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This information is current as of March 1, 2017.

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J Immunol published online 20 February 2017

<http://www.jimmunol.org/content/early/2017/02/18/jimmunol.1502361>

Supplementary Material

<http://www.jimmunol.org/content/suppl/2017/02/18/jimmunol.1502361.DCSupplemental>

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Peripherally Generated Foxp3⁺ Regulatory T Cells Mediate the Immunomodulatory Effects of IVIg in Allergic Airways Disease

Amir H. Massoud,^{*,†,1} Gabriel N. Kaufman,^{*} Di Xue,^{*} Marianne Béland,^{*} Marieme Dembele,^{*} Ciriaco A. Piccirillo,[‡] Walid Mourad,[†] and Bruce D. Mazer^{*}

IVIg is widely used as an immunomodulatory therapy. We have recently demonstrated that IVIg protects against airway hyper-responsiveness (AHR) and inflammation in mouse models of allergic airways disease (AAD), associated with induction of Foxp3⁺ regulatory T cells (Treg). Using mice carrying a *DTR/EGFP* transgene under the control of the *Foxp3* promoter (DEREG mice), we demonstrate in this study that IVIg generates a de novo population of peripheral Treg (pTreg) in the absence of endogenous Treg. IVIg-generated pTreg were sufficient for inhibition of OVA-induced AHR in an Ag-driven murine model of AAD. In the absence of endogenous Treg, IVIg failed to confer protection against AHR and airway inflammation. Adoptive transfer of purified IVIg-generated pTreg prior to Ag challenge effectively prevented airway inflammation and AHR in an Ag-specific manner. Microarray gene expression profiling of IVIg-generated pTreg revealed upregulation of genes associated with cell cycle, chromatin, cytoskeleton/motility, immunity, and apoptosis. These data demonstrate the importance of Treg in regulating AAD and show that IVIg-generated pTreg are necessary and sufficient for inhibition of allergen-induced AAD. The ability of IVIg to generate pure populations of highly Ag-specific pTreg represents a new avenue to study pTreg, the cross-talk between humoral and cellular immunity, and regulation of the inflammatory response to Ags. *The Journal of Immunology*, 2017, 198: 000–000.

Regulatory T cells (Treg) are specialized subsets of T lymphocytes that express the Foxp3 transcription factor and play a nonredundant role in maintaining immunological tolerance (1, 2), as illustrated by the severe autoimmune disease that develops in Foxp3-deficient neonatal mice and humans (3, 4).

Treg play an essential role in immune homeostasis. In particular, the presence of Treg in the lung is crucial for assuring normal lung health. In the steady-state, APCs such as tissue-resident macrophages appear to be programmed to interact with T cells, ensuring that inhalation of common particulate substances induces Treg responses and minimizing the inflammatory potential associated with elimination of these substances (5). In situations where an inflammatory response is required, such as in viral infections, the presence of Treg is required to ensure that the necessary host response does not lead to overt destruction of lung tissue (6). Treg

diminish cell proliferation and inflammatory cytokine production, thereby minimizing the damage caused by the antiviral response.

The mechanisms behind the induction of a Treg response and Treg-mediated suppression of inflammatory diseases are not entirely established. In allergic disease, individuals who are tolerant to inhaled allergens have higher peripheral blood Treg counts than do symptomatic asthmatic individuals (7), suggesting that a lack of Treg may play a role in the aberrant responses to allergens in asthma. Few human studies have directly characterized lung Treg, and these data are highly variable, mainly due to the ubiquitous use of corticosteroids in symptomatic asthmatics (8–11). In murine studies, infusion of Treg can inhibit allergen-driven allergic airways disease (AAD), and Treg induction may be an important part of abrogating allergic inflammation (12). Therefore, in allergen-driven diseases such as asthma, strategies to boost Treg

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Received for publication November 6, 2015. Accepted for publication January 25, 2017.

This work was supported by Grifols Bioscience (Clinical Investigation Program), Canadian Institutes for Health Research Grants ISO115295 (to B.D.M.) and MOP67211 (to C.A.P.), the Research Institute of the McGill University Health Centre, and by the Strauss Family Foundation. Infrastructure support was received from the Fonds de Recherche du Québec-Santé. G.N.K. is the recipient of a Canadian Institutes for Health Research Canada graduate scholarships doctoral research award. C.A.P. holds a Canadian Institutes for Health Research Canada Research Chair.

A.H.M., G.N.K., and B.D.M. designed the study; A.H.M., G.N.K., D.X., M.B., and M.D. performed the experiments and analyzed the data; A.H.M. and G.N.K. wrote the paper; G.N.K. and B.D.M. revised the paper; C.A.P. and W.M. provided important

critical comments; B.D.M. supervised the research group and takes responsibility for the integrity of the work; and all authors read and approved the final version of the manuscript.

Microarray data and Minimum Information about a Microarray Experiment compliance information have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71811>) under accession number GSE71811.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AAD, allergic airways disease; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; EGFP, enhanced GFP; GO, Gene Ontology; GzmA, granzyme A; GzmB, granzyme B; HSA, human serum albumin; iTreg, induced Treg; LAP, latency-associated peptide; MHC-II, MHC class II; Nrp1, neuropilin-1; PANTHER, Protein Analysis through Evolutionary Relationships; pTreg, peripheral Treg; RW, ragweed; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; Treg, regulatory T cell; tTreg, thymic Treg.

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are an important focus of research for development of novel Treg-based therapies (13).

We have demonstrated that IVIg is able to attenuate airway hyperresponsiveness (AHR) and pulmonary inflammation following sensitization and challenge to OVA or ragweed (RW) in BALB/cJ (14) and C57BL/6J (15) mice. The action of IVIg is associated with a significant increase in highly suppressive Treg, and it appears to be dependent on CD11c⁺ dendritic cells (DC). This finding concurs with the work of Trinath et al. (16). Treg generated following IVIg administration appear to be Ag specific, but it is not known whether this property is important in the action of IVIg in attenuating murine AAD. Additionally, it is not clear whether Treg generated by IVIg require the presence of thymic Treg (tTreg) or can be peripherally differentiated solely from pre-existing CD4⁺CD25⁺Foxp3⁺ T cells.

In the present study, we have used mice carrying a *DTR/EGFP* transgene under the control of the *Foxp3* promoter (DEREG mice), allowing for selective depletion of Foxp3⁺ Treg following injection of diphtheria toxin (DT). We demonstrate that in the complete absence of Treg following multiple DT doses, IVIg failed to attenuate AHR and alleviate airway inflammation. However, when pre-established Treg were depleted before, but not following, IVIg treatment, mice exhibited induction of Foxp3⁺ Treg following IVIg therapy, and they did not develop AHR and airway inflammation after allergen challenge. Using a heterologous Ag model, IVIg-generated peripheral Treg (pTreg) controlled allergic responses when adoptively transferred, in an Ag-specific manner. Thus, we have demonstrated that Ag-specific Treg can be induced from pre-existing CD4⁺ effector T cells in the absence of tTreg. IVIg-generated Ag-specific Treg suppress all aspects of Ag-driven airway inflammation. We further characterized these IVIg-generated pTreg by gene expression microarray. Gene expression profiling revealed that IVIg-generated pTreg upregulate genes associated with cell cycle, chromatin, cytoskeleton/motility, immunity, and apoptosis. This suggests that IVIg-generated pTreg are a metabolically/transcriptionally active and phenotypically plastic population, actively responding to inflammation by dampening the immune response.

Materials and Methods

Animal studies

All animal studies were conducted according to procedures reviewed and approved by the Faculty of Medicine's Animal Care Committee at McGill University, in compliance with Canadian Council on Animal Care guidelines.

Mouse strains

C57BL/6 depletion of Treg (DEREG) Foxp3-DT receptor (DTR)/enhanced GFP (EGFP) mice, expressing EGFP and DTR under control of the *Foxp3* promoter, were provided by A.Y. Rudensky (Memorial Sloan-Kettering Cancer Center) and were used to track and/or deplete Foxp3⁺ Treg (17, 18). OT-II transgenic mice expressing an MHC class II (MHC-II)-restricted, OVA-specific Vα2/Vβ5.1 TCR (19) were mated to Foxp3^{EGFP} reporter mice (20) to produce transgenic mice with OVA-specific Foxp3^{EGFP} Treg. Founder Foxp3^{EGFP} and OT-II strains on C57BL/6J backgrounds were obtained from The Jackson Laboratory. Male and female mice were bred and maintained in a specific pathogen-free vivarium at the Research Institute of the McGill University Health Centre and used at 6–8 wk of age.

AAD models and depletion of Treg

Mice were sensitized and challenged to OVA (14, 15) or RW (21) as previously described. One day prior to Ag challenge (day 28), mice were treated by i.p. injection with 2 g/kg IVIg (Grifols) or an equal volume of 5% human serum albumin (HSA) (Grifols). Foxp3⁺ Treg depletion was achieved by i.p. injection of 40 ng/g DT to DEREG mice as described (17).

Flow cytometric analyses

Cell staining for flow cytometry was performed as described previously (15). Cells were stained with a protein-binding dye to assess viability

(fixable viability dye eFluor 780; eBioscience), and Fc receptor binding was blocked using anti-CD16/32 Abs (clone 93; BioLegend). Cells were subsequently incubated with appropriate fluorochrome-labeled Abs for 30 min at 4°C in 100 μl of PBS with 0.2% (w/v) BSA. Ab clones, fluorochrome conjugations, and suppliers are specified in Supplemental Table I. Ab concentrations were determined by titration for optimal signal-to-noise ratio. Some samples were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) or transcription factor buffers (eBioscience) for intracellular cytokine and/or intranuclear transcription factor detection. Samples were acquired on a BD LSR II or FACSCanto II flow cytometer using FACSDiva 6.0.3 software. Data analysis was performed offline using FlowJo v10.0.8 software (Tree Star). Hierarchical gating strategies were specified manually for each cell population of interest.

AHR to methacholine

AHR was measured as described previously (14, 15) using the flexiVent small-animal ventilation system (Scireq). Briefly, mice were anesthetized, tracheotomized, paralyzed, and connected to the ventilator. Methacholine (16–256 mg/ml in saline) was aerosolized into the inspiratory stream, and lung resistance was measured after each dose.

Histological analysis

Lungs were fixed by inflation with 10% neutral buffered formalin, processed, and embedded in paraffin using standard protocols. Five-micrometer sections were stained with H&E and examined by light microscopy.

Preparation of lung and spleen cell suspensions

Lungs and spleens were digested with collagenase D (0.15 Wünsch U/ml) and DNase I (0.1 mg/ml) (both from Roche Life Science) in PBS, with 0.9 mM calcium chloride, 0.5 mM magnesium chloride hexahydrate, and 15 mM HEPES. Single-cell suspensions were resuspended in PBS without calcium or magnesium (for flow cytometric analyses) or complete medium consisting of RPMI 1640 (HyClone) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES, and 50 μM 2-ME for culture.

Treg adoptive transfer

In some experiments, pulmonary Treg were isolated from DT-depleted, IVIg-treated, and OVA-challenged mice by flow cytometric sorting, as detailed below. These cells were adoptively transferred by intratracheal infusion into recipient syngeneic mice (13), which were exposed to OVA or RW as above.

Coculture experiments

CD11c⁺ DC purified by magnetic bead sorting (MACS; Miltenyi Biotec) from lungs of DT-treated and OVA-exposed mice were cocultured at a 1:5 ratio with flow-sorted OT-II/Foxp3^{EGFP} viable CD4⁺Foxp3⁺ splenic T cells, which have TCRs specific for the OT-II peptide of OVA (aa 323–339) in the context of MHC-II and express EGFP in Foxp3-expressing cells. Cultures were pulsed with 1 mg/ml OVA and maintained for 4 d, followed by stimulation with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for the last 6 h of culture (15).

Multiplex cytokine assays

A mouse Th1/Th2 ultrasensitive multiplex kit from Meso Scale Diagnostics was used to measure murine IFN-γ, IL-4, IL-5, IL-10, IL-12, and TNF-α in bronchoalveolar lavage (BAL) according to the manufacturer's instructions. Meso Scale Diagnostics plates were analyzed on the Meso Scale Discovery 1250 plate imager. The concentrations of cytokines in test samples were determined by referencing their electrochemiluminescence responses against a standard curve generated from serially diluted calibrators.

ELISA

Total serum IgE and IL-13 in BAL were measured by ELISA (eBioscience) according to the manufacturer's protocols.

Flow cytometric sorting of Treg

Treg from spleens of IVIg- or HSA-treated mice, with or without Treg depletion, were flow-sorted using a BD FACSARIA III to isolate pure populations of IVIg-generated pTreg, mixed endogenous and IVIg-generated pTreg, and endogenous Treg. The flow gating strategy for the Treg sort was viable CD4⁺CD25^{high}Foxp3⁺ cells (depicted in Supplemental Fig. 1), as per the published gating strategy of d'Hennezel and Piccirillo (22) and d'Hennezel et al. (23). Postsort purity was verified to be 80–90%.

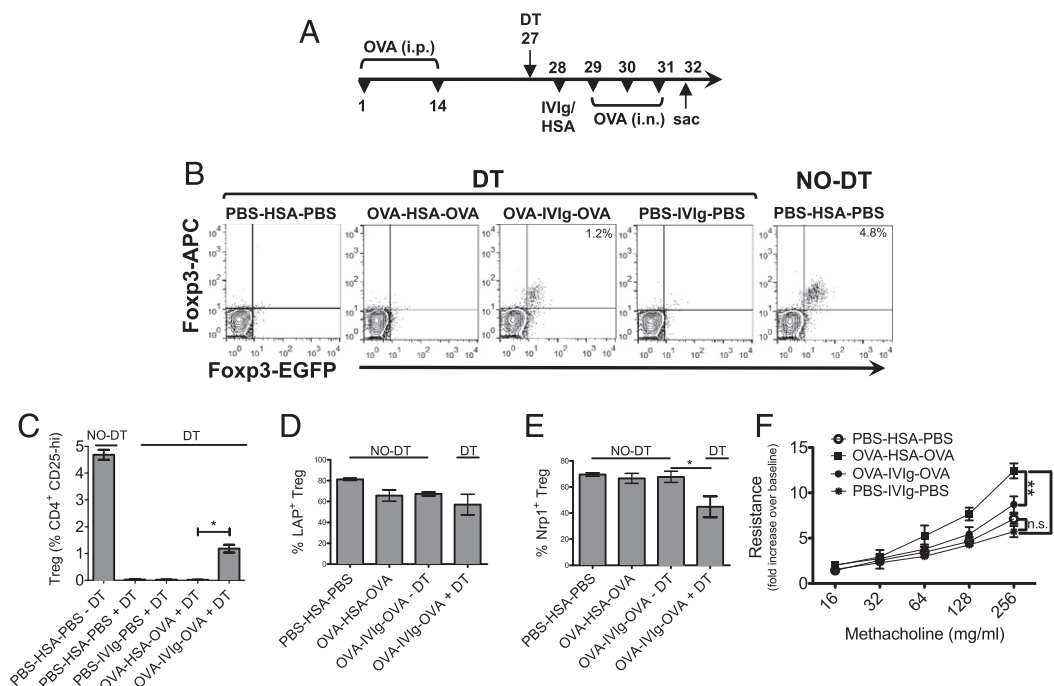


FIGURE 1. Peripheral induction of Treg by IVIg was sufficient to attenuate AHR. **(A)** Timeline of OVA sensitization, challenge, and depletion of endogenous Foxp3⁺ Treg in DEREG mice. Mice were sensitized (i.p.) to OVA/aluminum hydroxide on days 1 and 14, treated with IVIg or HSA on day 28, and challenged (intranasally [i.n.]) with OVA on days 29, 30, and 31. Foxp3⁺ Treg were depleted by one i.p. dose of DT 24 h prior to treatment (day 27). **(B and C)** Representative flow cytometric analysis (B) and bar graph (C) demonstrating efficient depletion of endogenous Treg in lungs upon DT injection prior to treatment. IVIg treatment of mice sensitized and challenged with OVA promoted the induction of Foxp3⁺ Treg in lung. For the gating strategy, viable CD4⁺CD25^{hi} cells were assessed for Foxp3^{EGFP} expression and Foxp3-allophycocyanin staining. *p < 0.05, ANOVA with a Tukey post hoc test (n = 4). **(D and E)** Bar graphs depicting the phenotype of IVIg-generated lung Treg: LAP (D) and Nrp1 (E) percentage expression were assessed on the viable CD4⁺CD25^{high}Foxp3⁺ population. For the gating strategy, viable CD4⁺CD25^{high}Foxp3⁺ lymphocytes were analyzed for LAP and Nrp1 expression. *p < 0.05, ANOVA with a Tukey post hoc test (n = 4). **(F)** IVIg treatment of DT-depleted, OVA-challenged mice was able to attenuate methacholine-induced AHR. **p < 0.01, repeated-measures ANOVA with a Tukey post hoc test, OVA-HSA-OVA plus DT versus OVA-IVIg-OVA plus DT for peak methacholine response (n = 6), ****p < 0.0001, repeated-measures ANOVA with a Tukey post hoc test, OVA-HSA-OVA plus DT versus PBS-HSA-PBS for peak methacholine response (n = 6). NO-DT, not treated with DT; n.s., no significant difference between PBS-HSA-PBS and PBS-IVIg-PBS groups (n = 6).

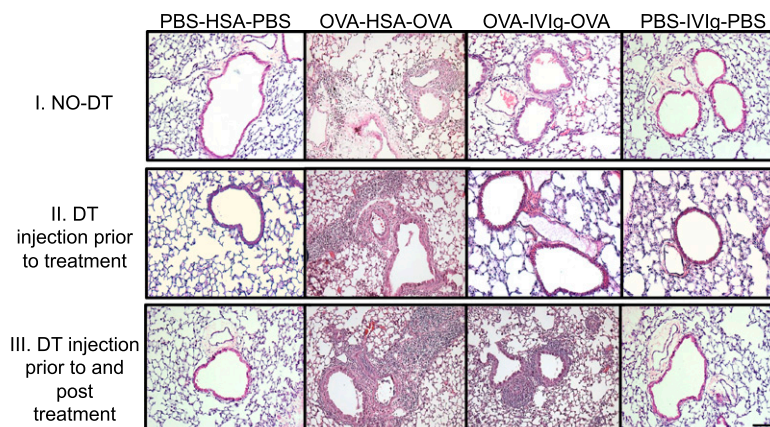
treatment diminished proinflammatory cytokines IL-4, IL-5, TNF- α , and IFN- γ in the BAL fluid, whereas Treg depletion completely reversed this effect. IL-10 was also significantly higher in the BAL of IVIg-treated mice in the presence of pTreg, whereas mice subjected to Treg depletion had attenuated IL-10 production (Fig. 4B).

IVIg-generated pTreg are Ag specific

To investigate whether Ag-specific pTreg were required for suppression of AHR, we used an adoptive transfer system (13)

whereby IVIg-generated Treg were purified from the lungs of DEREG mice treated with one dose of DT prior to IVIg administration and OVA challenge. These cells were adoptively transferred to either OVA- or RW-sensitized syngeneic mice, 1 d prior to challenge, instead of IVIg treatment. Subsequently, recipient mice were challenged with their sensitizing Ag. pTreg transfer from OVA-exposed and IVIg-treated mice led to marked attenuation of AHR (Fig. 5A), reduced total and OVA-specific serum IgE levels (Fig. 5B, 5C), decreased IL-13 production in BAL (Fig. 5D), and improved lung histopathology (Fig. 5E).

FIGURE 2. IVIg-generated Treg inhibited cellular influx in airways. Representative photomicrographs of inflammation analyzed on H&E-stained lung sections. Original magnification, $\times 200$; scale bar, 50 μ m. IVIg treatment of OVA-sensitized and challenged mice alleviated airway inflammation compared with non-IVIg-treated mice (row I). IVIg-generated pTreg, induced after endogenous Treg depletion prior to IVIg treatment, abrogated cellular influx to the lung in OVA-exposed animals (row II). Depletion of Treg prior to and during OVA challenge completely reversed the action of IVIg in preventing cellular influx to the lung (row III). NO-DT, not treated with DT.



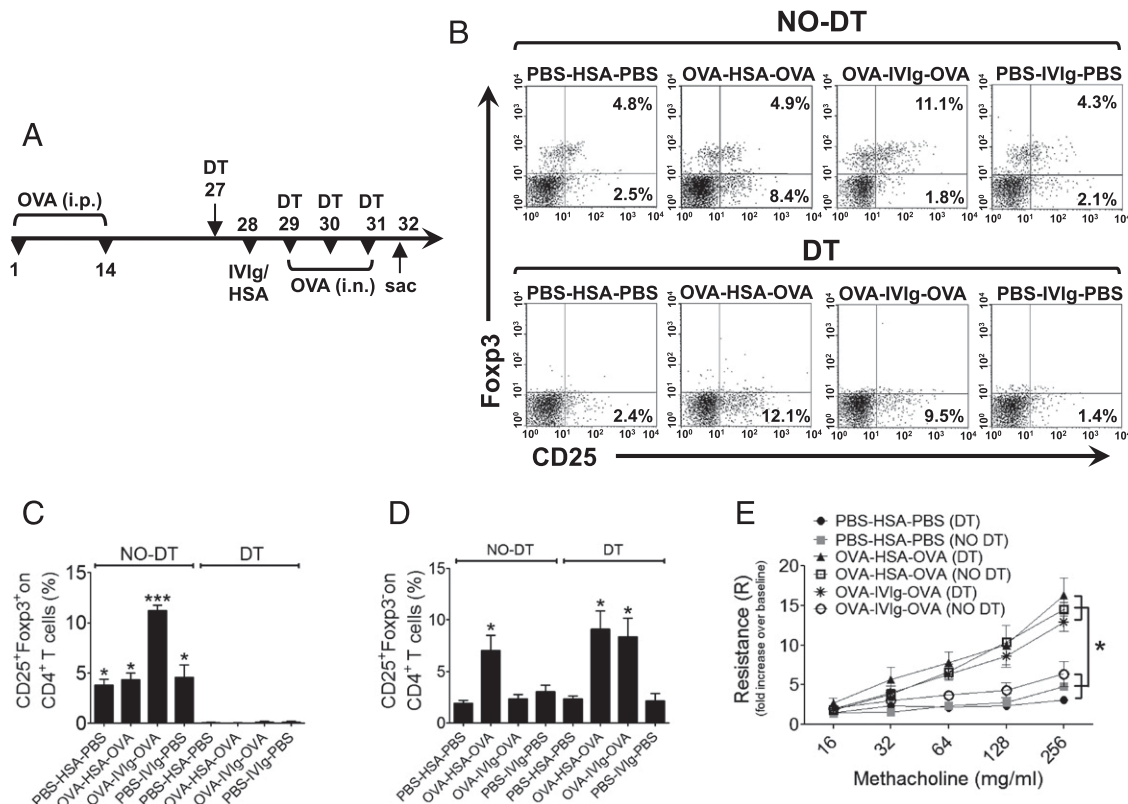


FIGURE 3. Repeated depletion of Foxp3⁺ Treg abolishes the immunomodulatory action of IVIg in airway inflammation. **(A)** Timeline of OVA sensitization and challenge, IVIg treatment, and depletion of Treg in DERE mice. Mice were sensitized (i.p.) to OVA/aluminum hydroxide on days 1 and 14, treated with IVIg or HSA on day 28, and challenged (intranasally [i.n.]) with OVA on days 29, 30, and 31. Fxop3⁺ Treg depletion was achieved with one dose of DT (i.p.) 24 h prior to IVIg/HSA treatment and three doses of DT concurrent with OVA challenge. **(B and C)** Representative flow cytometric dot plots (B) and frequency (C) of lung Treg. OVA-IVIg-OVA treatment of mice that were not Treg depleted with DT induced a 2-fold increase in the frequency of Fxop3⁺ Treg, compared with the other control groups (B, upper panel). DT treatment led to complete depletion of Fxop3⁺ Treg in all experimental groups (B, lower panel). For the gating strategy, viable CD4⁺ lymphocytes were analyzed for CD25 and Fxop3 staining. ****p* < 0.005, **p* < 0.05, ANOVA with a Tukey post hoc test (*n* = 6). **(D)** Frequency of CD4⁺CD25⁺Fxop3⁻ activated T cells. Undepleted OVA-IVIg-OVA mice demonstrated significantly lower frequencies of activated non-Treg T cells in lungs compared with the OVA-HSA-OVA group. Depletion of Treg in mice reversed this effect of IVIg. **p* < 0.05, ANOVA with a Tukey post hoc test (*n* = 6). **(E)** IVIg treatment of OVA-challenged mice attenuated methacholine-induced AHR, whereas Treg depletion reversed this effect. ***p* < 0.01, repeated-measures ANOVA with a Tukey post hoc test, PBS-HSA-PBS (DT, NO-DT) and OVA-IVIg-OVA (NO-DT) versus other control groups for peak methacholine response (*n* = 6). NO-DT, not treated with DT.

However, the transfer of OVA-specific pTreg into RW-sensitized and challenged mice did not inhibit AHR or other inflammatory parameters (Fig. 5). These data suggest that IVIg induces Ag-specific Treg and that Ag specificity is an important function of Treg in decreasing AHR.

IVIg modifies DC independent of the presence of Treg

DC and Treg are known to interact and mutually modulate function (35). We have demonstrated that IVIg confers tolerogenic properties to CD11c⁺ DC by decreasing their maturation and altering their cytokine production (14, 15). We questioned whether the regulatory effect of IVIg on DC required Treg. All endogenous and generated Treg were repeatedly depleted in DERE mice sensitized and challenged with OVA. CD11c⁺ DC were purified from the lungs of these mice, and their maturation status was determined by examining the expression of the costimulatory molecule CD80, as well as MHC-II. IVIg treatment decreased the expression of both CD80 and MHC-II in pulmonary DC, irrespective of the presence or absence of *in vivo* Treg (Fig. 6A, 6B). Thus, Treg are not required for the IVIg-mediated downregulation of CD80 and MHC-II.

We have demonstrated that IVIg can modify CD11c⁺ DC to induce Treg *ex vivo* (induced Treg [iTreg]) in an Ag-specific manner. To determine whether this required the presence of

in vivo Treg, purified pulmonary CD11c⁺ DC obtained from Treg-depleted, IVIg-treated mice were cocultured with OT-II/Foxp3^{EGFP} CD4⁺ T cells in the presence of OVA. OT-II/Foxp3^{EGFP} cells cultured with CD11c⁺ DC in the absence of IVIg generated T effector cells that proliferated and produced Th2 and Th17 cytokines, with minimal induction of Treg (data not shown). In contrast, CD11c⁺ DC from either DT- or non-DT-exposed mice treated with IVIg were equally able to induce Fxop3⁺ iTreg *in vitro* (Fig. 6C, 6D). This effect was associated with diminished Th1/Th2/Th17-type intracellular cytokine production by effector CD4⁺ T cells in the coculture (Fig. 7A–D). IL-10 levels were significantly higher in cocultures from IVIg-treated mice, compared with DC from nontreated animals. However, IL-10 production was attenuated in the absence of endogenous Treg (Fig. 7E). Overall, these results suggest that IVIg induces tolerogenic changes in DC even in the absence of Treg.

Differential gene expression of IVIg-generated pTreg

To investigate gene targets that were differentially expressed in pTreg generated by IVIg treatment, compared with endogenous Treg, we analyzed RNA from sorted Treg on gene-expression microarrays. Microarray data and Minimum Information about a Microarray Experiment compliance information have been deposited in the National Center for Biotechnology Information

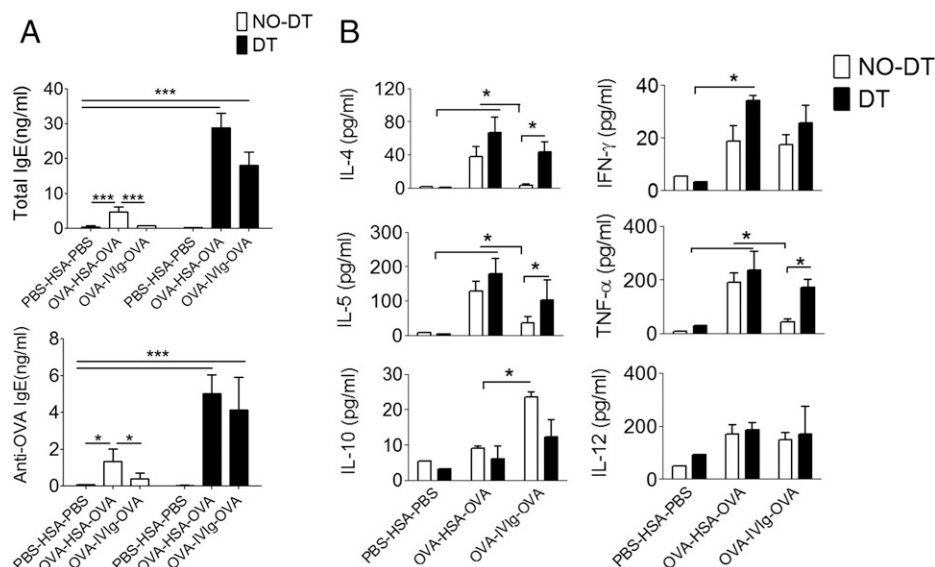


FIGURE 4. Modulation of proinflammatory mediators by IVIg was abrogated by repeated depletion of Foxp3⁺ Treg. **(A)** Total serum and OVA-specific IgE levels determined by ELISA 24 h after the last challenge. IVIg treatment lowered the elevated serum IgE levels in non-DT-treated OVA-sensitized and challenged mice, whereas Treg depletion not only abrogated this effect but substantially increased the IgE levels in serum of both the OVA-HSA-OVA and OVA-IVIg-OVA groups, compared with non-DT-treated mice. **(B)** BAL cytokine level determined by multiplex assay 24 h after the last challenge. IVIg significantly decreased BAL fluid IL-4, IL-5, TNF-α, and IFN-γ levels in non-DT-treated OVA-exposed mice, compared with other control groups. Treg depletion prior to and during OVA challenge abrogated this effect. IL-10 was significantly higher in the BAL of non-Treg-depleted OVA-IVIg-OVA mice. *** $p < 0.005$, * $p < 0.05$, ANOVA with a Tukey post hoc test ($n = 4$). NO-DT, not treated with DT.

Gene Expression Omnibus database (Gene Expression Omnibus accession no. GSE71811, accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71811>).

We found that 78 genes were differentially expressed by IVIg-generated pTreg, relative to endogenous Treg. We present the top 10 differentially expressed genes in Table I and the entire list of

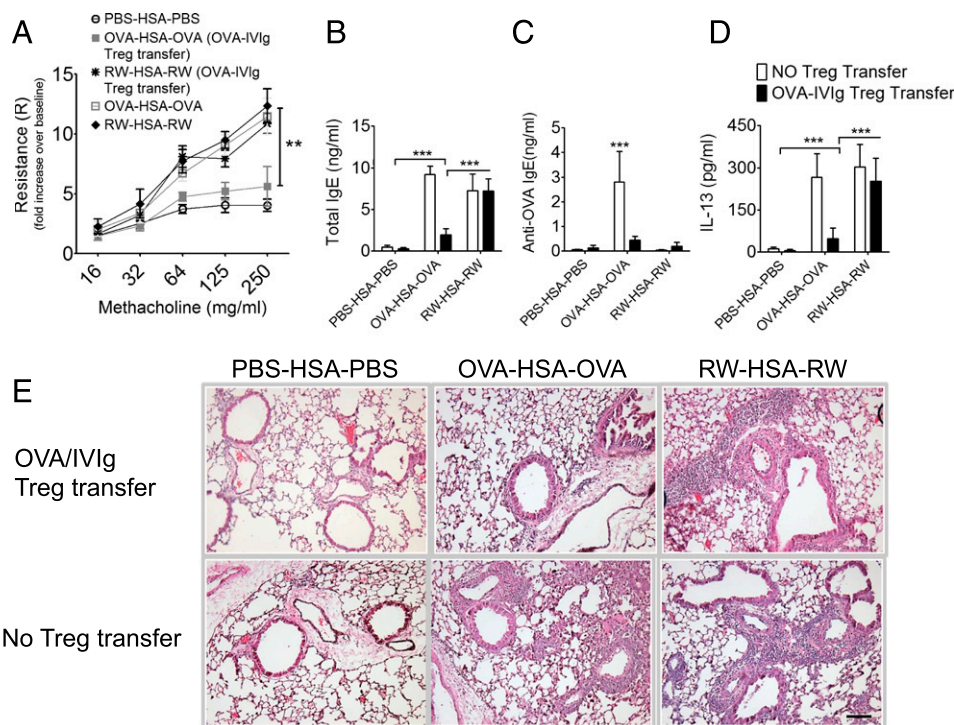
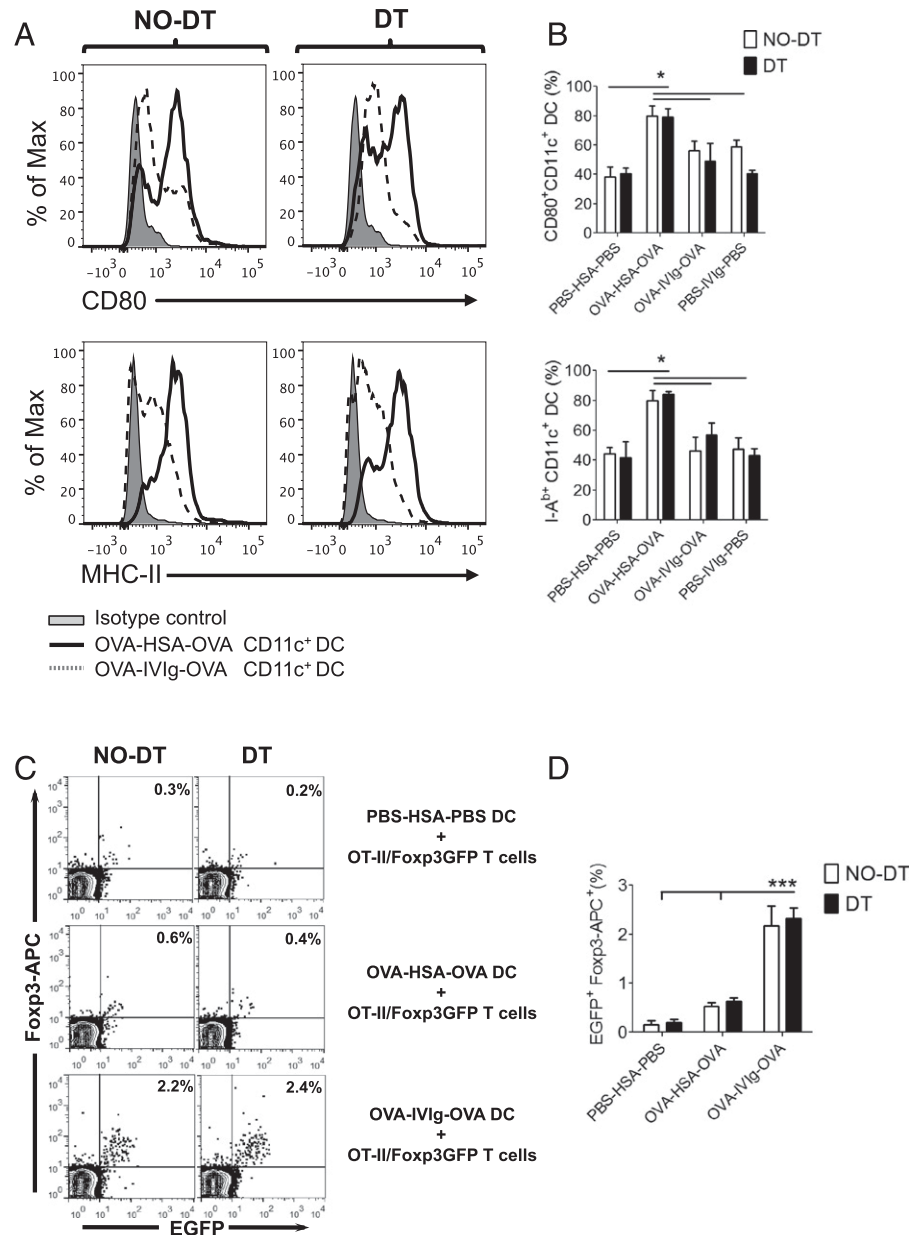


FIGURE 5. Adoptive transfer of OVA/IVIg-generated Treg suppressed pulmonary inflammation in an Ag-specific manner. **(A)** Adoptive transfer of pTreg obtained from OVA/IVIg-treated mice, in which endogenous Treg were depleted prior to IVIg treatment, to OVA-sensitized mice, attenuated methacholine-induced AHR after OVA challenge of the recipients, whereas OVA/IVIg pTreg were not protective in RW-exposed mice. *** $p < 0.01$; repeated-measures ANOVA with a Tukey post hoc test, negative control group and OVA-immunized pTreg recipients versus other control groups for peak methacholine response, $n = 4$. **(B–D)** Total serum IgE (B), OVA-specific serum IgE (C), and pulmonary IL-13 BAL levels (D) as determined by ELISA 24 h after the last challenge. Adoptive transfer of IVIg-generated pTreg suppressed total IgE (B) and IL-13 (D) production in an Ag-specific manner. * $p < 0.05$, ANOVA with a Tukey post hoc test, OVA-HSA-OVA versus other control groups ($n = 4$). **(E)** Representative photomicrographs of inflammation analyzed on H&E-stained lung sections. Original magnification, $\times 200$; scale bar, 50 μm . OVA/IVIg pTreg adoptive transfer inhibited inflammatory cell influx in OVA-immunized, but not RW-immunized, animals.

FIGURE 6. In vivo depletion of Treg did not alter the modulatory actions of IVIg on DC maturation or ex vivo Treg induction. **(A and B)** Representative flow cytometric analysis (A) and quantification (B) of CD80 and MHC-II expression on pulmonary CD11c⁺ DC. IVIg treatment of OVA-exposed mice decreased the expression of CD80 and MHC-II on pulmonary CD11c⁺ DC, with or without endogenous Treg depletion by DT. For the gating strategy, viable CD11c⁺ cells were analyzed for CD80 and MHC-II staining. **p* < 0.05, ANOVA with a Tukey post hoc test (*n* = 6). **(C and D)** Lung CD11c⁺ DC from Treg-depleted and non-Treg-depleted mice were cocultured with CD4⁺Foxp3⁺ OT-II/Foxp3EGFP T cells in the presence of OVA for 4 d. Representative flow cytometry analysis (C) and quantification (D) of Foxp3-allophycocyanin⁺EGFP⁺ Treg within total CD4⁺ T cells demonstrating that DC derived from OVA-IVIg-OVA mice, whether DT treated or untreated, were both able to significantly expand OVA-specific Foxp3⁺ Treg in coculture, compared with other control groups. For the gating strategy, viable CD4⁺ CD25^{high} cells were assessed for Foxp3⁺EGFP⁺ expression and Foxp3-allophycocyanin staining. ****p* < 0.001, ANOVA with a Tukey post hoc test (*n* = 6). NO-DT, not treated with DT.



differentially expressed genes in Supplemental Table II. Fig. 8A depicts the log fold change as a function of mean log expression level (M-A plot). When comparing mixed (endogenous and IVIg-generated) Treg versus endogenous Treg alone, we detected similar patterns of differential gene expression (data not shown), reflecting gene expression from both endogenous and IVIg-generated Treg.

Gene expression data were validated by quantitative real-time PCR for the genes *Gzma*, *Gzmb*, and *Ifitm2*, indicating significant increases in the IVIg-induced Treg population (Fig. 8E).

STRING database results

When examining the list of differentially expressed genes, the STRING database calculated 63 network nodes, which represent genes with known protein-coding abilities, in the list. A simple numerical comparison between the calculated and expected number of "edges," that is, known relationships between genes, yields 810 edges for the network of differentially expressed genes in IVIg-generated pTreg, as opposed to 33 expected edges, with an associated clustering coefficient of 0.898 and a protein-protein interaction *p* value of >0.001. This strongly suggests that the

network of gene products has significantly more interactions than expected, relative to a random set of similarly sized proteins across the mouse genome. Thus, it is highly likely the proteins in the network are biologically linked as a group.

IVIg-generated pTreg have restored granzyme A expression levels

To confirm the results of the gene array, we examined the levels of intracellular granzyme A (GzmA) protein expression by flow cytometry. Treg from negative-control animals displayed high GzmA mean fluorescence intensity, which was lowered by OVA exposure. IVIg treatment increased GzmA expression levels to near baseline (Fig. 8B).

IVIg-generated pTreg are a metabolically active population

We examined known gene function by in silico database analysis of our list of differentially expressed genes. PANTHER database hits for biological processes revealed that genes involved in cellular and metabolic processes (including *Cdk1*, *Rrm2*, *Arhgap19*, and *Anxa2*), biological regulation (including *Aurkb*, *Ccna2*, *Ccnb2*, *Ube2c*, and

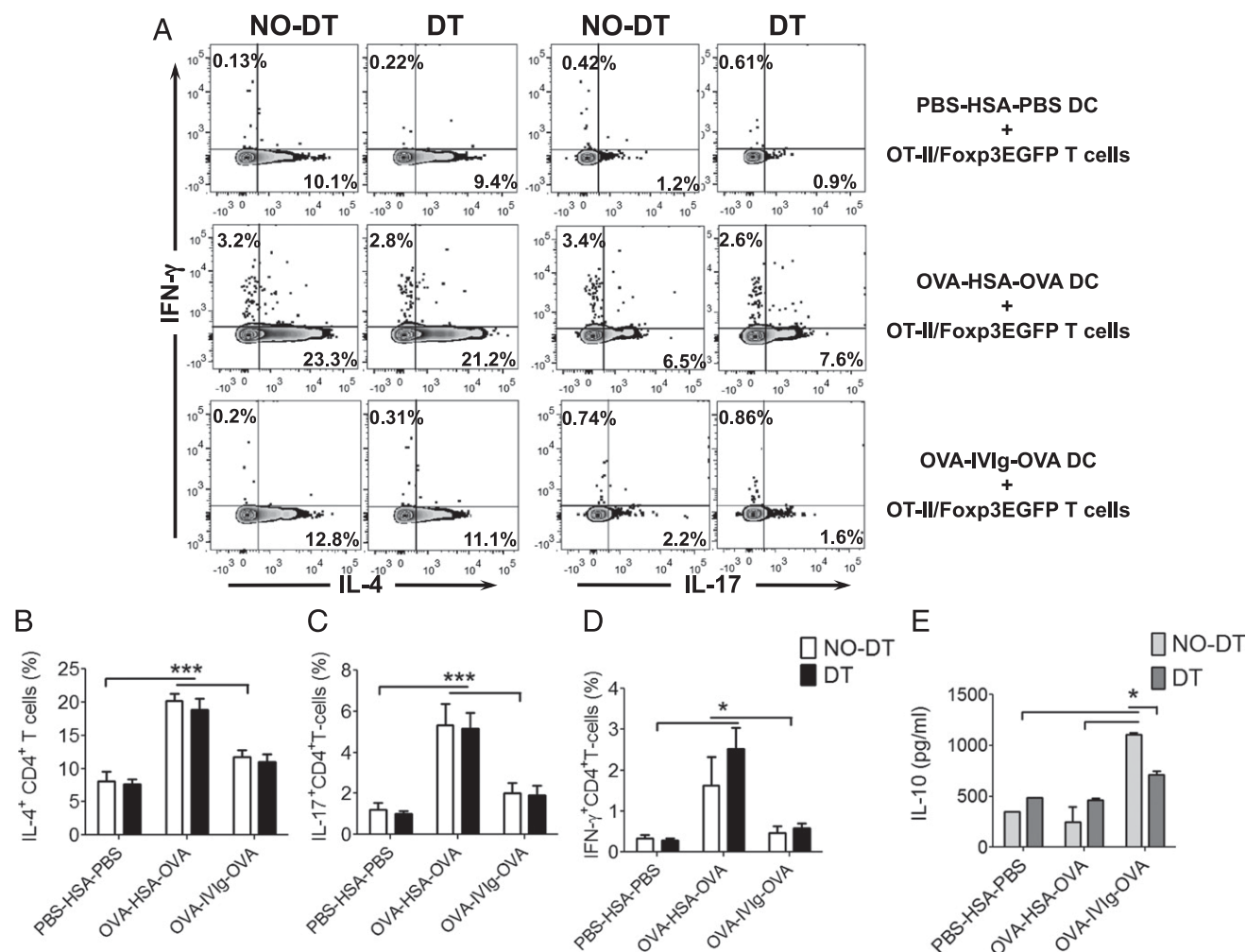


FIGURE 7. Depletion of endogenous Treg did not reverse the tolerogenic properties of IVIg-primed DC in regulation of cellular responses ex vivo. CD11c⁺ DC and OT-II/Foxp3^{EGFP} CD4⁺ T cells were purified, cocultured, and stimulated with ionomycin/PMA in the presence of GlogiPlug. Intracellular IL-4, IL-17, and IFN-γ production were analyzed in CD4⁺ T cells by flow cytometry. (A–D) Representative flow cytometric analyses (A) and quantification (B–D) of IL-4⁺, IL-17⁺, and IFN-γ⁺ cells within CD4⁺ T cells. For the gating strategy, viable CD4⁺ lymphocytes were analyzed for intracellular cytokine staining. *** $p < 0.005$, * $p < 0.05$, ANOVA with a Tukey post hoc test ($n = 4$). (E) IL-10 levels in coculture supernatant, as determined by ELISA. OVA-IVIg-OVA DC from non-DT-treated mice increased IL-10 levels in coculture compared with other groups. DC from OVA-IVIg-OVA mice treated with DT prior to and during OVA challenge were unable to induce IL-10 production in coculture. * $p < 0.05$, ANOVA with a Tukey post hoc test ($n = 4$). NO-DT, not treated with DT.

Gzma), immune system processes (including *Igi*, *Il12rb2*, *Ccr5*, and *Gzmb*), and apoptosis (*Birc5*) were enriched in our gene list (Fig. 8C). Protein classes represented included cytoskeletal and nucleic acid-binding proteins (including *Tpx2*, *Kif18b*, *Kif22*,

Kif23, *Top2a*, *E2f8*, *Hist1h2bk*, and *Hist1h1b*), enzyme modulators (including *Arhgap19*, *Arhgap11a*, *Ccnb2*, and *Ccna2*), and receptors and defense/immunity proteins (including *Il12rb2* and *Ccr5*) (Fig. 8D).

Table I. Top 10 differentially expressed genes in IVIg-generated iTreg versus endogenous Treg

Gene Symbol	Log ₂ FC	Average Expression	<i>t</i>	<i>p</i> Value	FDR <i>p</i> Value	<i>B</i>
Igkv10-96	4.219708628	8.168786494	2.31976789	0.038474312	0.247390427	-4.07776012
Gzmb	4.181552169	9.384794965	7.147125082	1.07×10^{-5}	0.004345594	3.756778046
Igk	4.01270105	7.019139998	3.940937957	0.001903012	0.042142496	-1.212521428
Hist1h2bk	3.116087063	7.878848707	4.664498275	0.000524374	0.020604625	0.038435769
Hist1h2ab	3.078058282	5.1489513	4.089289027	0.001453985	0.035997011	-0.951444191
Ighm	3.02809623	7.912686098	2.48994712	0.028161956	0.207180796	-3.789020284
Gm5593	3.008957503	7.092032121	5.340769276	0.000167017	0.012287079	1.145554211
Cdkn3	2.904471003	7.863296936	6.082340584	5.12×10^{-5}	0.007772727	2.27930145
1500009L16Rik	2.894095195	8.737148937	7.354114123	8.05×10^{-6}	0.004345594	4.024009575
Ifitm2	2.855573753	8.493691797	9.741504958	4.17×10^{-7}	0.002231589	6.687057179

Average expression, average log₂ expression for the probe over all arrays and channels; *B*, log odds that gene is differentially expressed; FDR *p* value, adjusted *p* value using false discovery rate multiple comparisons correction; gene symbol, official Mouse Genome Informatics database abbreviation for gene; Log₂ FC, log₂ fold change for contrast; *p* value, raw *p* value; *T*, moderated *t* statistic.

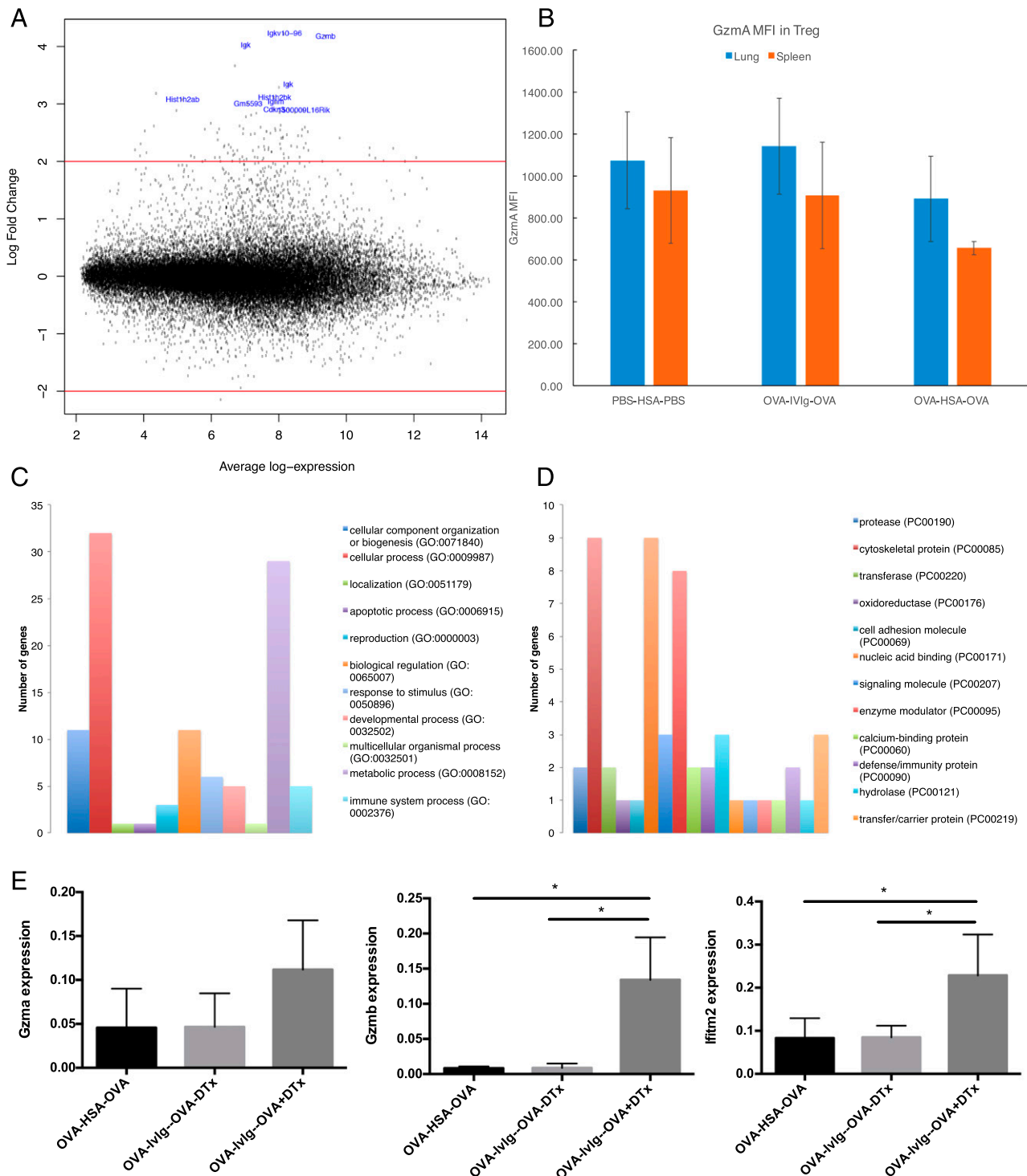


FIGURE 8. IVIg-generated Treg gene expression suggests a metabolically active and plastic phenotype. **(A)** $-\log_2$ fold change versus average log expression plot for the contrast of IVIg-generated pTreg versus endogenous Treg. Red lines indicate the threshold of 2-fold change for differential gene expression. The 10 most differentially expressed genes are labeled with their National Center for Biotechnology Information gene symbol. **(B)** GzmA mean fluorescence intensity (MFI) in lung and spleen Treg (viable CD4⁺CD25^{high}Foxp3⁺ cells) from sensitized, treated, and challenged animals. **(C and D)** PANTHER database search results. Annotation terms are plotted by number of genes per annotation term in the set of genes with a log₂ fold change FC of >2. Individual genes can have multiple annotations. **(B)** Biological processes represented by GO annotations; **(C)** protein classes represented by PANTHER annotations. **(E)** Quantitative real-time PCR validation of gene array results. Results are presented as fold change from control with normalization to *Gapdh* reference gene. * $p < 0.05$, ANOVA with a Tukey post hoc test ($n = 4$).

The Database for Annotation, Visualization, and Integrated Discovery functional annotation clustering yielded 23 clusters in total, 11 of which had statistically significant enrichments in annotation terms for cell cycle and cell division/mitosis (including *Cdk1*, *S100a6*, *Kif11*, *Prc1*, *Mki67*, *E2f8*, *Nuf2*, *Tpx2*, *Nusap1*, *Birc5*,

Cenpe, *Aurkb*, *Cep55*, *Racgap1*, *Ube2c*, *Cdkn3*, *Esco2*, *Ncaph*, *Ccnb2*, *Plk1*, *Bub1*, *Gm5593*, *H2afx*, and *Ccna2*), cytoskeleton (including *Kif23*, *Kif22*, *Kif11*, *Prc1*, *Kif15*, *Tpx2*, *Nusap1*, *Gm5593*, *Kif18b*, *Birc5*, *Cenpe*, *Cep55*, *Aurkb*, *Racgap1*, and *Anxa2*), chromosome/nucleosome (including *Hist1h2ab*, *Kif22*,

Mki67, Hist1h2bk, Hist1h1b, Hist1h1a, Nuf2, Bub1, H2afx, Hist1h2ak, Cenpe, Birc5, Aurkb, and Top2a), and histone (including *Hist1h2ab, Hist1h2bk, H2afx, and Hist1h2ak*) (Supplemental Table III).

Discussion

IVIg-generated Treg

As an immune modulator, IVIg has multiple mechanisms of action (12). We have previously demonstrated that IVIg is able to modify DC–T cell communication, leading to induction of highly suppressive Treg (15). In this study, we address the distinctive nature of the pTreg induced by treatment of allergen-sensitized mice with IVIg. The present work is paralleled by a number of murine studies that have demonstrated functional pTreg in vivo (36). However, a particularly unique aspect of the system described in the present study is the generation of a pure pTreg population, without endogenous Treg contamination. This allowed us to investigate differential expression of the various Treg compartments in a physiological setting of Ag-driven inflammation. It also allowed us to isolate sufficient Ag-specific pTreg generated in vivo for adoptive transfer experiments (37).

We have previously demonstrated (14, 15) that IVIg resolves Ag-induced airway inflammation and AHR in murine AAD via a mechanism associated with the peripheral induction of highly suppressive Foxp3⁺ Treg and their homing to pulmonary tissues. This mechanism depended on modification of CD11c⁺ DC and modulation of cytokine production. The most important finding of the present study was the requirement of Treg in IVIg-directed peripheral tolerance and protection against airway inflammation. pTreg were generated from naive or mature CD4⁺ effector T cells during T cell activation (38), and they were effective in preventing the inappropriate immune responses to inhaled OVA, due to their Ag specificity and their compartmentalization capability (39).

Cellular phenotype

When examining specific IVIg pTreg phenotypes, we determined that both IVIg pTreg and endogenous Treg express high levels of LAP, a marker associated with enhanced suppressive activity. Chen et al. (33) described a LAP-expressing Treg population that regulated the multiple sclerosis disease model experimental autoimmune encephalomyelitis in a TGF- β -mediated fashion, and it had greater suppressive ability than did other Treg subsets. Similarly, Duan et al. (40) found that LAP⁺ Treg abolish both induction and effector phases of AAD in a model of Ag tolerance. Sun et al. (41) suggested LAP as a marker for functional Treg in the context of cancer immunotherapy: this may provide an alternate biomarker for Treg induction in many disease states.

Conversely to LAP, we found that IVIg pTreg had lower Nr1p expression than did endogenous Treg. Nr1p has been recently described as a specific marker for tTreg (34), as opposed to canonical markers such as Foxp3, CD25, GITR, and CTLA-4. Weiss et al. (42) found that Nr1p^{low} Treg were undetectable in thymus but enriched in the intestinal mucosa, a major site of Treg induction. Interestingly, high Nr1p expression correlated well with Helios expression, although Helios is found on subsets other than tTreg (43). Yadav et al. (34) described Nr1p^{low} pTreg generated in vitro by CD3/CD28 stimulation or by Ag-loaded DC, T cell coculture, and in vivo by chronic Ag administration. Furthermore, Nr1p levels remained stable regardless of activation state. Nr1p has been implicated in sustaining interaction between DC and Treg and blocking effector T cell access (35). We speculate that pTreg, with low Nr1p expression, are potentially more available to traffic to inflammatory tissue and suppress immune

responses. This may explain our finding of greatly enhanced pTreg in pulmonary tissues from IVIg-treated mice (15).

Gene expression profile of IVIg-generated pTreg

When examining the gene expression profile of IVIg-generated pTreg, we determined that they represent a metabolically active, motile, and phenotypically plastic population, increasing the expression of genes associated with cell cycle, chromatin activity/histones, cytoskeleton, and apoptosis. This suggests several possible functions matching an active regulatory and phenotypically plastic phenotype, responding to inflammatory stimuli by modifying gene expression (44). The upregulated cytoskeleton-associated genes indicate that IVIg-generated pTreg are motile, homing to the inflammatory microenvironments in local and systemic tissues. The upregulated cell cycle/mitosis-associated and regulatory process genes reflect a potentially expanding population of functional regulatory cells. Furthermore, these cells are transcriptionally active, as evidenced by the upregulated genes associated with chromosome/nucleosome/histone. Beier et al. (45) reviewed *Foxp3* epigenetic modifications and explained that methylation of the normally demethylated *Foxp3* promoter Treg-specific demethylated region inhibits Treg function, whereas acetylation of Treg-specific demethylated region promotes Treg function. They therefore suggest that histone deacetylases are relevant targets for functional modulation. More specifically, Wang et al. (46) recently reported that histone deacetylase 3 modulates tTreg and pTreg development and function, and is essential for iTreg differentiation and suppressive function in vitro. Interestingly, we found that the *Foxp3* gene itself was not differentially expressed, with high average expression among all groups (Supplemental Table I). Bin Dhuban et al. (47) suggested that Foxp3 does not directly alter chromatin accessibility; rather, it modulates Treg transcriptional activity by binding to pre-established enhancers, such as the Foxo family of transcription factors.

We found that the second-highest differentially expressed gene in IVIg-generated pTreg was the serine protease granzyme B (GzmB); GzmA was also differentially expressed (Supplemental Table II). This is consistent with reported characterizations of Treg in other disease models. Velaga et al. (48) found that in a murine model of gastrointestinal graft-versus-host disease, Treg require GzmA to prevent graft-versus-host disease, perhaps due to their increased migratory efficiency, as GzmA cleaves extracellular matrix proteins. Using a murine model of respiratory syncytial virus infection, Loebbermann et al. (49) determined that Treg expressing GzmB are required to suppress cellular infiltration into the lung in respiratory syncytial virus infection; these cells degranulate after in vitro stimulation. They suggested that this requirement is likely due to the cytolytic action of GzmB on virus-infected cells, as well as increased migratory ability due to decorin cleavage in the extracellular matrix, which facilitates Treg homing to inflammatory sites. This is another line of evidence that may explain our finding of pTreg enrichment in lung tissues of IVIg-treated Ag-exposed mice (15).

DC and Treg induction

The requirement for DC in the induction of Treg in our model is in agreement with findings from other investigators, demonstrating the critical role of CD11c⁺ DC in elaboration of peripheral T cell tolerance through induction of Treg (50, 51). IVIg induces downregulation of costimulatory molecules and modulates cytokine secretion by DC (52). Nonetheless, the induction of tolerogenic DC was necessary but not sufficient for inhibition of AAD when Treg were eliminated by successive DT injections. In

addition to the AAD model, the implication of IVIg in induction and development of Treg has been demonstrated in experimental allergic encephalomyelitis (53), systemic lupus erythematosus (54), vasculitis (55), immune thrombocytopenic purpura (56), and Kawasaki disease (57). Notably, the deficiency of Foxp3⁺ Treg in patients with Kawasaki disease is associated with resistance to IVIg therapy (58). Taken together, these findings indicate that Treg play an integral role in mediating the immunomodulatory effects of polyclonal IgG.

IgE and Treg

Interestingly, our data showed that the depletion of all endogenous Treg just prior to allergen challenge did not lead to marked exacerbation of airway pathology and AHR, despite a striking increase in total serum IgE levels. IgE is responsible for initiation of allergic reactions and is important for the development of persistent airway inflammation (59, 60). Consistent with our findings, Baru et al. (61) demonstrated that depletion of Foxp3⁺ Treg during the course of allergen challenge did not lead to an aggravated allergic response, whereas Treg depletion in the priming phase of Ag immunization resulted in exacerbation of allergic airway inflammation.

In conclusion, taken together, our results show that IVIg generates a pure population of functional pTreg from non-Treg precursors. We demonstrate the absolute requirement for Foxp3⁺ Treg in the IVIg-mediated resolution of allergic airway inflammation, delineate the precise genetic signature and phenotype of this pure population of pTreg, and provide a rationale for potential application of IVIg in autoimmune and inflammatory conditions associated with Treg deficiencies. Further work will characterize the molecular mechanisms of pTreg function and DC tolerogenicity, along with Treg fate mapping to delineate the precise timing and stability of the Treg regulatory response. These studies will provide a more profound understanding of the induction of regulatory function.

Acknowledgments

We thank Camille Stegen of the McGill University Flow Cytometry Core facility for assistance with flow cytometric sorting. We thank the Gene Expression Analysis Platform of the McGill University and Génome Québec Innovation Centre for providing expertise and assistance with gene expression sample processing.

Disclosures

The authors have no financial conflicts of interest.

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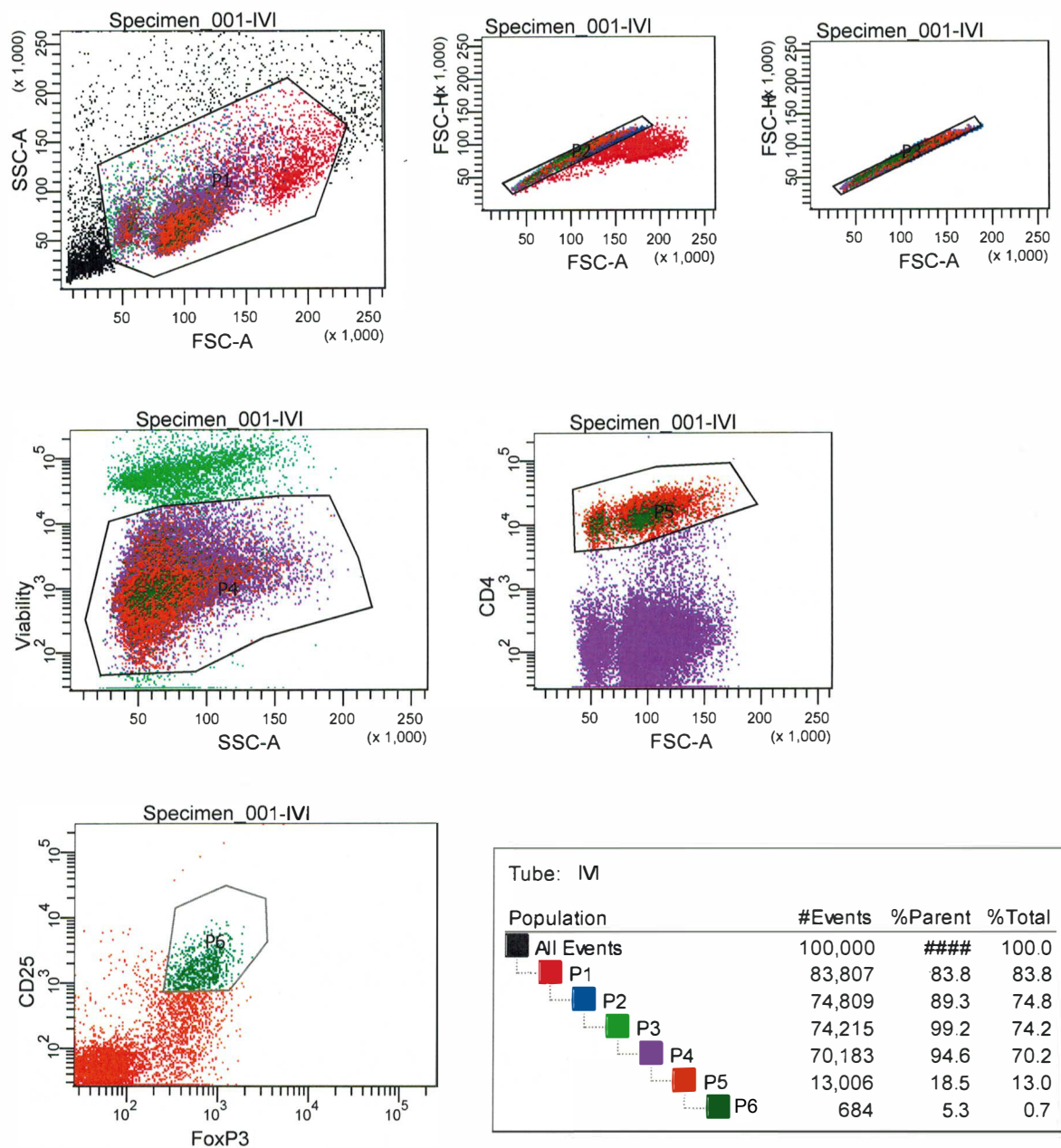
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Supplemental Table I. Antibodies used for flow cytometric analyses.

<u>Panel</u>	<u>Antigen</u>	<u>Fluorochrome</u>	<u>Manufacturer</u>	<u>Clone</u>
Treg	CD4	BV510	BioLegend	RM4-5
	CD25	PE	BioLegend	3C7
	Foxp3	APC	eBioscience	FJK-16s
	LAP	PerCP-Cy5.5	BioLegend	TW7-16B4
	Nrp-1	eFluor450	eBioscience	3DS304M
	GzmA	PerCP-eFluor710	eBioscience	GzA-3G8.5
	GzmB	PE-eFluor610	eBioscience	NGZB
DC	CD11c	PE-eFluor610	eBioscience	N418
	MHC-II	eFluor450	eBioscience	M5/114.15.2
	CD80	FITC	BD Pharmingen	16-10A1
	IL-4	PE	BioLegend	11B11
	IL-17	PerCP-Cy5.5	eBioscience	eBio17B7
	IFN- γ	APC	BioLegend	XMG1.2

FACSDiva Version 6.1.3



Supplemental Figure 1: Gating strategy for Treg flow cytometric sorting.