beckman Coulter) for surface expression was performed. Subse-

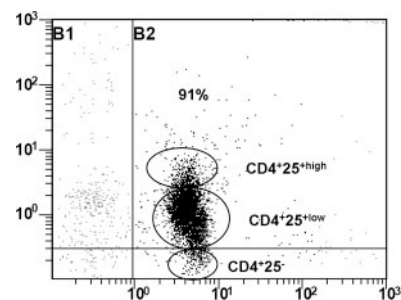


FIGURE 2. A representative dot plot FACS analysis of $CD4^+CD25^+$ T cells. Based on their CD25 expression, human $CD4^+$ T cells were divided into three populations: $CD25^{\text{high}}$, $CD25^{\text{low}}$, and CD25 negative cells. We considered Treg cells as those expressing a high level of CD25 as shown in the figure.

quently, cells were washed, fixed, permeabilized (cell permeabilization kit; Caltag Laboratories) and stained for the detection of intracellular cytokines using PE-conjugated anti-IL-10 (R&D Systems) or anti-TGF- β (IQ Products). Additionally, cells were washed, fixed, permeabilized, and stained

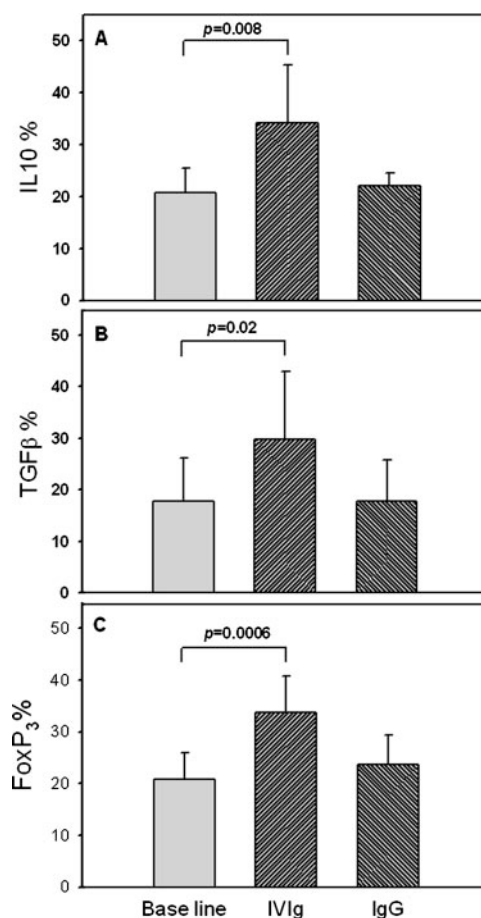
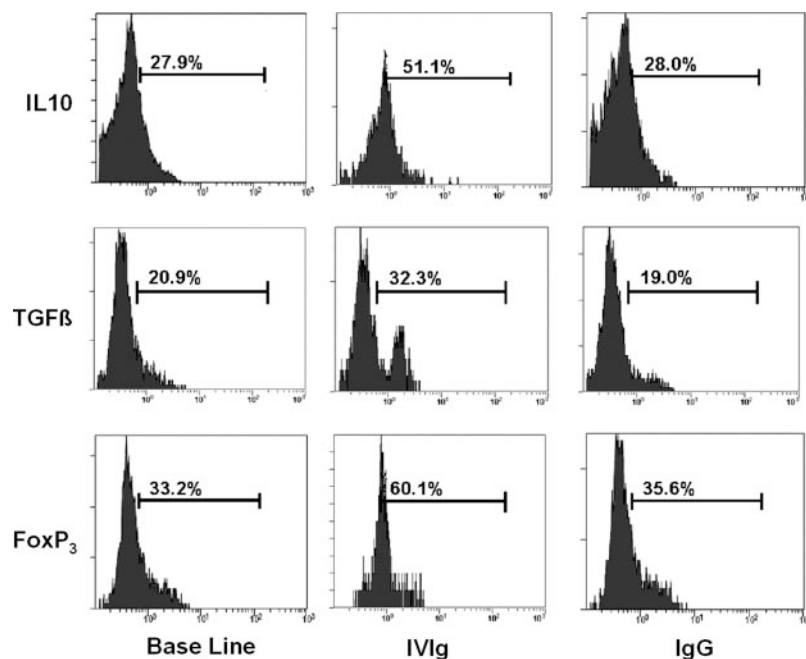


FIGURE 3. Summary of intracellular cytokine expression in Treg cells ($CD4^+CD25^{\text{high}}$), cultured during 12 h alone (baseline) or following the addition of IVIg (6 mg/ml) or human C-IgG (6 mg/ml). *A*, The expression of baseline IL-10 was significantly increased following the addition of IVIg to culture ($20.7 \pm 4.7\%$ vs $34.2 \pm 5.2\%$; $p = 0.008$), whereas IgG did not change significantly the extent of expression. *B*, A significant increase in the expression of TGF- β following the addition of IVIg ($17.7 \pm 8.5\%$ vs $29.8 \pm 13\%$; $p = 0.02$), contrary to IgG which did not affect this expression. *C*, Here also, the addition of IVIg increased FoxP3 expression ($20.8 \pm 5.2\%$ vs $33.7 \pm 5.9\%$; $p = 0.0006$) but IgG did not. Data are expressed as mean values \pm SD.

FIGURE 4. A representative figure of IL-10, TGF- β , and FoxP3 expressions at baseline and following IVIg and IgG additions. CD4⁺ T cells were cultured in 24-well plates at a final concentration of 1×10^6 cells/well in triplicates alone or supplemented with IVIg (6 mg/ml) or with human C-IgG (6 mg/ml) during 12 h. Cells were analyzed by flow cytometry. Gates were set so that the CD4⁺CD25⁻ population was based on the isotype control, whereas the CD25^{high} population was determined relative to the low intensity of CD25 staining found on non-CD4 T cells. Baseline IL-10, TGF- β , and FoxP3 expressions were increased following the addition of IVIg. However, the addition of C-IgG did not change baseline expression.



according to the manufacturer's instructions for Foxp3 (PE anti-human Foxp3 staining set; eBioscience). Gates were set so that the CD4⁺CD25⁻ population was based on the isotype control whereas the CD25^{high} population was determined relative to the low intensity of CD25 staining found on non-CD4 T cells. Cells were analyzed by flow cytometry (FACSCalibur, CellQuest software; BD Bioscience).

Cytokine detection

CD4⁺CD25⁻ T cells were cultured either alone or mixed at 1:4 ratio (10^5 cells/well) with CD4⁺CD25⁺ and stimulated with $10 \mu\text{g/ml}$ plate bound anti-CD3 and $1 \mu\text{g/ml}$ soluble anti-CD28 Abs (Bioscience). Cultures were set in triplicates in 96-well plates (Nunc) alone or supplemented with IVIg (6 mg/ml) or with C-IgG (6 mg/ml) and maintained for 16, 24, and 36 h. Supernatants were collected and analyzed for the presence of TNF- α .

Determination of TNF- α levels in culture supernatants

A sandwich ELISA (DuoSet; R&D Systems) was used to measure TNF- α levels in the culture supernatants. 96-well plates (Costar 3590) were utilized to coat the capture Ab. The substrate solution was tetramethylbenzidine (DakoCytomation). TNF- α levels were expressed as picograms of TNF- α per milligram of cell protein. Treg cells protein level was determined by the Lowry method (19). To normalize the results between different individuals, the pg/mg cell protein levels of TNF- α , measured in the supernatants of cultured cells, was considered as 100%. The change in TNF- α secretion by CD4⁺ T effector cells in various conditions was expressed as percentage of change from baseline.

Statistical analyses

Comparison of intracellular cytokine expression as well as of TNF secretion between each of the two conditions was performed using the paired Student *t* test or the paired Wilcoxon test as needed. Two tailed *p* values of 0.05 or less were considered to be statistically significant. Data are expressed as mean values \pm SD.

Results

Based on their CD25 expression, human CD4⁺ T cells can be divided into three populations: CD25^{high}, CD25^{low}, and CD25⁻ cells. The gate for CD4⁺CD25^{high} was drawn based on the top of CD4⁺ T cells expressing high levels of CD25 in the peripheral blood of each patient (Fig. 2).

Intracellular cytokine expression

Baseline expression of intracellular IL-10, TGF- β , and the transcription factor FoxP3 in CD4⁺CD25^{high} Treg cells (before the

incubation with IVIg, or C-IgG) was as follows: IL-10 (mean $20.7 \pm 4.7\%$, $n = 12$), TGF- β (mean $17.7 \pm 8.5\%$, $n = 12$), and FoxP3 (mean $20.8 \pm 5.2\%$, $n = 12$). The addition of IVIg to CD4⁺ T cells induced a significant elevation in the expression of intracellular cytokines in CD4⁺CD25^{high} Treg cells: IL-10 ($20.7 \pm 4.7\%$ vs $34.2 \pm 5.2\%$; $p = 0.008$), TGF- β ($17.7 \pm 8\%$ vs $29.8 \pm 13\%$; $p = 0.02$) and Foxp3 ($20.8 \pm 5.2\%$ vs $33.7 \pm 5.9\%$; $p = 0.0006$). In contrast to IVIg the addition of C-IgG into cultured CD4⁺ T cells, did not statistically modify the level of intracellular cytokines compared with that of the baseline. See a summary of 12 experiments (Fig. 3) and also a representative experiment (Fig. 4).

Cytokine assay

The ability of added CD4⁺CD25⁺ Treg cells to suppress TNF- α secretion by CD4⁺CD25⁻ effector T cells at 16, 24, and 36 h as described in the above methods was as follows: After 16 h, TNF- α secretion was suppressed by 4.8%; $p = 0.1$. When IVIg was added to CD25⁺ the suppression became significant (66%; $p < 0.01$). Contrary to IVIg, the addition of C-IgG to CD25⁺ inhibited the secretion of TNF- α by only 26% ($p = 0.1$; see Fig. 5A). When cells were cultured during 24 h, the addition of CD4⁺CD25⁺ induced a decrease of 62% ($p < 0.01$) in the production of TNF- α , whereas the addition of IVIg increased this suppression by another 22% ($p = 0.03$). Here also, the addition of C-IgG had no additional suppressive effect ($p = 0.4$; see Fig. 5B). After 36 h, TNF- α secretion (following the addition of CD4 + 25+) was diminished by 32% ($p < 0.01$). Again, the addition of IVIg induced an additional suppression of 26% ($p = 0.05$), whereas the addition of C-IgG increased this suppressive effect by only 9% ($p = 0.5$; see Fig. 5C).

Discussion

Among the several mechanisms that play role in maintaining peripheral self-tolerance is the existence of a unique CD4⁺CD25⁺ population of naturally occurring regulatory T cells that actively prevent both the activation and the effector function of autoreactive T cells that have escaped different mechanisms of tolerance (20–22).

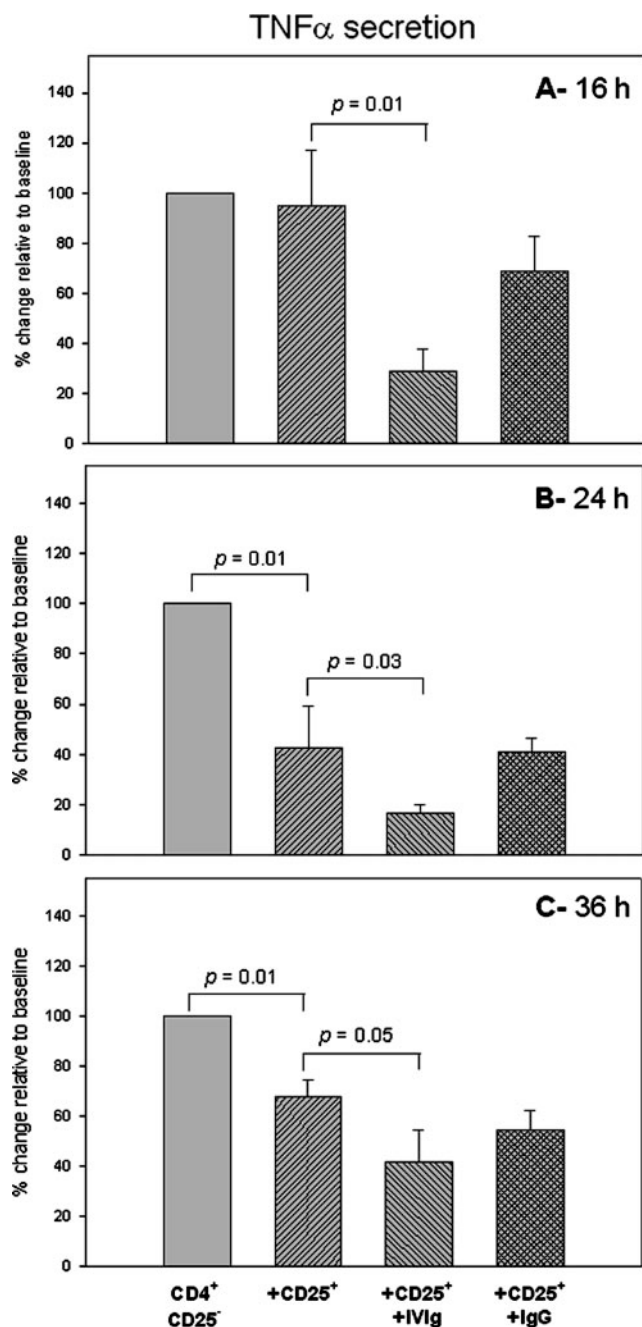


FIGURE 5. TNF- α production from cultured CD4⁺CD25⁻ effector T cells at 16, 24, and 36 h. Baseline levels of TNF- α were expressed as 100%. **A.** After 16 h, the addition of CD4⁺CD25⁺ Treg cells to effector T cells decreased TNF- α production by 4.8%. The addition of IVIg to effector T cells in the presence of Treg cells increased the suppression of TNF- α production (66%; $p < 0.01$). The addition of C-IgG did not suppress TNF- α production significantly (26%; $p = 0.1$). **B.** At 24 h, the addition of CD4⁺CD25⁺ Treg cells to effector T cells decreased TNF- α production by 62%; $p < 0.01$. When IVIg was added, TNF- α production was suppressed by another 22%; $p = 0.03$, whereas, the addition of C-IgG did not suppress TNF- α production. **C.** At 36 h, the addition of Treg cells to effector T cells diminished significantly the TNF- α production by 32%; $p < 0.01$. Again, the addition of IVIg increased this suppression by another 26%; $p = 0.05$, however, the addition of C-IgG did not suppress TNF- α production.

Many studies have established the occurrence of numeric decrease as well as the defective regulatory function of CD4⁺CD25⁺ Treg cells in patients with active SLE. These alterations were

mostly associated with a marked decrease in FoxP3 mRNA and protein expression in Treg cells from patients with active but not inactive SLE (23).

In a recent study, in vitro activation of CD4⁺CD25^{high} Treg cells from patients with active SLE, increased the expression of FoxP3 and restored their suppressive function, suggesting that strategies to enhance the function of these cells might benefit patients with autoimmune diseases (24). The idea of using various therapeutic regimens to restore the function of Treg cells mainly converting transient Treg activity into a stable phenotype was also reported in previous studies.

In agreement with these findings, the interaction of CD4⁺CD25⁺ Treg cells with activated monocytes in the joint of RA patients might lead to diminished suppressive activity of Treg cells in vivo, thus contributing to the chronic inflammation in RA (25). With these results in mind, and the fact that the blockade of TNF- α by therapy with anti-TNF has proven to be beneficial by inhibiting inflammation and preventing joint damage in RA patients, one may assume that this clinical effect might be mediated in part by a restoration of the defective function of CD4⁺CD25⁺ Treg cells in patients with active RA. In a previous study CD4⁺CD25⁺ Treg cells were shown to display increased proclivity to undergo spontaneous apoptosis in active RA. Alterations in CD4⁺CD25⁺ cell apoptosis and cell count were found to correlate with RA disease activity. Reversal of these deviations from normal was documented in association with the beneficial outcome of infliximab therapy (26).

In this regard, glucocorticoids were reported to affect the activity of Treg cells on the basis of FoxP3 and cytokine expression (27). FoxP3 mRNA expression was significantly increased in asthmatic patients receiving inhaled glucocorticoid treatment, systemic glucocorticoid treatment, or both. The frequency of CD25⁺ memory CD4⁺ T cells and transient FoxP3 mRNA expression by CD4⁺ T cells significantly increased after systemic glucocorticoid treatment. In addition, glucocorticoids induced IL-10 and FoxP3 expression in short-term and long-term cultures in vitro. This study showed that treatment with glucocorticoids may promote or initiate differentiation toward Treg cells by FoxP3-dependent mechanism. Thus, targeting these cells that aim to increase the expression of these molecules and their suppressive activity could become one of the tools by which self-tolerance is restored.

Here, and in agreement with all the above, we show for the first time that IVIg was proven by a unique mechanism to enhance the suppressive activity of CD4⁺CD25⁺ Treg cells. In this study we demonstrate that the addition of IVIg to CD4⁺ cells increased intracellular expression of IL-10, TGF- β and FoxP3 when we gated on CD4⁺CD25^{high} T cells, suggesting that IVIg have the properties of directly affecting Treg cells. We then established that the addition of IVIg to the culture of cells increased the suppressive function of Treg cells by further attenuating TNF secretion by CD4⁺ effector cells when IVIg was added to CD25⁺ cells.

The mechanisms by which IVIg could possibly affect the function of Treg cells is still not clear enough. Increased expression of intracellular IL-10 in Treg cells could inhibit the production of proinflammatory cytokines by Th1 such as TNF- α . In this regard, IVIg treatment resulted in the down-regulation of the Th1-type cytokine TNF- α , and the up-regulation of the Th2-type cytokine IL-10 (28). As supported by several experimental studies, IVIg regulate crucial steps of T cell-mediated immune responses. These effects involve the modulation of activation, proliferation, differentiation, apoptosis, and effector mechanisms of T cells. The pattern of IVIg-T cell interactions is complex, as IVIg may directly

bind to regulatory structures on T cells, or modulate T cell functions indirectly via soluble or cellular components of the immune system (29).

It is well established that autoantibodies directed against “public” idiotopes present in the first CDR (CDR1) and the third framework (FR3) of the $V\beta$ gene. Products are generated in response to the over-production of autodestructive T cells bearing particular $V\beta$ gene products and function to down-regulate the expression of these T cells. Specificity characterizations of polyclonal and monoclonal IgM and IgG autoantibodies from SLE patients were reported (30). Because Abs of these specificities are present in IVIg preparations, the immunomodulatory effects of Abs directed against TCR variable domains may account, at least in part, for the efficacy of IVIg in the therapy of autoimmune diseases. One may speculate that IVIg contains a wide range of natural autoantibodies that are directed against TCR variable domains on Treg cells, and that in contrast to effector $CD4^+$ T cells, they increase their activity by enhancing the FoxP3 expression or by increasing the activity of CTLA-4 on these cells. The fact that Ig influences the composition of T the cell repertoire by increasing its diversity allows for a much wider application of this molecule in the clinical practice, aiming to improve the diversity of the reconstituted immune repertoires (31).

Our present study reports on a unique effect of IVIg by which it restores the suppressive function of Treg cells which may add to its beneficial properties in the treatment of autoimmune diseases. The new role of the Ig molecule may help to explain several effects that IVIg has in the T cell compartment, such as modulation of the activation and function of Treg cells. Further studies are planned to better understand the precise mechanisms by which IVIg immunomodulate this unique and important subset of T cells.

Disclosures

The authors have no financial conflict of interest.

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