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# Patients treated with high-dose intravenous immunoglobulin show selective activation of regulatory T cells

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## Summary

**Intravenous immunoglobulin (IVIg) is used to treat autoimmune and systemic inflammatory diseases caused by derailment of humoral and cellular immunity. In this study we investigated whether IVIg treatment can modulate regulatory T cells ($T_{regs}$) in humans *in vivo*. Blood was collected from IVIg-treated patients with immunodeficiency or autoimmune disease who were treated with low-dose ($n = 12$) or high-dose ($n = 15$) IVIg before, immediately after and at 7 days after treatment. Percentages and activation status of circulating $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) $T_{regs}$ and of conventional $CD4^+FoxP3^-$ T-helper cells ($T_{conv}$) were measured. The suppressive capacity of $T_{regs}$ purified from blood collected at the time-points indicated was determined in an *ex-vivo* assay. High-dose, but not low-dose, IVIg treatment enhanced the activation status of circulating $T_{regs}$, as shown by increased FoxP3 and human leucocyte antigen D-related (HLA-DR) expression, while numbers of circulating $T_{regs}$ remained unchanged. The enhanced activation was sustained for at least 7 days after infusion, and the suppressive capacity of purified $T_{regs}$ was increased from 41 to 70% at day 7 after IVIg treatment. The activation status of $T_{conv}$ was not affected by IVIg. We conclude that high-dose IVIg treatment activates $T_{regs}$ selectively and enhances their suppressive function in humans *in vivo*. This effect may be one of the mechanisms by which IVIg restores imbalanced immune homeostasis in patients with autoimmune and systemic inflammatory disorders.**

**Keywords:** human studies, intravenous immunoglobulin, IVIg, regulatory T cells

## Introduction

Intravenous immunoglobulin (IVIg) was introduced initially as a replacement therapy for patients with immune deficiencies, but high-dose IVIg is now used widely for the treatment of autoimmune and systemic inflammatory diseases caused by autoantibodies and/or derailment of the cellular immune system [1,2]. Moreover, IVIg has shown efficacy in prevention and treatment of organ allograft rejection and graft-*versus*-host disease (GVHD) [3–6]. Several possible mechanisms of action that explain the beneficial effects of high-dose IVIg in autoantibody- and immune complex-mediated diseases have been unravelled during recent decades. These mechanisms include inhibition of the binding of immune complexes or cell-bound immunoglobulin (Ig)G to activating Fcγ receptors, saturation of the neonatal FcR resulting in an enhanced clearance of autoantibodies and up-regulation of the inhibitory FcγRIIb [1,2,7]. However, the mechanisms by which high-dose IVIg is effective in diseases which are caused mainly by cell-mediated immune responses, such as Kawasaki disease, dermatomyositis, GVHD and acute transplant rejection, have not yet been elucidated fully. Recent *in-vitro* and mice studies suggest that one of the mechanisms by which IVIg suppresses cellular immunity is by activating $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) regulatory T cells ($T_{regs}$) [8,9].

$T_{regs}$ are critical regulators of cell-mediated immune responses and suppress pathogenic immune responses in autoimmune diseases, transplantation and GVHD [10]. Current immunosuppressive drugs used to treat or to prevent these diseases exert generalized inhibition of the

**Table 1a.** Patient characteristics in low-dose intravenous immunoglobulin (IVIg)-treated patients.

| IVIg treatment indication | No. of patients | Age in years, median (range) | IVIg dose in g/kg, median (range) |
|---|---|---|---|
| Common variable immunodeficiency | 5 | 41 (20–57) | 0·38 (0·30–0·48) |
| Hypogammaglobulinaemia | 6 | 60 (45–77) | 0·39 (0·25–0·59) |
| Agammaglobulinaemia | 1 | 30 | 0·25 |

immune system, thereby disabling protective immunity against pathogens and malignancies. Therapeutic modalities that stimulate $T_{reg}$-mediated immune regulation without affecting global immune functions are attractive. Recently, we found that IVIg can activate both human and mouse $CD4^+CD25^+FoxP3^+$ $T_{regs}$ *in vitro* and increase their ability to suppress allogeneic T cell proliferation [11]. By triggering functional activation of $T_{regs}$, IVIg prevented graft rejection in a fully mismatched skin transplant model. In line with our data, it has been shown that IVIg can prevent mice from developing experimental autoimmune encephalomyelitis [12] and herpes simplex virus-induced encephalitis [13] by enhancing the suppressive capacity and stimulating the expansion of $T_{regs}$. In addition, IVIg was found to enhance the suppressive capacity of human $T_{regs}$ *in vitro* [14].

These data suggest that IVIg treatment can stimulate $T_{reg}$ expansion and function, and may therefore be an attractive tool to re-establish $T_{reg}$-mediated immune regulation. However, the data on the effects of IVIg on $T_{regs}$ are restricted to *in-vitro* and mice experiments and it is unknown whether IVIg treatment actually affects $T_{reg}$ expansion and function in patients. Therefore, in the present study we analysed systematically the effects of either low- or high-dose IVIg treatment on the percentages, activation status and suppressive capacity of circulating $T_{regs}$ in autoimmune and immunodeficient patients. We observed increased activation of $T_{regs}$ after high-dose IVIg treatment, which was not observed for conventional T cells ($T_{conv}$). Additionally, $T_{regs}$ isolated after high-dose IVIg treatment showed enhanced suppressive capacity *ex vivo*.

## Material and methods

### Patients

Twenty-seven patients (21 female/six male) who were treated with IVIg were included in this study. To assess whether IVIg had a dose-dependent effect on $T_{reg}$ activation, we subdivided the patients into two groups: those who received 'low-dose' IVIg and those who received 'high-dose' IVIg. Because patients with immunodeficiency started initially with a supplementary dose of 0·4–0·6 g/kg, we defined 'low-dose' IVIg as a dose $\leq$0·6 g/kg and 'high-dose' IVIg as a dose >0·6 g/kg. The median dose of IVIg received by low-dose patients was 0·39 g/kg (range 0·25–0·59) and that of high-dose patients was 0·98 g/kg (range 0·65–1·71). High-dose IVIg-treated patients showed a significantly higher rise in plasma IgG levels (+17·3 mg/ml) compared to low-dose IVIg-treated patients (+8·2 mg/ml) immediately after treatment ($P < 0·001$), as quantified by immunoturbometric analysis (Roche Diagnostics GmbH, Mannheim, Germany). At day 7, plasma IgG levels dropped again in both groups (data not shown). The indications for IVIg treatment in these patients are depicted in Table 1a,b. In the high-dose treatment group (Table 1b), five patients received IVIg for licensed indications, including common variable immunodeficiency ($n = 2$), hypogammaglobulinaemia ($n = 1$) and idiopathic thrombocytopenic purpura ($n = 2$). One patient with Good's syndrome received high-dose IVIg as supplementary therapy for hypogammaglobulinaemia. Other patients included in Table 1b received high-dose IVIg as anti-inflammatory therapy 'off-label', as they did not

**Table 1b.** Patient characteristics in high-dose intravenous immunoglobulin (IVIg)-treated patients.

| IVIg treatment indication | No. of patients | Age in years, median (range) | IVIg dose in g/kg, median (range) |
|---|---|---|---|
| Polymyositis† | 3 | 41 (33–56) | 1·00 (0·67–1·67) |
| Common variable immunodeficiency | 2 | 40 (37–43) | 0·68 (0·65–0·70) |
| Immune thrombocytopenic purpera | 2 | 68 (65–71) | 1·00 |
| Acquired von Willebrand syndrome† | 2 | 52 (40–63) | 0·95 (0·93–0·98) |
| Hypogammaglobulinaemia | 1 | 40 | 0·66 |
| Good's syndrome/hypogammaglobulinaemia | 1 | 69 | 0·86 |
| Polyserositis e.c.i. | 1 | 24 | 0·78 |
| Polychondritis† | 1 | 68 | 0·69 |
| Dermatomyositis† | 1 | 46 | 1·71 |
| Systemic vasculitis e.c.i.† | 1 | 64 | 1·4 |

†IVIg treatment was indicated based on unresponsiveness to conventional treatment. e.c.i., *e causa ignota*.

respond to conventional immunosuppressive treatment. The IVIg preparations used for treatment were Nanogam® ($n = 13$; Sanquin, Amsterdam, the Netherlands), Kiovig® ($n = 9$; Baxter, Deerfield, IL, USA), Flebogamma® ($n = 4$; Grifols, Barcolona, Spain) and Octagam® ($n = 1$; Octapharma, Lachen, Switzerland). With the exception of three patients, all patients had been treated previously with IVIg, with an average of 28 days (range 21–35 days) before this study. Twenty-one patients were receiving IVIg monotherapy and six patients received additional corticosteroid treatment. All patients showed clinical improvement after treatment.

### Ethics statement

The medical ethics committee of the Erasmus University Medical Centre approved the study, and written informed consent was obtained from all patients participating in the study.

### Sample collection and preparation

Heparin-decoagulated blood samples were collected from healthy blood bank donors and from patients immediately before IVIg infusion immediately after IVIg infusion (4–24 h after the start of the infusion) and 7 days after infusion. Due to practical issues, we were unable to obtain blood samples from one patient immediately after IVIg treatment and from two patients 7 days after treatment. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient sedimentation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Plasma was collected from the gradient and centrifuged at 1157 ***g*** for 10 min at 4°C to remove thrombocytes and debris. For storage of PBMCs, the cells were cryopreserved in RPMI-1640 (Gibco BRL Life Technologies, Breda, the Netherlands) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 10% dimethylsulphoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA). Until further analysis, the PBMC samples were stored at −135°C and plasma at −80°C. To minimize the possible effects of interassay variation, measurements in plasma and on PBMC obtained at different time-points from the same patient were performed on the same day.

### Antibodies and flow cytometry

For the dentification of T cell subsets, PBMCs were stained with anti-CD3-AmCyan, anti-CD4-allophycocyanin (APC)-H7 and CD8-Pacific blue (all from BD Biosciences, San Jose, CA, USA). Percentages and activation status of $T_{regs}$ were determined by surface labelling with anti-CD4-APC-H7, anti-CD25-phycoerythrin (PE)-cyanin 7 (Cy7) (both from BD Biosciences), anti-CD127-PE (Beckman Coulter, Immunotech, Marseille, France), anti-CD38-Pacific Blue (ExBio antibodies, Praha, Czech Republic), anti-human leucocyte antigen D-related (HLA-DR)-peridinin chlorophyll (PerCP) (eBioscience, San Diego, CA, USA) monoclonal antibody (mAb) and proper isotype controls. For the detection of memory ($CD45RO^+$) $T_{regs}$, we additionally labelled the cell surface using CD45RO-fluorescein isothiocyanate (FITC) mAb (Beckman Coulter). Cells ($1 \times 10^6$) were incubated with the mAb in 50 µl phosphate-buffered saline (PBS) (Lonza, Verviers, Belgium) for 30 min on ice and protected from light. Subsequently, intracellular $FoxP3^+$ staining was performed using anti- FoxP3–APC mAb (or rat IgG2a isotype control mAb) purchased from eBioscience, according to the manufacturer's instructions. To study proliferation, intracellular labelling with anti-Ki-67-FITC (or mIgG1 isotype control mAb) from BD Biosciences was performed. After staining, the cells were washed and resuspended in 100 µl PBS for measurement by flow cytometry (FACS Canto, BD Biosciences, San Jose, CA, USA). A minimum of $1{\cdot}5 \times 10^5$ mononuclear cells (MNC) were acquired. Analyses were performed by fluorescence activated cell sorter (FACS) Diva software (BD Bioscience). Viable MNC were gated based on forward-/side-scatter. For the calculation of percentages of cells expressing a certain marker, or for calculation of the geometric mean of fluorescence intensities (MFI), fluorescence of isotype-matched control mAb was subtracted from fluorescence of specific mAb.

### Suppression assay

To determine the effect of IVIg treatment on the suppressive capacity of $T_{regs}$ *ex vivo*, we collected additional blood from seven patients in the study who received high-dose IVIg. To purify $T_{regs}$ and T responder cells ($T_{resp}$), we first purified non-touched $CD3^+$ cells from thawed PBMCs from each time-point by performing immunomagnetic depletion of non-T cells using PE-conjugated antibodies against BDCA1 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD14, CD56 (both from BD Bioscience) CD19, CD56, CD123 (all from Beckman Coulter) as well as CD15- and CD235-microbeads (both from Miltenyi Biotec). The purified $CD3^+$ cells were then stained with anti-CD3-AmCyan, anti-CD4-APC-H7, anti-CD25-APC, 7-aminoactinomycin D (7-AAD) (all from BD Bioscience) and anti-CD127-PE mAb (Beckman Coulter Coulter). Subsequently, $T_{regs}$ were obtained by flow cytometric sorting $7\text{-}AAD^-CD3^+CD4^+CD127^-CD25^+$ cells using FACS Aria (BD Biosciences). Purity of the $T_{regs}$ (defined as $CD4^+CD25^+CD127^-$), as determined by flow cytometry, was 98±1%. $T_{resp}$ were obtained by sorting both $7\text{-}AAD^-CD3^+CD4^-$ and $7\text{-}AAD^-CD3^+CD4^+CD127^+CD25^-$ cells, which resulted in a purity of 99±0·4% (defined as $CD3^+CD127^+CD25^-$ cells), as determined by flow cytometry. Cells were recovered after cell sorting by incubation in culture medium [RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin

(100 μg/ml) and 10% fetal bovine serum (Hyclone, Logan, UT, USA)] at 37°C for at least 3 h prior to co-culture.

To compare their suppressive capacity, $T_{regs}$ purified from the patient blood collected before IVIg treatment, immediately after treatment and at day 7 after treatment were co-cultured with $T_{resp}$ ($5 \times 10^4$) obtained before treatment that were stimulated with 1 μg/ml phytohaemagglutinin (PHA) in the presence of 40 Gy-irradiated autologous PBMC ($5 \times 10^4$) at $T_{reg}$ : $T_{resp}$ ratios of 1:4 in culture medium in round-bottomed 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands). After 5 days, supernatants were collected and the cumulative interferon (IFN)-γ secretion by $T_{resp}$ was quantified by enzyme-linked immunosorbent assay (ELISA) (Ready-SET-Go®; eBioscience) according to the manufacturer's instructions. Each condition was tested at least in triplicate, from which means were calculated. These means were used to calculate the percentage of suppression using the formula: (IFN-γ $level_{Tresp}$–IFN-γ $level_{Tresp+Treg}$)/ IFN-γ $level_{Tresp} \times 100\%$.

### Statistical analyses

Differences in measured variables between time-points before and after IVIg treatment were analysed pairwise using the Wilcoxon signed-rank test. For analysis of the $T_{reg}$ suppression assay we used the paired Student's *t*-test, as the differences in IFN-γ production by $T_{resp}$ collected at different time-points before and after IVIg treatment were distributed normally. Two-sided *P*-values < 0·05 were considered significant. Statistical analysis was performed using GraphPad Prism version 5·0 (GraphPad Software, San Diego, CA, USA). In accordance with the statistical test used, all data presented in dot-plots show median values, but data on the suppressive capacity of $T_{reg}$ (Fig. 2b) are depicted as means ± standard error of the mean (s.e.m.).

## Results

### IVIg enhances selectively the activation status of $T_{regs}$ *in vivo*

IVIg treatment had no effect on the numbers of circulating $CD4^+$ and $CD8^+$ T cells (Fig. 1a). To study the effects of IVIg treatment on $T_{regs}$, we measured the percentages and immunophenotype of circulating $T_{regs}$. The gating strategy used to determine $CD4^+CD25^+FoxP3^+$ cells is shown in Fig. 1b. At baseline, we observed comparable percentages of circulating $T_{regs}$ in patients who were treated with low-dose IVIg ($n = 12$) or high-dose IVIg ($n = 15$) and in healthy controls ($n = 10$) (Fig. 1c). In addition, baseline activation status of the $T_{regs}$ in the low- and high-dose patients were comparable to healthy controls, as assessed by expression levels of FoxP3 (Fig. 1d) and HLA-DR (Fig. 1e).

IVIg treatment had no effect on the percentages of circulating $T_{regs}$ in both low- and high-dose IVIg-treated patients (Fig. 1f). Although low-dose patients showed a slight increase of $T_{reg}$ percentages, this increase was not significant. Additionally, Ki-67 staining in $T_{regs}$ showed no increased proliferation of $T_{regs}$ after IVIg treatment in both groups (data not shown). However, in patients treated with high-dose IVIg, we observed an increased expression level of FoxP3 in $CD4^+CD25^+FoxP3^+$ $T_{regs}$ immediately after IVIg treatment, with an average increase of 29% (Fig. 1b,g, $P = 0{\cdot}04$). This increased FoxP3 expression level was sustained on day 7 after infusion (+35%, $P = 0{\cdot}02$).

Next, we analysed the expression of the activation marker HLA-DR on $CD4^+CD25^+FoxP3^+$ $T_{reg}$, gated as shown in Fig. 1b. We found that the proportions of HLA-DR expressing $T_{reg}$ increased gradually after IVIg treatment, again only in high-dose-treated patients, and was 37% higher immediately after ($P = 0{\cdot}02$) and 39% at day 7 after treatment ($P = 0{\cdot}1$) (Fig. 1b,h). Prednisone treatment did not influence the expression of FoxP3 and HLA-DR, as all six patients who were co-treated with prednisone (low-dose $n = 3$, high-dose $n = 3$) showed the same patterns as patients without prednisone treatment (data not shown). In addition, the same trends of $T_{reg}$ activation were found in patients with autoimmune disease and immunodeficient patients who were treated with high-dose IVIg (Supplementary Fig. S1).

To determine whether IVIg also affects the activation status of $T_{conv}$, we measured HLA-DR expression on $CD4^+FoxP3^-$ $T_{conv}$ (Fig. 1b,i). Compellingly, HLA-DR expression remained low on $T_{conv}$ after IVIg treatment, even in high-dose IVIg-treated patients, which may suggest that IVIg treatment selectively activates $CD4^+CD25^+FoxP3^+$ $T_{regs}$, but not $CD4^+FoxP3^-$ $T_{conv}$ in humans *in vivo*.

To study the effect of IVIg on the differentiation stage of $T_{regs}$, we measured CD45RO expression on $T_{regs}$. We found no effect of IVIg treatment on numbers of $CD45RO^+$ $T_{regs}$ in peripheral blood in both groups, suggesting that IVIg did not drive conversion from naive into memory $T_{regs}$ within the time-frame of the study (data not shown).

### IVIg increases the suppressive capacity of $T_{regs}$ *ex vivo*

To investigate whether or not IVIg treatment stimulates the suppressive capacity of $T_{regs}$, we isolated $CD3^+CD4^+CD25^+CD127^-$ $T_{regs}$ and autologous $CD3^+CD25^-CD127^+$ $T_{resp}$ from PBMCs of seven patients who received high-dose IVIg that were collected before, after and at day 7 after IVIg treatment. The gating strategy for flow cytometric purification of these cells is depicted in Fig. 2a. $T_{resp}$ isolated from blood collected before IVIg infusion were stimulated with PHA and co-cultured with $T_{regs}$ purified from blood samples collected before, after and at day 7 after treatment in a $T_{reg}$ : $T_{resp}$ ratio of 1:4. After 5 days of culture, cumulative IFN-γ production by $T_{resp}$ was determined. $T_{regs}$ that were isolated before treatment showed suppression of IFN-γproduction of 41%, while $T_{regs}$ isolated at day 7 demonstrated a suppres-

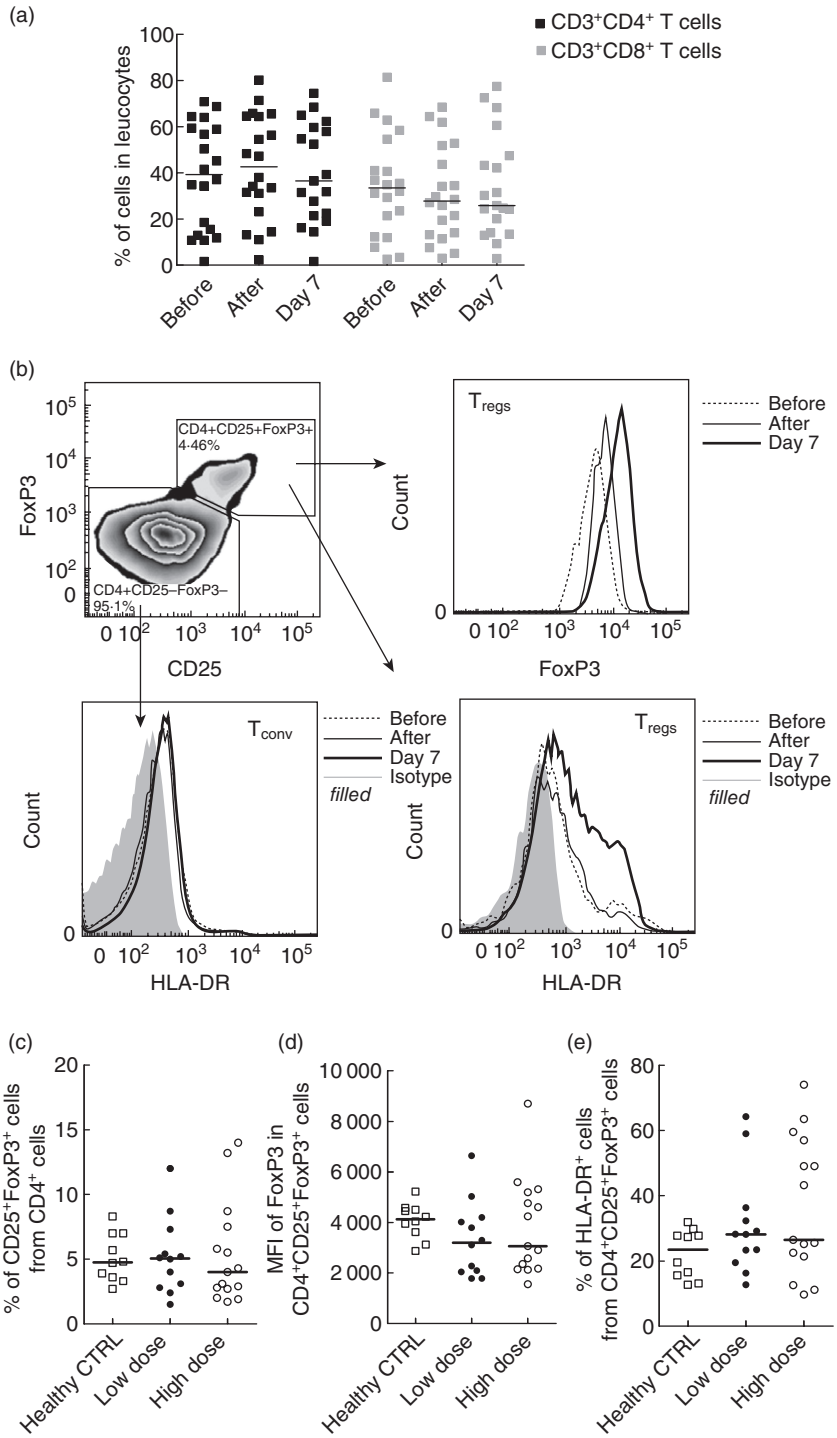


**Fig. 1.** Intravenous immunoglobulin (IVIg) treatment selectively activates circulating regulatory T cells ($T_{regs}$), but not conventional T helper cells ($T_{conv}$). (a) Percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ in peripheral blood mononuclear cells (PBMCs) did not alter after IVIg treatment. (b) Density plot shows the gating strategy of $T_{regs}$ and $T_{conv}$ after gating on $CD4^+$ cells. Histograms show intracellular forkhead box protein 3 (FoxP3) expression in $T_{regs}$ and surface expression of human leucocyte antigen D-related (HLA-DR) on $T_{regs}$ and $T_{conv}$ obtained before, immediately after and at day 7 after IVIg treatment of a representative patient. (c) Baseline percentages of $CD25^+FoxP3^+$ $T_{regs}$ within circulating $CD4^+$ T cells, (d) mean fluorescence intensity (MFI) of FoxP3 in $T_{regs}$ and (e) percentages of $T_{regs}$ expressing HLA-DR from healthy blood donors ($n = 10$) and patients treated with low-dose IVIg ($n = 12$) or high-dose IVIg ($n = 15$). (f) Percentages of $CD25^+FoxP3^+$ $T_{regs}$ within the $CD4^+$ population before, immediately after and 7 days after IVIg treatment in patients who received low-dose ($n = 12$) or high-dose IVIg ($n = 15$). (g) MFI of FoxP3 in $T_{regs}$, (h) percentages of $T_{regs}$ and (i) percentages of $T_{conv}$ expressing HLA-DR before, immediately after and 7 days after IVIg treatment in patients who received low-dose ($n = 12$) or high-dose IVIg treatment ($n = 15$). Horizontal lines represent median. $^*P < 0{\cdot}05$.

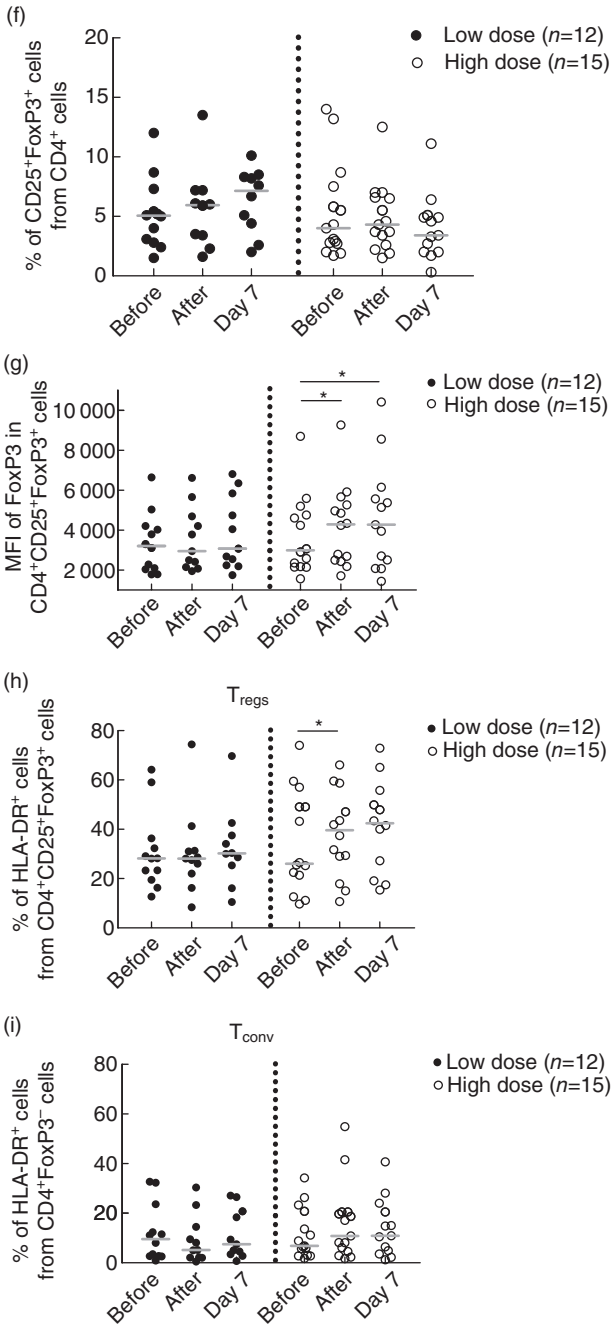


**Fig. 1.** *Continued*

sion of 70% ($P = 0{\cdot}001$, Fig. 2b). Thus, in accordance with the observed increased $T_{reg}$ activation status at day 7 after IVIg treatment, $T_{regs}$ isolated at 7 days also showed a higher suppressive capacity. These data suggest that IVIg treatment may stimulate the suppressive function of circulating $T_{regs}$ for up to at least 1 week after treatment.

## Discussion

Multiple ongoing studies are being performed to induce $T_{reg}$ expansion or stimulate $T_{reg}$ function in order to control T

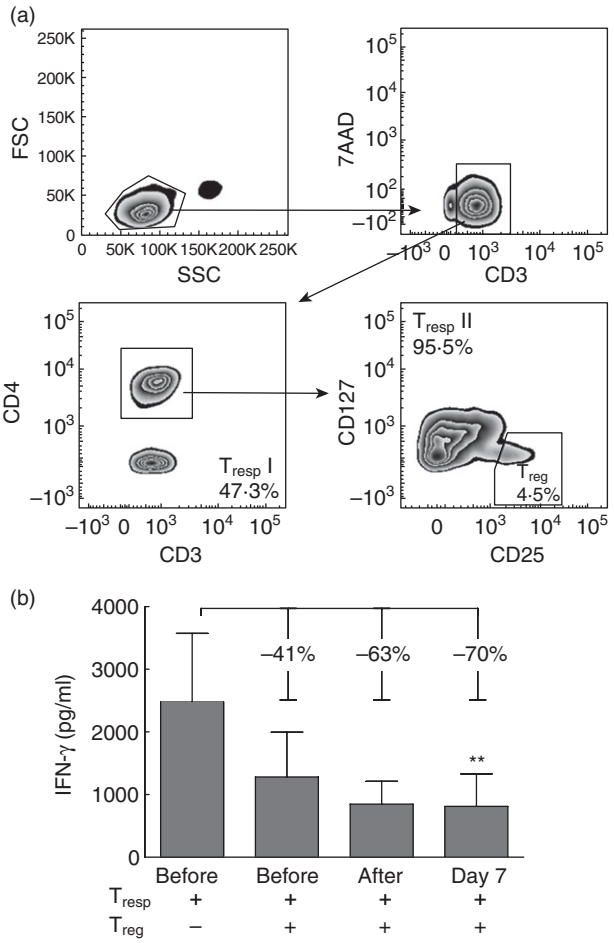


**Fig. 2.** Enhanced suppressive activity of regulatory T cells ($T_{regs}$) purified from blood after intravenous immunoglobulin (IVIg) treatment. (a) Regulatory T cells ($T_{regs}$) were obtained by flow cytometric sorting on 7-aminoactinomycin D (7-AAD)$^-$CD3$^+$CD4$^+$CD127$^-$CD25$^+$ cells ($T_{regs}$) from peripheral blood mononuclear cells (PBMC) collected from seven high-dose IVIg-treated patients before treatment, immediately after treatment and at day 7 after treatment, and T responder cells ($T_{resp}$) were obtained by sorting both 7-AAD$^-$CD3$^+$CD4$^-$ ($T_{resp}$ I) and 7-AAD$^-$CD3$^+$CD4$^+$CD127$^+$CD25$^-$ ($T_{resp}$ II) cells from autologous PBMCs collected before IVIg-treatment. (b) $T_{regs}$ purified from the patients before, after and at day 7 after treatment were co-cultured with $T_{resp}$ ($5 \times 10^4$) obtained before treatment that were stimulated with 1 μg/ml phytohaemagglutinin (PHA) in the presence of 40 Gy-irradiated autologous PBMCs ($5 \times 10^4$) at a $T_{reg}$ : $T_{resp}$ ratio of 1:4 in culture medium in round-bottomed 96-well plates. After 5 days, supernatants were collected and the cumulative interferon (IFN)-γ production by $T_{resp}$ was quantified by enzyme-linked immunosorbent assay. Each condition was tested at least in triplicate from which means were calculated. From each patient, these means were used to calculate the percentage of suppression using the formula: (IFN-γ $level_{Tresp}$–IFN-γ $level_{Tresp–Treg}$)/IFN-γ $level_{Tresp} \times 100\%$. Bar graphs represent mean ± standard error of the mean. $^{**}P < 0{\cdot}01$, compared to $T_{resp}$ alone.

cell-mediated disorders. Interestingly, the current study suggests that high-dose IVIg treatment may be useful to enhance $T_{reg}$ activation and suppressive function *in vivo*. We show that IVIg treatment enhances both the activation status and suppressive function of $T_{regs}$ in patients. Upon high-dose IVIg treatment, a gradual rise in FoxP3 and HLA-DR expression in circulating $CD4^+CD25^+FoxP3^+$ $T_{regs}$ was found, with the highest expression on day 7 after infusion. This finding indicates that IVIg treatment activates $T_{regs}$ for at least 1 week, when IVIg levels already declined. $T_{reg}$ activation was clearly dependent upon the IVIg dose, as patients who received low-dose IVIg ($\leq$0·6 g/kg) did not show significant effects. Activation of $T_{reg}$ was not restricted to patients with autoimmune disease, as immunodeficient patients who received high-dose IVIg also showed a rise in FoxP3 and HLA-DR expression on $T_{regs}$. Thus, our data support the generally accepted idea that a high-dose treatment regimen is required in order to gain anti-inflammatory activity [7].

Our *ex-vivo* functional assays showed that IVIg treatment may not only stimulate the activation status, but may also enhance the suppressive function of circulating $T_{regs}$. We observed an increased suppressive function of $T_{regs}$ that was highest on day 7 after IVIg treatment, which is in line with the time-dependent increase of the activation status after treatment. Supporting our data, previous studies have shown that $T_{regs}$ with a higher HLA-DR [11] and FoxP3 [15,16] expression exert higher suppressive capacity. Further studies are warranted to confirm this finding, as the suppressive function was studied in a small number of patients. Moreover, it will be extremely interesting to monitor the activation status and suppressive function of $T_{regs}$ after high-dose IVIg therapy over a longer time-period to study the durability of this effect.

In order to assess the suppressive capacity of $T_{regs}$, we did not use proliferation as the classical read-out, as interleukin (IL)-2 produced by $T_{resp}$ in the co-culture system may influence proliferation of $T_{regs}$, hence affecting overall [$^3$H]-thymidine incorporation [17]. Therefore, we analysed IFN-γ secretion by $T_{resp}$, which is independent of the extent of $T_{regs}$ hyporesponsiveness and may therefore be more unbiased.

While a few previous studies have shown an increase of circulating $T_{reg}$ numbers in patients treated with IVIg [18–21], we show an increase in both the activation status and functional suppressive capacity of $T_{reg}$ upon high-dose IVIg therapy in humans *in vivo*. Although the effects are moderate this finding may be of importance, as in several autoimmune diseases the numbers of $T_{regs}$ are normal, yet these cells display a functional defect [22–24]. A possible explanation for the fact that we did not find an increase in the circulating $T_{reg}$ numbers after IVIg treatment is that our patients did not have a deficit of $T_{reg}$ numbers compared to healthy subjects (Fig. 1c), while the previous studies included patients with acute inflammatory diseases which had decreased numbers of circulating $T_{regs}$ before IVIg treatment [18–20].

Compellingly, high-dose IVIg treatment selectively activated $CD4^+CD25^+FoxP3^+$ $T_{regs}$, but not $CD4^+FoxP3^-$ $T_{conv}$. This observation may be interesting for the clinic, as conventional immunosuppressive drugs used for treating autoimmune diseases and allograft rejection, in particular calcineurin inhibitors, do not only suppress $T_{conv}$, but also $T_{regs}$ [25–32]. By selective activation of $CD4^+CD25^+FoxP3^+$ $T_{regs}$, but not $T_{conv}$, IVIg may be superior to classical immunosuppressive drugs. Similar observations were also found *in vitro* and in mice models [11,12,14]. In addition, IVIg selectivity for $T_{regs}$ has also been suggested by Maddur and colleagues [33], who showed that IVIg inhibited T helper type 17 (Th17) cell differentiation and amplification, while it increased the numbers of $T_{regs}$ among memory T cells.

The mechanism by which IVIg selectively activates $T_{regs}$ is still unclear. Our previous data [11], together with the study by Ephrem *et al.* [12], suggest that the activation may be mediated by direct binding of IVIg to an unknown 'IVIg receptor' present on $T_{regs}$ but not on $T_{conv}$. Because we observed that human $T_{regs}$ do not express classical Fcγ receptors [11], this receptor must be a non-classical IgG-binding molecule. Alternatively, IVIg may bind to specific surface receptors by its F(ab′)2 fragment, as suggested by Maddur *et al.* [33], or IVIg may contain $T_{reg}$ epitopes that can activate $T_{regs}$ by presentation on major histocompatibility complex (MHC) $II^+$ antigen-presenting cells towards $T_{regs}$, as proposed by De Groot and colleagues [34,35].

The current study focused upon patients treated with IVIg for different indications. Although the underlying diseases are variable, the positive effect of high-dose IVIg on $T_{reg}$ activation is seen consistently in patients treated with high-dose IVIg. These results suggest that the effects of IVIg on $T_{reg}$ activation are not restricted to a certain disease, but to high-dose treatment. It would, however, be interesting to confirm the dose-dependent effect of IVIg in patients with the same treatment indication in the future.

In summary, we have demonstrated that high-dose IVIg therapy in humans *in vivo* can activate $T_{regs}$ selectively and enhance their suppressive capacity. As the effects in our patient cohort were moderate, we propose that $T_{reg}$ stimulation may be one of the mechanisms by which IVIg controls inflammation in patients with autoimmune and systemic inflammatory disorders, but other mechanisms are probably also required to restore imbalanced immune homeostasis. The data presented in this study underline the multi-faceted character of the immunomodulatory mechanisms of IVIg.

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## Disclosure

The authors declare no financial or commercial conflicts of interest.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1.** Regulatory T cell ($T_{reg}$) activation status after high-dose intravenous immunoglobulin (IVIg) treatment. Mean fluorescence intensity (MFI) of forkhead box protein 3 (FoxP3) expression in $T_{regs}$ from (a) autoimmune disease patients and (b) immunodeficient patients and percentages of human leucocyte antigen D-related (HLA-DR) expression on $T_{regs}$ in (c) autoimmune disease patients and (d) immunodeficient patients that are obtained before, immediately after and at day 7 after IVIg infusion. Horizontal lines represent median; n.s.: non-significant.