

Intravenous immunoglobulin attenuates airway inflammation through induction of forkhead box protein 3-positive regulatory T cells

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Background: Intravenous immunoglobulin (IVIG) is a frequently used disease-modifying therapy for a large spectrum of autoimmune and inflammatory conditions, yet its mechanisms of action are incompletely understood. Using a robust murine model of antigen-driven allergic airways disease, we have demonstrated that IVIG markedly improves ovalbumin (OVA)-induced airway hyperresponsiveness characterized by 4- to 6-fold enhancement in regulatory T (Treg) cells in pulmonary and associated lymphoid tissues.

Objective: We sought to determine whether IVIG induces antigen-specific Treg cells and to address cellular interactions that lead to induction of Treg cells by IVIG.

Methods: C57Bl/6 mice were sensitized and challenged by means of intranasal OVA exposure. IVIG or albumin control was administered 24 hours before challenge. Treg cells were tracked by using green fluorescent protein (GFP)-forkhead box protein 3 (Foxp3) knock-in reporter mice (Foxp3^{GFP}), and Treg cell and dendritic cell (DC) phenotypes and activities were elucidated by using coculture and flow cytometry.

Results: IVIG therapy of OVA-sensitized and OVA-challenged mice induced antigen-specific forkhead box protein 3 (Foxp3)-positive Treg cells from non-Treg cell precursors. The induced Treg cells home specifically to the lungs and draining lymph nodes and have greatly potentiated suppressive activity compared with that seen in Treg cells purified from control

mice. Induction of Treg cells is mediated by tolerogenic DCs generated after IVIG exposure. Compared with albumin-treated, OVA-exposed mice, IVIG-primed DCs express altered Notch ligands, including increased Delta-4 and reduced Jagged-1 levels, reflecting decreased T_H2 polarization. Furthermore, IVIG-primed DCs can stimulate Treg cell differentiation from uncommitted Foxp3⁺CD4⁺ T cells *ex vivo*, and adoptive transfer of IVIG-primed DCs abrogates airway hyperresponsiveness and induces Treg cells.

Conclusion: The anti-inflammatory effects of IVIG therapy can be mediated by the immunomodulation of DCs, creating a bridge that induces antigen-specific, highly suppressive Treg cells. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Intravenous immunoglobulin, asthma, regulatory T cells, dendritic cells, IgG, immune modulation, airway hyperresponsiveness

Intravenous immunoglobulin (IVIG) is the most commonly used therapeutic plasma derivative. It is the mainstay of therapy for primary and secondary immune deficiencies and is extensively used in the treatment of autoimmune and inflammatory diseases. Although IVIG therapy has been used for close to 3 decades, the mechanism of action is incompletely understood.¹ Direct effects of IVIG on B cells, T cells, dendritic cells (DCs), neutrophils, and natural killer cells have been described. Recent studies suggest its action might be mediated by the Fab portion through antigen binding, through the Fc portions of the IgG molecule by inducing FcγRIIb, or via glycosylation or sialylation of sites on the Fc chains.² Progress made in determining the structural basis for the action of IVIG must be complemented by further study into the cellular basis of IVIG-induced tolerance.

We have demonstrated that murine allergic airways disease is significantly attenuated by injection of therapeutic human IVIG,³ with significant inhibition of pulmonary inflammation and airway hyperreactivity.³ A most striking finding of this study was the highly significant increase in regulatory T (Treg) cell numbers in the lungs and draining lymph nodes (dLNs) of IVIG-treated mice. IVIG has been used successfully for the treatment of severe steroid-dependent asthma, but the reasons for its efficacy are not well defined, and further trials are required to better understand its potential use.^{4,5}

Treg cells are essential mediators of normal immune responses. Imbalances in Treg cell number or function are implicated in patients with autoimmune and allergic diseases. This is manifest in its extreme by the immune dysregulation, polyendocrinopathy,

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Abbreviations used

AHR:	Airway hyperresponsiveness
APC:	Allophycocyanin
CFSE:	Carboxyfluorescein succinimidyl ester
DC:	Dendritic cell
dLN:	Draining lymph node
IVIG:	Intravenous immunoglobulin
Foxp3:	Forkhead box protein 3
GFP:	Green fluorescent protein
HSA:	Human serum albumin
iTReg:	Induced regulatory T
LN:	Lymph node
OVA:	Ovalbumin
PE:	Phycoerythrin
Teff:	Effector T
Treg:	Regulatory T
WT:	Wild-type

enteropathy, X-linked syndrome, whereby mutations in the critical transcription factor forkhead box protein 3 (Foxp3) cause a syndrome characterized by severe skin, endocrine, and allergic manifestations and enteritis with X-linked inheritance.⁶ Induction of Treg cells is characteristic of successful injected allergen immunotherapy for airway disease⁷ and oral immunotherapy for food allergy.⁸ In murine models of allergic airways disease, infusion of Treg cells to sensitized mice is associated with abrogation of pulmonary inflammation and airways reactivity,^{9,10} and agents that induce Treg cells minimize allergic inflammation.¹¹ A goal of therapy in patients with severe chronic asthma is to design treatment approaches that can improve the balance between effector T (Teff) and Treg cell subsets.

IVIG therapy has been associated with increased numbers of Treg cells in both human diseases and murine models.¹²⁻¹⁴ We demonstrate, for the first time, that IVIG treatment promotes induction of Treg cells from nonregulatory precursors. These Treg cells are highly suppressive and antigen specific. IVIG-mediated induction of Treg cells is a direct consequence of its action on a subset of CD11c⁺ pulmonary DCs. Polyclonal IgG acts to bridge the innate and adaptive immune systems, modifying DC phenotype and generating Foxp3⁺ Treg cells, which can modulate subsequent inflammatory responses.

METHODS**Wild-type and reporter murine stains**

C57BL/6 mice were obtained from Charles River Laboratories (Senneville, Quebec, Canada). C57BL/6 green fluorescent protein (GFP)-Foxp3 knock-in reporter mice (B6.Foxp3^{GFP}) were kindly provided by A. Y. Rudensky (Memorial Sloan-Kettering Cancer Centre, New York, NY). OT-II transgenic mice expressing MHC class II-restricted, ovalbumin (OVA)-specific V α 2/V β 5.1 T-cell receptor were obtained from Taconic Laboratories (Hudson, NY) and were mated to Foxp3^{GFP} reporter mice to produce transgenic mice expressing OVA-specific Foxp3^{GFP} Treg cells. Mice were bred and maintained in a specific pathogen-free animal facility at the McGill University Health Center-Research Institute and used at 6 to 8 weeks of age according to procedures accepted by the Animal Ethics Committee at McGill University.

Murine model of allergic airway disease

Mice were sensitized intranasally to OVA as in McCusker et al,¹⁵ with modifications as shown in Kaufman et al.³ One day before antigen challenge (day 28), 2 g/kg IVIG (Gammunex; Talecris Biotherapeutics, Mississauga, Ontario,

Canada) or an equal volume of 3% human serum albumin (HSA; Talecris Biotherapeutics) control was administered intraperitoneally. Ragweed sensitization was performed by administering 2 intraperitoneal injections of ragweed (80 μ g per injection; Greer Laboratories, Lenoir, NC) in 25 μ L of Imject Alum (Pierce, Rockford, Ill) and 75 μ L of PBS 4 days apart. Seven days after the second intraperitoneal injection, mice were challenged by means of intranasal administration of 8 μ g of ragweed in PBS.

Histologic analysis

Lungs were fixed with 10% buffered formalin and embedded in paraffin. Sections of 0.5 μ m were stained with hematoxylin and eosin and examined by using light microscopy. For immunohistochemistry, antigen retrieval was performed in sodium citrate buffer, pH 6, heated in a microwave for 10 minutes. Tissues were permeabilized with 0.2% Triton 100X in Tris-buffered saline. Sections were immersed in serum-free protein block (Dako, Burlington, Ontario, Canada), followed by incubation with primary anti-GFP antibodies (Sigma-Aldrich Mississauga, Ontario, Canada) at 4°C overnight, followed by biotinylated anti-rabbit IgG antibody (Cell Signaling Technology, Boston, Mass) and streptavidin-horseradish peroxidase-streptavidin (Vector Laboratories, Burlingame, Calif) for 45 minutes. Peroxidase was developed with diaminobenzidine (DAB1, Dako) and then counterstained with hematoxylin.

Airway responsiveness to methacholine

Airway hyperresponsiveness (AHR) was measured as in Kaufman et al.³ Mice were exposed to saline, followed by doubling concentrations of aerosolized methacholine (16-256 mg/mL, Sigma-Aldrich) by using a flexiVent small-animal ventilator (Scireq, Montreal, Quebec, Canada). The relative peak response at each methacholine dose compared with baseline was calculated.¹⁶

Preparation of cell suspensions from lymphoid and nonlymphoid tissues

Lungs were perfused with PBS and inflated with 1 mL of collagenase (Type XI-S, Sigma-Aldrich) in PBS and 0.5 mmol/L Ca²⁺. Paratracheal and large peribronchial lymph nodes (LNs) were removed before lung digestion. The lungs were minced and incubated for 45 minutes at 37°C in 3 mL of collagenase, DNase (1000 unit/mL, Sigma-Aldrich) in PBS, and 0.5 mmol/L Ca²⁺ and then passed through a 40- μ m cell strainer. Spleens and other lymphoid organs were mechanically dissociated through a 90- μ m mesh. Single-cell suspensions were resuspended in complete medium consisting of RPMI-1640 (Invitrogen, Toronto, Ontario, Canada) supplemented with 10% FBS (Hyclone, Logan, Utah), L-glutamine, penicillin/streptomycin, HEPES, and β -mercaptoethanol.

Cell separation and adoptive transfer

CD4⁺ T cells were isolated from spleens and LNs of C57/B6.Foxp3^{GFP} or OT-II-Foxp3^{GFP} reporter mice by means of positive separation of magnetically labeled CD4⁺ cells (Miltenyi Biotec, Vancouver, British Columbia, Canada) followed by FACS sorting of GFP⁺ cells. CD4⁺Foxp3^{-GFP} (3×10^6) cells were adoptively transferred through the tail vein to wild-type (WT) mice. Plasmacytoid DCs were separated by means of positive separation with CD304 and CD141 (Miltenyi Biotec). Magnetically labeled CD11c⁺ DCs were then removed from plasmacytoid DC-depleted cells. The purity of isolated CD11c⁻ and CD11c⁺ cells was typically 95% or greater.

Flow cytometric analysis

Cells were washed in PBS containing 2% FBS, and Fc receptor binding was blocked with IVIG for 20 minutes (100 μ L/50 mL). Antibodies included anti-CD62 ligand-phycoerythrin (PE), anti-CCR7-PE, anti-CCR4-PE-Cy7, anti-CD11c-allophycocyanin (APC), anti-CD11c-fluorescein isothiocyanate, anti-CD8 α -fluorescein isothiocyanate, anti-Jagged-1-PE, and anti-Delta-4-APC, all of which were purchased from Becton Dickinson (Franklin Lakes,

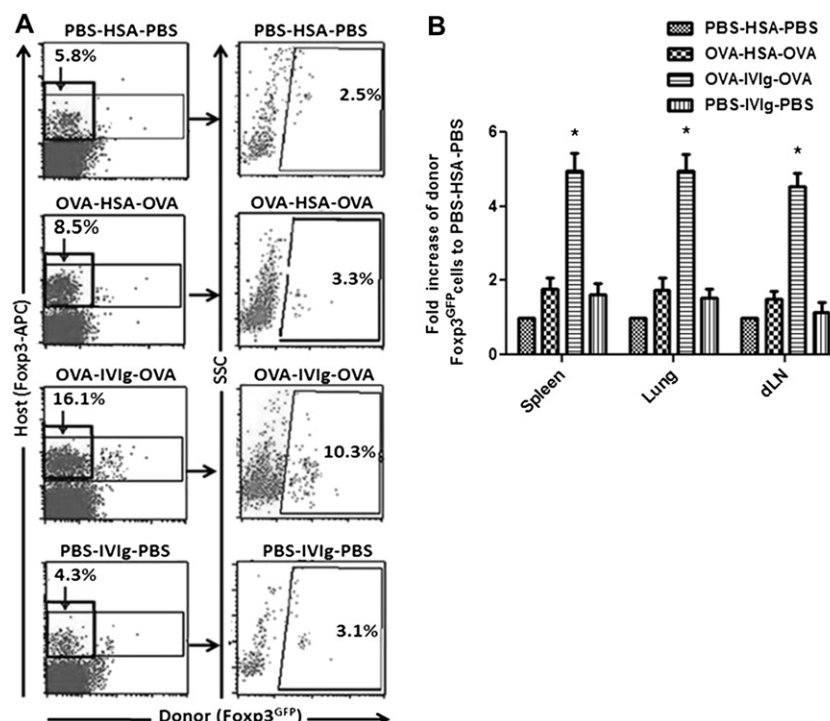


FIG 1. IVIG induces *de novo* Treg cells from Foxp3⁺ T cells. Representative flow cytometric analysis of endogenous host Treg cells stained with anti-Foxp3 antibodies conjugated to APC (Foxp3^{APC}+GFP⁻) and induced donor Foxp3^{APC}+GFP⁺ Treg cells within the lungs of mice with allergic airways disease is shown. **A**, Frequency of (host) Foxp3^{APC}+GFP⁻ Treg cells and (donor) Foxp3^{APC}+GFP⁺ cells. OVA-IVIG-OVA treatment induced Treg cells from Foxp3^{GFP}⁻ precursors. Results are representative of 3 identical studies (n = 9 for each condition). **B**, Bar graphs demonstrating the fold increase of induced Foxp3^{GFP}⁺ cells among all Treg cells within spleens, dLNs, and lungs. *P < .05. Results represent 3 identical experiments with 9 mice in each group.

NJ). Cells (10^6) were incubated with appropriate antibodies for 30 minutes at 4°C. Some samples were fixed and permeabilized with Cytofix/Cytoperm (eBioscience, San Diego, Calif) and incubated with anti-Ki67-PE (BD Biosciences) or anti-Foxp3-APC (eBioscience). Intracellular cytokine staining was performed on phorbol-12-13-dibutyrate (5 ng/mL)– and ionomycin (50 ng/mL, Sigma-Aldrich)–stimulated cells in the presence of GolgiPlug (BD Bioscience). After 6 hours of culture, cells were permeabilized and stained with anti-IL-10-APC or anti-IFN- γ -APC (BD Biosciences) for 30 minutes at 4°C. Relative fluorescence intensities were determined on a BD FACSCalibur flow cytometer.

Assessment of Treg cell suppressor activity

CD4⁺ T cells were purified from spleens and dLNs of naive OT-II mice and incubated with carboxyfluorescein succinimidyl ester (CFSE; 1 μ mol/L) for 5 minutes at 37°C. Cells were washed and resuspended in complete medium for 15 minutes. GFP⁺ Treg cells were recovered from lungs and dLNs by means of cell sorting. CFSE-labeled OT-II CD4⁺ T cells (responder T cells) were mixed with Treg cells in ratios of 1:1 to 1:16 and cocultured in 6-well plates. Cells were stimulated with 1 mg/mL OVA, with 2×10^5 purified CD11c⁺ DCs added. After 3 days, cells were harvested, and CFSE dilution was analyzed by means of flow cytometry.

RESULTS

IVIG induces Foxp3⁺ Treg cells from nonregulatory CD4⁺ T-cell precursors

To understand the observed increase in Treg cell numbers after IVIG treatment,³ we determined whether IVIG could induce Treg

cells from CD4⁺Foxp3⁺ precursors or whether treatment with IVIG was associated with expansion of preexisting Foxp3⁺ cells. Purified CD4⁺Foxp3⁺ T cells (3×10^6) were adoptively transferred to WT mice before sensitization. Twenty-four hours before antigen challenge, mice received either IVIG or HSA. After 5 days of OVA or sham challenge, mice were sacrificed, and expression of Foxp3^{GFP} was assessed by means of flow cytometry. IVIG treatment led to consistent 4- to 6-fold increases in expression of CD4⁺Foxp3⁺GFP⁺ cells within the spleen, lungs, and dLNs (Fig 1). There was also an increase in the percentage of endogenous (CD4⁺Foxp3⁺GFP⁻) Treg cells in IVIG-treated mice compared with that seen in the other groups (Fig 1). These results indicate that IVIG treatment can induce Treg cells from conventional CD4⁺Foxp3⁺ T cells as part of its immunoprotective effects *in vivo*.

The distribution of induced regulatory T (iTreg) cells in the lung tissue of recipient mice was determined histopathologically by detecting Foxp3^{GFP}⁺ cells in lung sections. As shown in Fig 2, there is a substantial reduction in the peribronchial cell infiltrate in OVA-IVIG-OVA mice compared with that seen in OVA-HSA-OVA mice. This was characterized by a 10-fold increase in Foxp3^{GFP}⁺ iTreg cell numbers within the lung infiltrate of OVA-IVIG-OVA mice (Fig 2, D). The striking increase in iTreg cells in IVIG-treated mice was accompanied by expression of homing receptors that predispose to migration from dLNs to pulmonary parenchyma. A majority of Treg cells found in dLNs expressed CD62 ligand in OVA-IVIG-OVA

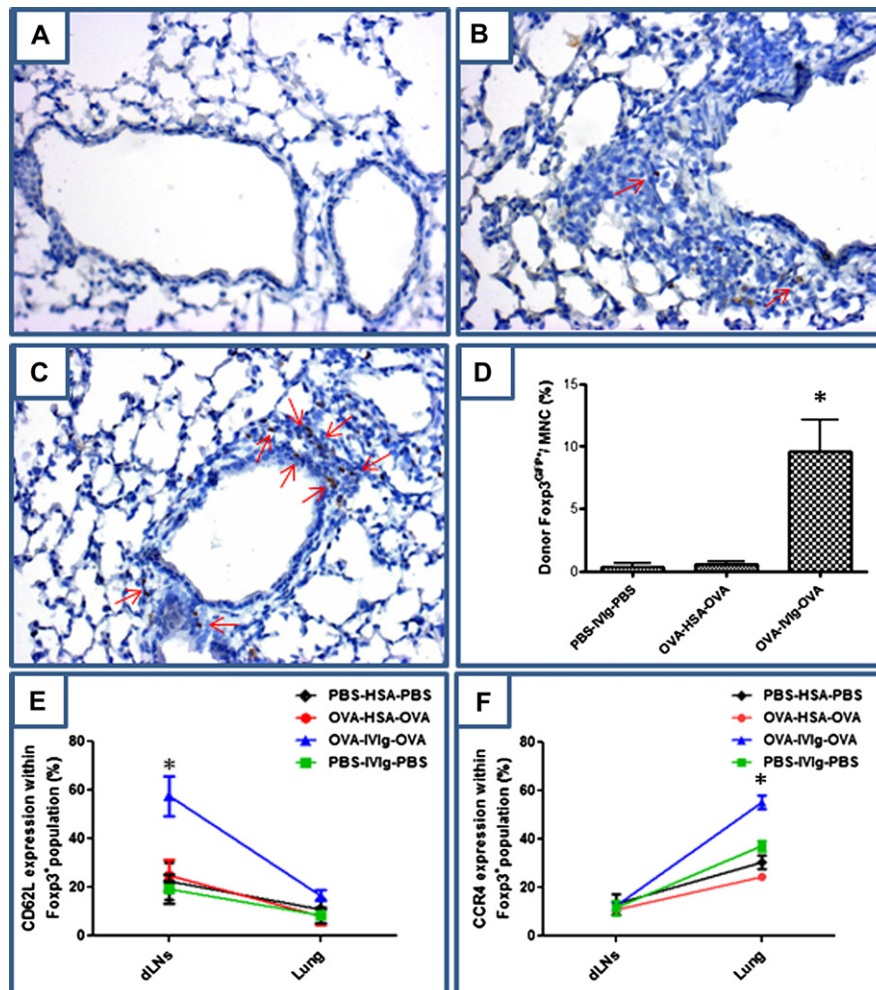


FIG 2. IVIG-induced Treg cells are greatly enriched in inflamed pulmonary tissues. Immunohistopathologic analysis of donor Foxp3^{GFP+} cells in lung sections by using anti-GFP antibody. **A**, Control (PBS-HSA-PBS) mice, **B** and **C**, OVA-IVIg-OVA mice (Fig 2, **C**) have decreased mononuclear cell infiltration compared with OVA-HSA-OVA mice (Fig 2, **B**). Within the mononuclear infiltrate, the frequency of induced GFP⁺ Treg cells in OVA-IVIg-OVA (Fig 2, **C**) mice is increased compared with that seen in the other groups. **D**, Histogram indicating the percentage of Treg cells within the mononuclear cell infiltrate (MNC) from each condition. Slides were prepared from 6 lungs for each experimental condition, representing 3 experiments. **E**, CD62L ligand (CD62L) expression was assessed on Treg cells from lungs and dLNs by using flow cytometry. IVIG-treated mice had diminished CD62L ligand expression on Treg cells in lungs compared with that seen in LNs. **F**, The expression of CCR4 was determined as above. Treg cell populations within the lungs of OVA-IVIg-OVA mice had significantly higher CCR4 expression than those in dLNs and compared with Treg cells in all other experimental conditions. **P* < .05. Representative of 2 separate experiments with 6 mice in each group.

mice, whereas expression of this receptor was downregulated on Foxp3⁺ Treg cells in the lung (Fig 2, **E**). Furthermore, iTreg cells detected in the peribronchial/perivascular infiltrate of IVIG-treated mice expressed 2- to 3-fold higher levels of CCR4, which is critical for localization of T cells into lung tissue (Fig 2, **F**). In parallel, expression of CCR4 on leukocytes from dLNs (Fig 2, **F**) and distal nondraining LNs remained at basal levels, and IVIG did not alter CCR7 expression in the lungs or dLNs (data not shown). iTreg cells from IVIG-treated mice showed increased proliferation with significantly higher Ki67 staining compared with that seen in HSA-treated mice (see Table E1 in this article's Online Repository at www.jacionline.org). These data suggest that IVIG induces iTreg cells and that CCR4 might be a key chemokine receptor,

mediating homing of iTreg cells to arrest OVA-induced pulmonary inflammation.

Induction of Foxp3⁺ Treg cells by IVIG is antigen specific

OT-II mice were bred onto the C57/B6.Foxp3^{GFP} reporter background to provide OVA-specific T cells expressing GFP if converted to Treg cells to investigate the action of IVIG in the induction phase of antigen-specific Treg cells. CD4⁺Foxp3^{-GFP-} cells were purified from OT-II.Foxp3^{GFP} mice and adoptively transferred to WT C57BL/6 syngeneic mice. The mice were sensitized and challenged with either OVA or ragweed, and IVIG or HSA was administered 1 day before challenge. Ragweed

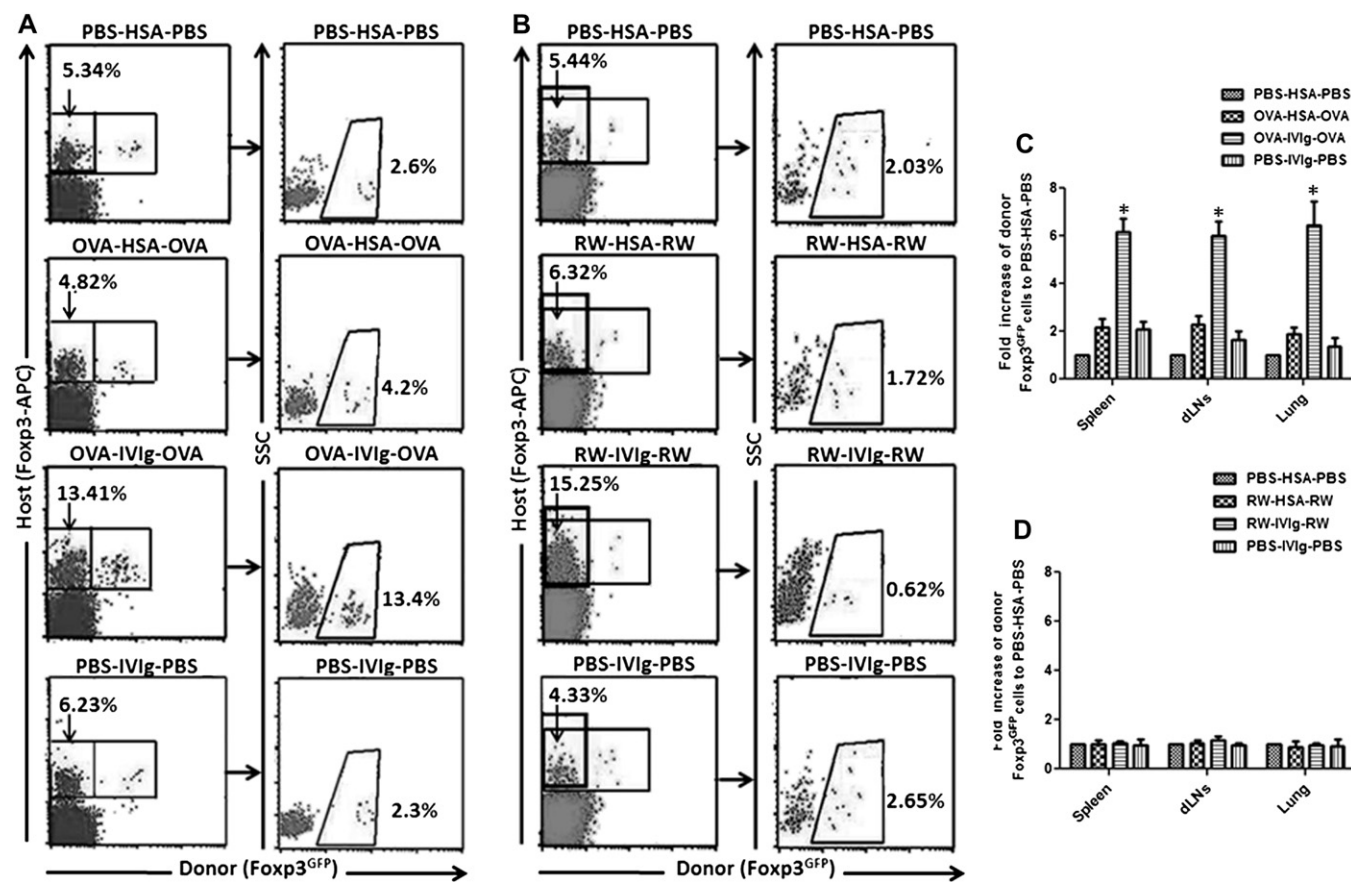


FIG 3. IVIG induces antigen-specific Treg cells. **A** and **B**, Representative flow cytometric plots of (donor) Foxp3^{GFP}+APC+ and (host) Foxp3^{APC}+GFP- Treg cells within the lungs of WT recipient mice. Before sensitization, all groups received 3×10^6 CD4^{GFP}- T cells from OT-II-Foxp3^{GFP} reporter mice. Only OVA-IVIG-OVA mice demonstrate an increase in OVA-specific CD4⁺Foxp3⁺GFP+ cell numbers (Fig 3, A) among the total Treg cells induced by IVIG. Ragweed (RW)-IVIG-RW mice (Fig 3, B) demonstrate an increase in CD4⁺Foxp3⁺ cell numbers compared with other groups but no increase in Foxp3⁺GFP+ cell numbers. **C** and **D**, Fold increase of OVA-specific Foxp3^{GFP}+ cell numbers among all Treg cells within the spleens, dLNs, and lungs compared with those seen in sham-treated PBS-HSA-PBS control mice. **P* < .05. Data are from 2 experiments (*n* = 6 mice per group).

sensitization and challenge induced significant pulmonary inflammation and increased AHR (data not shown). A 6-fold increase in Foxp3⁺GFP+ iTreg cells was observed after challenge in OVA-IVIG-OVA mice (Fig 3, A and C). Although there was a significant increase in endogenous Foxp3⁺ Treg cell numbers in ragweed-IVIG-ragweed mice (Fig 3, B), only baseline levels of GFP+ cells were detected in ragweed-challenged mice (Fig 3, B and D). This experiment indicates that IVIG treatment leads to preferential induction of antigen-specific Treg cells *in vivo*.

IVIG induces Foxp3⁺ Treg cells with increased suppression activity

Because IVIG therapy strongly influenced induction of Treg cells, the suppressive capacity of IVIG-induced iTreg cells was compared with that of Treg cells harvested from control groups by using an *ex vivo* suppression assay. As depicted in Fig 4, Treg cells obtained from OVA-IVIG-OVA mice were consistently more effective in suppressing proliferation of OVA-stimulated OT-II T cells than Treg cells from PBS-HSA-PBS or OVA-HSA-OVA mice. Treg cells from IVIG-treated mice suppressed OVA-induced CD4 proliferation at ratios of 1:1 to 1:16 (Fig 4).

Importantly, IVIG-induced iTreg cells demonstrated higher frequencies of IL-10⁺ and IFN- γ ⁺ cells (see Table E2 in this article's Online Repository at www.jacionline.org) compared with that seen in Foxp3⁺ Treg cells from control groups. Taken together, these data suggest that treatment with IVIG not only favors the development of iTreg cells but also robustly increases the potency of the effector phase of Treg cell activity.

IVIG primes CD11c⁺ DCs to generate Treg cells

Recent studies implicate DCs in the elaboration of peripheral T-cell tolerance through induction and expansion of antigen-specific Treg cells.^{17,18} Splenic CD11c⁺ DCs were purified from naive mice and primed with IVIG (10 mg/mL) for 3 hours and then incubated with OVA (2 mg/mL) for 3 hours to test the ability of IVIG to induce tolerogenic DCs. DCs primed with OVA alone or IVIG alone were used as controls. IVIG plus OVA-primed DCs cocultured with purified CD4⁺ T cells from OT-II.Foxp3^{GFP} mice generated a 2-fold increase in expression of CD4⁺Foxp3⁺GFP+ compared with that seen in DCs that were pulsed with OVA or IVIG alone or CD4⁺ cells cultured with IVIG alone (Fig 5, A). If T cells were similarly incubated with IVIG, washed, and

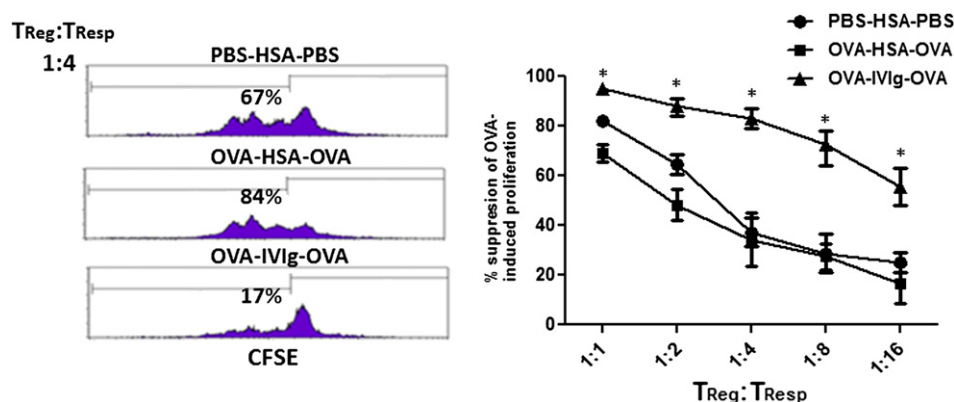


FIG 4. Treg cells from IVIG-treated mice are highly suppressive. The ability of Treg cells from the experimental animals to suppress OVA-induced proliferation was evaluated by using an *ex vivo* suppression assay (see the Methods section) and monitored by means of CFSE dilution. Foxp3^{GFP+} cells from OVA-IVIG-OVA mice suppressed the proliferation of responder T cells (T_{Resp}) to OVA stimulation to a greater degree at all ratios from 1:1 to 1:16 compared with those seen in the other control groups. The *left panel* is a representative histogram plot for data obtained at a ratio of 1 Treg cells to 4 responder T cells. The proliferating cells fall to the *left of the upper line*, and the percentage of proliferating cells are indicated. The *right panel* demonstrates the survival graph of the percentage suppression by donor Treg cells from each experimental group at 1:1 to 1:16 (Treg cell/responder T cell) ratios. * $P < .05$. Results are representative of 2 experiments ($n = 6$ mice per group).

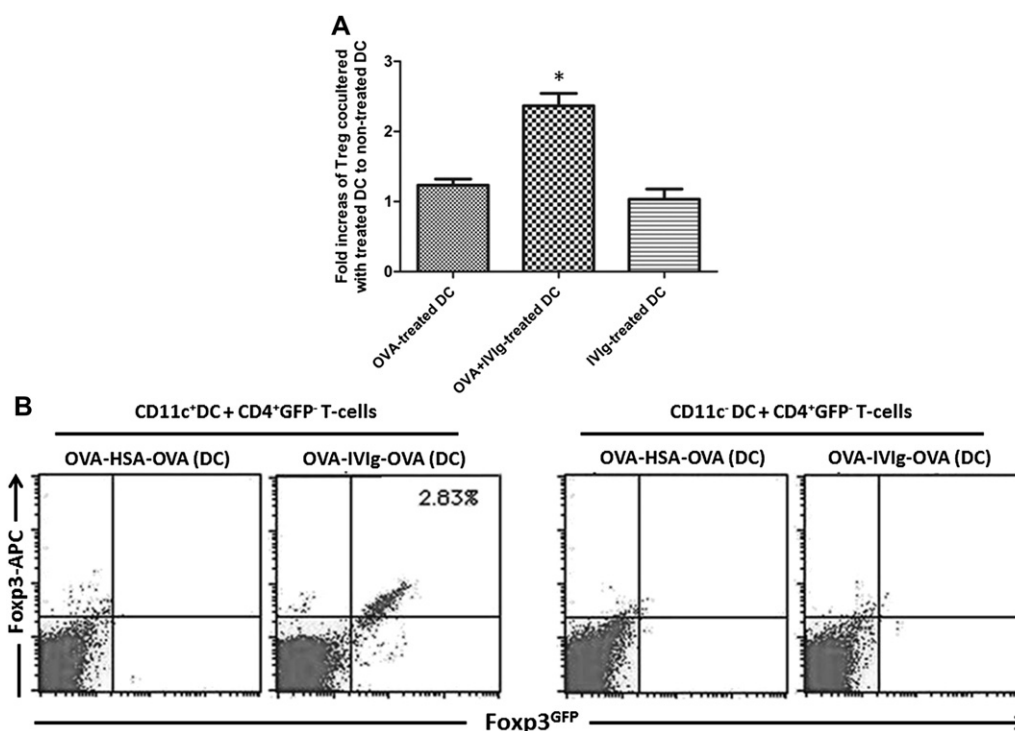


FIG 5. IVIG primes CD11c⁺ pulmonary DCs to induce Treg cells. **A**, DCs were purified from naive mice and pulsed with IVIG with and without OVA and then cocultured with CD4⁺ cells from OT-II-Foxp3^{GFP} reporter mice. The induction of *de novo* Treg cells was assessed by measuring the percentage of Foxp3^{GFP+} APC⁺ cells in the CD4⁺ population by means of flow cytometry. Only OVA-IVIG-pretreated DCs induced the expansion of Treg cells. **B**, Purified CD11c⁺ DCs or plasmacytoid CD11c⁺ DCs from the lungs of OVA-IVIG-OVA and OVA-HSA-OVA mice were cocultured with CD4⁺ GFP⁺ T cells from OT-II-Foxp3 reporter mice. Only pulmonary CD11c⁺ DCs from OVA-IVIG-OVA mice induced expression of Foxp3. * $P < .05$ ($n = 6$ mice for each experimental condition).

cocultured with DC plus OVA, there was no significant increase in Treg cell numbers from baseline (see Fig E1 in this article's Online Repository at www.jacionline.org). Thus iTreg cell induction is mediated, at least in part, by the interaction of DCs with IVIG,

and both antigen and IVIG are required for optimal Treg cell induction.

Subsequently, CD11c⁺ DCs purified from digested lungs of the 4 experimental groups were cocultured with 10⁶ Foxp3⁺ CD4⁺

TABLE I. Phenotypic characteristics of CD11c⁺DC indicating increased expression of IL-10, Delta-like-4, and CD8α following IVIG treatment

	PBS-HSA-PBS	OVA-HSA-OVA	OVA-IVIG-OVA	PBS-IVIG-PBS
IL-10	2.1% ± 1%	1.9% ± 1.3%	7.9% ± 2.1%*	9.3% ± 4.2%*
IFN-γ	0.8% ± 0.5%	7.9% ± 3.2%**	1.9% ± 1.5%	1.8% ± 1.2%
Delta-like-4	27.2% ± 4 %	28.5% ± 4.4%	42.5% ± 7.5%*	37% ± 5.4%*
Jagged-1	11.8% ± 3.5%	28.9% ± 5.5%**	16.4% ± 3.6%	8.5% ± 2.7%
CD8α	30% ± 5.5%	12% ± 4.2%	59% ± 10%*	14% ± 3.5%

IVIG also decreased Jagged-1 expression as well as IFN-γ compared to OVA-HSA-OVA mice.

**P* < .05 comparing IVIG to HSA-treated groups.

***P* < .05 for OVA-HAS-OVA compared to all other groups. N = 6 mice in each group.

T cells from OT-II-Foxp3^{GFP} mice and stimulated with OVA for 5 days. Only DCs harvested from IVIG-treated mice increased expression of Foxp3^{GFP+} in cultured CD4⁺ cells (Fig 5, B). We then fractionated CD11c⁺ and CD11c[−] DCs to determine which DC population was primarily responsible for the tolerogenic effects of IVIG.^{18–20} CD11c⁺ DCs from OVA-IVIG-OVA mice induced Treg cells from conventional T cells, whereas CD11c[−] DCs from OVA-IVIG-OVA mice did not (Fig 5, B). Furthermore, neither CD11c[−] DCs nor CD11c⁺ DCs from OVA-HSA-OVA mice could induce Foxp3 expression in CD4⁺ T cells (Fig 5, B). Collectively, our results show that myeloid CD11c⁺ DCs are selectively modulated by IVIG and are responsible for the generation of iTreg cells *in vivo*.

Tolerogenic DCs from IVIG-treated mice differed from other DC populations

IVIG-treated mice had increased numbers of pulmonary DCs expressing intracellular IL-10 and surface CD8α (Table I). Because IVIG treatment was associated with changes in Notch ligand genes, including increased Delta-4 mRNA and diminished Jagged-1 mRNA,³ pulmonary DCs were examined for Jagged and Delta isoforms by using flow cytometry. Expression of Delta-like 4 increased whereas Jagged-1 expression decreased in OVA-IVIG-OVA pulmonary DCs compared with that seen in OVA-HSA-OVA DCs (Table I). These results suggest that induction of Treg cells by IVIG might involve modulation of Delta-4 expression, activity, or both on tolerogenic CD11c⁺ DCs.

Adoptive transfer of DCs from OVA-IVIG-OVA mice recapitulates protective effects mediated by IVIG treatment *in vivo*

To determine whether induction of Treg cells by IVIG was mediated by its actions on DCs, we hypothesized that adoptive transfer of DCs from OVA-IVIG-OVA mice would inhibit AHR and induce Treg cells in OVA-sensitized and OVA-challenged mice. Pulmonary CD11c⁺ DCs from PBS-HSA-PBS, OVA-HSA-OVA, PBS-IVIG-PBS, and OVA-IVIG-OVA mice were purified and transferred intratracheally to WT syngeneic mice 24 hours before OVA challenge. DCs from OVA-IVIG-OVA mice induced Foxp3⁺ Treg cells in recipients, whereas adoptively transferred DC from OVA-HSA-OVA mice did not exhibit increased Treg cell numbers (10% to 11.5% vs 6.5% to 7.5%; Fig 6). Recipients of DCs from OVA-IVIG-OVA or PBS-IVIG-PBS mice also showed significantly reduced AHR to methacholine (Fig 7, F) and substantially lower inflammatory changes in the airways (Fig 7) compared with recipients of DCs from OVA-HSA-OVA

or PBS-HSA-PBS mice. The results indicate that the induction of Treg cells by IVIG can be mediated directly through DCs.

DISCUSSION

IgG is a key player in immune networks. Beyond its major property in defense against pathogens, IgG regulates immune function through idiotype–anti-idiotype networks, feedback inhibition of immunoglobulin production, and modulation of cytokine responses.²¹ Despite the consistent efficacy of polyclonal IgG in a large number of unrelated conditions, no unifying mechanism of action has been determined. Our study broadens comprehension of the regulatory actions of IVIG by dissecting links between IVIG and innate and adaptive immunity to ascertain its anti-inflammatory effects.

In this study we use a well-described model of allergic airways diseases.^{15,22} The allergic airway model has many advantages for evaluating immune-regulatory properties of polyclonal IgG, as it is antigen-driven, requires antigen-presentation, T-cell and B-cell responses, and examination of relevant tissues and physiological measurements are straightforward. Furthermore, small clinical trials make a case for studying IVIG for the treatment of patients with severe asthma.^{4,5} In this study we build on our previous observations³ and address the dynamics and cellular basis of Treg cell induction by IVIG *in vivo*. We investigate whether IVIG can generate Treg cells from conventional CD4⁺ T cells, the antigen specificity of IVIG-induced iTreg cells, and contributions of DCs to this process.

Our data clearly demonstrate that high-dose IgG induces antigen-specific Treg cells. Using adoptive transfer of CD4 cells from B6.Foxp3^{GFP} reporter mice, we demonstrate that IVIG can induce Treg cells *de novo* from Foxp3^{−GFP−} T cells, suggesting that there is not simply expansion of endogenous Treg cells. IVIG requires the presence of antigen to induce Treg cells; IVIG infusion alone modifies the DC phenotype (Table I) but does not increase Treg cell populations. Furthermore, adoptive transfer of CD4⁺ T cells from OT-II-Foxp3^{GFP} mice followed by exposure to a relevant (OVA) or irrelevant (ragweed) antigen revealed that IVIG only induced OT-II-Foxp3⁺GFP⁺ cells in the presence of OVA, the relevant antigen. Not surprisingly, total numbers of Treg cells were increased in tissues of mice sensitized and challenged to ragweed after IVIG treatment. However, there was a conspicuous absence of OVA-specific Foxp3⁺GFP⁺ cells in ragweed-sensitized mice. This implies that antigen specificity is a key feature of the anti-inflammatory effect of IVIG in this model.

The induction of antigen-specific Treg cells has important implications. It suggests that Treg cells interact with the same antigen as Teff cells at the DC interface, a juxtaposition that

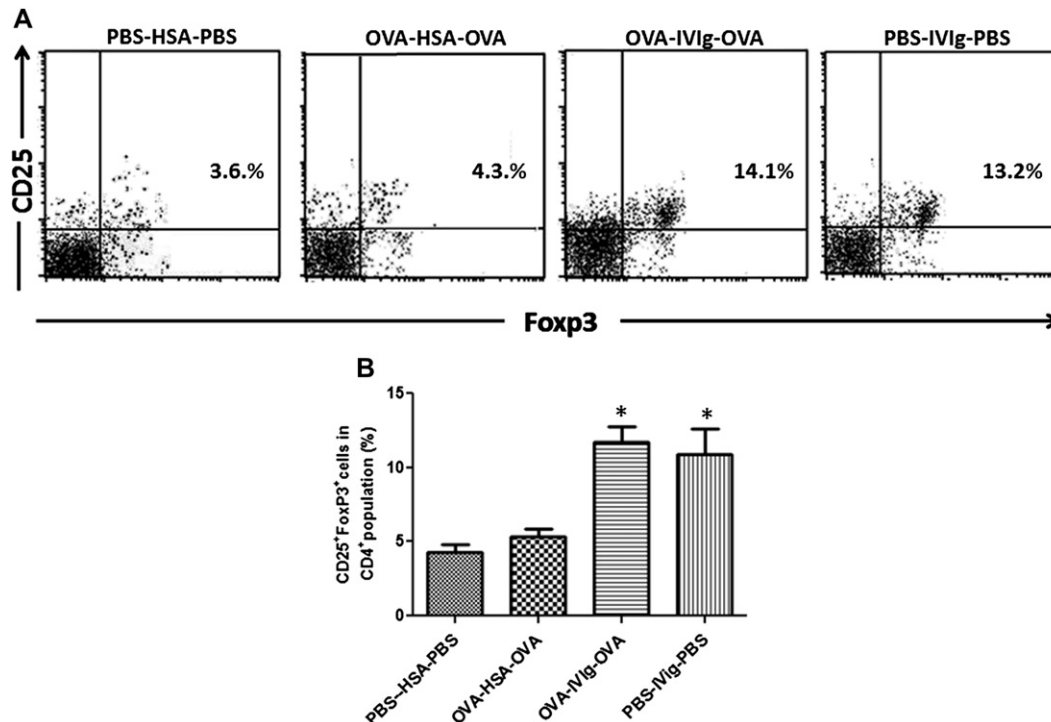


FIG 6. Adoptive transfer of pulmonary DCs from IVIG-treated mice recapitulates the protective effect of IVIG therapy by inducing Treg cells. Pulmonary DCs were purified from the lungs of mice from each experimental group after the completed challenge protocol. Recipient mice received adoptive transfer of purified DCs 1 day before challenge (day 28) in place of IVIG or HSA treatment. **A** and **B**, Representative flow cytometric plots (Fig 6, **A**) and quantification of CD4⁺CD25⁺Foxp3⁺ Treg cells (Fig 6, **B**) within the digested lung tissue of recipient mice after challenge. Adoptive transfer of pulmonary DCs from OVA-IVIG-OVA and PBS-IVIG-PBS mice to OVA-sensitized animals induced Treg cells comparably to IVIG treatment. * $P < .05$. Results are representative of 2 experiments ($n = 6$ mice for each experimental group).

encourages targeted, antigen-specific suppression. This is emphasized by the inhibition of OVA-induced proliferation in T cells from IVIG-treated mice, whereas mitogen-induced T-cell proliferation was not affected.³ Antigen specificity favors induction and migration of Treg cells to antigen-exposed sites, in this case the airways and dLNs. Moreover, the pattern of IVIG-induced chemokine receptor expression, with increased CCR4 and decreased L-selectin in the lung, predisposed to homing to inflamed lungs. LN-associated Treg cells expressed higher levels of L-selectin, as well as CCR7. It remains to be determined whether the trafficking of Treg cells is from the inflamed tissues to the dLNs, as proposed by Zhang et al,²³ and what other factors facilitate this pattern of circulation.

The highly suppressive effects of IVIG-induced Treg cells on Teff cells (Fig 5) provide an explanation for the observed marked decrease in pulmonary inflammation.³ IVIG-induced Treg cells suppressed OVA-mediated proliferation of OT-II cells at a 4- to 8-fold lower dilution compared with identically prepared Treg cells from other control mice. Exposure to high levels of polyclonal IgG is not simply associated with increased numbers of iTreg cells but also an increased suppressive potency. The increase in potency might be due to the increased percentage of antigen-specific Treg cells in the CD4 population or to a higher percentage of Treg cells expressing IL-10 in IVIG-treated mice. This implies that on exposure to antigen, more Treg cells will be activated by OVA in IVIG-treated mice.

The second novel finding is the crucial link between IVIG-primed DCs and induction of Treg cells. Previous work²⁴⁻²⁸

documented that IVIG modifies maturation and surface receptor expression on DCs. Our data indicate that IVIG-primed DCs, both *ex vivo* or adoptively transferred to OVA-sensitized mice, can replace IVIG, diminishing AHR and increasing Treg cell populations in the lungs. IVIG-treated DCs have several important properties that might predispose to induction of Treg cells. DCs harvested from IVIG-treated mice expressed lower levels of the costimulatory molecules CD80 and CD86 and reduced class II HLA molecules.^{3,25} Diminished levels of costimulatory molecules are associated with immature or tolerogenic DCs, which can induce anergy and increase iTreg cell numbers.^{29,30} It remains to be demonstrated whether the DC phenotype after exposure to IVIG represents a true arrest of maturity or strictly an alteration of characteristics that predispose to tolerogenic activity. Significant changes in the expression of Notch ligands are also induced by IVIG, including decreased Jagged-1 mRNA³ and surface expression and reciprocal increases in Delta-like 4 mRNA and protein. Jagged-1 and Jagged-2 induce T_H2 responses,^{31,32} whereas Delta-4 can induce regulatory responses. Thus the overall phenotype consistently reflects the ability of IVIG to power tolerogenic responses.

The receptor with which IVIG interacts to induce tolerogenic DC populations is unknown. Previous work suggests that FcγRs are important for its anti-inflammatory action in patients with immune thrombocytopenic purpura. Prime receptor candidates include the inhibitory receptor FcγRIIb and the activating receptor FcγRIII. Using rabbit IgG, Yamamoto et al³³ abrogated AHR in a murine model of allergen-induced reactive airways disease in

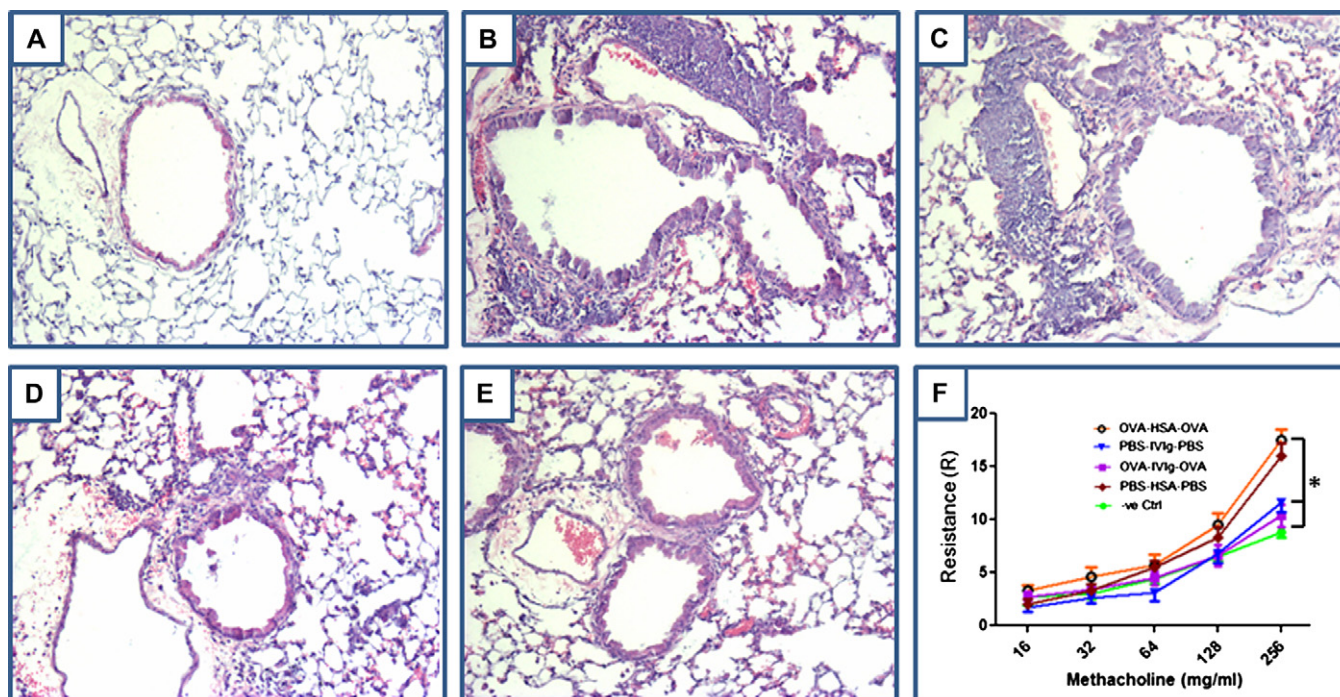


FIG 7. Adoptive transfer of pulmonary DCs from IVIG-treated mice protects against increased AHR and pulmonary inflammation. Pulmonary DCs were purified from the lungs of mice from each experimental group after the completed challenge protocol. Recipient mice received adoptive transfer of purified DCs 1 day before challenge (day 28) in place of IVIG or HSA treatment. **A-E**, Histologic examination of hematoxylin and eosin–stained representative lung sections, demonstrating the protective effect of DCs adoptively transferred from IVIG-treated mice to OVA-HSA-OVA mice: untreated control mice (Fig 7, A), DCs from PBS-HSA-PBS mice (Fig 7, B), DCs from OVA-HSA-OVA mice (Fig 7, C), DCs from OVA-IVIG-OVA mice (Fig 7, D), and DCs from PBS-IVIG-PBS mice (Fig 7, E). **F**, AHR to methacholine (pulmonary resistance) was measured by using the flexiVent, as shown in the Methods section. DCs from IVIG-treated mice substantially attenuated methacholine-induced AHR. * $P < .05$. Results are representative of 2 experiments ($n = 6$ mice for each experimental group).

an Fc γ RIIb-dependent manner. In contrast, adoptive transfer of IVIG-treated splenocytes or DCs from Fc γ RIIb^{-/-} mice is reproducibly able to duplicate the action of IVIG.^{24,34-36} Immune complexes can also duplicate the action of IVIG, suggesting interaction with activating Fc γ R on DCs can potentially induce anti-inflammatory responses.^{27,37} IVIG might regulate immune responses through specific epitopes on Fc receptors (“Tregitopes”) that directly activate naturally occurring Treg cells and inhibit antigen-specific serologic and T-cell responses.³⁸ Glycosylated regions of the Fc receptor, such as sialylation of ASP297, might be crucial in conferring the anti-inflammatory action of IVIG in *in vivo* models, such as immune thrombocytopenic purpura and serum-induced arthritis^{2,24,39-41} and inhibition of B-cell activation.⁴² In the K/BxN model of serum-induced arthritis, the ligation of the inhibitory molecule specific intracellular adhesion molecule-3 grabbing nonintegrin related molecule 1 (or the human homolog dendritic cell specific-intracellular adhesion molecule-3 grabbing nonintegrin) on splenic marginal-zone macrophages mediates the action of IVIG, potentially through an IL-4-mediated pathway.^{39,43} In contrast, in our T_H2-driven model, IL-4 levels are diminished,³ and pulmonary DCs appear to be critical. Further comparisons are warranted.

The action of IVIG in this model of allergic airways disease demonstrates an important link between innate and adaptive immunity. IVIG acts to bridge the development of tolerogenic DCs and the production of targeted, antigen-specific Treg cells.

Therapeutically, this represents an important paradigm to comprehend how so-called tolerogenic DCs develop and what crucial signals are necessary for the induction and expansion of antigen-specific Treg cells. In addition, the crucial role of humoral immunity in mediating inflammatory responses but perhaps more importantly in affecting the balance between inflammation and essential regulatory events is now being elucidated.

Clinical implications: Use of IVIG might improve antigen-driven conditions, such as autoimmune disease and severe asthma, through induction of highly suppressive, antigen-specific Treg cells.

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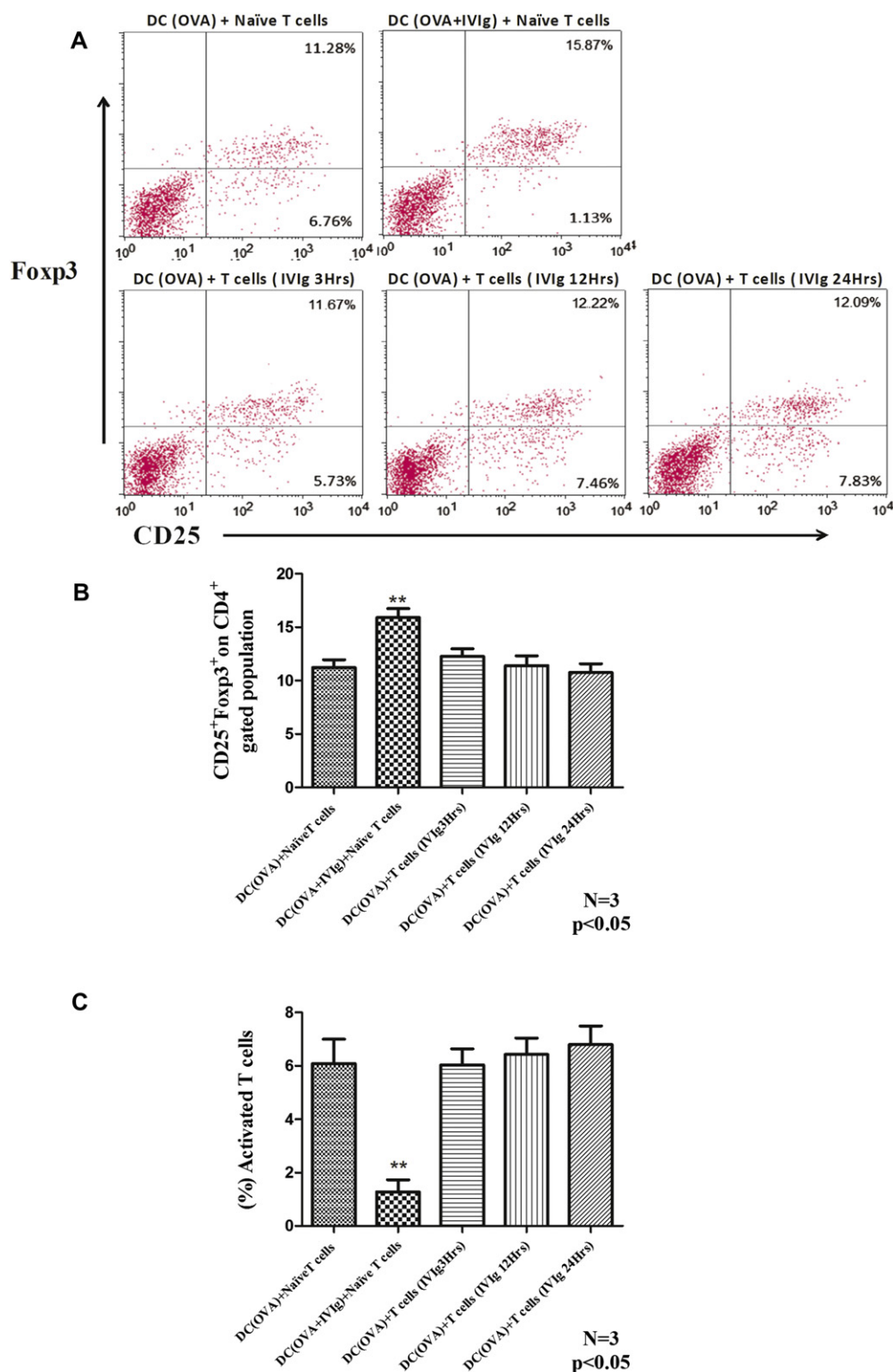


TABLE E1. Ki67 expression monitored by means of flow cytometry on CD4⁺CD25⁺Foxp3⁺ Treg cells from the lungs under each experimental condition (n = 3 experiments with 3 mice in each group)

Condition	Ki67 expression
PBS-HSA-PBS	16.7 ± 1.43
OVA-HSA-OVA	15.67 ± 2.03
OVA-IVIG-OVA	30.67 ± 2.96*
PBS-IVIG-PBS	14.83 ± 0.73

IVIG-treated mice had consistently higher Ki67 expression.

**P* < .05.

cell percentages *in vitro*. ***P* < .05 (n = 3). **C.** Summary of activated T cells in coculture, as delineated by CD25⁺Foxp3⁻ cells detected in *right lower quadrant* of Fig E1, A. Only DCs preincubated with IVIG and OVA decreased the numbers of activated T cells in culture, suggesting induction of highly suppressive Treg cells by IVIG. Preincubation of T cells with IVIG before washing did not decrease the number of activated T cells on exposure to OVA-pulsed DCs. ***P* < .05 (n = 3).

TABLE E2. Cytokine production from induced Treg cells (n = 6 mice in each group)

Cytokine	PBS-HSA-PBS	OVA-HSA-OVA	OVA-IVIG-OVA	PBS-IVIG-PBS
IL-10	0.41% $\pm$ 0.1%	0.55% $\pm$ 0.2%	3.2% $\pm$ 1%*	0.25% $\pm$ 0.15%
IFN- γ	0.21% $\pm$ 0.07%	0.1% $\pm$ 0.07%	1.8% $\pm$ 1.3%*	0.12% $\pm$ 0.05%

Cells from digested pulmonary tissue and dLNs from recipient mice were assessed for the production of IL-10 and IFN- γ by means of flow cytometry. The frequency of IFN- γ ⁺ and IL-10⁺ cells was significantly higher within donor Foxp3^{GFP+} cells from OVA-IVIG-OVA mice compared with those from the other experimental groups.

**P* < .05.