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Glatiramer Acetate Treatment Directly Targets CD11b$^+$Ly6G$^-$ Monocytes and Enhances the Suppression of Autoreactive T cells in Experimental Autoimmune Encephalomyelitis

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Abstract

Glatiramer acetate (GA) is used for the treatment of relapsing-remitting multiple sclerosis (MS) and can suppress experimental autoimmune encephalomyelitis in animals. Effective GA treatment is associated with the induction of anti-inflammatory T_H2 responses and antigen-specific expansion of CD25$^+$/Foxp3$^+$ Tregs through the modulation of antigen-presenting cells. Here, we show that intravenous injection of fluorochrome-labelled GA resulted in rapid and specific binding of GA to CD11b$^+$ F4/80^{lo} Ly6G$^-$ blood monocytes via an MHC class II–independent mechanism. Intravenous GA treatment enhanced the intrinsic capability of these monocytes to directly suppress T cell proliferation *in vitro*. The suppressive function correlated with reduced proliferation of myelin-specific T cells *in vivo* after intravenous GA treatment. In contrast, subcutaneous treatment with GA inhibited the pro-inflammatory IFNγ-producing T cell phenotype rather than suppressing T cell proliferation. These data indicate that (1) GA engages directly with circulating monocytes to induce type II monocyte suppressor function; and (2) the therapeutic efficacy of GA may be expanded by employing different routes of GA administration to engage alternative mechanisms of suppression of autoreactive T cells in MS.

Introduction

Multiple sclerosis (MS) is an autoimmune disease where the central nervous system (CNS) is attacked by the host immune system [1]. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS that is induced by immunization with myelin oligodendrocyte glycoprotein peptides ($MOG_{35–55}$) or other myelin components [2]. The pathogenesis of both MS and EAE is initiated by myelin-specific CD4 T cells whereby both T_H1 and T_H17 cells contribute to pathogenic processes [3–5]. In this context, activated CD4 T cells infiltrate the tissue of the CNS and generate a local inflammatory environment resulting in the recruitment of the monocyte, macrophage and CD8 T cell populations that are responsible for the damage to CNS tissue [3, 6].

Glatiramer acetate (GA) is a randomly associated copolymer comprised of L-alanine, L-tyrosine, L-glutamic acid and L-lysine in a defined molar ratio [7]. Although previous studies have shown that GA relieves clinical symptoms in patients with MS and suppresses EAE in mice, the mechanism of action is not yet fully understood. It has been shown that T cell phenotype skewing from T_H1 to T_H2 [8, 9], decreased T_H17 inflammation [10] and antigen-specific expansion of Foxp3$^+$ T regulatory cells (Treg) [11] can contribute to disease suppression. In addition, increased lymphocyte apoptosis, enhanced neuronal repair and T cell receptor (TCR) antagonism to myelin components are also associated with GA treatment [12–14]. It is therefore likely that GA treatment does not depend on a single mechanism, but alters the dysregulated immune system in multiple ways to suppress autoimmunity.

It has been recently reported that blood monocytes from naïve mice exhibit the ability to suppress T cell function and that this suppressor function is lost upon induction of EAE [15]. These findings identify monocytes as a potential therapeutic target for controlling autoimmunity. *In vitro* studies have shown that GA can alter the activation state and cytokine pattern of a variety of different antigen-presenting cells (APCs) [16–19]. In fact, monocytes from GA-treated patients and mice produce elevated levels of anti-inflammatory factors [11, 20]. Furthermore, subcutaneous GA treatment has been shown to induce type II suppressor monocyte in a model of EAE [11]. Together, these data suggest that GA has the potential to directly target monocytes *in vivo* and may contribute towards protection from autoimmunity by re-establishing monocyte-dependent suppression of autoreactive T cells. However, whether GA acts directly on the monocyte population or through promiscuous modulation of multiple APC subsets to induce type II suppressor function *in vivo* is yet to be determined.

To expand our understanding of the suppressive mechanisms of GA and elucidate whether GA targets specific subsets of APC, we investigated the association between GA treatment and blood monocyte function. We found that following intravenous administration, GA directly and selectively targeted blood monocytes *in vivo* without the requirement for MHC class II. GA^+ monocytes exhibited enhanced suppression of T cell proliferation *in vitro*. Upon intravenous GA treatment, proliferation of myelin-specific T cells was also impaired *in vivo*. Interestingly, although subcutaneous GA treatment afforded protection from EAE, protection was associated with selective inhibition of IFN-γ production, rather than IL-17 or suppression of T cell proliferation. Our findings not only provide further examples of the mechanisms involved in GA-dependent suppression of autoimmune reactivity but also illustrate that the different routes of GA administration engage different immunosuppressive pathways.

Materials and methods

Mice. Breeding pairs of C57BL/6J ($CD45.2^+$) mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and the congenic $CD45.1^+$ mice (B6.SJLPtprca/Pepcb/BoyJ) were from the Animal Resource Centre (Canning Vale, WA, Australia). MHC class II–deficient $B6Aa^0/Aa^0$ mice were obtained from Dr H. Bluethmann (Hoffmann-La Roche, Basel, Switzerland). 2D2 mice ($CD45.2^+$) expressing transgenic TCRs specific for the $MOG_{35–55}$ peptide (MEVGWYRSPFS RVVHLYRNGK) presented by IAb were obtained from Harvard Medical School (Boston, MA, USA) and derived as described [21] All mice were maintained at the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. Experimental protocols were approved by the Victoria University of Wellington Animal Ethics Committee and performed according to their guidelines. Sex- and age-matched mice were used between 8 and 12 weeks of age for all experiments.

Immunizations and treatment. Experimental autoimmune encephalomyelitis was induced by subcutaneous immunization with 50-μg $MOG_{35–55}$ (synthesized by Mimotopes, Clayton, Vic., Australia) emulsified in complete Freund's adjuvant (CFA) containing 500 μg heat-killed *Mycobacterium tuberculosis,* followed by intraperitoneal injections of 250-ng pertussis toxin 1 day after immunizations. Mice were treated with GA simultaneously for EAE induction according to Gilgun-Sherki *et al.* [22], by immunization with a single emulsion containing both $MOG_{35–55}$ and 500 μg GA (Teva Pharmaceutical, Petach Tikva, Israel).

For intravenous treatment, mice were injected with 100 μg GA in 100 μl PBS into the tail vein. Blood monocytes were purified for flow cytometric analysis or tissue culture between 20 min and 3 h after GA injections.

Cell purification. Peripheral blood mononuclear cells (PBMCs) were prepared from whole mouse blood by density gradient centrifugation (Lympholyte®-M; Cedarlane, Burlington, ON, Canada). Monocytes were enriched with PBMCs by magnetic sorting using PE-conjugated anti-CD11b antibody and anti-PE magnetic beads (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were ≥80% $CD11b^{hi}$ $Ly6G^-$. $CD4^+$ cells were purified from whole splenocyte suspensions with the Dynabeads® FlowComp™ Mouse CD4 kit (Invitrogen, Carlsbad, CA, USA) and were ≥95% $CD4^+$.

Proliferation and suppression assays. For *in vitro* proliferation assays, draining lymph node cells were isolated from mice previously immunized with antigen. The lymph node cells were incubated with serial dilutions of antigen, and proliferation was measured by the incorporation of [^{3}H]-thymidine (GE Healthcare, Piscataway, NJ, USA). For *in vitro* suppression assays, splenocytes or lymphocytes were co-cultured with enriched monocytes in the presence of anti-CD3/anti-CD28-coated beads (Invitrogen) or $MOG_{35–55}$, respectively, and proliferation was measured as mentioned previously. For *in vivo* suppression assays, $MOG_{35–55}$-specific $CD4^+$ T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE), purified and adoptively transferred to $CD45.1^+$ congenic mice (2×10^6 cells per mouse). $MOG_{35–55}$ and GA were either intravenously injected together with the cells or subcutaneously administered in CFA. CFSE dilution of donor cells was analysed in various tissues of the recipients 2–5 days after cell transfer by flow cytometry.

Cytokine measurements. Culture supernatants were tested for secreted cytokines using the Bio-Plex™ cytokine assay (Bio-Rad, Auckland, New Zealand).

Monocyte depletion. Dichloromethylene diphosphonate (Cl_2MDP)-loaded liposomes were prepared as described earlier [23]. For depletion of blood monocytes, mice were intravenously injected with 200 μl of Cl_2MDP liposomes 18 h prior to EAE induction and GA treatment.

Fluorophore labelling of proteins. Proteins were resuspended in freshly made 0.1 M $NaHCO_3$ and incubated with 10 μg Alexa Fluor 488 (Invitrogen) or FITC (Sigma-Aldrich, St. Louis, MO, USA) per 50 μg of protein for 8 min. Then, 0.1 volume of 1 M Tris-Cl (pH 8.5) was added, and excess fluorophore was removed using Vivaspin 5 kDa MWCO polyethersulfonate columns (Sartorius, Göttingen, Germany).

Statistical analysis. Statistical significance on two data sets was tested using unpaired, two-tailed *t*-tests. For testing three or more data sets, ANOVA or repeated measures ANOVA was performed followed by Tukey's multiple comparisons test. Differences were considered significant at a value of $P < 0.05$.

Results

Glatiramer acetate binds to blood monocytes *in vivo*

To identify the *in vivo* target cell population of GA, GA was labelled with Alexa Fluor® 488 (Alexa488) and injected intravenously to C57BL/6J mice. Analysis of blood cells from injected mice showed that GA associated with a mononuclear $CD11b^{hi}$ cell population (Fig. 1A, left panels). This association was specific for GA, because Alexa488-OVA did not bind to these cells. Alexa488 staining on $CD11b^{hi}$ cells was also observed when GA-Alexa488 was injected into MHC class II–deficient mice (Fig. 1A, right panels), showing that MHC class II was not necessary for targeting of GA to these cells *in vivo*.

Further characterization of the cell surface markers on GA^+ cells from both wild-type and MHC class II–deficient mice identified them as $F4/80^{lo}/Ly6G^-$, consistent with a monocyte phenotype (Fig. 1B and data not shown). GA-$Alexa488^+$ monocytes were observed within 20 min of GA administration, and >95% monocytes were GA^+ after 3–6 h (Fig. 1C). Taken together, our findings showed that GA rapidly and specifically targets blood monocytes after intravenous administration.

Intravenous GA treatment enhances the ability of monocytes to suppress T cell proliferation

Previous work in our group has shown that naïve blood $CD11b^{hi}$ $F4/80^{lo}$ $Ly6G^-$ cells exhibit the capacity to suppress T cell proliferation *in vitro* [15]. In this study, co-culture with blood monocytes from naïve mice also suppressed T cells stimulated with anti-CD3/anti-CD28-coated beads, and this effect was enhanced in monocytes isolated from mice that had been treated with GA (Fig. 2A). GA-treated monocytes also exhibited enhanced suppression of antigen-specific proliferation of CD4 T cells (Fig. 2B).

To determine whether intravenous GA treatment could suppress T cell proliferation *in vivo*, CFSE-labelled, MOG-specific TCR transgenic CD4 T cells were adoptively transferred into $CD45.1^+$ congenic mice. T cells were transferred in the presence of either $MOG_{35–55}$ alone or $MOG_{35–55}$ and GA, and 2–4 days later, *in vivo* T cell proliferation was measured by flow cytometry. As shown in Fig. 2C, *in vivo* T cell proliferation was reduced in GA-treated mice in comparison with mice injected with $MOG_{35–55}$ alone. Taken together, these findings showed that intravenous GA treatment greatly delayed T cell proliferation *in vivo*, which is likely due to the enhanced capability of blood monocytes to suppress antigen-specific T cell proliferation.

Subcutaneous GA treatment suppresses IFNγ production but not T cell proliferation

Subcutaneous administration of GA is commonly used for MS treatment and has been shown to suppress EAE [7]. To address the question of whether suppression of pathogenic T cell proliferation by monocytes was also contributing to the efficacy of subcutaneous GA treatment, we adopted a co-immunization model of EAE treatment modified from Gilgun-Sherki *et al.* [22]. Mice were injected subcutaneously with a CFA emulsion containing combinations of the disease-causing $MOG_{35–55}$ peptide and GA.

To investigate antigen-specific T cell expansion, CFSE-labelled MOG-specific TCR transgenic cells were adoptively transferred into congenic mice, and the recipients immunized with $CFA^+MOG_{35–55}$ peptide with or without GA. As shown in Fig. 3A, $MOG_{35–55}$-specific T cell proliferation was not reduced in the draining lymph nodes or the spleens of co-immunized mice, compared to immunization with $MOG_{35–55}$ alone.

Next, we looked for an explanation for the lack of effect on T cell proliferation in this subcutaneous model of GA treatment. We observed that the percentage and absolute number of $CD11b^{hi}$ $Ly6G^-$ monocytes remained unchanged in draining lymph nodes and spleens of immunized mice (Fig. 3B), suggesting that migration of blood monocytes into lymphoid organs did not take place during the time studied. To confirm this, we used dichloromethylene diphosphonate (Cl_2MDP)-loaded liposomes to deplete monocytes prior to immunization [24]. Depletion of blood monocytes had no effect on EAE suppression following subcutaneous GA treatment (Fig. 3C), indicating that blood monocytes did not play a significant role in the suppression of T cell proliferation in the subcutaneous co-immunization model of GA treatment.

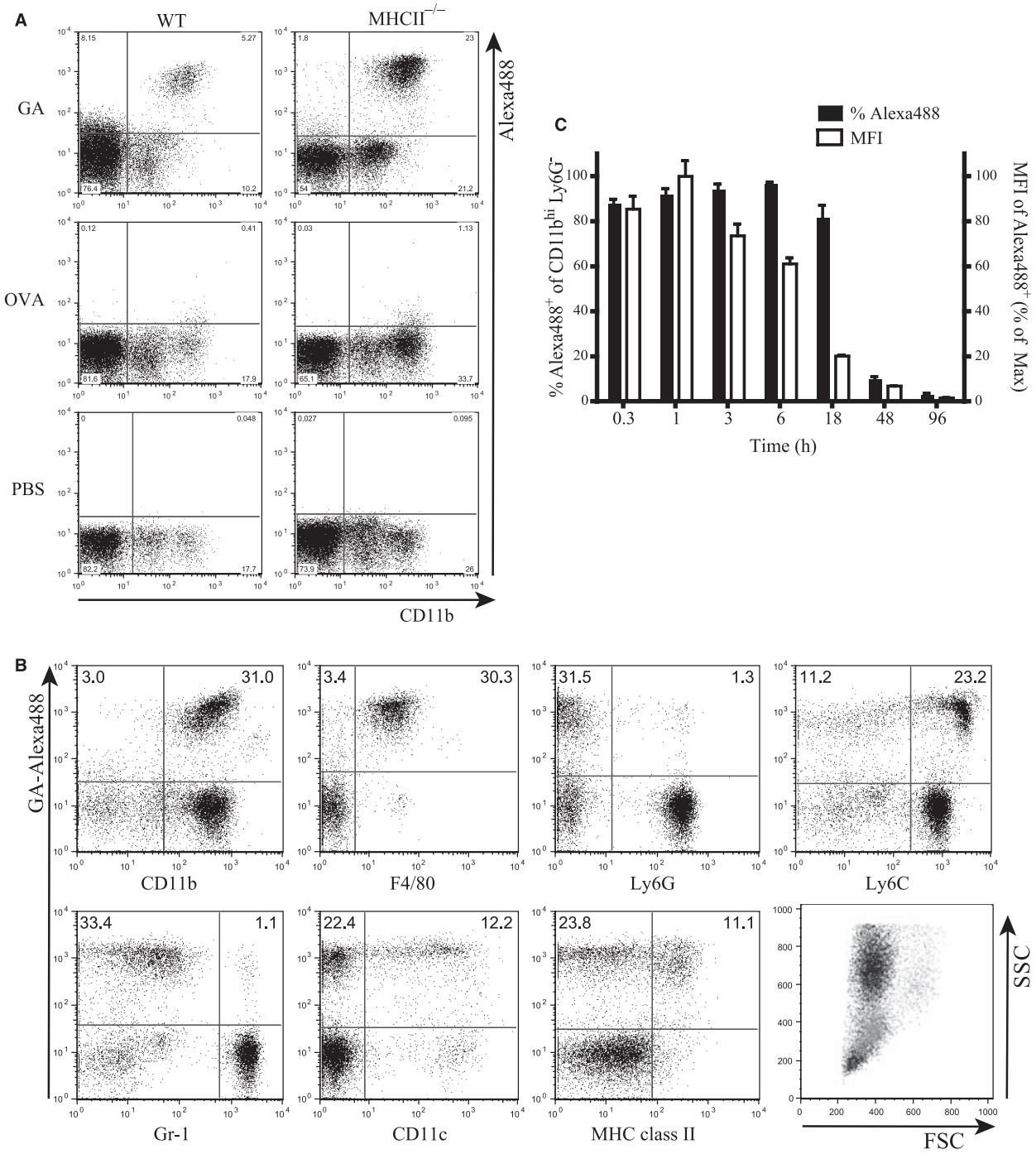

Figure 1 Glatiramer acetate (GA) binds to blood monocytes after intravenous injection. (A) C57BL/6J (WT) or B6Aa^0/Aa^0 (MHCII$^{-/-}$) mice were intravenously injected with 50 μg of Alexa488-labelled GA or OVA, or with PBS only. Blood was analysed by flow cytometry 1 h after the injections. Plots are gated on mononuclear cells. (B) Cell surface marker expression on GA$^+$ cells. Bottom right panel shows forward/side scatter profile of GA$^+$ cells (grey) in comparison with the whole white blood cell fraction (black). Blood cells were enriched for CD11b-expressing cells before analysis. (C) C57BL/6J mice received intravenous injections of GA-Alexa488, and white blood cells of individual mice were analysed over time (mean ± SEM of n = 5 mice). Plotted data show the percentage of CD11b^{hi} Ly6G$^-$ cells that show Alexa488 fluorescence and the geometric mean fluorescence intensity (MFI) of the Alexa488$^+$ population. Data represent one of at least two independent experiments.

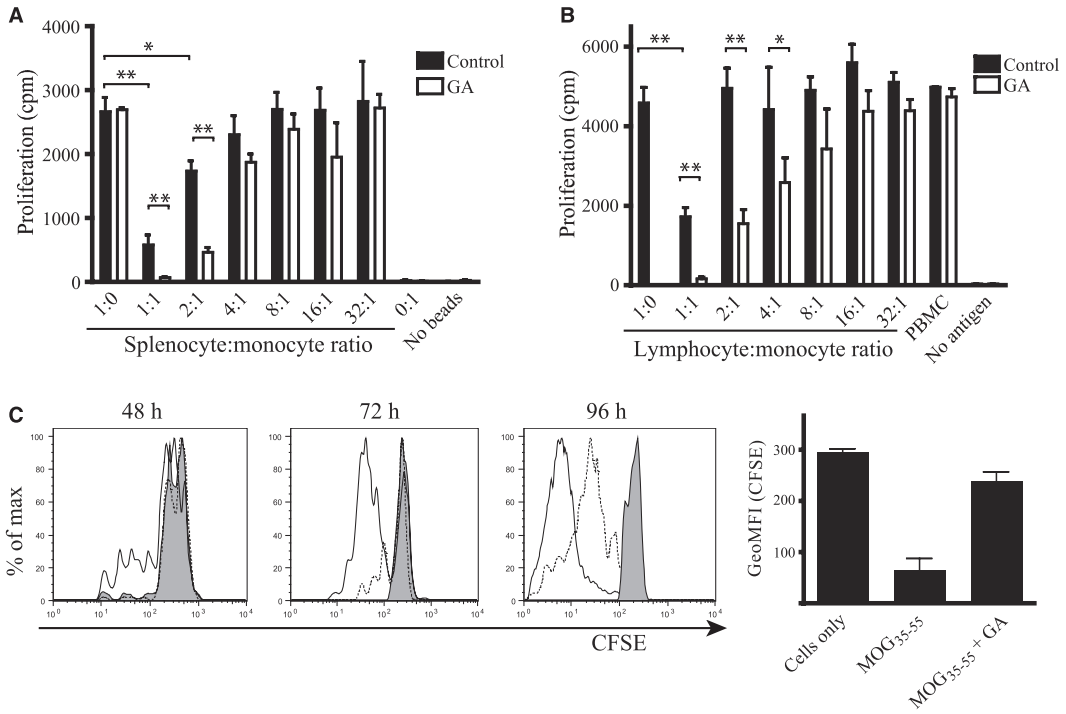

Figure 2 Intravenous Glatiramer acetate (GA) treatment enhances the intrinsic ability of monocytes to suppress T cell proliferation. (A) Monocytes were enriched from the blood of C57BL/6J mice that had been intravenously treated with 50 μg GA or from untreated controls (>80% CD11b^{hi} Ly6G^{-}). Serial dilutions of monocytes were co-cultured with 5×10^4 autologous splenocytes in the presence of anti-CD3/anti-CD28-coated beads. Proliferation was measured by the incorporation of [^{3}H]-thymidine. (B) Lymphocytes were isolated from myelin oligodendrocyte glycoprotein peptides (MOG_{35-55}) TCR transgenic mice and incubated in the presence of 10 μg/ml MOG_{35-55}. Monocytes were purified from GA-treated and control mice as in (A), and serial dilutions of monocytes were added to the lymphocyte culture 24 h later. *$P < 0.05$, **$P < 0.01$; two-tailed t-test (mean ± SEM of triplicate analysis). (C) CD4 T cells were purified from MOG_{35-55} TCR transgenic mice (>95% CD4$^+$) and adoptively transferred into CD45 congenic non-transgenic mice (2×10^6 cells per mouse). The cells were either injected alone (shaded histogram), together with 10 μg MOG_{35-55} (solid histograms), or co-injected with MOG_{35-55} and 100 μg GA (dotted histograms). Carboxyfluorescein succinimidyl ester (CFSE) dilution in the responder cells was analysed 2–4 days after cell transfer. Bar graph shows geometric mean intensity of CFSE fluorescence of $n = 3$ mice at the 72-h time point. Results shown represent one of at least two independent experiments.

Antigen-specific IFN-γ secretion is reduced after co-immunization with GA

Next, we looked for other possible mechanisms involved in protection from EAE after subcutaneous administration of GA. Consistent with unaffected T cell proliferation *in vivo* (Fig. 3A), the proliferative capacity of draining lymph node cells from mice co-immunized with MOG_{35-55} and GA was not reduced upon *ex vivo* re-stimulation with MOG_{35-55} (Fig. 4A). However, the draining lymph node cells exhibited an antigen-specific reduced capacity to secrete IFN-γ (Fig. 4B), suggesting that subcutaneous GA treatment protected the mice by reducing the generation of key pro-inflammatory T cells. Interestingly, the reduced secretion of pro-inflammatory cytokines was not universal, as IL-17 levels were unaffected in cells from GA-treated mice (Fig. 4B).

Expansion of Treg has been demonstrated in GA-treated mice [11, 25], and the efficacy of GA treatment partially depends on the presence of CD25$^+$ Foxp3$^+$ Tregs [26]. Consistent with this, neutralizing CD25/Foxp3$^+$ Treg [27, 28] using anti-CD25 mAbs (clone PC61.5) eliminated the majority, but importantly, not all of the suppressive effect of GA treatment (Fig. 4C). Nevertheless, the reduced capability of draining lymph node cells to secrete IFN-γ was independent of the presence of CD25$^+$/Foxp3$^+$ Tregs (Fig. 4D). Together, our findings confirmed that suppression of EAE in the co-immunization model of GA treatment partially depends on functional CD25$^+$/Foxp3$^+$ Tregs and, importantly, we have identified a Treg-independent inhibition of antigen-specific IFN-γ-secreting T_H1 cells as a new mechanism contributing to the suppression of EAE following subcutaneous GA treatment.

Discussion

Glatiramer acetate has been approved for the treatment of relapsing-remitting MS for over a decade, but its mechanism of action is not fully understood. It is thought to

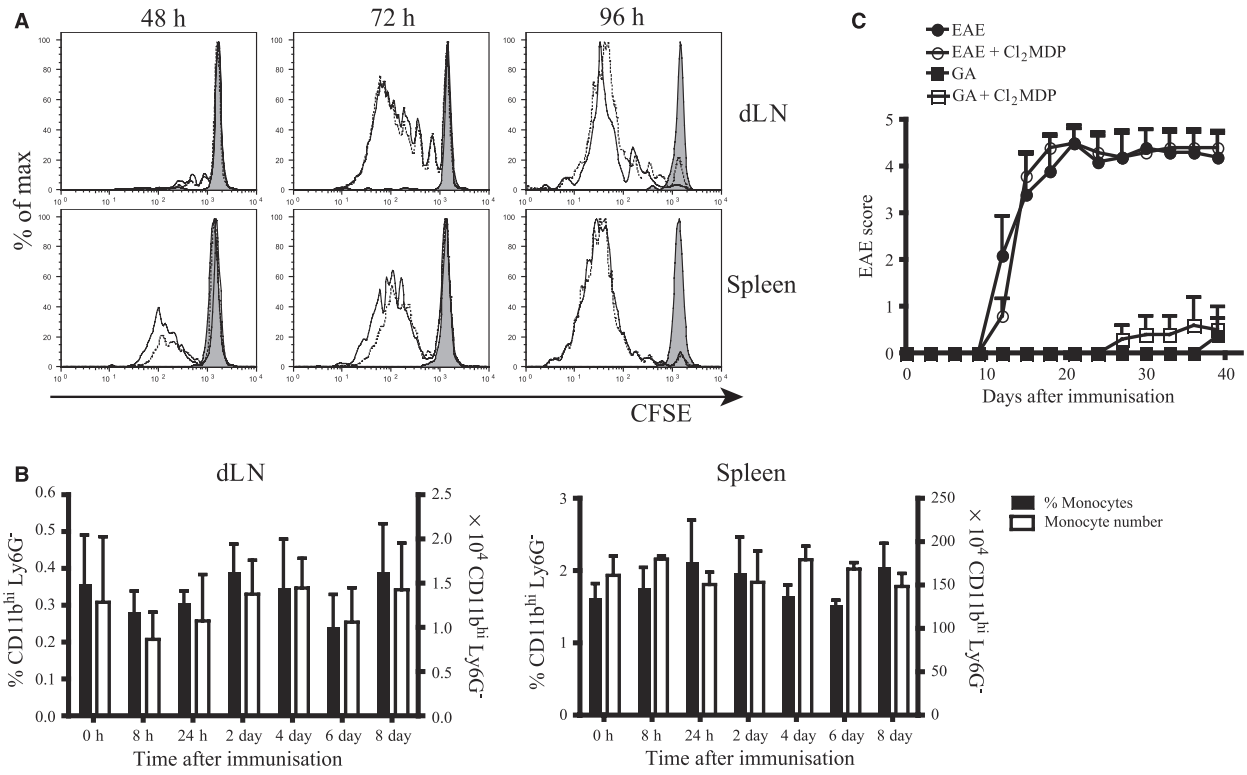

Figure 3 Myelin oligodendrocyte glycoprotein peptides ($MOG_{35–55}$)-specific T cell proliferation is not impaired in a Glatiramer acetate (GA) co-immunization model of Experimental autoimmune encephalomyelitis (EAE) treatment. (A) $MOG_{35–55}$-specific CD4 T cells (>95% pure) were adoptively transferred to $CD45.1^+$-congenic mice. One day after cell transfer, recipients were immunized with complete Freund's adjuvant (CFA) only (shaded histograms), $MOG_{35–55}$ in CFA (solid histograms), or $MOG_{35–55}$ and GA in CFA (dotted histograms). Draining lymph nodes (dLN) and spleens were removed 2–4 days after immunizations, and proliferation of donor cells was analysed by flow cytometry. Results shown in the histograms are gated on $CD45.2^+$ $CD4^+$ cells (n = 3 mice per group). (B) Percentage and number of $CD11b^{hi}$ $Ly6G^-$ monocytes in draining lymph nodes and spleens over time following immunizations (n = 3 mice per group). (C) Blood monocytes were temporarily depleted with Cl_2MDP-loaded liposomes prior to EAE induction or co-immunization treatment with GA (n = 5 mice per group). Data represent one of at least two independent experiments.

act by modulating APC function to induce anti-inflammatory/regulatory T cells [11, 17]. GA treatment can either downregulate the production of pro-inflammatory molecules (TNF-α, IL-1β, IL-12 or RANTES) and/or enhance the production of anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13 and sIL-1Ra) by a variety of APC, including DC, monocytes, macrophages and microglia *in vitro* [16–20, 29, 30]. Monocytes expressing an anti-inflammatory phenotype have been observed *in vivo* [11, 20]. Whether GA induces anti-inflammatory monocyte phenotypes directly or via modulation of other cell types has been unclear. Previous reports show that stimulation of anti-inflammatory/regulatory T cells by GA-modulated APC depends on MHC class II–restricted antigen presentation. However, MHC class II is not required to facilitate GA-dependent anti-inflammatory monocyte functions, suggesting that induction of anti-inflammatory monocyte function by GA does not require T cells [11].

Our data show that GA is able to further reduce proliferation of self-reactive T cells by directly enhancing T cell suppression by monocytes. Monocyte-like cells with the ability to suppress immune responses have been described in a variety of experimental models including tumours [31], allograft rejection [32], experimental autoimmune myocarditis [33] and EAE [34]. Furthermore, freshly isolated naïve blood monocytes [15] as well as monocytes generated in culture from naïve bone marrow [33] exhibit the ability to suppress *in vitro* T cell proliferation. Here, we show that GA directly modulates monocytes *in vivo* in an MHC class II–independent manner, resulting in enhanced T cell suppressive function. Importantly, this suppressive ability does not depend on the presence of antigen in the culture, thus expanding on the findings of Weber *et al.* [11] concerning the role of monocytes in counteracting autoimmunity during GA treatment.

Autoimmunity is associated with a break in tolerance resulting in the inappropriate expansion of self-reactive T cells. It has recently been shown that loss of constitutive monocyte-dependent suppression of autoreactive T cell activation may be a contributing factor in the development

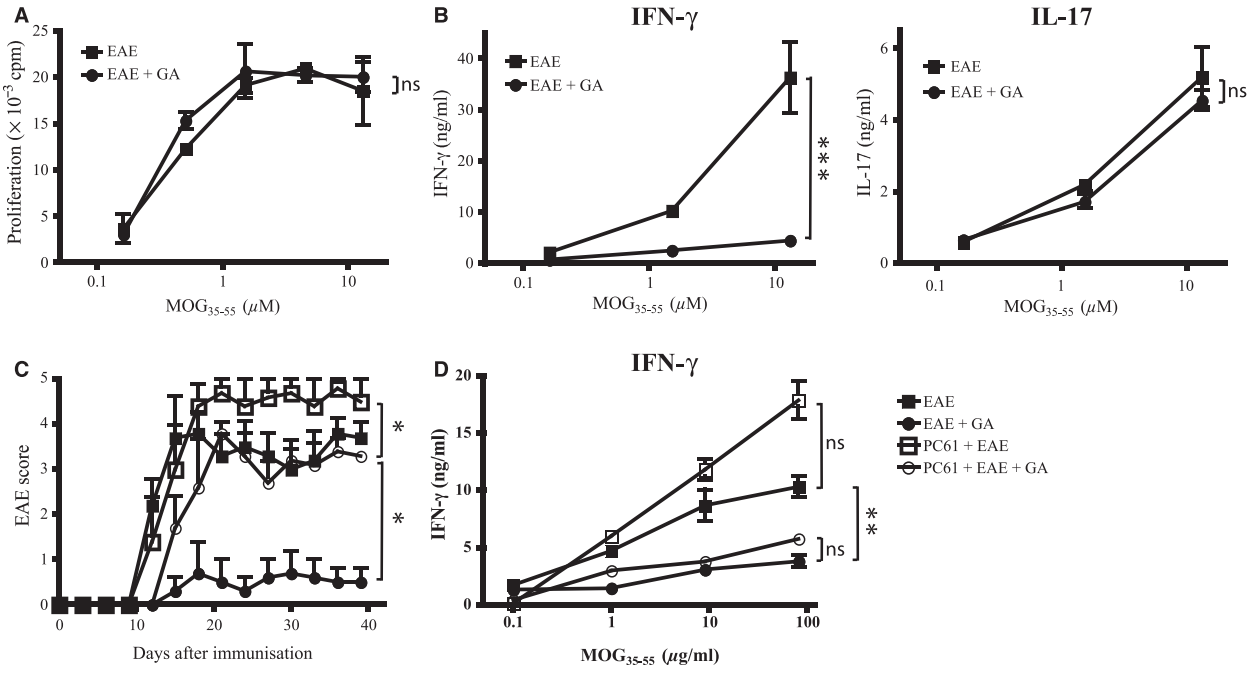

Figure 4 Experimental autoimmune encephalomyelitis (EAE) suppression by co-immunization with myelin oligodendrocyte glycoprotein (MOG) and Glatiramer acetate (GA) depends on Treg and downregulation of IFN-γ. (A) Draining lymph node cells from mice previously immunized with MOG_{35-55} alone or with MOG_{35-55} and GA were restimulated with MOG_{35-55} 7 days after immunization (mean ± SEM of triplicate analysis). (B) The supernatants of the lymphocyte cultures in (A) were analysed for the production of pro-inflammatory cytokines (mean ± SEM of duplicate analysis). (C) C57BL/6J mice received intraperitoneal injections of 200 μg of anti-CD25 antibody (PC61) 3 days before EAE induction and GA treatment. (D) Draining lymphnode cells were isolated. Restimulated with MOG35-55 *ex vivo* and IFN-γ levels in supernatants analysed by ELISA. (n = 5 mice per group). ns $P > 0.05$, *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$; two-tailed t-test. Data represent one of two independent experiments.

of EAE in mice [20]. Interestingly, a reduction in T cell proliferation has been suggested to be part of the mechanism by which GA ameliorates MS. In the light of current and earlier findings [11], it appears that GA treatment plays a key role in re-establishing type II suppressor function as well as the ability to directly suppress T cell proliferation by monocytes and thereby recover the tolerance to self-antigens.

Previous *in vitro* studies have provided evidence of direct binding of GA to MHC class II [35], although the functional relevance of this binding is controversial. Our data show that MHC class II is not required for either GA binding or enhanced suppressor function of blood monocytes *in vivo* following intravenous GA administration. The fast rate of binding of GA to the blood monocytes indicates that GA uptake is likely to be cell surface receptor mediated rather than via less specific mechanisms such as macropinocytosis. Although GA binding to $\alpha_M\beta_2$ integrin on human monocytes has been reported *in vitro* [36], in this study, we only observed binding of GA to blood monocytes *in vivo*. It is known that upon activation by cytokines, chemokines, complement and bacterial products, β_2-integrins undergo a conformational change from a closed to an open and active state [37]. Therefore, the absence of GA binding to blood monocytes *in vitro* may be due to activation-induced conformational changes of the $\alpha_M\beta_2$ integrin during monocyte purification. Further research into the mechanism of GA binding to monocytes *in vivo* is required and has the potential to reveal novel targets for the development of immunosuppressive therapies for the treatment of autoimmune disorders.

It is interesting to note that protection from EAE in the subcutaneous co-immunization model of GA treatment was not associated with reduced T cell proliferation or the presence of GA^+ monocytes in the blood or lymphoid tissue. GA is administered daily via the subcutaneous route to patients with MS. This treatment has systemic effects on the adaptive immune response and has been shown to cause sustained monocyte modulation [8, 20]. Hence, long-term GA treatment may affect blood monocytes in a sustained manner and promote monocyte-mediated suppression of pathogenic T cells in patients with MS. This effect was not observed in our study in mice immunized with strong pro-inflammatory adjuvants like CFA.

Instead, our data indicate that EAE suppression by GA treatment via the subcutaneous route involves both the inhibition of IFN-γ responses and the stimulation of Treg. Although Treg-dependent protection appears to be a characteristic feature of GA treatment in EAE, the results of this study and others [26] also suggest that GA

can differentially regulate IFN-γ and IL-17 responses. It is possible that these IFN-γ and IL-17 responses are controlled by different GA-modulated APC working in concert to induce T cell-mediated protection [11, 17, 19].

We propose that there are two different mechanisms by which GA can affect monocytes/APC leading to protection from EAE, depending on the route of GA administration. First, direct modulation of blood monocytes by GA through a receptor-mediated pathway increases the ability of the monocytes to suppress autoreactive T cell proliferation. Second, modulation of APC and a subsequent cytokine shift associated with reduced activation of Th1 cells and the induction of T_H2 and Treg [11, 17, 19]. Finally, this study highlights the potential for utilizing alternative routes for GA administration to engage additional immunosuppressive pathways and thereby enhance the therapeutic efficacy of GA in the treatment of MS.

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