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Type II monocytes modulate T cell–mediated central nervous system autoimmune disease

Martin S Weber[1], Thomas Prod'homme[1], Sawsan Youssef[2], Shannon E Dunn[2], Cynthia D Rundle[1], Linda Lee[1], Juan C Patarroyo[1], Olaf Stüve[3], Raymond A Sobel[4], Lawrence Steinman[2] & Scott S Zamvil[1]

Treatment with glatiramer acetate (GA, copolymer-1, Copaxone), a drug approved for multiple sclerosis (MS), in a mouse model promoted development of anti-inflammatory type II monocytes, characterized by increased secretion of interleukin (IL)-10 and transforming growth factor (TGF)-β, and decreased production of IL-12 and tumor necrosis factor (TNF). This anti-inflammatory cytokine shift was associated with reduced STAT-1 signaling. Type II monocytes directed differentiation of T_H2 cells and $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}) independent of antigen specificity. Type II monocyte–induced regulatory T cells specific for a foreign antigen ameliorated experimental autoimmune encephalomyelitis (EAE), indicating that neither GA specificity nor recognition of self-antigen was required for their therapeutic effect. Adoptive transfer of type II monocytes reversed EAE, suppressed T_H17 cell development and promoted both T_H2 differentiation and expansion of T_{reg} cells in recipient mice. This demonstration of adoptive immunotherapy by type II monocytes identifies a central role for these cells in T cell immune modulation of autoimmunity.

Glatiramer acetate (GA, Copolymer-1, Copaxone), a synthetic random basic copolymer composed of tyrosine, glutamic acid, alanine and lysine, is prescribed for treatment of relapsing-remitting multiple sclerosis (MS)[1]. Many investigations have addressed the immunological basis for the clinical effects of GA in MS and MS models[2,3]. Although different potential mechanisms have been considered, most have attributed activity of GA to perturbations in T cell–antigen reactivity, focusing on its influence on the adaptive immune response. *In vitro* studies established that GA can bind to major histocompatibility complex (MHC) class II molecules (MHC II) and suggested that GA might preferentially alter presentation of myelin antigens to autoreactive T cells[4,5]. Studies from humans with MS have shown that GA treatment promotes development of T_H2-polarized GA-reactive $CD4^+$ T cells[6–8]. Currently, it is assumed that these T_H2 cells traffic to the central nervous system (CNS), where they release anti-inflammatory cytokines[6,9–11] and neurotrophic factors[12] upon cross-recognition with myelin autoantigens, a process known as bystander suppression[10].

Recent reports indicate that GA treatment may also exert immunomodulatory activity on antigen-presenting cells (APCs)[13–17]. The aim of this investigation was to evaluate the influence of GA on APCs and its significance for GA-mediated T cell immune modulation and clinical protection in EAE. GA treatment in mice promoted the development of monocytes that secreted an anti-inflammatory 'type II' cytokine pattern. Type II monocytes directed differentiation of naive T (T_H0) cells into T_H2 and $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}) independent of antigen specificity. Cross-reactivity with myelin antigen was not required for these T cells to mediate clinical benefit, as adoptive transfer of type II monocyte–induced regulatory T cells specific for a non–self-antigen ameliorated established EAE. Adoptive transfer of type II monocytes into mice with EAE reversed paralysis, reduced CNS infiltration and induced T cell immune modulation. These results indicate that APCs are the primary target for GA-mediated immune modulation *in vivo*. Using adoptive transfer of APCs[18], as we show for GA-induced type II monocytes, provides a useful paradigm for studying how other therapeutics for autoimmune diseases might influence APC function and APC–T cell interaction *in vivo*.

RESULTS

GA treatment induces type II monocytes

In treatment of MS, GA is administered daily in aqueous solution by subcutaneous injection. In EAE, GA has been traditionally administered in incomplete Freund's adjuvant (IFA) once before disease induction[9,10]. To study the influence of GA on APC immune modulation in a manner that more closely reflects its use in MS therapy, and to eliminate potential immune deviation caused by the use of adjuvant[19], we subcutaneously injected GA dissolved in phosphate-buffered saline (PBS) daily. GA treatment initiated before EAE induction prevented development of relapsing-remitting EAE (**Fig. 1a**) or reversed EAE when treatment was started after onset of paralysis (**Fig. 1b**). GA treatment was associated with a T_H2 bias of myelin-reactive T cells (**Fig. 1c**) and expansion of T_{reg} (**Fig. 1d**).

[1]Department of Neurology and Program in Immunology, University of California, San Francisco, 513 Parnassus Avenue, S-268, San Francisco, California 94143-0435, USA. [2]Department of Neurology and Neurological Sciences, Interdepartmental Program in Immunology, Stanford University, Stanford, California, USA. [3]Neurology Section, Veterans Affairs North Texas Health Care System, Medical Service, Dallas, Texas, USA. [4]Department of Pathology, Stanford University, Stanford, California, USA. Correspondence should be addressed to S.S.Z. (zamvil@ucsf.neuroimmunol.org).

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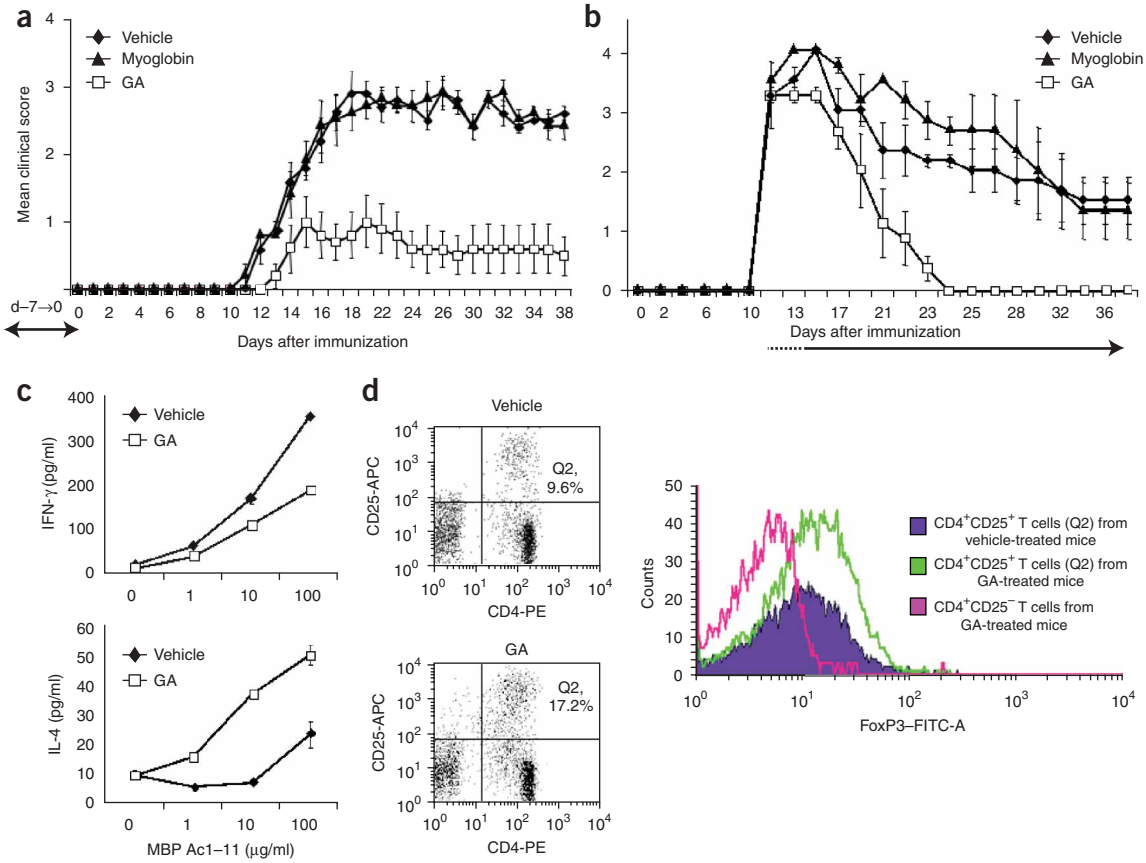

Figure 1 GA prevents and reverses established EAE, promoting a T_H2 bias and development of $CD4^+CD25^+FoxP3^+$ regulatory T cells. (**a**) Daily subcutaneous GA injections prevent EAE. $(PL/J \times SJL/J)F_1$ mice were injected with GA (150 μg) or myoglobin (150 μg) or vehicle (PBS) alone over 7 d before immunization with MBP Ac1-11. Solid arrow indicates treatment period. (**b**) Daily subcutaneous GA injections reverse EAE. Mice were randomized to treatment groups when they developed a clinical EAE score ≥2. Dotted line indicates randomization period and solid line indicates continuous treatment with GA, myo or vehicle alone. (**c**,**d**) GA treatment promotes a T_H2 bias and development of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}). (**c**) Twelve days after immunization, splenocytes from mice treated with either GA or PBS over 7 d before immunization were evaluated for secretion of IFN-γ and IL-4 upon restimulation with MBP Ac1-11. (**d**) Splenocytes from treated mice were evaluated for T_{reg} cells by FACS. The percentage of $CD4^+CD25^+$ T_{reg} cells (Q2) within the population of $CD4^+$ T cells is indicated. Intracellular FoxP3 expression by $CD4^+CD25^+$ T cells (Q2) is shown in the right panel.

We isolated $CD11b^+$ monocytes from GA-treated mice and evaluated them for secretion of proinflammatory and anti-inflammatory cytokines. In comparison with proinflammatory type I monocytes from control (vehicle)-treated mice, monocytes from GA-treated mice secreted less TNF and IL-12, but more IL-10 and TGF-β in the absence of stimulation or upon re-stimulation with interferon (IFN)-γ (**Fig. 2a**,**b**). Thus, the beneficial clinical effects of GA treatment were associated with development of anti-inflammatory type II monocytes. Monocytes from GA-treated mice consistently showed a modest decrease in expression of CD40 and CD80, costimulatory molecules involved in T_H1 responses[20], and a mild increase of CD86, a costimulatory molecule associated with T_H2 responses[21]. PD-L1, a negative costimulatory molecule involved in maintaining peripheral tolerance[22], was also upregulated. In contrast, MHC II expression was unaffected (**Fig. 2c**).

Similar to activation with IFN-γ, type II monocytes stimulated with lipopolysaccharide (LPS), another proinflammatory stimulus, also secreted an anti-inflammatory cytokine pattern. STAT-1 is a transcription factor that augments production of several proinflammatory cytokines, including TNF[23] and IL-12 (ref. 24), while repressing production of anti-inflammatory cytokines, such as IL-10 (ref. 25). As shown in **Figure 2d**, LPS-induced STAT-1 phosphorylation (activation) was reduced in type II monocytes. Thus, one important signaling pathway involved in proinflammatory innate immune responses was altered during type II monocyte differentiation.

Some data suggest that T_H2 cells induced by GA treatment facilitate type II differentiation of APCs[14]. Therefore, we examined whether T_H2 cells were required for development of type II monocytes. Monocytes from GA-treated STAT6-deficient mice, which cannot generate IL-4–producing T_H2 cells[26], secreted a type II cytokine pattern in a manner similar to monocytes from GA-treated wild-type mice (**Fig. 2a**). Monocytes isolated from GA-treated RAG-1–deficient mice, which lack mature B and T lymphocytes[27], secreted this anti-inflammatory cytokine pattern. Therefore, type II monocyte induction by GA treatment *in vivo* did not require T_H2 cells, or T cells in general. Further, GA-mediated type II monocyte differentiation did not require MHC II expression, as monocytes from GA-treated MHC II–deficient mice also secreted a type II cytokine profile (**Fig. 2b**). Thus, GA exerted immunomodulatory effects on APCs that did not require binding to MHC II, as would be expected if GA functioned solely as an altered peptide ligand.

Type II monocytes direct T cell immune modulation

As studies indicate that antigen-specific T cell lines generated from GA-treated humans show T_H2 polarization[6–8], we addressed whether type II monocytes could direct T cell deviation. Type II monocytes isolated from GA-treated mice were cocultured with untreated naive T (T_H0) cells from T cell receptor (TCR) transgenic mice with their respective antigen. When stimulated by type II monocytes, naive T cells specific for myelin basic protein (MBP) Ac1-11 preferentially differentiated into IL-4–secreting T_H2 cells, whereas naive T cells cocultured with monocytes from vehicle- or myoglobin (control)-treated mice predominantly differentiated into IFN-γ–secreting T_H1 cells (**Fig. 3a**). The T_H2 differentiation driven by type II monocytes was not selective for MBP-specific T cells or myelin-specific T cells in general, as it occurred similarly with naive T cells specific for myelin oligodendrocyte glycoprotein (MOG) p35–55 or ovalbumin (OVA), a non-myelin control antigen.

$CD4^+CD25^+$ T_{reg} cells are an important subclass of regulatory cells that can attenuate auto-reactive T cell responses[28,29]. Deficiencies in T_{reg} cells have been identified in several autoimmune conditions, including MS[30,31], and some investigations indicate GA treatment of MS can restore the number and function of T_{reg} cells[32,33]. As GA treatment of EAE similarly promoted development of $CD4^+CD25^+FoxP3^+$ T_{reg} cells (**Fig. 1d**), we tested whether type II APCs could be responsible for induction of T_{reg} cells. GA-treated monocytes, but not vehicle-treated monocytes, directed expansion of T_{reg} cells (**Fig. 3b**). Intracellular staining of responding T cells showed that these T_{reg} cells were distinct from IL-4–producing T_H2 cells (**Fig. 3c**). Thus, GA-induced type II APCs promoted differentiation of two non-overlapping T cell populations, T_H2 and T_{reg} cells.

GA treatment does not require T cell myelin recognition

Studies have suggested the beneficial effects of GA-induced regulatory T cells relates to their capability to cross-react with myelin antigen[6,9]. As we observed that type II monocytes induced T_H2 cells and T_{reg} cells independent of their T cell antigen specificity, we tested whether (1) myelin cross-reactivity of GA-reactive T cells or (2) GA specificity itself was required for the treatment effect of GA in EAE. GA-reactive T cells isolated from GA-treated mice, which were restricted by MHC II (**Fig. 4a**), proliferated and secreted IL-4 in response to GA, but did not cross-react with myelin antigens either by proliferation or cytokine

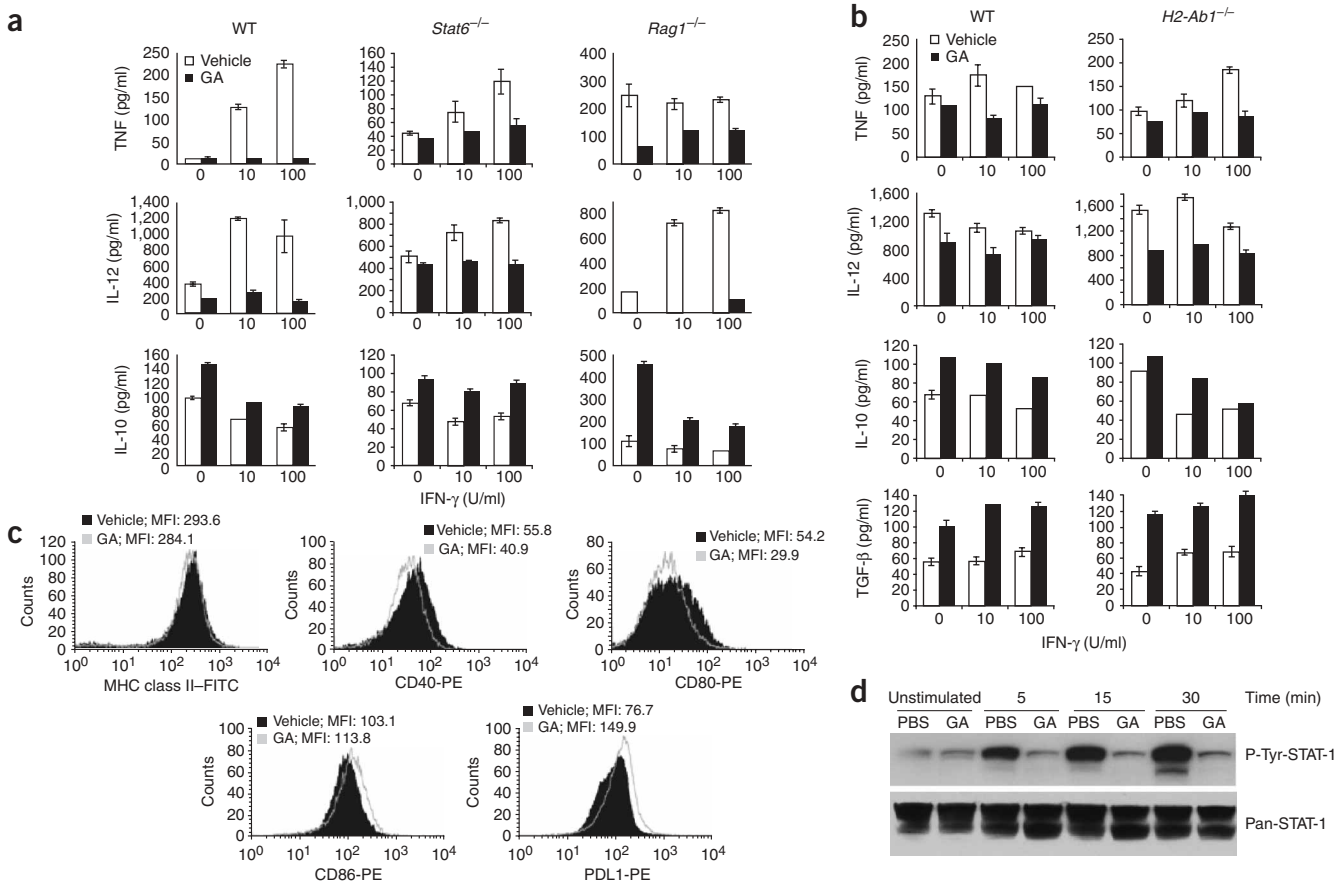

Figure 2 GA treatment induces type II monocytes. (**a**) Development of type II monocytes, which secreted less proinflammatory TNF and IL-12, and more anti-inflammatory IL-10 and TGF-β, occurred independent of T_H2 cytokines or T cells. $CD11b^+CD11c^-$ monocytes (purity, >98%) were isolated from C57BL/6 wild-type (WT), STAT6-deficient C57BL/6 (*Stat6*$^{-/-}$) or RAG-1–deficient C57BL/6 mice (*Rag1*$^{-/-}$) after *in vivo* GA (150 μg/d) or vehicle (PBS) treatment over 7 d. (**b**) Type II monocyte development did not require MHC II expression as monocytes from GA-treated MHC II–deficient mice (*H2-Ab1*$^{-/-}$) secreted the type II cytokine profile. (**c**) Expression of MHC II, CD40, CD80 (B7-1), CD86 (B7-2) and PD-L1 on monocytes was evaluated by FACS. Data are representative of three separate experiments. (**d**) Phosphorylation of STAT-1 is reduced in activated type II monocytes. Bone marrow–derived monocytes were cultured over 6 d in the presence or absence of GA. Monocytes were washed and stimulated with LPS (10 ng/ml) for the indicated duration. Proteins were separated by 4–20% SDS-PAGE electrophoresis and membranes were probed for phosphorylated (P-Tyr-) and total (pan-) STAT-1. Data are representative of three separate experiments.

production (**Fig. 4b**). To further investigate the possibility of T cell cross-reactivity between GA and myelin antigens, we immunized mice with GA, intact MBP, MBP Ac1-11 (data not shown), PLP 139–151 or MOG p35–55 and performed reciprocal recall assays to each of these antigens. T cells responded exclusively to the priming antigen (**Fig. 4c**). To test whether GA recognition by T cells was required for mediating the clinical benefit in GA treatment, type II monocyte–induced regulatory $CD4^+$ T cells specific for the foreign nonencephalitogenic control antigen OVA were adoptively transferred into mice with established EAE. Although these T cells did not cross-react with GA (**Supplementary Fig. 1a,b** online), they suppressed EAE in a manner similar to GA-reactive T cells[34] (**Fig. 3d**). Further, type II monocyte–induced regulatory T cells from TCR-α–deficient OT-II mice, which contain OVA 323–339–specific T cells that cannot rearrange endogenous TCR-α genes for recognition of other antigens, including GA or myelin, also reversed EAE (**Fig. 3d**). These findings question the relevance of both myelin cross-reactivity and GA specificity in immune modulation by GA-induced regulatory T cells.

GA-treated type II monocytes reverse established EAE

Our data indicate that the APC is the driver for GA-mediated immunomodulation. To investigate whether GA-treated APCs could mediate immune modulation *in vivo*, we adoptively transferred type II monocytes into recipient mice with established EAE. Before transfer, we confirmed that GA-treated donor monocytes secreted an anti-inflammatory type II cytokine pattern, even upon LPS stimulation (**Fig. 5a**). We further established that these cells secreted less IL-6 and IL-23, two proinflammatory cytokines that participate in

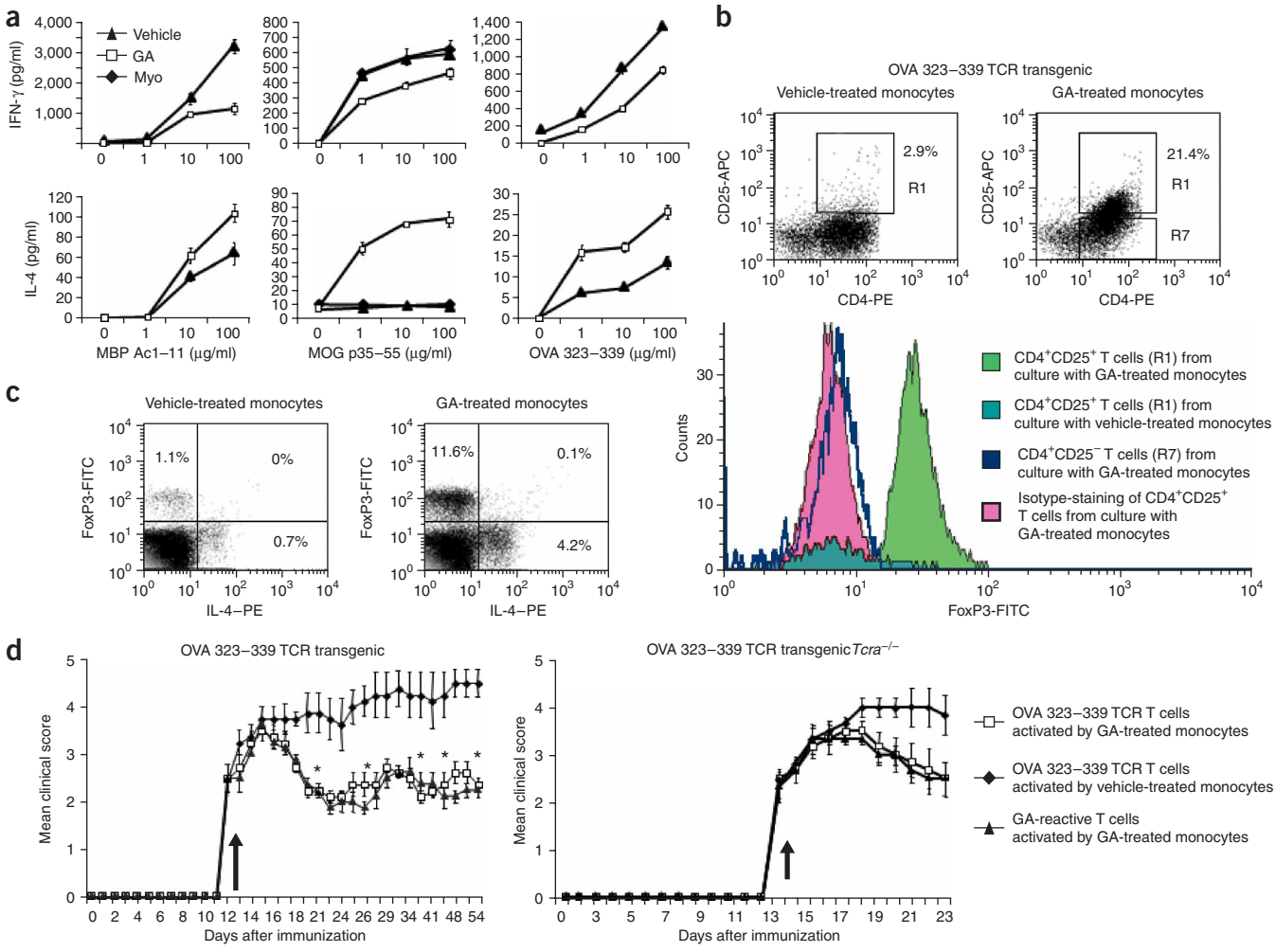

Figure 3 Type II monocytes direct naive T cells to differentiate into T_H2 cells and $CD4^+CD25^+FoxP3^+$ regulatory T cells independent of antigen specificity. (**a**) Monocytes from GA-treated mice induce T_H2 differentiation. Splenic $CD11b^+CD11c^-$ monocytes from mice treated with GA (150 μg/d), myoglobin (myo, 150 μg/d) or vehicle (PBS) over 7 d were cocultured with naive transgenic T_H0 cells specific for MBP Ac1-11, MOG p35–55 or OVA 323–339 and the respective antigen. T_H1-T_H2 differentiation was evaluated by secretion of IFN-γ or IL-4. (**b**) Monocytes from GA-treated mice induce $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg}). Percentage of $CD4^+CD25^+$ T_{reg} cells within all $CD4^+$ T cells is indicated. Intracellular FoxP3 expression by $CD4^+CD25^+$ T cells is shown in the right panel. (**c**) Type II monocyte–induced T_H2 and T_{reg} cells are two distinct populations. OVA 323–339–specific T cells rested for 12 d after stimulation were evaluated for intracellular FoxP3 and IL-4 expression (gated on $CD4^+$ T cells). (**d**) Regulatory T cells induced by GA-treated type II monocytes reverse EAE independent of antigen specificity. Monocytes were cocultured with untreated naive OVA 323–339–specific T cells from OT-II (left panel) or OT-II TCR-α–deficient mice (OT-II *Tcra*$^{-/-}$, right panel) and OVA 323–339. We transferred 5×10^6 T cells into C57BL/6 mice when they developed an EAE score ≥2 (arrow indicates timepoint). GA-specific T cells stimulated by GA-treated monocytes with GA were transferred as a positive control. Mean group scores are indicated ± s.e.m. $^*P < 0.05$.

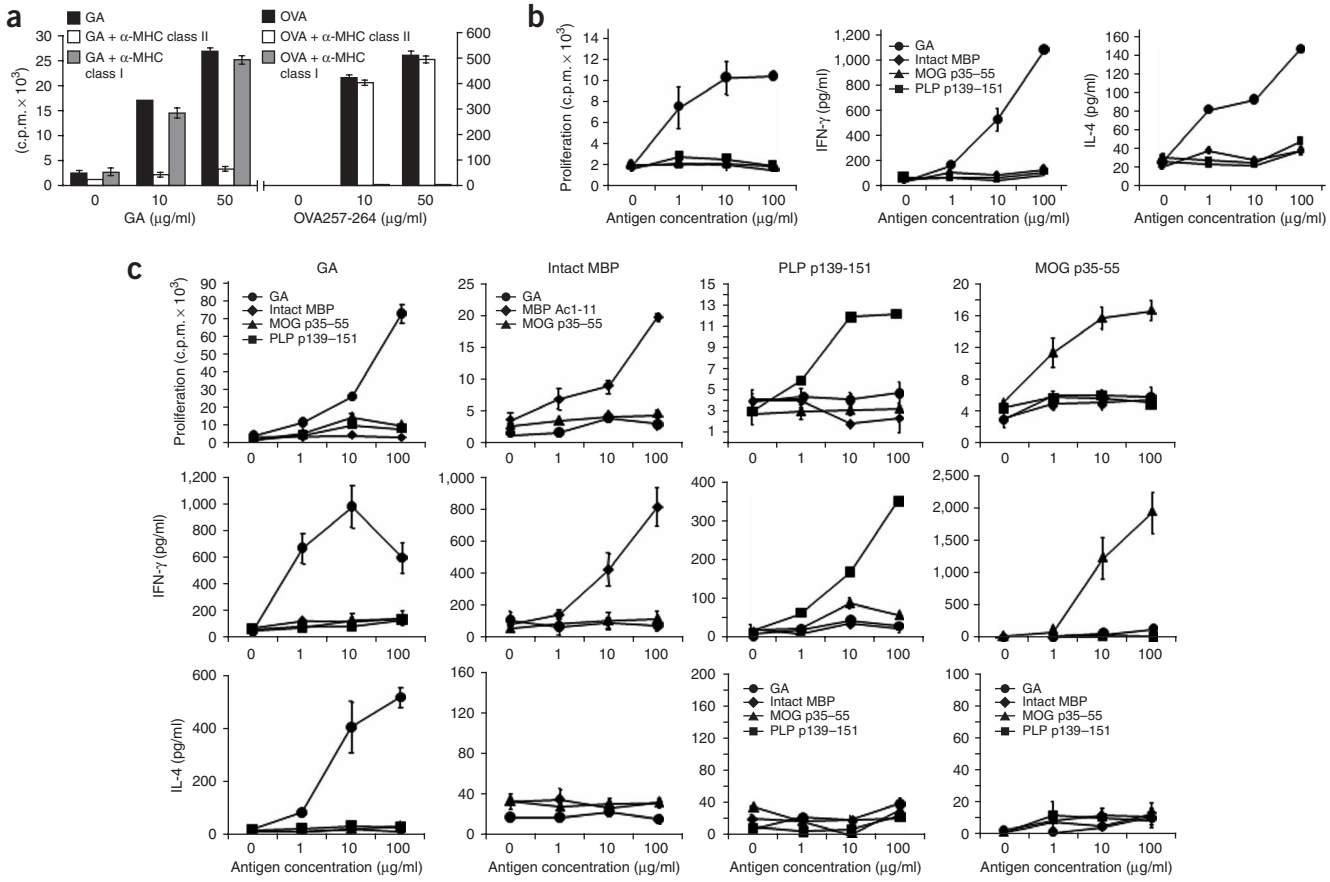

Figure 4 GA treatment induces GA-reactive T cells that do not cross-react with myelin antigens. (**a**) Proliferation of T cells isolated from GA-treated mice (150 μg/d over 7 d) was inhibited by addition of a monoclonal antibody to MHC II (M5/114), but not by addition of an antibody to MHC I (28-14-8) (left panel). As control, antibody to MHC I inhibited the proliferative response of splenic MHC class I–restricted OT-I transgenic T cells (right panel). (**b**) GA-reactive T cells from GA-treated mice did not cross-react with intact MBP, MOG p35–55 or PLP p139–151. (**c**) Splenic T cells from mice immunized with GA ((PL/J × SJL/J)F_1), intact MBP ((PL/J × SJL/J)F_1), PLP p139–151 (SJL/J) or MOG p35-55 (C57BL/6) reacted exclusively to the antigen used for immunization, both by proliferation and cytokine secretion.

differentiation of pathogenic T_H17 cells[35,36]. In adoptive transfer, GA-treated but not vehicle-treated monocytes reversed clinical EAE (**Fig. 5b**), which was associated with reduction in number of CNS parenchymal inflammatory lesions (**Fig. 5c,d**). This clinical and histologic benefit was not attributed to carryover of GA bound to MHC II on donor monocytes. First, donor GA-treated monocytes did not activate $CD4^+$ GA-reactive T cells (**Supplementary Fig. 2** online). Second, we did not detect a GA-reactive T cell response in recipients (data not shown). The possibility that donor monocytes were contaminated with T cells, which could have contributed to the clinical benefit, was also excluded; we did not detect $CD3^+$ T cells among donor monocytes (data not shown) and, more definitively, type II monocytes derived from RAG-1–deficient mice, which lack mature T and B cells, suppressed EAE in a manner similar to wild-type type II monocytes (see below).

Using green fluorescent protein $(GFP)^+$ or $CD45.1^+$ donor monocytes, we examined their migration pattern in recipient mice with EAE. Both GA-treated and vehicle-treated donor monocytes were detected in lymphoid tissue on day 2 (**Supplementary Fig. 3a** online) and within CNS parenchymal inflammatory lesions at day 5 (**Fig. 5e**). Donor monocytes remained $CD11b^+$, $CD11c^-$ and $CD8^-$. A similar percentage of vehicle- or GA-treated $CD45.1^+$ monocytes were detected among CNS-infiltrating cells of CD45.2 recipient mice, suggesting there was no preferential CNS recruitment of either type I or type II monocytes (**Supplementary Fig. 3b**).

Whether donor monocytes directed T cell immune modulation *in vivo* was next investigated. Proliferation and secretion of T_H1 cytokines IFN-γ, IL-12 and TNF by myelin-reactive T cells were reduced in recipients of GA-treated type II monocytes (**Fig. 6a**). IL-17 secretion was also reduced. Conversely, secretion of T_H2 cytokines IL-4 and IL-10 was increased in comparison with that in recipients of control type I monocytes. Type II monocyte treatment was associated with an increased frequency of T_{reg} cells (**Fig. 6b**). These observations suggested that T cells might be the effector cells of adoptively transferred type II monocytes. Further, data indicate that generation of T_{reg} cells is MHC II dependent[37]. Therefore, we investigated whether MHC II expression on donor type II monocytes was required to ameliorate EAE in recipient mice by transferring GA-treated MHC II–deficient monocytes. In contrast to wild-type type II monocytes, donor MHC II–deficient type II monocytes did not reverse EAE (**Fig. 6c**) or promote development of T_H2 cells or T_{reg} cells in recipient mice (**Supplementary Fig. 4a,b** online). Thus, EAE attenuation by type II monocytes required induction of MHC II–restricted host regulatory T cells.

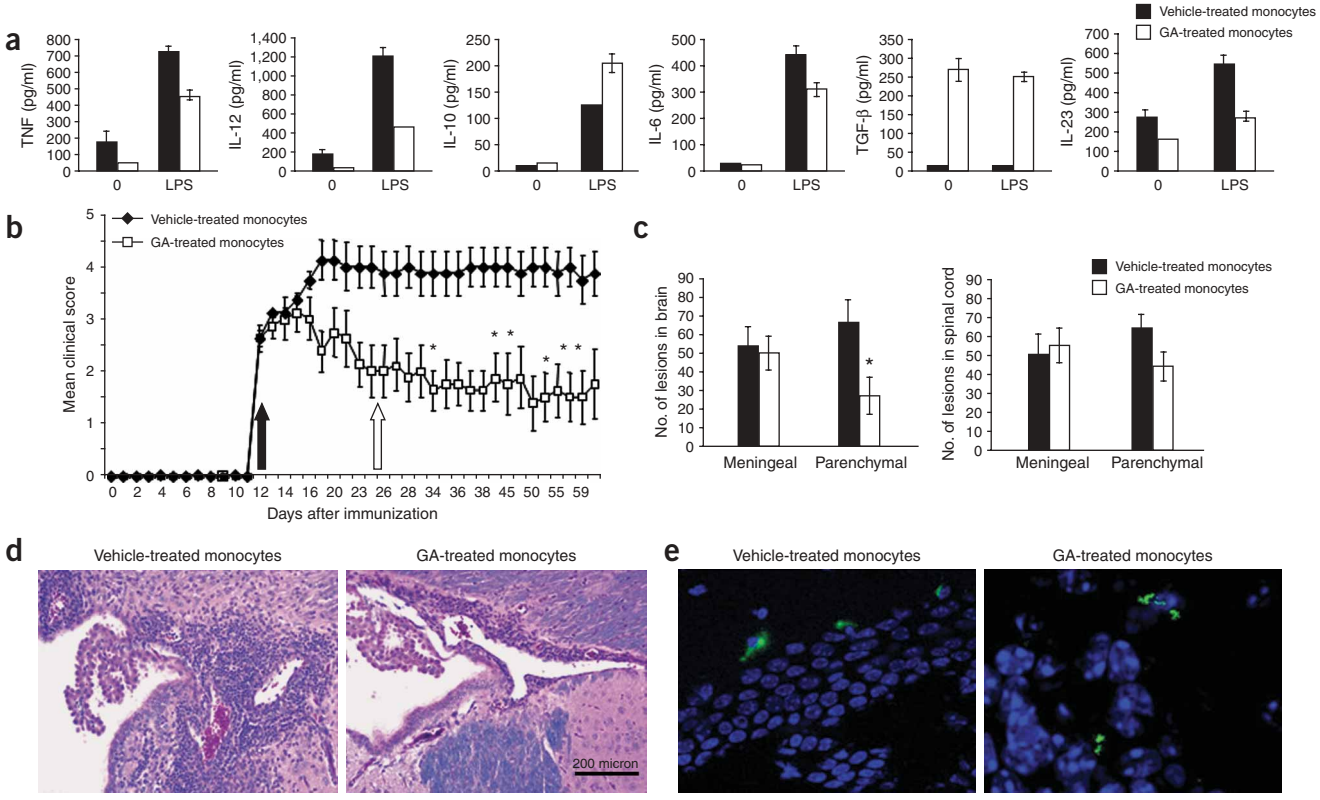

Figure 5 GA-induced type II monocytes reverse clinical and histologic EAE in recipient mice. Bone marrow–derived monocytes were cultured in the presence of GA (50 μg/ml) and IFN-γ (100 U/ml) for 6 d. FACS analysis for CD11b expression showed >99% purity. These $CD11b^+$ monocytes were $CD11c^-$, $CD8^-$ and $CD115^+$. (**a**) Before adoptive transfer, GA-treated type II monocytes were evaluated for secretion of TNF, IL-12, IL-10, IL-6, TGF-β and IL-23 without or with LPS stimulation (1 μg/ml). (**b**) We injected 5×10^6 unstimulated monocytes intravenously into recipient C57BL/6 mice immunized with MOG p35–55 after they developed a disease grade ≥2 (black arrow indicates timepoint of adoptive transfer, five mice per group). Mean group scores are indicated by ± s.e.m. *$P < 0.05$ was considered significant. Data are representative of four separate experiments. (**c**,**d**) Histologic analysis showed reduced parenchymal inflammatory CNS infiltration in recipients of GA-treated monocytes (white arrow indicates timepoint of analysis). (**c**) Lesion quantification showed reduced total number of parenchymal inflammatory foci in recipients of GA-treated monocytes. * $P < 0.05$. (**d**) A representative brain periventricular region from a recipient of GA-treated monocytes showed decreased parenchymal inflammation compared with the corresponding region in a recipient of vehicle-treated monocytes (original magnification, ×240). (**e**) Both GA-treated and vehicle-treated green fluorescent protein (GFP)-positive monocytes were visualized within inflammatory lesions (DAPI, blue) in the CNS.

DISCUSSION

Like several MS therapies, GA is only partially effective[1]. It is recognized that the magnitude of clinical benefit observed in EAE does not necessarily correlate to a drug's potency in MS, a more complex disease. EAE is useful for evaluating the mechanism of action of established MS treatments and for preclinical assessment of new therapeutic approaches considered for development in MS[38]. We have shown that GA-induced type II monocytes directed naive T cells to differentiate into both T_H2 and T_{reg} cells and, when transferred into mice with established EAE, reversed paralysis and induced T cell immune modulation. Cell-based strategies that promote antigen-specific T cell responses are being considered in cancer treatment[39]. Identifying type II monocytes as a vehicle for T cell immune modulation may be relevant to treatment of MS and other autoimmune diseases.

Our findings indicate that APCs may be the primary target for GA-mediated immune modulation. GA treatment promoted development of type II monocytes independent of T cells or T cell–derived cytokines. It is not clear whether the random basic copolymer GA or its byproducts mediates type II differentiation through binding a particular target molecule. In this regard, the type II anti-inflammatory cytokine secretion pattern similarly occurred in monocytes isolated from GA-treated MHC II–deficient mice, suggesting that it is not the binding of GA to MHC II that mediates type II monocyte development. Possibly, type II differentiation interferes with multiple proinflammatory signaling pathways in monocytes. STAT-1 is a transcription factor that participates in expression of several costimulatory molecules and proinflammatory cytokines. Some data suggest that phosphorylated STAT-1 is increased in monocytes in patients with active MS[40]. Our results show that STAT-1 phosphorylation in type II monocytes is reduced, identifying a novel property of GA that could contribute to its anti-inflammatory benefit.

Type II monocytes may induce immune regulation within the CNS. EAE reversal by type II monocytes was associated with reduced CNS parenchymal inflammation in recipient mice. Some studies have shown that *in vivo* depletion of macrophages selectively suppressed parenchymal CNS infiltration, which correlated with reduced EAE severity[41]. In our studies, type II monocytes were capable of migrating to the CNS and were primarily observed in parenchymal lesions, where they may have promoted local immunomodulation. Alternatively, although not mutually exclusive, type II monocytes could act through peripheral T cell immune modulation. In this regard, in addition to secreting a T_H2 polarizing cytokine pattern, donor type II monocytes produced TGF-β but not IL-6,

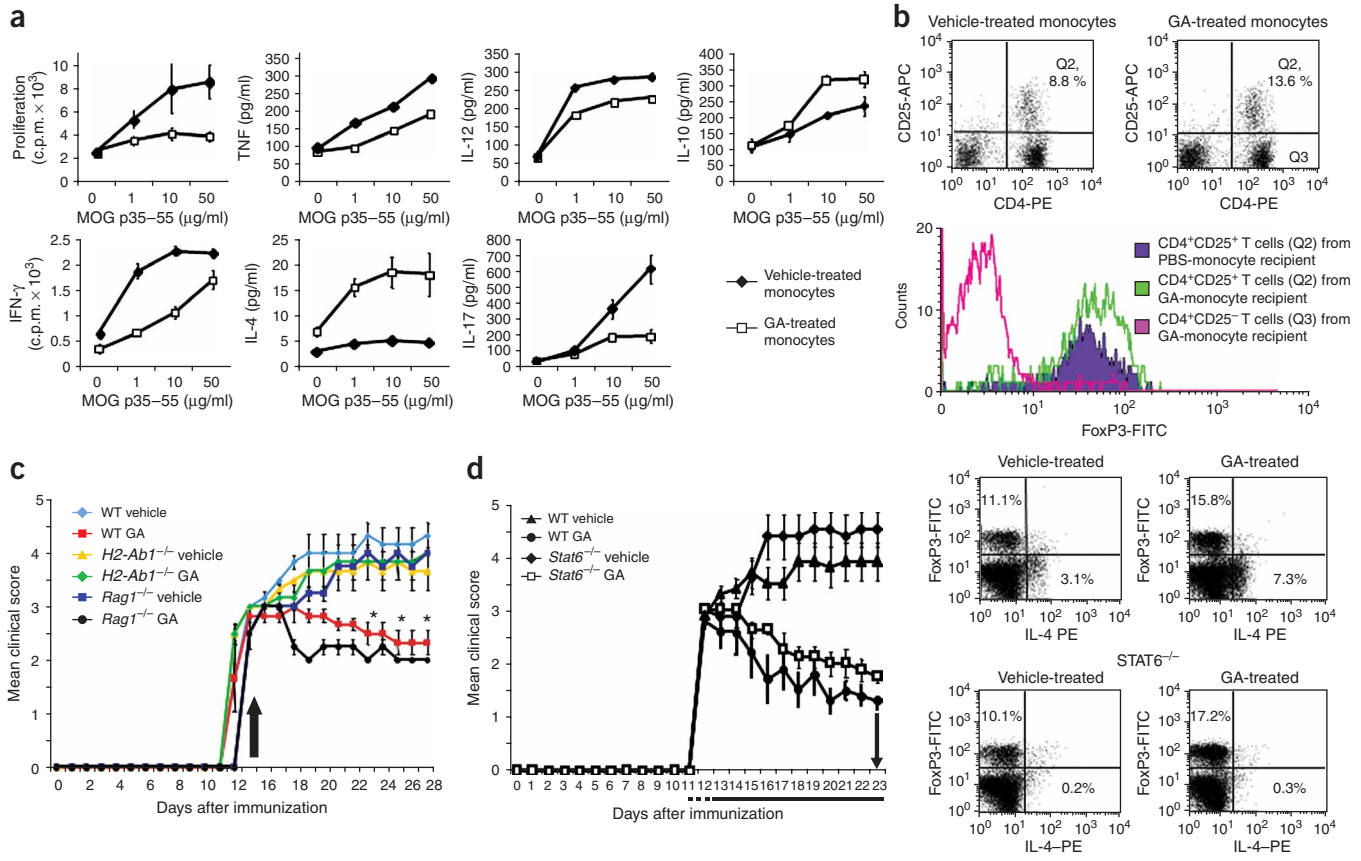

Figure 6 GA-induced type II monocytes direct T cell immune regulation *in vivo*. (**a**) GA-treated monocytes inhibited proliferation, induced a T_H2 bias and reduced development of IL-17–secreting T cells in recipient mice with established EAE. Splenocytes were isolated 13 d after adoptive transfer of GA- or vehicle (PBS)-treated monocytes (white arrow in **Fig. 5b**) and evaluated for cytokine secretion upon restimulation with MOG p35–55. (**b**) GA-treated monocytes induced expansion of $CD4^+CD25^+FoxP3^+$ T_{reg} cells in recipient mice. The percentage of $CD4^+CD25^+$ T_{reg} cells within the population of $CD4^+$ T cells is indicated. (**c**) GA-treated type II monocytes from RAG-1-deficient (*Rag1*$^{-/-}$), but not from MHC II–deficient (*H2-Ab1*$^{-/-}$), mice reversed established EAE. We injected 5×10^6 monocytes intravenously into recipient C57BL/6 mice immunized with MOG p35–55 after they developed a disease grade ≥ 2 (arrow indicates timepoint of adoptive transfer, four mice/group). Mean group scores are indicated by ± s.e.m. $^*P < 0.05$ was considered significant. (**d**) Daily GA treatment reversed EAE and induced $FoxP3^+$ T_{reg} cells in STAT6-deficient mice. EAE was induced by immunization with MOG p35–55. When mice developed a clinical EAE score ≥ 2, they were randomized to receive daily GA or vehicle (PBS). Dotted line indicates randomization period and solid line indicates continuous treatment with GA, or vehicle (PBS) alone (left panel). GA treatment of STAT6-deficient mice was associated with induction of $CD4^+CD25^+FoxP3^+$ T_{reg} cells, but not IL-4–producing T_H2 cells. The percentage of $FoxP3^+$ T_{reg} or IL-4–producing T_H2 cells among all $CD4^+$ T cells (pregated) is indicated.

facilitating development of T_{reg} cells and inhibiting T_H17 differentiation[35,36]. Activation of antigen-specific $CD4^+$ T cells requires MHC II expression on APCs. Notably, MHC II–deficient monocytes did not promote T cell immune deviation or ameliorate EAE. Thus, whereas induction of type II monocytes by GA did not require MHC II expression, the efferent arm of this immunomodulatory response, both induction of T_{reg} and T_H2 cells, as well as the associated clinical benefit, was MHC II dependent. Further, adoptive transfer of type II monocyte-induced regulatory T cells alone also reversed EAE. Together, these findings underscore the importance of MHC II expression on APCs for induction of T_H2 and T_{reg} cells *in vivo* and support the role of T cells as effector cells in GA-mediated immune modulation. Adoptive transfer of type II monocytes may provide a paradigm to characterize other gene products that act at the APC–T cell interface to promote development of regulatory T cells *in vivo*.

Whether T_H2 or T_{reg} cells, which are both induced during GA treatment of MS and EAE or by adoptive transfer of GA-induced type II monocytes, are equally relevant to GA's clinical benefit is not clear. Therefore, we tested GA treatment in STAT6-deficient mice, which do not generate IL-4–secreting T_H2 cells[26]. GA treatment reversed EAE and was associated with induction of T_{reg} cells, but not T_H2 cells (**Fig. 6d**). Like other results[42], these data emphasize a role for T_{reg} cells, but do not eliminate the possibility that both T_H2 and T_{reg} cells contribute substantially to the therapeutic effect of GA in EAE and MS.

Immune modulation by GA in MS treatment has been attributed to generation of GA-reactive T_H2 cells that cross-react with myelin antigen. Through reactivation within the CNS, they are thought to mediate bystander suppression of CNS inflammation. This concept was advanced by two observations. First, GA-reactive T_H2 cells could be identified in the CNS of mice protected from EAE[10]. Second, in some, but not all studies[43], a small number of GA-specific T_H2 cell lines generated from MS patients or mice could cross-react with MBP primarily at the level of cytokine secretion, but not by proliferation[6,9–11,34]. Our data indicate that T cell–myelin cross-reactivity is not required for GA-mediated immune modulation. It was only after six cycles (>10 weeks) of *in vitro* stimulation with GA that we detected secretion of small amounts of cytokines by

GA-reactive T-cell lines when stimulated with intact MBP (**Supplementary Fig. 5** online). However, these observations from long-term cultures of GA-specific T cell lines should not account for the clinical benefit that was evident soon after GA treatment onset. Notably, type II monocyte–induced regulatory T cells specific for a foreign control antigen also adoptively transferred EAE protection, consistent with some observations that T_{reg} cells can modulate immune responses in an antigen-independent manner[44]. Thus, whereas recognition of self myelin antigen is required for induction of CNS autoimmunity[45], regulation of EAE does not necessarily require T cell recognition of self-antigen. Our results indicate that the traditional paradigm that GA-reactive T cells can only mediate clinical benefit by cross-reacting with myelin antigen should be revisited. Although approved specifically for MS therapy, our finding that GA-induced type II monocytes promote T cell immune modulation independent of antigen specificity suggests that GA could be beneficial in other human inflammatory conditions. In this regard, GA has been effective in treatment of models of arthritis, uveoretinitis[46], inflammatory bowel disease[47] and graft rejection[48], findings which are unlikely to be attributed to T cell–antigen cross-reactivity within the target organ.

Our new insights derived from studying the mechanism of action of GA show a pivotal role of type II monocytes in immune modulation of CNS autoimmunity and highlight the importance of these cells as a target for pharmacologic intervention in autoimmune diseases. The model established in this report, adoptive transfer of type II monocytes, provides a valuable approach to study communication between APCs and T cells that leads to induction of regulatory T cells *in vivo*.

METHODS

Mice. We purchased (PL/J × SJL/J)F_1, B10.PL, C57BL/6, C57BL/6 RAG-1–deficient (B6.129S7), C57BL/6 transgenic (ACTB-eGFP)1Osb/J, C57BL/6 OVA 257–264–specific TCR transgenic (OT-1) and C57BL/6 MHC II–deficient female mice from the Jackson Laboratory. We obtained STAT6-deficient C57BL/6 mice from S.J. Khoury (Harvard). B10.PL MBP Ac1–11–specific TCR transgenic mice[49] and C57BL/6 MOG 35–55–specific TCR transgenic mice[50] were provided by V.K. Kuchroo (Harvard), C57BL/6 OVA 323–339–specific TCR transgenic (OT-II) mice from M.F. Krummel (University of California, San Francisco) and OT-II TCR-α-deficient mice from A. Weiss (University of California, San Francisco).

Peptides. MBP peptide Ac1–11 (Ac-ASQKRPSQRHG), proteolipid protein peptide 139–151 (HCLGKWLGHPDKF) and OVA 257–264 (SIINFEKL) were synthesized by Quality Control Biochemicals. MOG peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Auspep. We purchased guinea pig MBP from Sigma. OVA 323–339 (ISQAVHAAHAEINEAGR) was synthesized by Abgent.

EAE induction. We injected (PL/J X SJL/J)F_1 mice subcutaneously with MBP Ac1-11 in CFA. We induced EAE in C57BL/6 and C57BL/6 STAT6-deficient mice with MOG p35–55 in CFA. After immunization and 48 h later, mice received an intravenous injection of 300 ng pertussis toxin. We scored mice as follows: 0, no symptoms; 1, decreased tail tone; 2, mild monoparesis or paraparesis; 3, severe paraparesis; 4, paraplegia and/or quadraparesis; and 5, moribund or death. We conducted at least two independent experiments with ≥10 mice/group. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

GA treatment. Mice received daily subcutaneous injections of 150 μg GA (Teva Neuroscience, Inc.) or myoglobin suspended in PBS or PBS alone before EAE induction or after they developed a clinical score ≥2.

Monocyte isolation and coculture with naive T cells. We MACS-separated splenic monocytes from GA- or myoglobin-treated mice. We evaluated monocyte preparation for CD11b, CD11c, B220, CD3, CD115, MHC II, CD40, CD80, CD86 and PD-L1 (Pharmingen). Monocytes were >99% $CD11b^+$. We cocultured monocytes with T cells from different TCR transgenic mice and their respective antigen. We evaluated T_H1-T_H2 differentiation by cytokine production (ELISA or intracellular FACS). We evaluated $CD4^+CD25^+FoxP3^+$ T_{reg} cells by FACS staining (eBiosience).

GA-reactive T cell lines. We generated GA-reactive T cell lines by immunization with GA in IFA. We cultured draining lymph node cells with GA for 10 d. We used irradiated splenocytes as APCs for restimulations.

Proliferation assays. We evaluated proliferation 12 d after immunization. We cultured spleen cells with antigen for 72 h, pulsed cultures with [^{3}H]thymidine and harvested cells 16 h later. We calculated mean [^{3}H]thymidine incorporation for triplicate cultures.

Cytokine analysis. We performed cytokine analysis by ELISA at 48 h (IL-12), 72 h (TNF, IFN-γ, IL-17, IL-6, TGF-β) and 120 h (IL-4 and IL-10). Results shown are average of triplicates ± s.e.m.

Adoptive transfer of regulatory T cells. We transferred 5×10^6 OVA 323–339–specific T cells derived from coculture with GA-treated monocytes into recipient mice with EAE. We transferred OVA-specific T cells cocultured with vehicle-treated monocytes and GA-reactive T cells stimulated by GA-treated monocytes in parallel.

Adoptive transfer of monocytes. We isolated monocytes from bone marrow of 8–12-week-old mice. We flushed femurs with PBS and passed cells through a 40 μm strainer, resuspended them in medium containing M-CSF (10 ng/ml) and IFN-γ (100 U/ml), then cultured them for 6 d with GA (50 μg/ml) or PBS. We washed monocytes three times. Cells were >99% $CD11b^+$ and were $CD11c^-$. We immunized recipient mice with MOG p35–55, randomized them at EAE score ≥ 2 and injected them intravenously with 5×10^6 GA- or PBS-treated monocytes.

Western blot. We resuspended cell pellets from bone marrow–derived monocytes stimulated with LPS in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% aodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM Na3VO4, 1 mM NaF) containing protease inhibitors (Roche). We separated proteins by SDS-PAGE and electroblotted them on nitrocellulose membranes (Amersham BioSciences). We incubated membranes with antibody specific for phosphorylated STAT-1 and antibody to STAT-1 (Upstate Biotechnologies) and developed them.

Histopathology. We fixed brains and spinal cords of recipient mice of GA- or PBS-treated monocytes in formalin. We stained sections with Luxol fast blue-hematoxylin and eosin. We counted meningeal and parenchymal inflammatory lesions as described[17,20]. We perfused anesthetized recipients of GA- or PBS-treated $eGFP^+$ monocytes with 4% paraformaldehyde (PFA). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize accumulation of inflammatory cells within CNS lesions. We evaluated sections of brain, spinal cord, spleen and cervical lymph nodes by confocal microscopy.

FACS analysis of CNS-infiltrating cells. We incubated CNS tissue in Hank Buffered Saline Solution containing collagenase for 1 h. We isolated cells by Percoll gradient and washed them twice before FACS staining.

Statistical analysis. Data are mean ± s.e.m. We examined significance between groups using the Mann-Whitney *U* test. A value of $P < 0.05$ was considered significant. Other statistical analysis was performed using a one-way multiple-range analysis of variance test (ANOVA) for multiple comparisons. A value of $P < 0.01$ was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.S.W. conducted most of the experiments, prepared the figures and participated in writing the manuscript. T.P., S.Y. and S.E.D. performed the western blot analysis for STAT1 phosphorylation. C.D.R., L.L. and J.C.P. participated in the characterization of type II monocytes *in vitro* and *in vivo*. O.S. and L.S. participated in the experimental design and in editing the manuscript. R.A.S. conducted the histological analyses. S.S.Z. initiated and supervised the project and participated in writing the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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1. Johnson, K.P. *et al.* Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind, placebo-controlled trial. *Neurology* **45**, 1268–1276 (1995).
2. Neuhaus, O., Farina, C., Wekerle, H. & Hohlfeld, R. Mechanisms of action of glatiramer acetate in multiple sclerosis. *Neurology* **56**, 702–708 (2001).
3. Farina, C., Weber, M.S., Meinl, E., Wekerle, H. & Hohlfeld, R. Glatiramer acetate in multiple sclerosis: update on potential mechanisms of action. *Lancet Neurol.* **4**, 567–575 (2005).
4. Fridkis-Hareli, M. *et al.* Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells–specificity and promiscuity. *Proc. Natl. Acad. Sci. USA* **91**, 4872–4876 (1994).
5. Teitelbaum, D., Fridkis-Hareli, M., Arnon, R. & Sela, M. Copolymer 1 inhibits chronic relapsing experimental allergic encephalomyelitis induced by proteolipid protein (PLP) peptides in mice and interferes with PLP-specific T cell responses. *J. Neuroimmunol.* **64**, 209–217 (1996).
6. Neuhaus, O. *et al.* Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *Proc. Natl. Acad. Sci. USA* **97**, 7452–7457 (2000).
7. Dhib-Jalbut, S. Mechanisms of action of interferons and glatiramer acetate in multiple sclerosis. *Neurology* **58**, S3–S9 (2002).
8. Duda, P.W., Schmied, M.C., Cook, S.L., Krieger, J.I. & Hafler, D.A. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J. Clin. Invest.* **105**, 967–976 (2000).
9. Aharoni, R., Teitelbaum, D., Sela, M. & Arnon, R. Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA* **94**, 10821–10826 (1997).
10. Aharoni, R. *et al.* Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1. *Proc. Natl. Acad. Sci. USA* **97**, 11472–11477 (2000).
11. Chen, M. *et al.* Glatiramer acetate induces a Th2-biased response and crossreactivity with myelin basic protein in patients with MS. *Mult. Scler.* **7**, 209–219 (2001).
12. Ziemssen, T., Kumpfel, T., Klinkert, W.E., Neuhaus, O. & Hohlfeld, R. Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. Brain-derived neurotrophic factor. *Brain* **125**, 2381–2391 (2002).
13. Weber, M.S. *et al.* Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity in vitro and in vivo. *Brain* **127**, 1370–1378 (2004).
14. Kim, H.J. *et al.* Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis. *J. Immunol.* **172**, 7144–7153 (2004).
15. Vieira, P.L., Heystek, H.C., Wormmeester, J., Wierenga, E.A. & Kapsenberg, M.L. Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells. *J. Immunol.* **170**, 4483–4488 (2003).
16. Stasiolek, M. *et al.* Impaired maturation and altered regulatory function of plasmacytoid dendritic cells in multiple sclerosis. *Brain* **129**, 1293–1305 (2006).
17. Stuve, O. *et al.* Immunomodulatory synergy by combination of atorvastatin and glatiramer acetate in treatment of CNS autoimmunity. *J. Clin. Invest.* **116**, 1037–1044 (2006).
18. Takahashi, K., Prinz, M., Stagi, M., Chechneva, O. & Neumann, H. TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med.* **4**, e124 (2007).
19. Zamora, A., Matejuk, A., Silverman, M., Vandenbark, A.A. & Offner, H. Inhibitory effects of incomplete Freund's adjuvant on experimental autoimmune encephalomyelitis. *Autoimmunity* **35**, 21–28 (2002).
20. Kuchroo, V.K. *et al.* B7–1 and B7–2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**, 707–718 (1995).
21. Ranger, A.M., Das, M.P., Kuchroo, V.K. & Glimcher, L.H. B7–2 (CD86) is essential for the development of IL-4-producing T cells. *Int. Immunol.* **8**, 1549–1560 (1996).
22. Salama, A.D. *et al.* Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **198**, 71–78 (2003).
23. Wesemann, D.R. & Benveniste, E.N. STAT-1 alpha and IFN-gamma as modulators of TNF-alpha signaling in macrophages: regulation and functional implications of the TNF receptor 1:STAT-1 alpha complex. *J. Immunol.* **171**, 5313–5319 (2003).
24. Flesch, I.E. *et al.* Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon gamma and tumor necrosis factor alpha. *J. Exp. Med.* **181**, 1615–1621 (1995).
25. VanDeusen, J.B. *et al.* STAT-1-mediated repression of monocyte interleukin-10 gene expression in vivo. *Eur. J. Immunol.* **36**, 623–630 (2006).
26. Chitnis, T. *et al.* Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *J. Clin. Invest.* **108**, 739–747 (2001).
27. Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869–877 (1992).
28. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061 (2003).
29. Brunkow, M.E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* **27**, 68–73 (2001).
30. Viglietta, V., Baecher-Allan, C., Weiner, H.L. & Hafler, D.A. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* **199**, 971–979 (2004).
31. Kukreja, A. *et al.* Multiple immuno-regulatory defects in type-1 diabetes. *J. Clin. Invest.* **109**, 131–140 (2002).
32. Hong, J., Li, N., Zhang, X., Zheng, B. & Zhang, J.Z. Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3. *Proc. Natl. Acad. Sci. USA* **102**, 6449–6454 (2005).
33. Putheti, P., Soderstrom, M., Link, H. & Huang, Y.M. Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis. *J. Neuroimmunol.* **144**, 125–131 (2003).
34. Aharoni, R., Teitelbaum, D., Sela, M. & Arnon, R. Bystander suppression of experimental autoimmune encephalomyelitis by T cell lines and clones of the Th2 type induced by copolymer 1. *J. Neuroimmunol.* **91**, 135–146 (1998).
35. Harrington, L.E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123–1132 (2005).
36. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238 (2006).
37. Shimoda, M. *et al.* Conditional ablation of MHC-II suggests an indirect role for MHC-II in regulatory CD4 T cell maintenance. *J. Immunol.* **176**, 6503–6511 (2006).
38. Steinman, L. & Zamvil, S.S. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann. Neurol.* **60**, 12–21 (2006).
39. Nestle, F.O. Dendritic cell vaccination for cancer therapy. *Oncogene* **19**, 6673–6679 (2000).
40. Frisullo, G. *et al.* pSTAT1, pSTAT3, and T-bet expression in peripheral blood mononuclear cells from relapsing-remitting multiple sclerosis patients correlates with disease activity. *J. Neurosci. Res.* **84**, 1027–1036 (2006).
41. Tran, E.H., Hoekstra, K., van Rooijen, N., Dijkstra, C.D. & Owens, T. Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J. Immunol.* **161**, 3767–3775 (1998).
42. Jee, Y. *et al.* Do Th2 cells mediate the effects of glatiramer acetate in experimental autoimmune encephalomyelitis? *Int. Immunol.* **18**, 537–544 (2006).
43. Lisak, R.P., Zweiman, B., Blanchard, N. & Rorke, L.B. Effect of treatment with Copolymer 1 (Cop-1) on the in vivo and in vitro manifestations of experimental allergic encephalomyelitis (EAE). *J. Neurol. Sci.* **62**, 281–293 (1983).
44. Thornton, A.M. & Shevach, E.M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* **164**, 183–190 (2000).
45. Zamvil, S. *et al.* T cell clones specific for myelin basic protein induce relapsing EAE and demyelination. *Nature* **317**, 355–358 (1985).
46. Zhang, M. *et al.* Copolymer 1 inhibits experimental autoimmune uveoretinitis. *J. Neuroimmunol.* **103**, 189–194 (2000).
47. Gur, C. *et al.* Amelioration of experimental colitis by Copaxone is associated with class-II-restricted CD4 immune blocking. *Clin. Immunol.* **118**, 307–316 (2006).
48. Arnon, R. & Aharoni, R. Mechanism of action of glatiramer acetate in multiple sclerosis and its potential for the development of new applications. *Proc. Natl. Acad. Sci. USA* **101**(suppl. 2), 14593–14598 (2004).
49. Hardardottir, F., Baron, J.L. & Janeway, C.A., Jr. T cells with two functional antigen-specific receptors. *Proc. Natl. Acad. Sci. USA* **92**, 354–358 (1995).
50. Bettelli, E. *et al.* Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J. Exp. Med.* **197**, 1073–1081 (2003).