Intravenous Immunoglobulin Treatment in Humans Suppresses Dendritic Cell Function via Stimulation of IL-4 and IL-13 Production

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Intravenous Immunoglobulin Treatment in Humans Suppresses Dendritic Cell Function via Stimulation of IL-4 and IL-13 Production

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High-dose i.v. Ig (IVIg) is a prominent immunomodulatory therapy for various autoimmune and inflammatory diseases. Recent mouse studies suggest that IVIg inhibits myeloid cell function by inducing a cascade of IL-33–Th2 cytokine production causing upregulation of the inhibitory FcγRIIib, as well as by modulating IFN-γ signaling. The purpose of our study was to explore whether and how these mechanisms are operational in IVIg-treated patients. We show that IVIg in patients results in increases in plasma levels of IL-33, IL-4, and IL-13 and that increments in IL-33 levels correlate with rises in plasma IL-4 and IL-13 levels. Strikingly, no upregulation of FcγRIIib expression was found, but instead a decreased expression of the activating FcγRIIa on circulating myeloid dendritic cells (mDCs) after high-dose, but not after low-dose, IVIg treatment. In addition, expression of the signaling IFN-γR2 subunit of the IFN-γR on mDCs was downregulated upon high-dose IVIg therapy. In vitro experiments suggest that the modulation of FcγRs and IFN-γR2 on mDCs is mediated by IL-4 and IL-13, which functionally suppress the responsiveness of mDCs to immune complexes or IFN-γ. Human lymph nodes and macrophages were identified as potential sources of IL-33 during IVIg treatment. Interestingly, stimulation of IL-33 production in human macrophages by IVIg was not mediated by dendritic cell–specific intercellular adhesion molecule-3–grabbing nonintegrin (DC-SIGN). In conclusion, high-dose IVIg treatment inhibits inflammatory responsiveness of mDCs in humans by Th2 cytokine-mediated downregulation of FcγRIIa and IFN-γR2 and not by upregulation of FcγRIIib. Our results suggest that this cascade is initiated by stimulation of IL-33 production that seems DC-SIGN independent.

I

travenous Ig (IVIg) is used for the treatment of several autoimmune and systemic inflammatory diseases caused by autoantibodies and/or derangement of the cellular immune system (1–7). Importantly, the anti-inflammatory effects of IVIg require treatment with high doses, which exceed those used for replacement therapy in immune deficiencies (3, 8–10). Although the clinical benefit of IVIg is evident, the mechanisms by which IVIg can suppress different types of immune responses have not yet been fully elucidated. Effects of IVIg on Ab-mediated immune responses include prevention of immune complex binding to activating FcγRs and enhanced clearance of autoantibodies by saturation of the neonatal FcR (4, 5, 8). The beneficial effects of IVIg on diseases caused by hyperactivity of cellular immunity are mediated by modulation of regulatory T cells (5, 11–14), macrophages (15, 16), and dendritic cells (12, 17, 18).

In several mouse models of Ab-induced inflammatory diseases, the anti-inflammatory effects of IVIg are initiated by binding of 2,6-sialylated IgG molecules to specific intracellular adhesion molecule-grabbing nonintegrin R1 on splenic marginal zone macrophages, resulting in upregulation of the inhibitory Fcγ-R1IB on macrophages at the site of inflammation, thereby preventing their activation by pathogenic immune complexes (IC) (19). Recent research has uncovered the missing link between these two types of macrophages in a serum-induced arthritis model by showing that IVIg stimulates IL-33 production in the spleen, which induces production of the Th2 cytokines IL-4 and IL-13 by basophils, which in their turn upregulate expression of the inhibitory Fcγ-R1IB on effector macrophages in the joints (20). However, the question whether and how this anti-inflammatory mechanism of IVIg also plays a role in humans is actually far from trivial. Firstly, the structural organization of the human spleen differs from the murine spleen, especially with regard to the marginal zone. The marginal zone macrophage, the postulated source of IL-33 production upon binding of 2,6-sialylated IgG molecules to SIGNR1, is absent in the human spleen (21). Secondly, splenectomized idiopathic thombocytopenic purpura (ITP) patients do still respond to IVIg therapy, arguing against an indispensable role for the spleen in mediating the protective effects of IVIg in humans (22). Thirdly, recent studies in ITP mouse models demonstrated that the inhibitory effects of 2,6-sialylated IgG molecules were not impaired upon
splenectomy (23, 24) and were even independent of IL-33 and IL-4 signaling (23). Collectively, these observations warrant a study to determine whether IVIg stimulates the IL-33–Th2–FcγRIIB pathway in humans.

Therefore, the first aim of the current study was to investigate the effects of IVIg therapy on IL-33 and Th2 cytokines as well as its effects on the expression of FcγRs on myeloid cells in humans in vivo. Because IVIg treatment has been shown to downregulate expression of the signaling IFN-γR2 unit on murine macrophages, thereby rendering them refractory to activation by IFN-γ (16), we also studied the effects of IVIg treatment on IFN-γR expression on circulating myeloid cells. To discern whether the effects of IVIg depend on treatment dose, we included patients treated with a high or low dose of IVIg in our study. In the second part of the study, we investigated whether a causative relation may exist among IVIg, IL-33, Th2 cytokines, receptor expression on myeloid cells, and the functional responsiveness of these cells, using human cells in vitro.

Materials and Methods

Patients

Twenty-nine patients (22 female and 7 male) with either immunodeficiency or autoimmune disease and treated with IVIg were included in this study and subdivided into two groups: those who received low-dose IVIg (LD) and those who received high-dose IVIg (HD). Because supplemental-dose treatment started initially with 0.4–0.6 g/kg, we defined LD as ≤0.6 g/kg and HD as >0.6 g/kg. The indications for IVIg treatment in these patients are depicted in Table I. Twenty-two patients were on IVIg monotherapy, and 6 patients received additional corticosteroid treatment. All patients are depicted in Table I. Twenty-two patients were on IVIg monotherapy, and 6 patients received additional corticosteroid treatment. All patients showed clinical improvement after treatment. After approval by the local institutional ethical review board, written informed consent was obtained from all participants.

IVIgs

The IVIg preparations received by the patients were Nanogam (n = 13; Sanquin, Amsterdam, The Netherlands), Kiovig (n = 11; Baxter, Deerfield, IL), Fiebigamma (n = 4, Grifols, Barcelona, Spain), and Octagam (n = 1; Octapharma, Lachen, Switzerland).

For in vitro experiments, we used Intratect (Biotest Pharma, Dreieich, Germany), which was a kind gift from the company. IVIg was diazylated against large volumes of RPMI at 4°C using Slide-A-Lyzer γ-irradiated dialysis cassettes (Pierce, Rockford, IL) to remove stabilizing agents and obtain neutral pH. In all experiments, IVIg was used at a concentration of 10 mg/ml, which is similar to the median increment in serum IgG concentration we observed in the IVIg-treated patients. To avoid artificial IC-mediated activation of mDCs due to adsorption of the IgG molecules to the culture plates, we precoated round-bottom 96-well cell-culture plates with FCS to prevent IgG binding (25). After 24 h, the plates were extensively washed with PBS, remaining liquid was removed, and mDCs, splenocytes, or lymph node cells were cultured as described below. To control for the elevated levels of protein upon addition of IVIg to cell cultures, we used equimolar amounts of human serum albumin (Sanquin) as described previously (6).

Sample collection and preparation

Heparin-decaagulated blood samples were collected from healthy blood donors and patients immediately before IVIg infusion, immediately after IVIg infusion (LD 4–6 h and HD 24–30 h after the start of the infusion), and 7 d after infusion. Plasma and PBMCs were isolated from whole blood by density-gradient sedimentation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Until further analysis, plasma samples were stored at −80°C and PBMC samples at −135°C. To minimize possible interassay variation, measurements on PBMCs and plasma obtained at different time points from the same patient were performed on the same day. Because we did not have sufficient PBMCs from all patients, measurements of different receptor expression on mDCs were performed on 10 LD and 11 HD patient samples.

Human splenic tissue (n = 6) and hepatic lymph nodes (n = 6) were obtained during organ procurement from liver donors. Lymph nodes were dissected from the hepatoduodenal ligament. Spleen tissue and lymph nodes were sliced into small pieces and passed over a 100-μm nylon mesh filter to obtain a single-cell suspension. Mononuclear cells were then obtained by Ficoll-Paque density centrifugation.

Abs and flow cytometry

For identification of CD20+ blood DC Ag-1 (BDCA1)+ mDC in blood of patients, PBMCs were stained with anti–CD20-Pacific Blue (eBioscience, San Diego, CA) and anti–BDCA1-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and for identification of monocytes with anti–CD14 PE-Cy7 (eBioscience). To detect expression of FcγRIIIa and FcγRIIB on their surface, we used FITC-conjugated or Alexa Fluor 488–conjugated anti–CD32b/FcγRIIB-FITC (clone ch2B6N297Q (26, 27), which was kindly provided by MacroGenics (Rockville, MD). IFN-γ chain 2 was stained by using anti–IFN-γR chain 1 mAb (BioLegend, San Diego, CA). A minimum of 3 × 10⁴ mononuclear cells was acquired. Analyses were performed by FACSdiva software (BD Biosciences). Viable mononuclear cells were gated based on forward/side scatter and exclusion of 7-AAD. For each sample, we used matched isotype control mAb to set gates for analysis.

ELISA

Plasma and/or culture media concentrations of IL-33, IL-5 (BioLegend), IL-4, IL-13, IL-6 (Pelikine; Sanquin, Amsterdam, The Netherlands), IFN-γ (Abcam, Cambridge, U.K.), TNF-α, IL-10 (Quantikine HS; R&D Systems, Minneapolis, MN), and IFN-γ–induced protein 10 (IP-10; Invitrogen, Toronto, Ontario, Canada) were measured by ELISA according to the manufacturer’s instructions. IVIg present in plasma did not interfere with cytokine detection, because addition of IVIg (10 mg/ml) to samples with known cytokine levels did not alter the measured concentrations.

mDC isolation and macrophage generation

BDCA1+CD20+ mDCs were purified from fresh heparinized blood of healthy volunteers by positive selection with PE-conjugated anti–CD1c mAb and anti–PE MACS beads, after depletion of B cells with CD19-conjugated MACS beads (Miltenyi Biotec) (18). The purity of mDCs as analyzed by flow cytometry was 92 ± 8%. Macrophages were generated in vitro by culturing human monocytes, which were purified by positive selection using anti–CD14 MACS beads (Miltenyi Biotec) with M-CSF (20 ng/ml) for 6 d. Fresh medium and cytokines were replenished every 2 d.

Effect of cytokines on receptor expression and function in vitro

To analyze the effects of IVIg, IL-33, IL-4, and IL-13, mDCs or macrophages (1 × 10⁶/200 μl) were cultured in 96-well round-bottom plates (Costar, Cambridge, MA) with IVIg (10 mg/ml), human rIL-33 (50 ng/ml; Enzo Life Sciences, Farmingdale, NY), rIL-4 (10 ng/ml; add to cells immediately after addition of IVIg), rIL-13 (100 ng/ml; Invivogen, San Diego, CA), or rIL-13 (100 ng/ml; Invitrogen, San Diego, CA) in RPMI supplemented with penicillin, streptomycin, and 10% FBS for 24 h.

To assess the responsiveness of mDCs to IC, IgG was immobilized by overnight incubation of IVIg (1 mg/ml) in 200 μl PBS in wells of 96-well round-bottom plates at 4°C for 24 h (26), and mDCs either pretreated or not with IL-4 or IL-13 were cultured in these plates for another 48 h. To determine their responsiveness to IFN-γ, pretreated mDCs were recultured for 24 h in the presence of IFN-γ (250 U/ml; Miltenyi Biotec). Maturation of DCs was stimulated by addition of 50 ng/ml LPS (derived from S. minnesota, ultrapure; Invivogen, San Diego, CA).
**Effect of IVIg on cytokine gene expression in vitro**

To identify the source of IL-33 or IL-4 production induced by IVIg, freshly isolated human splenocytes or lymph node cells (2 x 10^7/2 ml) or M-CSF–induced monocyte-derived macrophages (1 x 10^7/2 ml) were cultured with or without IVIg (10 mg/ml) in six-well plates in RPMI supplemented with penicillin, streptomycin, and 10% FBS. LPS was used at 1 μg/ml to stimulate the cells. Cells were harvested after 24 h, and relative IL-33 mRNA levels were determined by quantitative RT-PCR. RNA was isolated from the cells using a Machery-Nagel NucleoSpin RNA II kit (Bioké, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). cDNA was prepared from total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Stanford, CA). TaqMan gene expression assays were performed to measure the mRNA levels of IL-33 or IL-4 as the gene of interest and GAPDH as the housekeeping gene using primer/probe sets from Applied Biosystems (Foster City, CA). As positive control for IL-33, we used RNA isolated from tonsils obtained during routine tonsillectomies, which was a kind gift from Dr. T. Cupedo (Hematology Department, Erasmus MC, Rotterdam, The Netherlands). As positive control for IL-4, we used RNA isolated from CD3+ T cells stimulated with PMA and ionomycin. All reactions were performed in duplicate. Relative expression was calculated by the cycling threshold method as 2^-ΔΔ threshold.

**Statistical analyses**

Differences in measured variables in blood between time points before and after IVIg treatment were pairwise analyzed using the Wilcoxon signed-rank test. Differences in measured variables obtained from cell cultures were pairwise analyzed using the Student t test, because the differences were normally distributed. Regression analyses were performed by using the Spearman correlation test. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). For all tests, p values <0.05 were considered as significant.

**Results**

**Increased IL-33, IL-4, and IL-13 plasma concentrations after IVIg treatment in vivo**

LD patients, defined as patients who were treated with IVIg dose ≤0.6 g/kg, received a median dose of 0.38 g/kg (range 0.25–0.59), and HD patients, defined as patients who were treated with IVIg dose >0.6 g/kg, received a median dose of 0.90 g/kg (range 0.65–1.71). The indications of IVIg treatment in LD and HD patients are depicted in Table I. Plasma IgG levels increased to significantly higher concentrations in HD patients compared with LD patients (data not shown).

Following IVIg treatment, plasma IL-33 significantly increased for at least 7 d in both LD (215% immediately after and 169% at day 7) and HD patients (152% immediately after and 70% at day 7) (Fig. 1A). However, the rise of plasma IL-33 in LD patients did not exceed that of the baseline levels of HD patients, and posttreatment levels were significantly higher in the HD patients compared with those in LD patients. The higher baseline IL-33 level in HD patients (3-fold compared with LD patients) may be explained by the active inflammation in these patients. Likewise, plasma IL-4 concentrations significantly increased for at least 7 d after IVIg infusion in both LD (115% immediately after and 52% at day 7) and HD patients (227% immediately after and 136% at day 7) (Fig. 1B) and also reached significantly higher posttreatment concentrations in HD than in LD patients. IL-13 plasma levels were significantly elevated in HD patients after IVIg therapy (574% immediately after and 524% at day 7) but not in LD patients (Fig. 1C). In contrast, we could not detect IL-5 in plasma in any of the patients (Human IL-5 ELISA; BioLegend; sensitivity 2 pg/ml). Among HD patients, 11 were treated with IVIg for autoimmune disease and 5 for immunodeficiency, and elevations in IL-33 and Th2 cytokine levels were observed for both patient groups (data not shown).

The rise in plasma IL-4 and IL-13 significantly correlated with the rise in IL-33 levels (Figs. 1D, 1E), supporting the hypothesis that IL-33 stimulates Th2 cytokine production in humans in vivo. To elucidate whether IVIg therapy selectively enhances plasma IL-33 and Th2 cytokine concentrations, we measured plasma levels of Th1 cytokines and the anti-inflammatory cytokine IL-10. IL-6 plasma level did not increase upon IVIg treatment, whereas transient increases were observed for TNF-α in HD patients, IFN-γ in both LD and HD patients, and IL-10 levels in LD patients, but all of these cytokines returned to baseline levels at day 7 after treatment (Fig. 1F–I). Together, these data show that IVIg therapy upregulates IL-33, IL-4, and IL-13 plasma levels up to at least 1 wk after IVIg infusion, whereas not or only transiently enhancing plasma levels of Th1 cytokines and IL-10.

**IVIg treatment reduces expression of activating FcγRIIa and IFN-γR2 on circulating mDCs in vivo**

We hypothesized that the sustained increase in Th2 cytokine levels induced by IVIg treatment might modulate the expression of FcγRIIb or FcγRIIa on myeloid cells, as has been previously shown in mice (20, 29) and human monocytes in vitro (30, 31). Therefore, we measured the expression of the inhibitory FcγRIIb and activating FcγRIIa on circulating mDCs and monocytes, defined as CD20+ BDCA1+ and CD14+ leukocytes, respectively. IVIg treatment did not affect FcγRIIb or FcγRIIa expression on circulating monocytes (data not shown). However, clear effects were observed on the expression of these receptors on circulating mDCs in HD patients, but not in LD patients. FcγRIIb expression decreased directly after infusion, but expression was restored at day 7 (Fig. 2A, 2B). In contrast, a stepwise decline of the activating FcγRIIa expression was observed, which became statistically

| Table I. Patient characteristics of LD- and HD-treated patients |
|-------------------------|-------------------------|-------------------------|-------------------------|
| IVIg Treatment Indication | No. of Patients | Age (y), Median (Range) | IVIg Dose (g/kg), Median (Range) |
| LD patients | Common variable immunodeficiency | 5 | 33 (20–57) | 0.38 (0.30–0.48) |
| Hypogammaglobulinemia | 7 | 60 (40–77) | 0.39 (0.25–0.59) |
| Agammaglobulinemia | 1 | 30 | 0.25 |
| HD patients | Refractory polymyositis* | 3 | 41 (33–56) | 1.00 (0.67–1.67) |
| Hypogammaglobulinemia | 3 | 68 (40–69) | 0.86 (0.66–0.86) |
| Common variable immunodeficiency | 2 | 40 (37–43) | 0.68 (0.65–0.70) |
| Acquired von Willebrand syndrome* | 2 | 68 (65–71) | 1.00 |
| Polyserositis e.c.i.* | 1 | 24 | 0.78 |
| Polychondritis* | 1 | 68 | 0.69 |
| Refractory dermatomyositis* | 1 | 46 | 1.71 |
| Systemic vasculitis e.c.i.* | 1 | 64 | 1.4 |

*IVIg treatment was indicated based on unresponsiveness to conventional treatment.
significant (−30%) at day 7 after IVIg treatment (Fig. 2A, 2C). These decreases were not attributable to interference of IVIg with anti-FcγRIIa or anti-FcγRIIb mAb binding (Supplemental Fig. 1A, 1B).

Next, we studied IFN-γR expression on mDCs and monocytes. A median of 97% (range 84–100) of mDCs expressed the ligand-binding IFN-γR1 subunit at baseline, which did not change after IVIg treatment (data not shown). In contrast, expression of the signaling IFN-γR2 subunit, which was restricted to 4.9% of total mDCs at baseline, stepwise declined after treatment in HD patients (251% at day 7), but not in LD patients (Fig. 2A, 2D). This decrease was not attributable to interference of IVIg with anti–IFN-γR2 mAb binding (Supplemental Fig. 1C). A trend toward reduction of IFN-γR2 expression was also observed for monocytes on day 7, but did not reach statistical significance (p = 0.11) (data not shown). Similar modulation of FcγRIIb, FcγRIIa, and IFN-γR on mDCs was observed in HD patients that received IVIg for either autoimmune or immunodeficient conditions (Supplemental Fig. 1D–F). Prednisone treatment did not influence the effect of IVIg on FcγRIIb, FcγRIIa, and IFN-γR expression on mDCs, because all six patients who were cotreated with prednisone (low dose, n = 2; high dose, n = 4) showed comparable patterns to those observed in patients without prednisone treatment (data not shown).

Together, these data show that upon HD, but not LD treatment, FcγRIIa and IFN-γR2 expression on circulating mDCs is sustainably suppressed, whereas FcγRIIb expression is only temporarily reduced.

IL-4 and IL-13 reduce FcγRIIb, FcγRIIa, and IFN-γR2 expression on human mDCs in vitro

To assess whether the decreased expression of FcγRIIb, FcγRIIa, and IFN-γR2 on circulating mDCs upon IVIg therapy may have been caused by the observed increase in IL-33, IL-4, or IL-13 levels or by IVIg itself, we cultured mDCs purified from blood of healthy donors with IVIg, rIL-33, rIL-4, or rIL-13 in vitro for 24 h. Interestingly, FcγRIIb, FcγRIIa, and IFN-γR2 expression was reduced on mDCs cultured with rIL-4 or rIL-13, whereas their expression was not affected by rIL-33 or by IVIg (Fig. 3A–C, Supplemental Fig. 2A–C). IL-4 and IL-13 showed redundant roles in the modulation of these receptors, as their expression did not further decrease in the presence of both cytokines in comparison with either cytokine alone (Supplemental Fig. 3A). Next, we assessed whether IL-4 and IL-13 could support downregulation of these receptors during LPS-induced mDC maturation. mDCs were cultured for 24 h in the presence of IL-4 or IL-13, followed by LPS stimulation for an additional 24 h. LPS downregulated the expression of FcγRIIb, FcγRIIa, and IFN-γR2 expression on mDCs, and we found that IL-4, IL-13, and LPS additively downregulated FcγRIIb, FcγRIIa, and IFN-γR2 expression (Supplemental Fig. 3B). Collectively, these data suggest that the enhanced levels of IL-4 and IL-13 after IVIg infusion in vivo are primarily responsible for the modulation of FcγRIIb, FcγRIIa, and IFN-γR2 expression on circulating mDCs.
Because the beneficial effects of IVIg in mice have been attributed to Th2 cytokine-mediated upregulation of FcγRIIb expression on macrophages (20), we also cultured human monocyte-derived macrophages with rIL-4 or rIL-13 for 24 h. However, Th2 cytokines did not significantly affect FcγRIIa or FcγRIIb expression on human macrophages (Supplemental Fig. 4A, 4B).

IL-4 and IL-13 reduce responsiveness of human mDCs to IC and IFN-γ in vitro

The balance between the expression of FcγRIIa and FcγRIIb establishes a threshold for mDC activation by IC (26). To determine the functional effects of the downregulation of both activatory FcγRIIa and inhibitory FcγRIIb by IL-4 or IL-13, we cultured purified mDCs with rIL-4 or rIL-13 for 24 h and subsequently recultured them in the presence of immobilized IgG for 48 h. Both rIL-4 and rIL-13 pretreatment suppressed IC-mediated maturation, as assessed by CD83 upregulation (Fig. 4A) and repressed proinflammatory IL-8 (Fig. 4B) and TNF-α (Fig. 4C) production after IC challenge. To determine the role of FcγRIIa and FcγRIIb in this effect, we blocked FcγRIIa or FcγRIIb ligation. Blockade of FcγRIIa prevented activation of mDCs by IC. Interestingly, blocking of FcγRIIb during IC stimulation of IL-4- or IL-13-pretreated mDCs resulted in a greater rise in CD83 expression (Fig. 4A) and cytokine production (Figs. 4B, C) compared with FcγRIIb blockade of control mDCs, indicating that FcγRIIb is relatively dominant over FcγRIIa in regulating the response of IL-4- or IL-13-treated mDCs to IC. Thus, downregulation of FcγRIIa by IL-4 or IL-13 reduces the responsiveness of mDCs to IC, despite simultaneous suppression of inhibitory FcγRIIb expression.

To establish the functional effect of IFN-γR2 downregulation by IL-4 and IL-13, we cultured purified mDCs with rIL-4 or rIL-13 for 24 h, followed by IFN-γ stimulation for an additional 24 h. In contrast to mDCs preincubated in medium, mDCs pretreated with rIL-4 and rIL-13 could not upregulate CD86 (Fig. 5A), CCR7 (Fig. 5B), and CD38 (Fig. 5C) expression after IFN-γ stimulation. Moreover, the production of IP-10/CXCL10, an important mediator of chemotaxis, apoptosis, cell growth, and angiostasis (32), was significantly reduced in mDCs in response to IFN-γ after treatment with rIL-4 or rIL-13 (Fig. 5D). Of note, IFN-γ stimulation did not affect the expression of FcγRIIa, FcγRIIb, and IFN-γR2 on mDCs, suggesting that the transient increase of IFN-γ in plasma directly after IVIg administration in our patient cohort was not the cause for the observed modulation of these receptors on circulating mDCs (data not shown).

Collectively, our data indicate that IL-4 and IL-13 treatment suppresses IC- and IFN-γ–mediated mDC maturation and cytokine/chemokine production by suppression of FcγRIIa and IFN-γR2 expression, respectively.

Human lymph node is a potential source for IL-33 production

We made a first attempt to identify the source of the enhanced IL-33 plasma levels upon IVIg infusion in humans. We hypothesized that IVIg may induce IL-33 production in secondary lymphoid tissues, because IL-33 is expressed in human secondary lymphoid tissues (33), and upregulation of this cytokine has been shown in murine spleen upon IVIg treatment (20). Therefore, we cultured freshly isolated human splenocytes or lymph node cells in vitro with or without IVIg. Because IL-33 expression can be induced by TLR4.
ligation (34–36), cells were cultured in the absence or presence of LPS. We detected no \textit{IL-33} mRNA in splenocytes, but in lymph node cells, \textit{IL-33} mRNA was detectable and markedly increased after incubation with IVIg in the presence of LPS (Fig. 6A). However, IL-33 protein could not be detected in supernatants from the cell cultures by ELISA (BioLegend; sensitivity 4 pg/ml), indicating no or very low IL-33 secretion by the cells in vitro. No \textit{IL-4} mRNA was found in splenocytes or lymph node cells upon IVIg treatment (data not shown).

**FIGURE 3.** IL-4 and IL-13 treatment reduces FcyRIIib, FcyRIIa, and IFN-γR2 expression on mDCs in vitro. Freshly isolated BDCA1+CD20+ mDCs (1 \times 10^5/200 μl) from blood of healthy volunteers were cultured in 96-well round-bottom plates with human rIL-33 (50 ng/ml), rIL-4 (10 ng/ml) or rIL-13 (100 ng/ml) for 24 h. The expression of FcyRIIib (A), FcyRIIa (B), and IFN-γR2 (C) on mDCs was measured by flow cytometry as indicated in Fig. 2A. Bars represent mean ± SEM of eight independent experiments. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) as compared with control (Ctrl).

**FIGURE 4.** IL-4– and IL-13–treated human mDCs are less responsive to immune complexes in vitro. Purified BDCA1+CD20+ mDCs (1 \times 10^5/200 μl) from blood of healthy volunteers were cultured with medium or rIL-4 (10 ng/ml) or rIL-13 (100 ng/ml) for 24 h, extensively washed, and recultured in 96-well round-bottom plates in the presence of immobilized human IgG for another 48 h. After culture (A), the expression of maturation marker CD83 on mDCs was determined by flow cytometry, and secretion of IL-8 (B) and TNF-α (C) in the supernatants was assessed by ELISA. Percentages depict upregulation of described marker when comparing conditions treated with anti-FcγRIIib + IC to IC alone. Bars represent mean ± SEM of four independent experiments. *\( p < 0.05 \), **\( p < 0.01 \) as compared with control (CTRL) + IC.

Stimulation of IL-33 production in human macrophages by IVIg is dendritic cell–specific intercellular adhesion molecule-3–grabbing nonintegrin independent

We hypothesized that a potential source of IL-33 production in lymph nodes could be macrophages, as it was shown that macrophages in human placenta are IL-33 producers (37). Because isolation of macrophages from lymph nodes was practically unattainable, we differentiated human macrophages from monocytes in the presence of M-CSF in vitro and assessed whether these cells
produced IL-33 mRNA. Similar to the observations on human lymph node cells, we found that human macrophages produced high levels of IL-33 mRNA in the presence of LPS and IVIg, but hardly any production was observed with LPS or IVIg alone (Fig. 6B). As these macrophages express dendritic cell–specific intercellular adhesion molecule–3–grabbing nonintegrin (DC-SIGN) (data not shown), a previously identified IVIg-binding target (20), we wondered whether the IVIg-mediated IL-33 production was dependent on DC-SIGN. Using an approved DC-SIGN–blocking Ab (AZN-D1) (38), we surprisingly found that IL-33 mRNA levels in human macrophages in the presence of IVIg and LPS were not affected when DC-SIGN was blocked (Fig. 6B). Collectively, these data identify human macrophages as a potential source of IVIg-induced IL-33 production that is independent of DC-SIGN.

Discussion

Although HD is increasingly being used to treat various autoimmune and inflammatory diseases, its mode of action is not fully understood. Recent mice studies have shown an essential role for a pathway involving IVIg-induced IL-33–IL-4 production that ultimately leads to upregulation of FcγRIIB expression on effector macrophages (20). However, whether this pathway is operational in humans is unknown and also questionable due to several fundamental physiological differences between mice and men. In the current study, we demonstrate that IVIg treatment in human results in a robust and selective increase of plasma levels of IL-33 and the Th2 cytokines IL-4 and IL-13, that lasts for at least 1 wk. Although a causative relationship between IL-33 and Th2 cytokine production in mice is difficult to establish, the observed associations between the rise of IL-33 and the increases in Th2 cytokines plasma levels support the hypothesis that IVIg can stimulate the IL-33–Th2 axis in humans, as has been previously observed in mice (20).

In contrast to mouse studies showing a stimulatory effect of IVIg treatment on inhibitory FcγRIIB expression on myeloid cells (4, 19, 20), we observed a rapid but transient decrease of FcγRIIB expression on circulating mDCs upon HD treatment in humans. Several studies have shown that FcγRIIB expression on circulating monocytes was not affected by IVIg treatment in humans (39–41), which we were able to confirm. In one human study, an increase of FcγRIIb expression was observed on monocytes and B cells after IVIg treatment. It has to be noted that in this study, FcγRIIb expression levels prior to IVIg treatment were in most cases diminished in comparison with healthy individuals, suggesting that IVIg treatment may enhance expression levels of FcγRIIb only when they were initially decreased (42).

However, HD therapy reduced expression of the activatory FcγRIIa and IFN-γR2 on mDCs until at least 1 wk after treatment. The discrepancies between the effect of IVIg on FcγRII expression between humans and mice may be related to the presence of both FcγRIIa and FcγRIIb in humans, whereas mice have only FcγRIIb (43, 44). Thus, due to major structural differences in FcγR expression between mice and humans, the mechanism of FcγR modulation found in mice may not be fully recapitulated in humans. Receptor downregulation was only observed in patients treated with HD, which corresponds with the insufficiency of LD therapy for effective anti-inflammatory treatment. The plasma IL-4 levels reached in LD patients did not exceed the baseline levels of HD patients and may therefore have been insufficient to downregulate receptor expression on mDCs.

In vitro experiments revealed that both IL-4 and IL-13, but not IL-33 or IVIg itself, can reduce the expression of FcγRIIb, FcγRIIa, and IFN-γR2 on human mDCs. To our knowledge, we are the first to show an effect of Th2 cytokines on FcγR and IFN-γR expression on human mDCs. Direct suppression of IFN-γR2 expression on macrophages, as was observed in a previous study (16), was probably caused by adsorbance of IgG to the culture plastic (25), which was prevented in our experiment by precoating of the culture wells with FCS. Our results reveal that IVIg represses IFN-γR2 on mDCs indirectly via stimulation of Th2 cytokine production. Downregulation of IFN-γR2 expression on mDCs prevented upregulation of CD86, CCR7, and CD38 as well as production of IL-33 mRNA.
is rather heterogeneous, clinical outcomes cannot be standardized for all patients, and a correlation study associating outcomes with cytokine or receptor levels is not reliable. However, in all patients included in the high-dose group, clinical improvement was reported, whereas increases of IL-33, IL-4, and IL-13 (except for one patient) were consistently observed in these patients. Although we cannot perform direct correlation testing, contribution of the increased IL-33 and Th2 cytokines to the clinical improvement of patients is not unlikely.

Previous studies have reported that human basophils stimulated with IL-33 are able to produce IL-4 and IL-13 in vitro (45–47), and we have found similar results (data not shown). Moreover, basophils in mice can also produce IL-4 and IL-13 when stimulated with IL-33 (20). T cells (48), mast cells (49), and macrophages (50) are a less likely sources, because IL-33 induces production of IL-5 and IL-13, but not IL-4, in these cell types.

Although it has been shown in mice that IgGs having 2,6-sialylated Fc induce IL-33 production by marginal zone macrophages in the spleen, recent studies show that the anti-inflammatory effects mediated by these IgG molecules in mouse ITP models were independent of IL-33 and the presence of the spleen (23). Furthermore, the spleen has been found dispensable for the anti-inflammatory effects of IVIg in humans, whereas a human counterpart of the murine marginal zone macrophage has not been identified (21, 22). Nevertheless, we found that IVIg infusion in humans enhanced IL-33 levels in plasma. In a first attempt to identify the source of IL-33 production, we found that IVIg induces IL-33 gene expression in human lymph node cells, but not in human splenocytes in vitro, although additional TLR4 ligation by LPS was needed.

We hypothesized that macrophages in lymph nodes may potentially be able to produce IL-33 in response to IVIg because recent data on human placenta revealed that placental and decidual macrophages are able to produce IL-33 (37). Because it was not feasible to isolate sufficient numbers of macrophages from lymph nodes, we differentiated human monocytes toward macrophages in vitro and found that these cells produced high levels of IL-33 mRNA upon culture with IVIg and LPS. Hence, we suggest that macrophages are potential sources of IVIg-induced IL-33 in humans, although fibroblasts (51) and epithelial cells (52, 53) may be other sources of IL-33 production. Surprisingly, IL-33 production in our macrophage experiments was not dependent on DC-SIGN. In concert with recent findings by others (54–56), these data ask for a re-evaluation of the role for DC-SIGN and 2,6-sialylated Fc in the anti-inflammatory effects of IVIg in humans. Moreover, further research is required to establish the role for and source of IL-33 production upon IVIg treatment in humans.

In the current study, we show that the recently identified IL-33–Th2 pathway by which IVIg inhibits myeloid cells functions in mice may also, with some variations, be operational in humans. Instead of upregulating FcγRIIb, as has been observed in mice, IVIg downregulates FcγRIIa and IFN-γR2 on circulating mDCs in humans and stimulates IL-33 production by human macrophages probably via a DC-SIGN–independent mechanism. The current study, together with our previous report showing that HD therapy activates regulatory T cells in humans (14), provides evidence that important anti-inflammatory mechanisms of action of IVIg identified in animal models are operational in humans. These modes of action observed in patients may at least partially explain the beneficial effects of HD treatment in various autoimmune diseases.

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