Expansion of CD4$^+$CD25$^+$ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis

Amal Ephrem,[1-4] Souleima Chamat,[4] Catherine Miquel,[5] Sylvain Fisson,[1-3] Luc Mouthon,[6] Giuseppina Caligiuri,[1-3] Sandrine Delignat,[1-3] Sriramulu Elluru,[1-3] Jagadeesh Bayry,[1-3] Sebastien Lacroix-Desmazes,[1-3] José L. Cohen,[7,8] Benoît L. Salomon,[7,8] Michel D. Kazatchkine,[1-3] Srini V. Kaveri,[1-3] and Namita Misra[1-3]

[1]Centre de Recherche des Cordeliers, Université Pierre et Marie Curie–Paris 6, Unité Mixte de Recherche (UMR) S 872, Paris, France; [2]Université Paris Descartes, UMR S 872, Paris, France; [3]Inserm U872, Paris, France; [4]Laboratory of Applied Immunology, Faculty of Public Health, Lebanese University, Beirut, Lebanon; [5]Inserm U752, University of Paris-5, Hôpital Sainte-Anne, Paris, France; [6]Unité Propre de la Recherche et de l'Enseignement Supérieur Equipe d'Accueil (UPRES-EA) 4058, Paris-Descartes University, Faculty of Medicine, Department of Internal Medicine, Cochin Hospital, Assistance Publique Hôpitaux de Paris, Paris, France; [7]Université Pierre et Marie Curie–Paris6, UMR 7087, Paris, France; and [8]Centre National de la Recherche Scientifique (CNRS), UMR 7087, Paris, France

The clinical use of intravenous immunoglobulin (IVIg) based on its immunomodulatory and anti-inflammatory potential remains an ongoing challenge. Fcγ receptor-mediated effects of IVIg, although well elucidated in certain pathologies, cannot entirely account for its proven benefit in several autoimmune disorders mediated by autoreactive T cells. In this study, we show that prophylactic infusion of IVIg prevents the development of experimental autoimmune encephalomyelitis (EAE), an accepted animal model for multiple sclerosis (MS). The protection was associated with peripheral increase in CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cell (Treg) numbers and function. The protection was Treg-mediated because IVIg failed to protect against EAE in mice that were depleted of the Treg population. Rather than inducing de novo generation from conventional T cells, IVIg had a direct effect on proliferation of natural Treg. In conclusion, our results highlight a novel mechanism of action of IVIg and provide a rationale to test the use of IVIg as an immunomodulatory tool to enhance Treg in early onset MS and other autoimmune and inflammatory conditions. (Blood. 2008;111:715-722)

Introduction

Natural CD4$^+$CD25$^+$ regulatory T cells (nTreg) expressing the lineage marker Foxp3 are the key players in controlling immune responses and in maintenance of T-cell homeostasis.[1,2] Therapeutic induction of the Treg represents a novel approach in the treatment of autoimmune pathology.[3,4] CD4$^+$CD25$^+$Foxp3$^+$ nTreg develop in thymus, in contrast to "adaptive" or "induced" Treg that develop in peripheral lymphoid tissues from CD4$^+$ conventional T cells (Tconv) and are frequently Foxp3$^-$. Although studies have highlighted the role of cytokines interleukin-2 (IL-2), transforming growth factor-β (TGF-β), and IL-10 in Treg development, other factors or mechanisms crucial to Treg homeostasis are not elucidated.[5] Intravenous immunoglobulin (IVIg) is an established therapy for several immune disorders.[6-9] Several mutually nonexclusive mechanisms have been proposed to explain the beneficial effect of IVIg[7,8]; however, the issue remains debated and an ongoing challenge. For instance, the FcγR-mediated effects of IVIg[10-12] cannot entirely account for its proven benefit in several peripheral and central demyelinating diseases such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), and relapsing-remitting multiple sclerosis (MS), which are primarily mediated by autoreactive T cells.[6,7,13,14] Because the T cells do not express FcγR,[15] the observed effects raise certain speculations, that is, if these effects could be attributed to a direct interaction of the variable region of the immunoglobulin (Ig) G molecules with the T cell or an indirect influence via other cell types such as dendritic cells (DC).

During the induction phase of experimental autoimmune encephalomyelitis (EAE), myelin reactive proinflammatory CD4$^+$ T cells proliferate in the periphery, infiltrate the central nervous system (CNS) during the effector phase and, in concert with other inflammatory mediators, lead to demyelination characterized by a progressive paralysis.[16] Natural remission and recovery from relapse in EAE is associated with the recruitment or generation of Treg in the CNS.[17,18] We and others have shown that IVIg protects against EAE development only when administered prophylactically.[14,19] We reasoned that IVIg manifests its protective effect in EAE through an early modulation of autoreactive T cells, and therefore we investigated the regulatory mechanisms, particularly the effect of IVIg on regulatory T cells.

Methods

Animals, antigen, and tissue culture medium

We purchased C57BL/6J mice (females, 6-8 weeks of age) from Charles River Laboratories (L'Arbresle, France), and all animal-handling procedures conformed to European Union guidelines. The MOG-$_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) peptide was purchased from NeoMPS (Strasbourg, France). Tissue culture medium was RPMI 1640 supplemented with

Submitted March 13, 2007; accepted September 23, 2007. Prepublished online as *Blood* First Edition paper, October 11, 2007; 10.1182/blood-2007-03-079947.

An Inside *Blood* analysis of this article appears at the front of this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin from Gibco (Cergy Pontoise Cedex, France), and 10% fetal calf serum from Eurobio (Les Ulis, France). The T-cell receptor (TCR)–hemagglutinin (HA) transgenic mice, expressing HA epitope of influenza virus, were kindly provided by Dr Benoît Salomon (Unité Mixte de Recherche 7087, Paris, France), and 6-week-old BALB/cByJ (BALB/c) mice were from Charles River Laboratories. The mice were bred in our animal facility under specific pathogen-free conditions.

EAE induction, assessment, and IVIg treatment

C57BL/6J mice (weighing approximately 20 gm) were immunized with 200 μg MOG_{35-55} peptide emulsified in Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St. Quentin Fallavier, France) 1:1 by volume containing 800 μg of nonviable desiccated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, L'Arbresk, France). A final volume of 200 μL was injected subcutaneously at 4 sites over the flanks. In addition, 300 ng of Pertussis toxin (List Biologic Laboratories, Meudon, France) was given intravenously on the same day and 2 days later. Clinical signs of EAE were assessed daily by the following scoring system: 0, no signs; 1, hindlimb weakness; 2, hindlimb weakness and tail paralysis; 3, hindlimb and tail paralysis; 4, hindlimb and tail paralysis and forelimb weakness; 5, moribund; and 6, death. From the day of the immunization until the peak of the disease (day 21-25), mice received daily intraperitoneal injections of 16 mg (0.8g/kg) IVIg (Sandoglobulin or a next-generation IVIg in development, IgPro10, both from CSL Behring) reconstituted at 50 mg/mL in phosphate-buffered saline (PBS). The control groups received either PBS alone or human serum albumin (HSA; Vialebex, LFB Biotechnologies, Les Ulis, France) intraperitoneally at equivalent molar amounts.

Preparation of cells from the blood, spleen, lymph nodes, and CNS

Blood of anesthetized mice was collected from the right ventricle in a syringe containing heparin. Mononuclear cells were isolated by floatation over Ficoll-Histopaque d = 1.077 (Sigma-Aldrich). Mice were perfused with cold PBS, followed by removal of spleen and draining lymph nodes (LN) and mechanical disaggregation. The brain and the spinal cord were mechanically disaggregated, and mononuclear cells of the CNS were isolated using 37.5% Percoll (Sigma-Aldrich).

Fluorescence activated cell-sorter analysis

For the analysis of cell surface marker expression, Abs: anti–CD4-fluorescein isothiocyanate (FITC) or anti–CD4-phosphatidylethanolamine (PE)-Cy5, anti-CD25 APC (clone PC61) or relevant isotype controls (all from BD Pharmingen, Le Pont de Claix, France) and PE-conjugated antihuman IgG (Beckman Coulter) were used. AntiFoxP3-PE and isotype control (rat IgG2a-PE; eBiosciences, Montrouge, France) were used per manufacturer's instructions. Cells were acquired and analyzed using fluorescence activated cell sorter (FACS)Calibur cytometer and the CellQuest Pro Software (BD Biosciences, Le Pont de Claix, France).

Measurement of secreted interferon-γ, IL-10, and TGF-β

Spleen cells were cultured in triplicates alone, with 5 μg/mL MOG_{35-55} or 1 μg/mL concanavalin A. Supernatants were collected after 24 hours, and cytokine concentration in the supernatant of cell culture was measured in sandwich enzyme-linked immunosorbent assay (ELISA; DuoSet; R&D Systems, Lille, France) according to the manufacturer's instructions.

Adoptive transfer of the protection

Donor C57BL/6J mice were subjected to an EAE induction and treated or not with IVIg. At the peak of the disease (day 21), mice were killed and single-cell suspensions were prepared from the spleen, and the draining LN (axillary and inguinal). $CD25^+$ T cells were enriched using anti-CD25 mAb (7D4; BD Biosciences) and antibiotin-microbeads (Miltenyi Biotec, Paris, France). The enriched fraction was labeled with streptavidin-PE and anti-CD4-FITC (3M4–5; BD Biosciences) and sorted for $CD4^+CD25^+$ high cells using FACSAria Cell Sorting System (BD Biosciences). The purity was more than 98%. Recipient mice (n = 4) received 0.25 or 0.5 million cells intravenously from IVIg-treated or untreated mice. After 24 hours, mice were subjected to an EAE induction as described in "EAE induction, assessment, and IVIg treatment."

Purification of $CD4^+CD25^+$ T cells, in vitro suppression, and proliferation assay of Treg

Single-cell suspension was prepared from the spleen. $CD4^+CD25^+$ T cells were isolated using $CD4^+CD25^+$ Treg-isolation-kit (Miltenyi Biotec) per manufacturer's instructions. For suppression assay, triplicate cultures of 5×10^4 $CD4^+CD25^-$ cells, 1 μg/mL anti-CD3-plate bound (clone 145.2C11), 1 μg/mL anti-CD28, and the indicated number of $CD4^+CD25^+$ cells were incubated in complete medium. After 4 days, the cells were pulsed for 16 hours with 1 μCi (37×10^3 becquerel) of [^{3}H] thymidine. Radioactive incorporation was measured by standard liquid scintillation counting, and results were expressed as counts per minute (cpm). For proliferation, Treg (5×10^4) were stimulated with 10 ng/mL rmIL-2, plate-bound anti-CD3, and soluble 1 μg/mL anti-CD28 Abs, with or without IVIg as indicated. Cultures were pulsed and harvested as explained above in this section.

Depletion of Treg in vivo

In vivo depletion of Treg was performed as previously described.[20] Briefly, mice were injected with 100 μg of anti-CD25 antibody, PC61 intraperitoneally at day 10 before EAE induction. Depletion was confirmed by flow cytometry. Mice were injected with IVIg or PBS as explained in "EAE induction, assessment, and IVIg treatment."

Tracking of T cells in vivo after IVIg administration

Brachial, axillary, cervical, and inguinal LN and spleen of TCR-HA-transgenic mice were harvested. $CD25^+$ cells were enriched as explained in "Purification of $CD4^+CD25^+$ T cells, in vitro suppression, and proliferation assay of Treg." Purity was 65% of $CD25^+$, more than 95% of them being $Foxp3^+$. $CD4^+$ cells were purified from the $CD25^-$ fraction using the mouse $CD4^+$ T-cell enrichment set (BD Biosciences) per manufacturer's instructions. To follow cell division, $CD25^-$ and $CD25^+$ cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and BALB/c mice were injected intravenously with either 2×10^6 $CD25^+$ cells or 10^6 $CD25^-$ cells or coinjected with 0.9×10^6 $CD25^-$ cells and 0.1×10^6 $CD25^+$ cells. The next day, mice were immunized with a subcutaneous injection of 2 μg $HA_{126-138}$ peptide in CFA in footpad. Mice received similar dose of IVIg intraperitoneally as in EAE for 10 days. Control group received PBS. At day 10, mice were killed, and the draining LN (popliteal), nondraining LN (axillary), and the spleen were mechanically dissociated, and cells were labeled with anti-CD4-PerCp, anti-$CD25^-$ APC (PC61) and anti-Foxp3-PE (FJK-16s) and analyzed as described previously.

Histology

Two mice from each group were killed by intracardiac perfusion with 4% paraformaldehyde. Brain and spinal cord were rapidly dissected, fixed with 4% paraformaldehyde for overnight at 4°C, incubated for 2 days in 30% sucrose, and then frozen in optimal cutting temperature (OCT). Six-micrometer cryostat serial sections were prepared, fixed in acetone for 10 minutes, rinsed in PBS, and stained with haematoxylin/eosin. The tissue sections were examined by light microscopy in a blinded manner and were evaluated for the extent of inflammation.

Slides were viewed with a Nikon Eclipse E600 microscope (Nikon, Champigny-sur-marne, France) using a Plan Fluor lens at 10×/0.25 and 40×/0.75 and Mount Medium (Merck, Darmstadt, Germany). Images were acquired using a Nikon DXM 1200 digital camera and were processed with a LUCIA G (version 4.71; Nikon) image analysis software and Adobe Photoshop version 7.0 software (Adobe Systems, San Jose, CA).

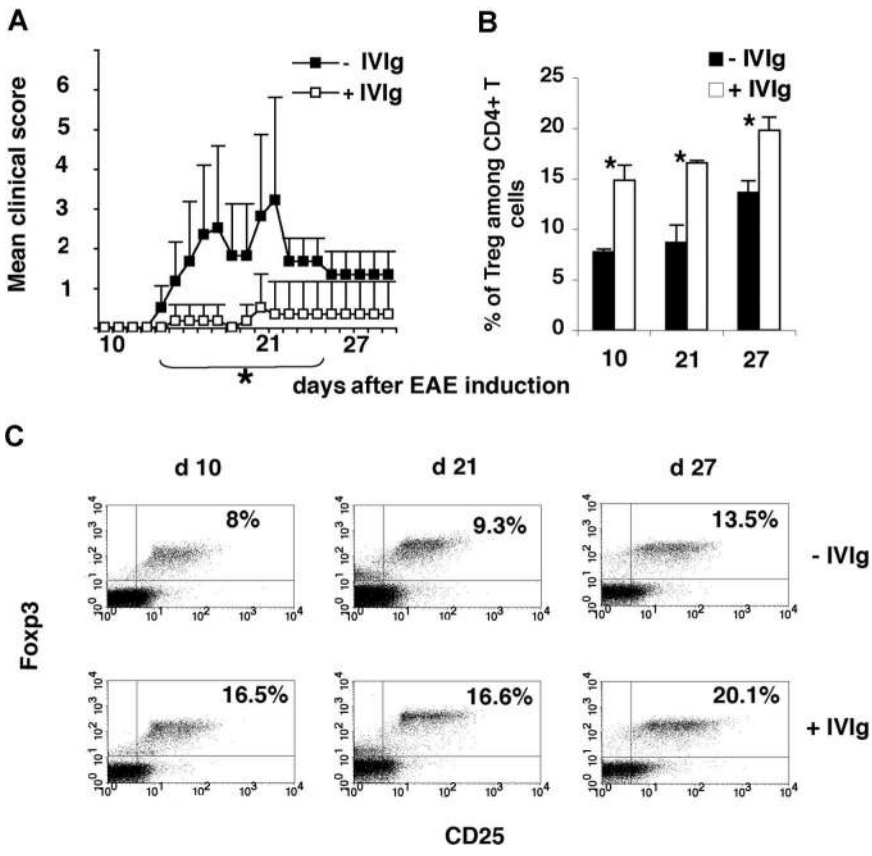

Figure 1. Protection of mice against EAE by IVIg is associated with an expansion of Treg in the peripheral lymphoid organs. (A) IVIg protects mice from EAE: EAE was induced in female C57Bl/6J mice by immunization with MOG_{35-55}. From day 0 of immunization until the peak of disease (day 21–25), mice received daily intraperitoneal injections of IVIg (□) or PBS alone (■). The severity of the disease was evaluated daily using a clinical score as explained in "EAE induction, assessment, and IVIg treatment." Mice: n = 6 to 8 for each group from 4 independent experiments. Data are the means plus or minus SD (*$P < .05$). (B) Increase in percentage of Treg: spleen cells from 3 PBS-treated mice (■), and 3 IVIg-treated mice(□) (open bars) were analyzed at days 10, 21, and 27 after immunization by flow cytometry for the expression of CD4, CD25, and Foxp3. Results of 1 experiment of 4 are shown. Data are the means plus or minus SD (*$P < .05$). (C) Representative plots from individual mice from each group at day 10, 21, and 27, gated on $CD4^+$. The number in each quadrant represents $CD25^+Foxp3^+$ gated on $CD4^+$.

Statistical analysis

Analysis of variance (ANOVA) or one-way between-groups ANOVA was used to determine the statistical significance of the data. A value of P less than .05 was considered to be statistically significant.

Results

IVIg protects against EAE via an expansion of peripheral regulatory T-cell population

We induced EAE in C57BL/6J mice and treated them with IVIg, PBS, or HSA as explained in "EAE induction, assessment, and IVIg treatment." The symptoms of EAE were observed in the PBS group from day 14 onward (Figure 1A); the disease typically peaked between day 21 and day 25 postimmunization, with an average clinical score of 3.2. Mice receiving IVIg from day 0 were almost completely protected from EAE (Figure 1A). The average clinical score was only 0.3 ($P < .05$), indicating a 10-fold decrease in the severity of the disease. Similar results were also observed using 2 different preparations of IVIg (Sandoglobulin and a next-generation IVIg in development, IgPro10, both from CSL Behring, Zurich, Switzerland). Although disease development was slightly slower in the HSA-treated group, compared with the PBS group, the maximal mean clinical score did not change significantly (3.4).

To determine whether the mechanism by which IVIg protection involves Treg, we studied the changes in $CD4^+CD25^+Foxp3^+$ T cells in treated and untreated mice by flow cytometry at different time points of the disease progression. We observed an increase in percentage of the $CD4^+CD25^+Foxp3^+$ T cells as early as day 10 in the IVIg-treated group (Figure 1B,C). The increase was observed in LN and blood, but interestingly, it was most significant in the spleen, where a 2-fold increase (7.78 ± 0.26% in untreated vs 14.87 ± 1.48% in IVIg-treated group; $P < .001$) was observed. This enhancement was maintained in periphery during all the phases of the disease. Thus, IVIg clearly induced or expanded Treg population throughout the clinical phases of EAE. Interestingly, no Treg expansion was observed in a control group of mice that were infused with IVIg in the absence of MOG immunization (data not shown), indicating that this expansion is specific to T cells that are activated through TCR stimuli.

IVIg enhances the suppressive capacity of Treg in vivo and in vitro

To study whether IVIg-expanded Treg had an enhanced suppressor function in vivo, we compared the capacity of Treg from treated and untreated group to protect recipient mice against EAE. In general, mice that were reconstituted with Treg from IVIg-treated or untreated mice developed milder EAE compared with nonreconstituted mice (Figure 2A). Further, mice that received 0.25 million Treg from IVIg-treated donor showed a mean clinical score of 0.5 plus or minus 1 compared with 1.25 plus or minus 0.8 in mice that received equal number of Treg from untreated mice (Figure 2A). The incidence of EAE development was 25% in the former group versus 75% in the latter group. Protective effect of the 2 Treg populations after a transfer of 0.5 million cells could not be discriminated because EAE development was totally inhibited in both cases (data not shown).

We further show that the expanded Treg population from IVIg-treated group was more efficient in suppressing the in vitro

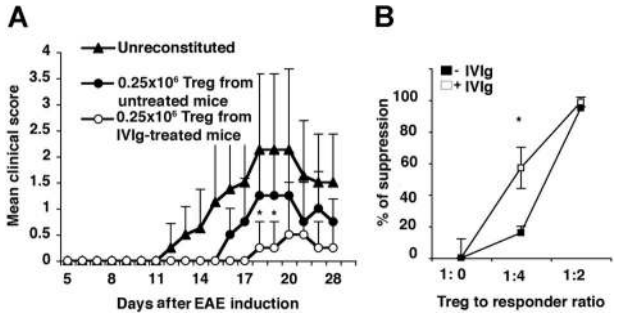

Figure 2. Enhanced function of IVIg-expanded Treg. (A) C57BL/6J mice (4 per group) were reconstituted (●) or not (▲) with 0.25 million Treg from IVIg-treated mice or (○) untreated mice. After 24 hours, recipient mice were subjected to an EAE induction. Data are the means plus or minus SD (*$P < .05$). (B) Suppression by IVIg-expanded Treg in vitro: 5×10^4 $CD4^+CD25^-$ cells isolated from the splenocytes of diseased mice were stimulated to proliferate for 96 hours with or without various concentrations of $CD4^+CD25^+$ cells isolated from the splenocytes of IVIg-treated (□) or PBS-treated (■) mice cells in round-bottom 96-well plates coated with anti-CD3 Abs and with soluble anti-CD28 Abs (1 μg/mL). Consistent data were obtained in 2 independent experiments.

response of TCR-stimulated $CD4^+CD25^-$ T cells (Figure 2B) compared with Treg from control group. At a Treg versus responder ratio of 1:4, the Treg suppression capacity increased from 16% in the control group to 57% in IVIg-treated group. However, at a ratio of 1:2, we did not observe a difference in suppressive capacity of Treg among 2 groups because of a saturation in percentage of suppression (approximately 100%). Together, these in vivo and in vitro results give a clear indication that in addition to an increase in numbers, IVIg-mediated protection also correlates with a functional enhancement of Treg.

IVIg-expanded Treg do not express TGF-β or IL-10

Suppressor cytokines like TGF-β and IL-10 are implicated in some models in the mechanism of action of Treg. To study the effect of IVIg treatment on secreted cytokine profiles, splenocytes of IVIg-treated and -untreated mice were prepared and stimulated with MOG_{35-55}, and culture supernatants were analyzed for IL-10 and TGF-β. We observed a 4-fold increase in IL-10 level (200 pg/mL and 50 pg/mL in treated and untreated group, respectively) and no significant difference in TGF-β levels (Figure 3A,B). To identify the source of IL-10 in the culture supernatants, we studied intracellular IL-10 expression in splenocytes and found that IL-10 was not produced from $CD4^+$ T cells (data not shown).

Downmodulation of pathogenic T cells in the periphery by IVIg-expanded Treg

Our results showing early expansion of $CD4^+CD25^+Foxp3$ cells suggested that the regulation of autoreactive T cells took place in peripheral lymphoid organs rather than in the CNS (ie, IVIg-induced Treg prevent CNS damage by restricting or energizing encephalitogenic T cells at the site of initiation of the immune response). To investigate this hypothesis, we compared the amount of secreted interferon-γ (IFN-γ) by splenocytes purified from IVIg-treated or untreated mice after MOG_{35-55} stimulation in vitro. Figure 3C shows that the culture supernatants of MOG_{35-55}-stimulated lymphocytes from treated mice showed significantly reduced IFN-γ compared with those of untreated mice (700 pg/mL vs 3000 pg/mL, respectively).

Absence of CNS inflammation in IVIg-treated mice

The data strongly indicate that Treg, if induced early during EAE, could prevent the activation/generation of effector T cells in lymphoid organs. We investigated the impact of this early suppression on subsequent lymphocytic infiltration and inflammation of the CNS, using histologic studies and flow cytometry. Although CNS tissue sections from untreated mice showed multiple foci of inflammation including lymphocytes and macrophages in perivascular and subpial areas, no histologic evidence of mononuclear cell infiltration was found in the treated group (Figure 4A). Further, we analyzed the T-cell infiltration in the CNS during peak and remission phase of EAE by flow cytometry. We observed a 6-fold increase of $CD4^+$ T cells in the CNS of untreated mice compared with naive mice (Figure 4B); of these, 23% were Treg (Figure 4B,C). The $CD4^+$ T cell numbers remained unchanged in IVIg-treated mice, and no Treg were detected. Because neither $CD4^+$ T-cell infiltration nor tissue inflammation was detected in IVIg-treated mice, it was not surprising that Treg were not recruited in the CNS. Our results indicate that when given prophylactically, IVIg regulated the inflammatory process in periphery, resulting in an absence of infiltration of effector cells in the CNS. However, we cannot rule out its local effect in the CNS in conditions other than those presented in this study.

Depletion of Treg before EAE induction abrogates IVIg-mediated protection

To further evaluate the implication of Treg in IVIg-mediated protection in EAE, we depleted mice of natural Treg population using a monoclonal antibody, PC61,[20] 10 days before EAE induction and treatment with IVIg. Antibody PC61 depleted $CD4^+CD25^+Foxp3^+$ T cells for a period of approximately 30 days. Depletion of Treg abolished the protective effect of IVIg, and the mice developed EAE of same severity as the control group (Figure 5A). The rate of reappearance of Treg was faster in immunized mice; however, their percentages were comparable in IVIg-treated and -untreated groups at the onset of EAE (Figure

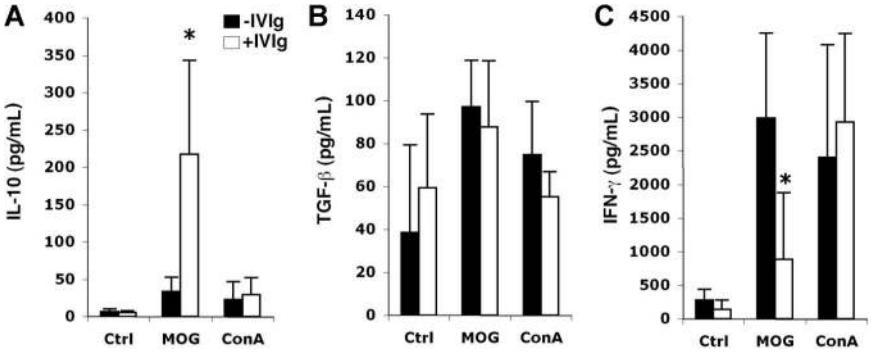

Figure 3. Cytokine secretion profiles after IVIg treatment. Effect of IVIg treatment on (A) IL-10, (B) TGF-β, and (C) IFN-γ secretion was studied. Mice that received PBS or IVIg were killed at day 10 (n = 4 per group), and spleens were harvested. Single-cell suspensions from the spleen were cultured in triplicates alone, in the presence of 5 μg/mL of MOG_{35-55}, or 1 μg/mL of ConA. Supernatants were collected after 24 hours, and cytokines were measured in sandwich ELISA. Results (means ± SD) of 1 experiment of 2 are shown (*$P \leq .05$).

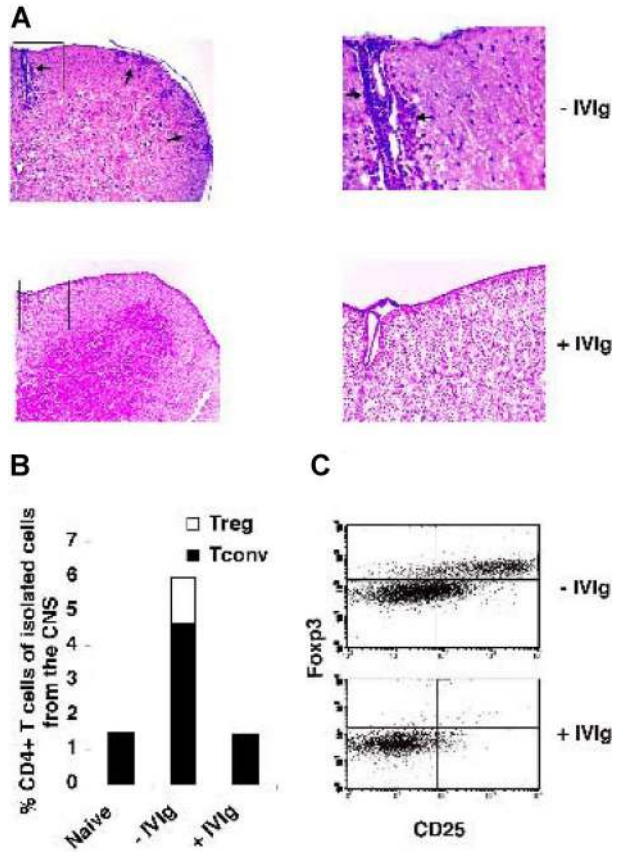

Figure 4. IVIg prevents CNS infiltration by T cells. (A) Histologic analysis of the cervical spinal cord of untreated (top) and IVIg-treated (bottom) mice stained with haematoxylin/eosin. Arrows indicate areas of spinal cord infiltrated by perivascular and subpial leukocyte aggregates. Left, 100×, right, 400× views of regions corresponding to boxed areas. (B) CNS cells isolated by percoll from 3 naive, IVIg-treated and -untreated mice were stained on day 27 after EAE induction and analyzed by FACS. Results are presented as percentage of Treg (□) and Tconv (■) $CD4^+$ T cells among the cells isolated from the CNS (mean of 3 mice). (C) Representative plots from individual mice, gated on $CD4^+$.

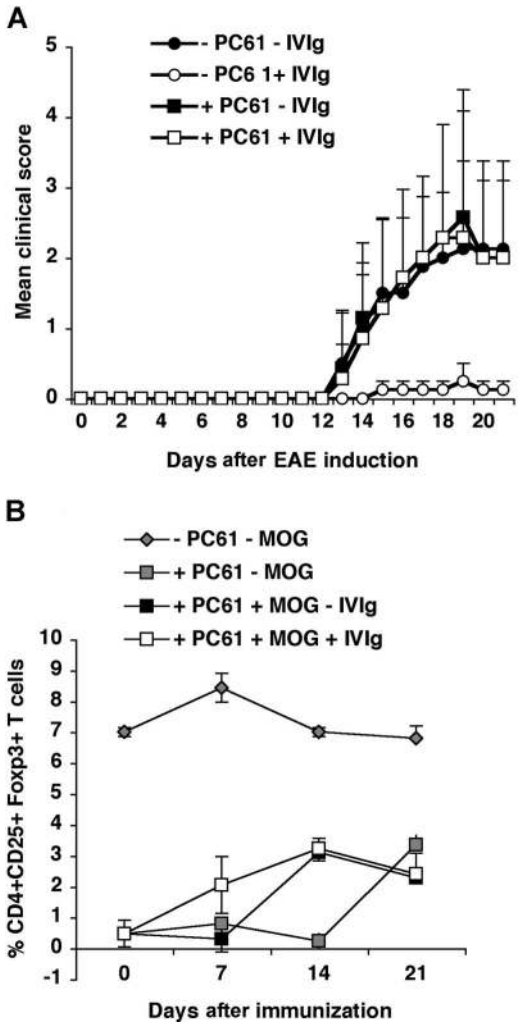

Figure 5. IVIg-mediated protection against EAE depends on the presence of Treg. (A) IVIg failed to protect mice that were depleted of Treg. Mice n = 7 in each group. For Treg depletion, animals received 100 μg of antibody PC61 intraperitoneally 10 days before EAE induction (squares). The control groups were not depleted (circles). The mice were treated (open symbols) or not (solid symbols) with IVIg. All mice were immunized with MOG_{35-55}. Clinical EAE was assessed as mentioned previously. (B) Reappearance of Treg in the blood of depleted mice, after EAE induction. Mice were depleted of T cells and immunized with MOG_{35-55} in the presence (□) or absence of IVIg (■). They were bled at intervals of 7 days after immunization (n = 5 for each group). Two control groups of naive untreated animals, which were Treg depleted (▨) or not (◈) were included. The percentage of cells coexpressing CD25 and Foxp3 in the CD4 population (Treg) was assessed in the blood using flow cytometry, and the mean percentages (± SD) for 5 mice in each group are represented. Similar results were obtained in 2 independent experiments. Data are the mean plus or minus SD.

5B). These results further indicate that IVIg-mediated protection in EAE was mediated by Treg.

Mechanisms underlying the IVIg-mediated expansion of Treg

To further explore the mechanisms that lead to IVIg-induced Treg expansion, we used an adoptive transfer model of TCR-transgenic T cells specific for a peptide of influenza virus HA ($HA_{126-138}$).[21] We studied 3 different conditions of adoptive transfer in BALB/c mice in the presence or absence of IVIg infusion: CFSE-labeled HA-specific $CD4^+CD25^-$ Tconv alone, $CD4^+CD25^+$ T cells alone (Treg), and a mixture of $CD4^+CD25^-$ T cells (90%) with $CD4^+CD25^+$ T cells (10%; Tconv + Treg). Cell transfer was followed by immunization with $HA_{126-138}$ peptide in CFA. Mice were killed 10 days after immunization, and the $CFSE^+$ T cells from the draining LN were gated and analyzed as shown in Figure 6A. Neither an induction of $CD4^+CD25^+Foxp3^+$ T cells in the Tconv group nor an increase of their proliferation in the Treg group was observed by IVIg treatment (Figure 6B). We reason that because of the strong activation context, IVIg effect on Treg could not be distinguished. However, an increase in $CD4^+Foxp3^+$ proliferation in the presence of IVIg was observed when Treg were coinjected with Tconv (Figure 6B). Next, we assessed the proliferation of Tconv cells. As shown in Figure 6C, IVIg did not suppress the proliferation of Tconv in the absence of Treg. The coinjection of Treg had a mild suppressive effect on the proliferation of Tconv (13%) in the absence of IVIg. Interestingly, the coinjection of Treg and IVIg together yielded a significant reduction (30%) in the proliferation of Tconv ($P < .001$). Thus, the presence of IVIg enhanced the suppressive capacity of the Treg.

In parallel, we studied the effect of IVIg on the proliferation of Treg in vitro. We purified $CD4^+CD25^-$ T cells (Tconv; > 95% pure) and $CD4^+CD25^+$ T cells (Treg; > 80% pure) from naive mice, stimulated them with a mixture of IL-2, anti-CD3, and anti-CD28 Abs, and observed their proliferation in the presence or absence of IVIg. Although IVIg did not have any effect on the

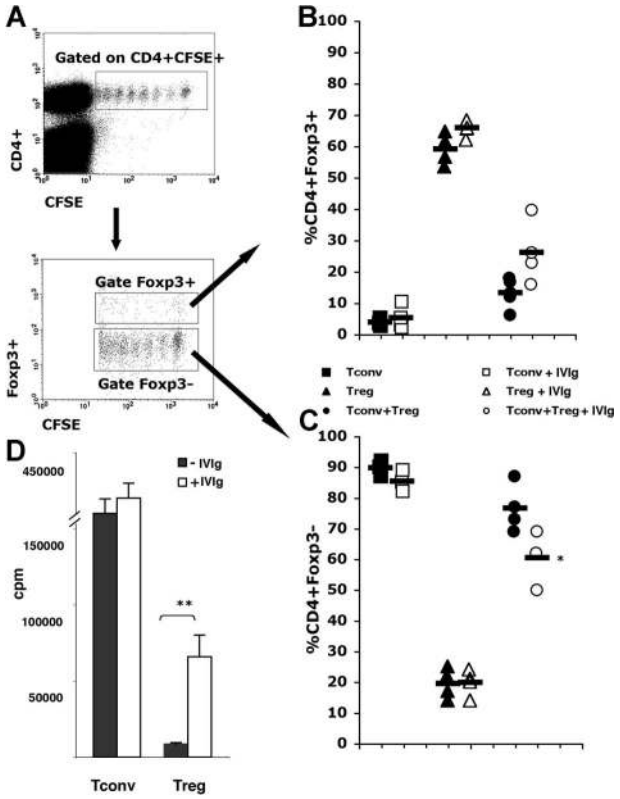

Figure 6. IVIg induces a mild proliferation of Treg rather than their de novo generation. TCR-HA transgenic T cells were fractioned by cell sorting into a $CD4^+CD25^-$ Tconv population and a $CD4^+CD25^+$ population (Treg) and labeled with CFSE. Three groups of BALB/c mice received intravenous injections of Tconv cells, Treg cells, or a mixture of both (90% Tconv + 10%Treg). Mice were injected with 2 μg HA peptide in footpad the next day and received daily infusion of IVIg (open symbols) or PBS (solid symbols). They were killed 10 days later, and cells of the draining LN were analyzed by flow cytometry. (A) Cells were gated on $CD4^+CFSE^+$ population, and the mean percentages of (B) $Foxp3^+$ and (C) $Foxp3^-$ cells were compared ($^*P \leq .05$). (D) IVIg induces in vitro proliferation of Treg. 5×10^4 cells/well of either Treg or Tconv were stimulated with 1 μg/mL of coated anti-CD3 Ab and 10 ng/mL of IL-2 and in the presence (open bars) or absence (solid bars) of IVIg. Proliferation was assessed by [^{3}H] thymidine incorporation ($^{**}P < .001$). Data are representative of results from 3 experiments. Error bars represent SD.

proliferation of Tconv, a significant increase ($P < .001$) was observed in Treg cultures (Figure 6D).

We further investigated whether IVIg bind directly to Treg. As shown in Figure 7, IVIg directly interacted with $CD4^+$ and $CD4^-$ cells, as revealed by flow cytometry. However, within the $CD4^+$ cells, IVIg binding to Treg was higher (mean fluorescence intensity [MFI] = 272) than that to Tconv (MFI = 132). To get an insight into the molecular interaction of the IgG molecule with cellular targets, we studied the protective effect of equimolar infusions of $F(ab)'_2$ and Fc preparations of IVIg. We did not observe a difference in protection against EAE and the Treg induction compared with intact IgG preparation (data not shown).

Discussion

Initially used in primary and secondary immune deficiencies, IVIg is increasingly being used for the treatment of autoimmune and systemic inflammatory diseases. Although considerable progress has been made in understanding the mechanisms by which IVIg exerts immunomodulatory functions in autoimmune diseases, these remain not fully elucidated. In the current study, we demonstrate a novel mechanism of action of IVIg in controlling the disease progression of EAE through upmodulation of Treg. We show that IVIg, when given prophylactically, prevents T-cell infiltration in the CNS through regulation of autoreactive T cells in the periphery, thus preventing the onset of irreversible neurologic damage. We believe that our results are extremely relevant for shaping future clinical strategies for the treatment of autoimmune diseases.

The etiology of autoimmune disease is often associated with dysregulation of the Treg compartment.[22] Indeed, regulatory T cells in patients suffering from autoimmune disease are either functionally defective or reduced in numbers in periphery.[23] Investigations to develop immune-mediated therapies have pursued 2 main lines: targeting autoimmune effector cells and reestablishing tolerance to autoantigens. In the EAE model, we show that IVIg belongs to the second category by modulating the population of Treg. By extrapolation, we can speculate that the beneficial effect of IVIg in treating relapsing-remitting MS (RRMS) may be related to the reestablishment of the Treg compartment, which is reported to be defective in this pathology.[24] Furthermore, similar mechanisms of action of IVIg may be operational in pregnancy- and postpartum-related relapses,[25] which are also believed to be associated with a variation in pregnancy-related Treg.[26] In a recent study on the effects of IVIg in experimental autoimmune myasthenia gravis (EAMG), Treg expansion was not observed.[27] Because IVIg was given during chronic disease and not prophylactically, as in our EAE model, we believe that the kinetics of disease progression and regulation would take a different course.

Spontaneous recovery in MS is rare when neurologic deficits have persisted for longer than 6 months, and there are no established therapies that promote neural regeneration and reverse neurologic deficits. Therefore, disease-modifying therapies at an early stage are crucial before neurologic deficits have set in. In our study, we observed that after prophylactic IVIg treatment, the proliferation and function of myelin-specific T cells were suppressed in the peripheral lymphoid organs. There was a decrease in the secretion of pro-inflammatory cytokine IFN-γ. Thus, the IVIg-expanded Treg prevented the proliferation and trafficking of pathogenic T cells toward the CNS, resulting in an absence of encephalomyelitis. This is an important observation that further validates IVIg testing in suitable subsets of early onset MS.[28] Indeed, the cumulative probability of developing clinically definite MS was significantly lower in IVIg-treated persons presenting with first neurologic event in a randomized, double-blind, placebo-controlled trial.[29]

Previous studies have shown that IVIg can modulate diverse lymphoid and myeloid cell populations.[30,31] Thus, at therapeutic concentrations used in autoimmune diseases (0.15 mM), IVIg induces a downmodulation of monocyte-derived DC, which in turn fail to support T-cell proliferation.[30] We asked whether the mechanism whereby IVIg leads to an expansion of Treg is central to the protection against EAE or if it is a mere bystander effect. Our results show that IVIg failed to protect mice that were depleted of Treg before EAE induction, thereby establishing unequivocally that the presence of natural Treg is crucial to the IVIg-mediated effect.

To get an insight into the mechanisms that led to Treg expansion by IVIg, we used adoptive transfer of HA-TCR transgenic T cells in wild-type (WT) recipients. The results clearly rule out a de novo generation of Treg. This conclusion is also supported by the observation that IVIg did not enhance TGF-β secretion that could have differentiated $CD4^+$ T cells into Treg in WT-EAE experiments. Therefore, the proliferation of nTreg is the most probable explanation for the peripheral expansion of Treg in EAE. This

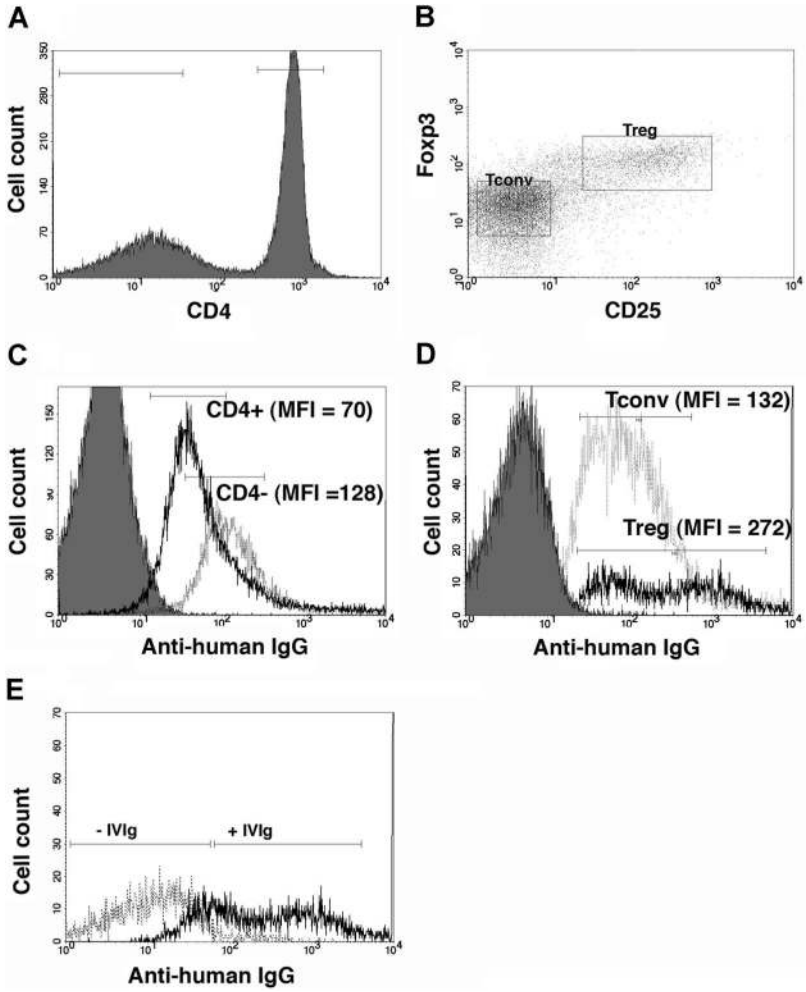

Figure 7. Interaction of IVIg with Treg and Tconv cells. Single-cell suspension was prepared from the spleen and the LN of C57BL/6J mice and incubated for 45 minutes at 4°C with 10 mg/mL of IVIg or monoclonal M protein (IgG purified from the serum of Multiple Myeloma patients). IVIg binding was revealed using a PE-conjugated antihuman IgG. (A) Lymphocytes were first gated on $CD4^+$ T cells that were further gated on (B) $CD25^-$ $Foxp3^-$ as Tconv and $CD25^+Foxp3^+$ as Treg. (C) Binding of IVIg to the $CD4^-$ cells and $CD4^+$ cells was analyzed based on MFI. (D) Within $CD4^+$ cells, binding of IVIg to Treg and Tconv cells. At the same concentration, monoclonal M protein did not bind to the cells. (E) Treg incubated with or without IVIg.

interpretation is supported by in vitro results. Furthermore, IVIg interacted directly with the Treg and other cell types, including Tconv and $CD4^-$ splenocytes. This is not surprising because IVIg is a source of polyreactive natural antibodies that interact with surface molecules of different T-cell types, contributing to its diverse mechanism of action. Previous reports have shown that IVIg bind to DC[30] and to activated T-cell lines specific for myelin binding protein.[32] It would be interesting to identify the cell surface molecules on Treg that interact with IVIg.

As a first step to understand the molecular interaction of the IgG molecule with cellular targets, we studied the protective effects of $F(ab)'_2$ and Fc preparations of IVIg. We did not observe a difference in protection against EAE and the Treg induction compared with intact IgG preparation. These questions may be better answered by studying protective effects of $F(ab)'_2$ and Fc preparations of IVIg against EAE in Fc receptor knockout mice. Mice lacking expression of FcγRIIB ($Fc\gamma RIIB^{-/-}$) have been successfully used in understanding the mechanisms of action of IVIg in ameliorating idiopathic thrombocytopenic purpura.[10-12]

In conclusion, we have demonstrated that Treg are implicated in the IVIg-mediated protection against EAE. An increase in the population of $CD4^+CD25^+Foxp3^+$ Treg is associated with the protection against EAE. Our observations explain the beneficial effects of IVIg in diverse autoimmune pathologies because Treg are pivotal in controlling auto reactivity. From the therapeutic perspective, our observations open up possibilities for testing IVIg in suitable subsets of early-onset MS. It has been observed that an absence of circulating IgG in XLA patients renders them susceptible to autoimmune pathologies.[33] Similarly, B cell–deficient mice, when induced with EAE, fail to recover, unlike their WT counterparts.[34] These observations indicate a dysregulation of the Treg compartment in the absence of IgG. Thus, the effect of therapeutic IgG on Treg modulation could reflect an important physiologic phenomenon.

Acknowledgments

We thank Drs D. Pham-Dinh and B. Bellon for assistance with EAE model; J.P. Duang Van Huyen, A. Nicoletti, P. Salameh, and H. Bouharoun-Tayoun for constructive suggestions at various stages of the study; and S. Graff-Dubois, A. Varthaman, J. Khallou-Laschet, A.T. Gaston, B. Wootla, and O. Cena for technical assistance.

This work was supported by Inserm; CNRS; CSL Behring, Zurich, Switzerland, Lebanese National Council for Scientific Research (LNCSR); and Coopération pour l'évaluation et le développement de la Recherche (CEDRE) Beirut, Lebanon. A.E. received a fellowship from CEDRE.

Authorship

Contribution: A.E. performed laboratory experiments, analyzed data, and wrote the report. S.C. helped in data analysis, writing the report, and acquiring funding. C.M. performed the histology experiments. S.F., J.L.C., and B.L.S. furnished PC61 and TCR-HA transgenic mice and contributed to data analysis. G.C., S.D., S.E., J.B., and S.L.-D. performed laboratory experiments. L.M. contributed to writing the report. M.D.K. and S.V.K. contributed to conception of the study, acquisition of funding, and writing the report. N.M. designed and supervised the study, analyzed the data, and wrote the report.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Srini V. Kaveri, Inserm UMR S 872, Centre de Recherche des Cordeliers, Equipe 16 Immunopathology and therapeutic immunointervention, 15, Rue de l'Ecole de Médecine,75006 Paris, France; e-mail: srini.kaveri@crc.jussieu.fr; or Namita Misra, Inserm U764, 32, Rue des Carnets, 92140 Clamart, France; e-mail: namita.misra@u-psud.fr.

References

1. Misra N, Bayry J, Lacroix-Desmazes S, et al. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. J Immunol. 2004;172:4676-4680.
2. Shevach EM, DiPaolo RA, Andersson J, et al. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev. 2006; 212:60-73.
3. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol. 2007; 7:585-598.
4. Tang Q, Bluestone JA. Regulatory T-cell physiology and application to treat autoimmunity. Immunol Rev. 2006;212:217-237.
5. Shevach EM. From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity. 2006;25: 195-201.
6. Achiron A, Gabbay U, Gilad R, et al. Intravenous immunoglobulin treatment in multiple sclerosis. Effect on relapses. Neurology. 1998;50:398-402.
7. Gold R, Stangel M, Dalakas MC. Drug insight: the use of intravenous immunoglobulin in neurology-therapeutic considerations and practical issues. Nat Clin Pract Neurol. 2007;3:36-44.
8. Bayry J, Lacroix-Desmazes S, Kazatchkine MD, et al. Monoclonal antibody and intravenous immunoglobulin therapy for rheumatic diseases: rationale and mechanisms of action. Nat Clin Pract Rheumatol. 2007;3:262-272.
9. Shoenfeld Y, Katz U. IVIg therapy in autoimmunity and related disorders: our experience with a large cohort of patients. Autoimmunity. 2005;38: 123-137.
10. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science. 2006;313:670-673.
11. Samuelsson A, Towers T, Ravetch J. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. Science. 2001;291:484-486.
12. Siragam V, Crow AR, Brinc D, et al. Intravenous immunoglobulin ameliorates ITP via activating Fc gamma receptors on dendritic cells. Nat Med. 2006;12:688-692.
13. Humle Jorgensen S, Sorensen PS. Intravenous immunoglobulin treatment of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. J Neurol Sci. 2005;233:61-65.
14. Pashov A, Dubey C, Kaveri SV, et al. Normal immunoglobulin G protects against experimental allergic encephalomyelitis by inducing transferable T cell unresponsiveness to myelin basic protein. Eur J Immunol. 1998;28:1823-1831.
15. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol. 2001;19:275-290.
16. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol. 2006;60:12-21.
17. Liu Y, Teige I, Birnir B, et al. Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. Nat Med. 2006;12: 518-525.
18. McGeachy MJ, Stephens LA, Anderton SM. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol. 2005;175:3025-3032.
19. Achiron A, Margalit R, Hershkoviz R, et al. Intravenous immunoglobulin treatment of experimental T cell-mediated autoimmune disease. Upregulation of T cell proliferation and downregulation of tumor necrosis factor alpha secretion. J Clin Invest. 1994;93:600-605.
20. Ait-Oufella H, Salomon BL, Potteaux S, et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med. 2006;12:178-180.
21. Terrada C, Fisson S, De Kozak Y, et al. Regulatory T cells control uveoretinitis induced by pathogenic Th1 cells reacting to a specific retinal neoantigen. J Immunol. 2006;176:7171-7179.
22. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004;22:531-562.
23. Lan RY, Ansari AA, Lian ZX, et al. Regulatory T cells: development, function and role in autoimmunity. Autoimmun Rev. 2005;4:351-363.
24. Viglietta V, Baecher-Allan C, Weiner HL, et al. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med. 2004;199:971-979.
25. Achiron A, Kishner I, Dolev M, et al. Effect of intravenous immunoglobulin treatment on pregnancy and postpartum-related relapses in multiple sclerosis. J Neurol. 2004;251:1133-1137.
26. Sánchez-Ramón S, Navarro AJ, Aristimuno C, et al. Pregnancy-induced expansion of regulatory T-lymphocytes may mediate protection to multiple sclerosis activity. Immunol Lett. 2005;96:195-201.
27. Zhu KY, Feferman T, Maiti PK, et al. Intravenous immunoglobulin suppresses experimental myasthenia gravis: immunologic mechanisms. J Neuroimmunol. 2006;176:187-197.
28. Pittock SJ, McClelland RL, Achenbach SJ, et al. Clinical course, pathologic correlations, and outcome of biopsy proved inflammatory demyelinating disease. J Neurol Neurosurg Psychiatry. 2005;76:1693-1697.
29. Achiron A, Kishner I, Sarova-Pinhas I, et al. Intravenous immunoglobulin treatment following the first demyelinating event suggestive of multiple sclerosis: a randomized, double-blind, placebo-controlled trial. Arch Neurol. 2004;61:1515-1520.
30. Bayry J, Lacroix-Desmazes S, Carbonneil C, et al. Inhibition of maturation and function of dendritic cells by intravenous immunoglobulin. Blood. 2003;101:758-765.
31. Bayry J, Lacroix-Desmazes S, Donkova-Petrini V, et al. Natural antibodies sustain differentiation and maturation of human dendritic cells. Proc Natl Acad Sci U S A 2004;101:14210-14215.
32. Achiron A, Mor F, Margalit R, et al. Suppression of experimental autoimmune encephalomyelitis by intravenously administered polyclonal immunoglobulins. J Autoimmun. 2000;15:323-330.
33. Etzioni A. Immune deficiency and autoimmunity. Autoimmun Rev. 2003;2:364-369.
34. Dittel BN, Urbania TH, Janeway CA Jr. Relapsing and remitting experimental autoimmune encephalomyelitis in B cell deficient mice. J Autoimmun. 2000;14:311-318.

2008 111: 715-722
doi:10.1182/blood-2007-03-079947 originally published online October 11, 2007

Expansion of CD4$^+$CD25$^+$ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis

Amal Ephrem, Souleima Chamat, Catherine Miquel, Sylvain Fisson, Luc Mouthon, Giuseppina Caligiuri, Sandrine Delignat, Sriramulu Elluru, Jagadeesh Bayry, Sebastien Lacroix-Desmazes, José L. Cohen, Benoît L. Salomon, Michel D. Kazatchkine, Srini V. Kaveri and Namita Misra

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.