SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
NEUROIMMUNOLOGY
IMMUNOLOGY

Received
12 October 2014

Accepted
12 December 2014

Published
16 January 2015

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Integrin alpha L controls the homing of regulatory T cells during CNS autoimmunity in the absence of integrin alpha 4

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Experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), results from an autoimmune attack of the central nervous system (CNS) by effector T helper (Th) 1 and Th17 cells. Regulatory T cells (Treg) can control effector T cells and limit the progression of CNS autoimmunity. Integrin alpha 4 (Itga4) is critical for the entry of Th1 but not Th17 cells into the CNS during EAE. Whether Itga4 controls the homing of Tregs in the CNS and whether Tregs can limit Th17-mediated EAE has, however, not been addressed. Through selective elimination of Itga4 in Foxp3-expressing cells, we show here that Tregs can suppress Th17-mediated EAE and enter into the CNS independently of Itga4. Furthermore, similarly to Th17 cells and in contrast to Th1 cells, Tregs depend on LFA-1 for their entry into the CNS in the absence of Itga4. Therefore, these data suggest that the efficacy of Itga4 neutralization on MS progression may be associated with the prevention of Th1 cells and the maintenance of Tregs migration into the CNS.

Multiple sclerosis (MS) is an inflammatory autoimmune disorder of the central nervous system (CNS). A large part of its clinical and histological features can be modeled in experimental autoimmune encephalomyelitis (EAE), an autoimmune disease of the CNS induced by immunization of mice with myelin autoantigens or the transfer of myelin-specific $CD4^+$ T cells[1]. Experiments carried out in EAE demonstrated that Th1 and Th17 cells are pathogenic cells[1,2]. Th1 and Th17 cells have been reported to induce distinct clinical signs, histopathological changes and lesion distribution[2,3]. $Foxp3^+$ regulatory T cells (Treg)[4] are believed to ameliorate disease progression[5,6] through the control of effector T cells[6]. To date, it is unresolved whether Th17-dominated autoimmune responses can be controlled by regulatory T cells.

Homing of $CD4^+$ T cells from the periphery into the CNS during MS and EAE involves specific adhesion molecules including integrin alpha 4 (Itga4)[7]. Based on this property, monoclonal antibodies targeting Itga4 have been developed. In mice, they prevent the development of EAE[7] and in MS patients Natalizumab is used as a second line of disease modifying therapy[8]. While clinical trials showed a drastic reduction in the relapse rate, a number of Natalizumab treated patients developed lethal progressive multifocal leukoencephalopathy (PML), a serious opportunistic brain infection caused by a neurotropic strain of the JC virus[8]. Lack of CNS immune surveillance is believed to account for the severe and often fatal CNS infection caused by this virus[8]. Despite its long-term use in the clinic, knowledge of the biological effects of anti-Itga4 antibody on different immune cell populations *in vivo* is still limited. Recent studies from our laboratory and others support the hypothesis that Itga4 blockade does not uniformly block lymphocyte homing and function[9,10]. Indeed, we have recently shown that conditional deletion of Itga4 on T cells leads to a Th17-mediated form of EAE, because Itga4 is specifically required for the homing of Th1 but not Th17 cells into the CNS[9]. Whether Itga4 blockade or elimination can differentially modulate the homing of effector versus regulatory T cells in the CNS has not been addressed.

In this study, we determined that Tregs can limit Th17-driven EAE. We further established that conditional deletion of Itga4 on $Foxp3^+$ T cells does not affect their homing into the CNS and/or their functions during EAE. Therefore, Tregs can patrol and function in the CNS during Itga4 blockade or neutralization. Importantly, in the absence of Itga4 we show that Tregs use LFA-1 (CD11a/CD18) to migrate into the CNS and control EAE progression.

Results

Tregs can control Th17-mediated EAE. The type of effector population (Th1 vs Th17 cells) driving the immune response has been proposed to determine the efficacy of Treg-mediated regulation[11]. To understand the effects of Itga4 modulation on T cell populations and in particular Treg populations during the course of CNS autoimmunity, we used mice with selective deletion of Itga4 on T cells ($CD4^{Cre}$ $Itga4^{fl/fl}$). Consistent with our previous study[9], we observed milder EAE signs in $CD4^{Cre}$ $Itga4^{fl/fl}$ mice (Figure 1A) and limited infiltration of Th1 cells into the CNS (Figure 1B), compared to $Itga4^{fl/fl}$ mice. In contrast, there were similar numbers of Th17 cells infiltrating the CNS of $CD4^{Cre}$ $Itga4^{fl/fl}$ and control mice. The milder disease observed in $CD4^{Cre}$ $Itga4^{fl/fl}$ mice suggested that the CNS-infiltrating effector population Th17 cells could be controlled by regulatory T cells. To address this hypothesis, we deleted Tregs in $CD4^{Cre}$ $Itga_4^{fl/fl}$ mice prior EAE induction by injection of anti-CD25 specific antibody. Treatment of $CD4^{Cre}$ $Itga_4^{fl/fl}$ mice with anti-CD25 antibody prior to immunization led to a significant decrease in the percentage of Tregs present in the blood of mice anti-CD25 treated compared to mice treated with isotype control (9.6% ± 1.24 for anti-CD25 treated mice vs 2.43% ± 0.39 for isotype treated mice, Figure 1C and D). Importantly, the elimination of Tregs in $CD4^{Cre}$ $Itga4^{fl/fl}$ mice led to exacerbated disease compared to $CD4^{Cre}$ $Itga4^{fl/fl}$ mice receiving isotype control antibody (Figure 1E) and restored an EAE course with severity similar to C57BL/B6 mice. This highlighted the active role that Tregs play in controlling Th17-driven disease in $CD4^{Cre}$ $Itga4^{fl/fl}$ mice (Figure 1) and indicated that Th17 cell-driven pathology can be controlled by Tregs.

Generation of Tregs does not require Itga4. It is unclear whether the control of effector T cell populations (Teff) occurs in the peripheral organs or in the CNS. To address this question and look at the specific effect of Itga4 on Tregs, we crossed $Itga4^{fl/fl}$ mice with $Foxp3^{Cre\text{-}YFP}$ mice[11,12]. In these animals, Cre-mediated deletion of Itga4 was efficient and specific to $Foxp3^+$ regulatory T cells (Figure 2B), leaving intact Itga4 expression by effector T cells (data not shown). Because Itga4 has been implicated in various processes including T cell migration and activation[13,14], we examined the effect of Itga4 deletion on Treg distribution in lymphoid organs. Deletion of Itga4 on Treg cells did not alter their thymic and peripheral distribution (Figure 2A). We also investigated whether Itga4 deletion could modulate the phenotype of Treg cells and the arsenal of surface molecules that they express. We observed similar levels of CD103, CD25, CD11a, GITR, CD62L, Helios, CTLA4 and CCR6 in Itga4-competent or -deficient (YFP^+) Tregs (Figure 2B). Next, we determined whether Itga4 could inhibit the function of Tregs. To address this possibility, we compared the capacity of Itga4-deficient or -competent Tregs to suppress the proliferation of effector T cells *in vitro*. Both WT and Itga4-deficient Tregs were equally effective at controlling the proliferation of Teff cells (Figure 2C), indicating that Itga4 neutralization do not limit the function of Tregs.

Itga4 is dispensable for the entry of Tregs in the CNS. Next, we evaluated the effects of Treg specific deletion of Itga4 on EAE development and Treg migration in the CNS. $Foxp3^{Cre}$ $Itga4^{fl/fl}$ and control $Foxp3^{Cre}$ mice were immunized with MOG_{35-55} for the development of EAE. Ninety six percent of the mice in the control group developed classical EAE with appearance of ascending paralysis between day 8 and 12 (Figure 3A). Interestingly, the deletion of Itga4 on Tregs did not affect the onset, severity, and/or the recovery of EAE (Figure 3A). We characterized Tregs present in the CNS at the peak of the disease (day 18–20) and confirmed that CNS-infiltrating Tregs in $Foxp3^{Cre}$ $Itga4^{fl/fl}$ mice were Itga4 deficient (Figure 3B). We also observed similar numbers of $Foxp3^+$ regulatory T cells infiltrating the CNS of $Foxp3^{Cre}$ and $Foxp3^{Cre}$ $Itga4^{fl/fl}$ mice, both at the peak (Figure 3C) and during the recovery phase of the

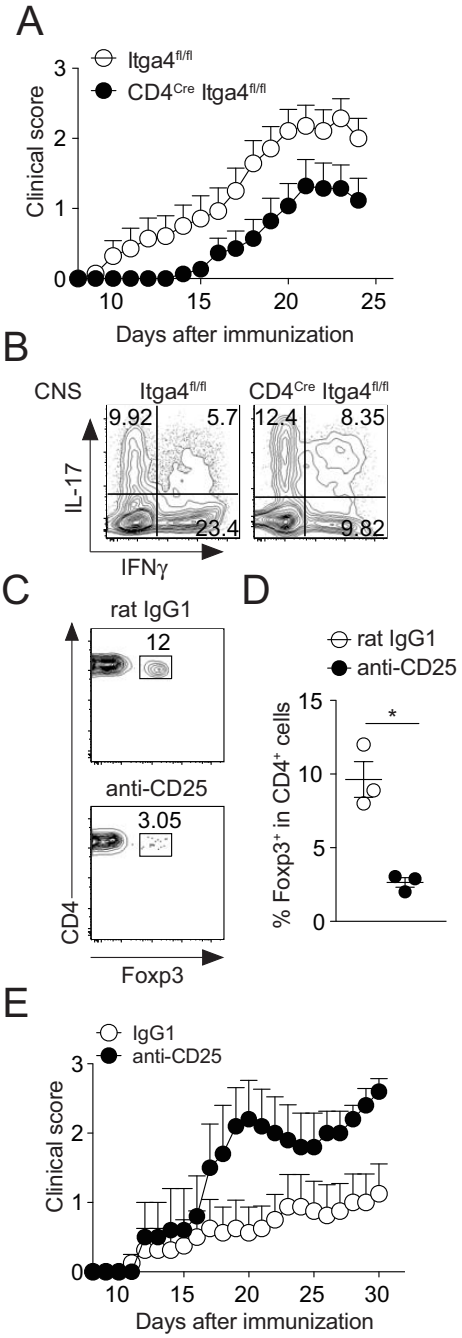

Figure 1 | Tregs control Th-17 mediated EAE. EAE was induced in $Itga4^{fl/fl}$ (open circles) and $CD4^{Cre}$ $Itga4^{fl/fl}$ mice (filled circles). (A) Shown is the mean clinical score for each group over time for 8 mice (±SEM). Data are representative of 4 experiments with 20 mice per group. (B) At the peak of the disease, CNS infiltrating mononuclear cells were collected and an intracellular cytokine staining was performed. The plots show the percentage of $IL17^+$ and $IFN\gamma^+$ cells gated on $CD4^+$ T cells. (C)–(E) $CD4^{Cre}$ $Itga4^{fl/fl}$ mice were treated twice with anti-CD25 or rat IgG1 antibodies prior immunization for EAE development. (C) The percentage of $CD4^+$ $Foxp3^+$ cells in the blood of each mouse was determined by flow cytometry prior immunization. The plots show the percentage of $CD4^+$ $Foxp3^+$ T cells. (D) Mean percentage of $Foxp3^+$ cells among $CD4^+$ T cells in the blood for both groups (* $p < 0.05$). (E) EAE clinical course in rat IgG1 treated (open circles) and anti-CD25 treated (filled circles) $CD4^{Cre}$ $Itga4^{fl/fl}$ mice is presented (n = 7 mice per group).

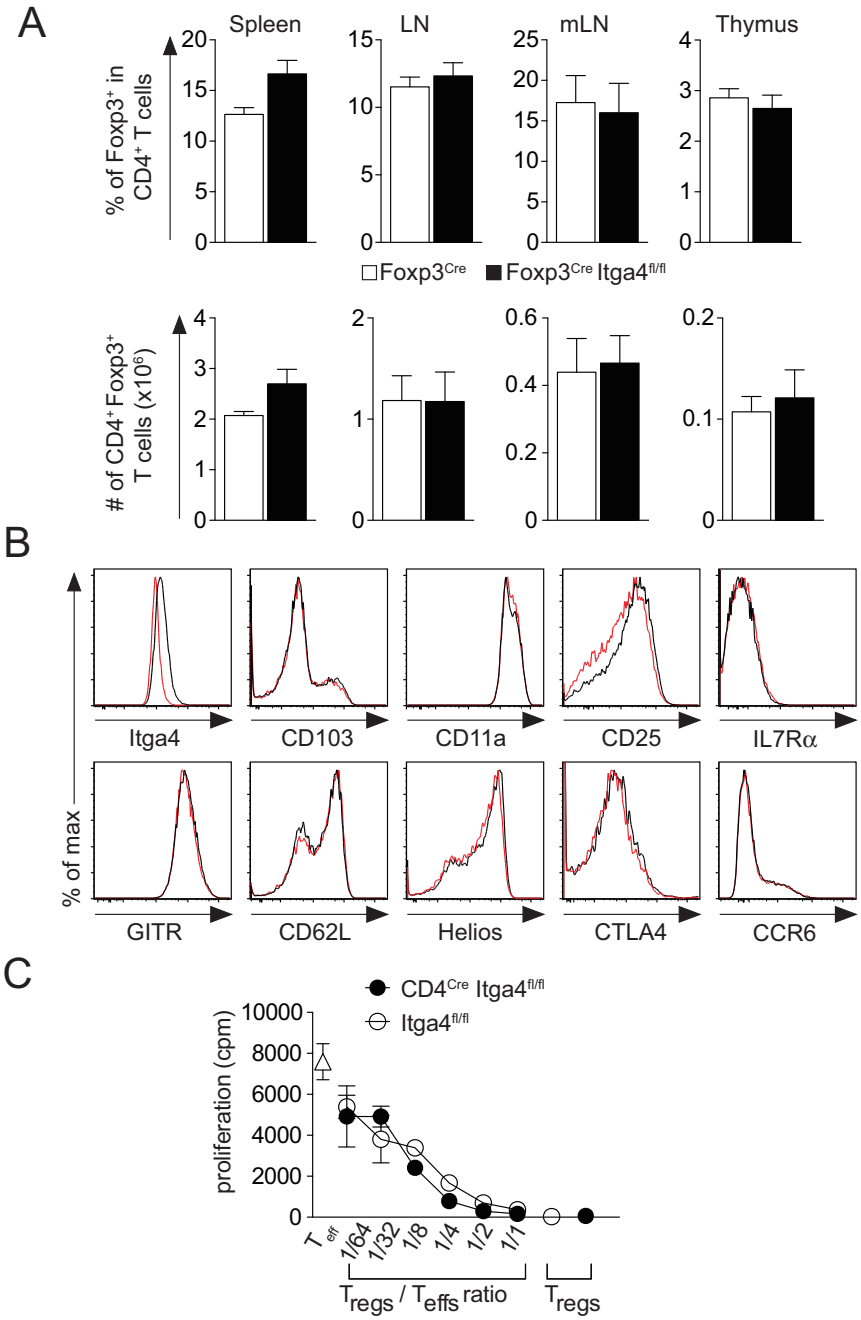

Figure 2 | Deletion of Itga4 in Foxp3$^+$ cells does not interfere with Tregs differentiation and functions at steady state. (A) and (B) Spleen, LN, mesenteric LN (mLN) and Thymus were collected from Foxp3^{Cre} and Foxp3^{Cre} Itga4$^{fl/fl}$ mice. (A) Frequency of Foxp3$^+$ cells among CD4$^+$ T cells (Top) and absolute numbers of CD4$^+$ Foxp3$^+$ regulatory T cells (Bottom) from these tissues were calculated from two independent experiments with 4–6 mice per group. (B) Expression of selective markers on CD4$^+$ Foxp3$^+$ T cells from Foxp3^{Cre} (dark line) and Foxp3^{Cre} Itga4$^{fl/fl}$ mice (red line). (C) Tregs from Itga4$^{fl/fl}$ (open circles) and CD4^{Cre} Itga4$^{fl/fl}$ mice (filled circles) were tested for their suppressive activity on CD4$^+$ CD25$^-$ cells proliferation *in vitro*. Mean proliferation (cpm ± SD) of Tregs alone, Teffs alone (open triangles) or Teffs in the presence of different ratios of Tregs/Teffs is shown and representative of 2 independent experiments.

disease (data not shown), showing that Tregs could enter the CNS independently of Itga4 (Figure 3 A–C). Importantly, Itga4-deficient Tregs, similarly to Itga4 competent Tregs, were MOG specific as shown by MOG_{38-49} I-Ab tetramer staining and upregulate CD103 and GITR in the CNS of mice during EAE (Figure 3D). Therefore, Tregs are able to enter and function in the CNS when Itga4 is neutralized or eliminated.

Itga4 deficient Treg cells use LFA-1 to control EAE development. If Tregs could access the CNS in the absence of Itga4 expression, which homing receptor did they use? To address this question, we analyzed the expression of integrin alpha chains in Tregs isolated from the LN of MOG_{35-55}-immunized mice and observed that ItgaL mRNA was one of the three integrin RNAs highly expressed by Tregs (Figure 4A). Integrins are heterodimeric integral membrane proteins composed of an alpha and a beta chain. ItgaL (CD11a) combines with Itgb2 (CD18) to form the lymphocyte function-associated antigen-1 (LFA-1) which is expressed by all leukocytes. Interestingly, LFA-1 deficient mice have been shown to have fewer Treg in the spleen and thymus and to be more susceptible to EAE upon immunization with

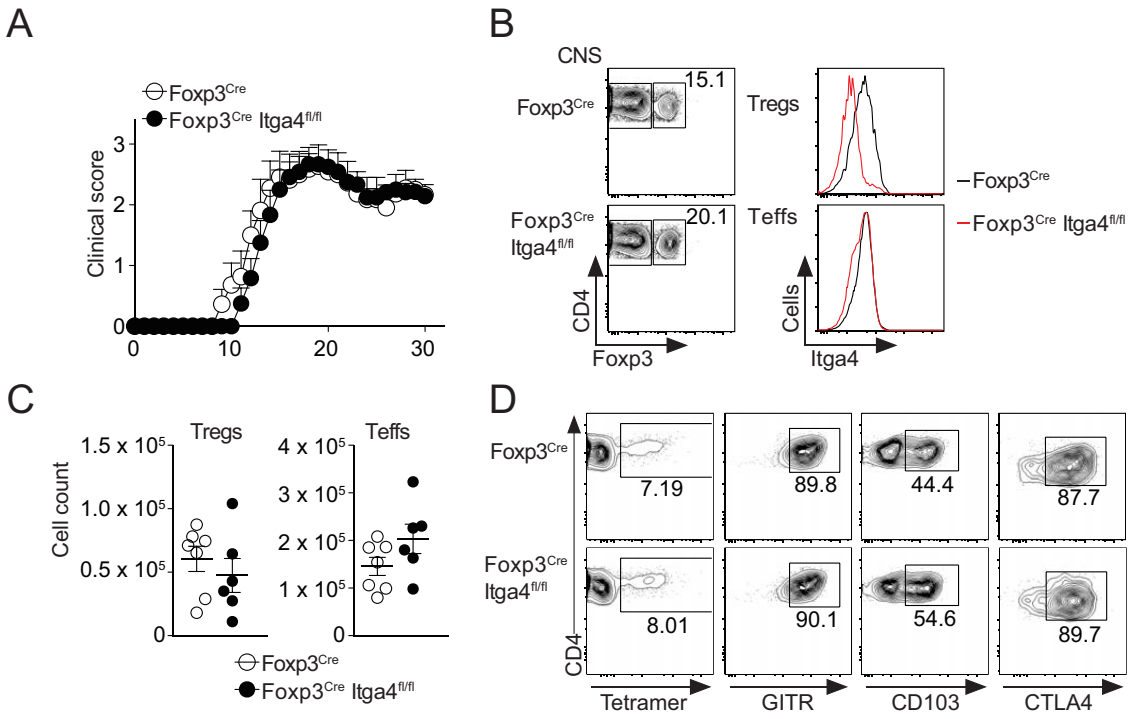

Figure 3 | Tregs can migrate in the CNS independently of Itga4 during EAE. (A)–(D) EAE was induced in Foxp3^{Cre} and Foxp3^{Cre} Itga4$^{fl/fl}$ mice. (A) EAE clinical course in Foxp3^{Cre} (open circles) and Foxp3^{Cre} Itga4$^{fl/fl}$ mice (filled circles) is presented and representative of 4 experiments with 24 mice per group. (B) Itga4 expression gated on Foxp3^{+} regulatory T cells identified by YFP expression (YFP^{+}) and effector T cells (YFP^{-}) from the CNS of Foxp3^{Cre} (black line) and Foxp3^{Cre} Itga4$^{fl/fl}$ (red line) mice at the peak of EAE (day 18–20). Data are representative of 2 independent experiments with 7 mice per group. (C) Mean number of Tregs (CD4^{+} YFP/Foxp3^{+}) and Teffs (CD4^{+} YFP/Foxp3^{-}) in the CNS of Foxp3^{Cre} and Foxp3^{Cre} Itga4$^{fl/fl}$ mice determined from 2 independent experiments with 7 mice per group. (D) Expression of selective markers (CTLA4, GITR, CD103) and MOG-IAb tetramer staining on CNS-infiltrating CD4^{+} Foxp3^{+} regulatory T cells from Foxp3^{Cre} (top) and Foxp3^{Cre} Itga4$^{fl/fl}$ mice (bottom) at the peak of the disease. All plots are representative of two independent experiments with 7 mice per group.

MOG_{35-55} in comparison to WT animals[15]. This suggested that LFA-1 could contribute to the migration of Tregs into the CNS. However, because LFA-1 deficient mice have profound defects in many immune cell populations[15,16] and because the number of peripheral Tregs is already low in LFA-1 deficient mice, the contribution of LFA-1 in the migration of Tregs into the CNS during EAE has been difficult to address using this model. Importantly, we observed that CD11a (ItgaL) was significantly upregulated on Tregs compared to Teffs (Figure 4B) after immunization with MOG_{35-55}. Therefore, we tested whether LFA-1 could be critical for the entry of Tregs into the CNS. To test our hypothesis, we treated Foxp3^{Cre} Itga4$^{fl/fl}$ and Foxp3^{Cre} control mice with anti-CD11a or isotype control antibodies after the priming of pathogenic CD4^{+} T cells (starting at day 8 post-immunization). As predicted, isotype control treatment (rat IgG2a) of Foxp3^{Cre} control and Foxp3^{Cre} Itga4$^{fl/fl}$ mice led to a rapid disease development followed by a recovery phase (Figure 4C). Consistent with the previously reported blockade of Th17 cell migration into the CNS by anti-CD11a antibody[10], the blockade of CD11a in Foxp3^{Cre} control mice resulted in a delayed onset of EAE but did not affect the recovery phase of the disease when Tregs play an important immunosuppressive role (Figure 4C) suggesting that WT Tregs can use alternative integrins to access the CNS. In contrast, disease severity was significantly increased in Foxp3^{Cre} Itga4$^{fl/fl}$ mice treated with anti-CD11a and these mice reached a maximal clinical score higher than anti-CD11a-treated Foxp3^{Cre} mice (Figure 4C and D). These results suggest that once Itga4 is blocked or eliminated, ItgaL is critical for Tregs entry in the CNS during EAE. To establish that the differences observed during the recovery phase of the disease were due to defective Treg homing, we quantified the percentage of Tregs present in the CNS of these different groups of mice using YFP expression (Figure 4E and F). Whereas isotype-treated Foxp3^{Cre} Itga4$^{fl/fl}$ and Foxp3^{Cre} mice had similar percentages of CNS-infiltrating Tregs, anti-CD11a-treated Foxp3^{Cre} Itga4$^{fl/fl}$ mice had significantly fewer Tregs infiltrating the CNS than anti-CD11a treated Foxp3^{Cre} mice (Figure 4E and F). These data indicate that upon blockade or in the absence of Itga4, Tregs use ItgaL to enter the CNS and control disease progression. Therefore, while Itga4 blockade did not significantly impair the migration and function of Tregs in the CNS, CD11a blockade in association with Itga4 elimination profoundly restricted Treg entry into the CNS and their control of EAE recovery.

Discussion

Recent work supports the hypothesis that Itga4 elimination or neutralization does not uniformly block lymphocyte extravasation but instead acts via selective modulation of its cellular target[17,18]. We and others have previously reported that elimination of Itga4 on T cells selectively blocks the entry of Th1 but not Th17 cells in the CNS[9,10]. Here, we have determined the effects of Itga4 elimination on Treg function and EAE development.

Natalizumab, a monoclonal humanized antibody (mAb) targeting the alpha-4 chain of α4β1 and α4β7 integrins on the surface of leukocytes has beneficial therapeutic effects in MS patients[8,17]. In rare cases, however, Itga4 neutralization has led to the development of PML, a severe infection of the CNS caused by the JC virus, which might be the result of poor immune-surveillance of the CNS by T cells and/or the presence of an immunosuppressive environment in the CNS. Despite the long-term use of Itga4-neutralizing antibody in MS, little is known about the cell-specific effects of Itga4 neutralization or elimination. Regulatory T cells accumulate in the CNS of mice during the progression of EAE and can ameliorate disease progres-

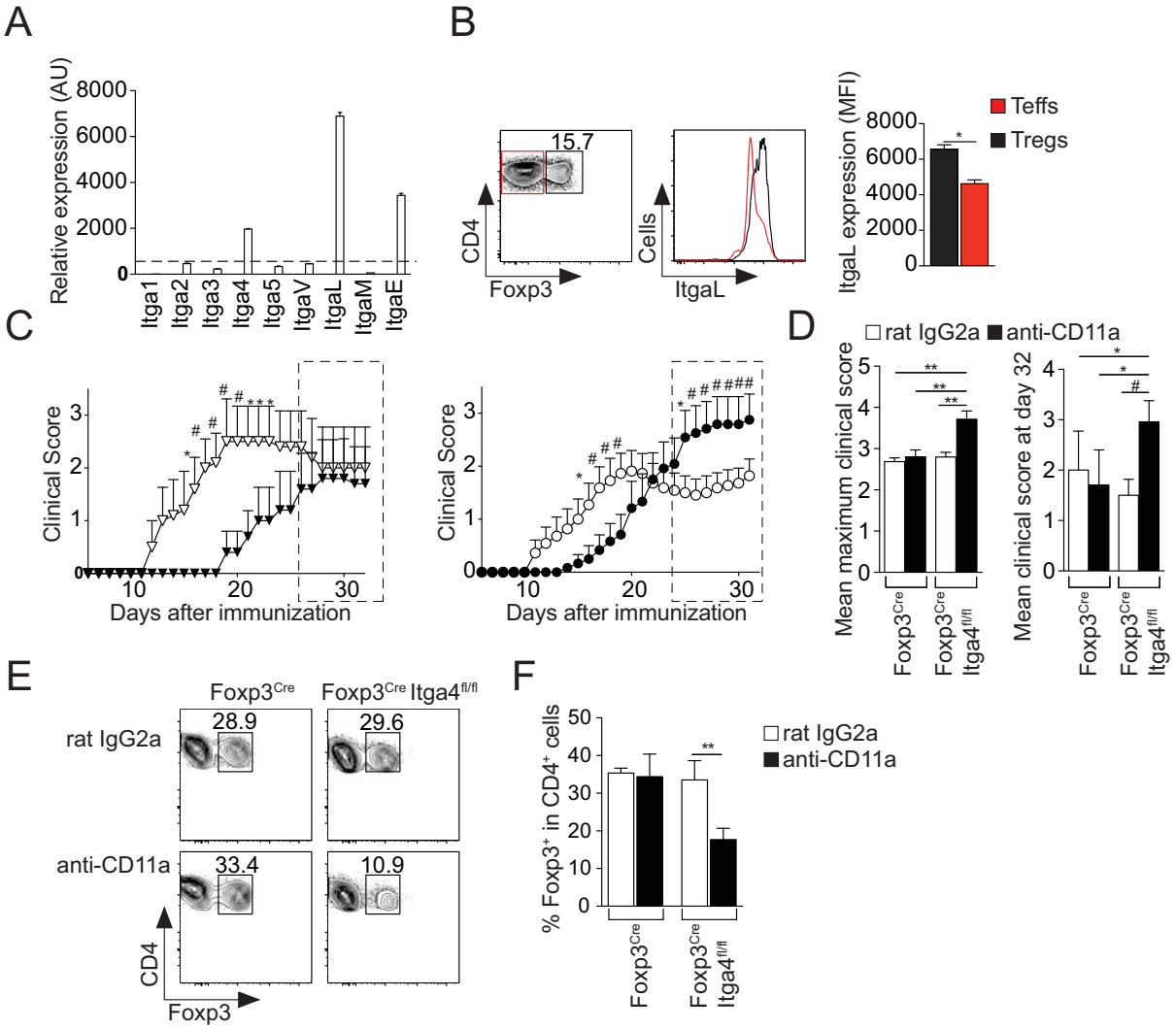

Figure 4 | ItgaL drives the homing of Treg cells into the CNS in the absence of Itga4. (A and B) EAE was induced in Foxp3^{Cre} mice. (A) Tregs were isolated from the spleen 10 days after immunization (before any clinical signs) based on their YFP expression. Relative expression of integrin alpha chains in Tregs was analyzed by RT-PCR. (B) ItgaL (CD11a) expression on splenic Teffs (red) and Tregs (black). (C)–(F) Foxp3^{Cre} and Foxp3^{Cre} Itga$_4$ $^{fl/fl}$ mice were immunized for EAE development and treated 8 days after immunization with anti-CD11a or isotype antibodies as described in Material and Methods. (C) Mean clinical score for each group over time is presented (±SEM). (D) Mean maximum clinical score and mean clinical score at the end of the experiment (day 32) were calculated for each group. (E)–(F) CNS were collected at day 32 and mononuclear cells isolated. The plots show representative Foxp3$^+$ T cells frequencies among CNS-infiltrating CD4$^+$ T cells from each group. Mean percentages of Tregs (CD4$^+$ Foxp3$^+$) in the CNS of mice were determined from 2 independent experiments (n = 7 mice per group) (*p < 0.05; **p < 0.01 and #p < 0.001).

sion in mice through the control of effector T cells[19–21]. Unfortunately, regulatory T cells have been shown to be defective in MS patients[22]. Itga4 exhibits multiple biological effects ranging from cell migration to modulation of T cell polarization and activation[13,23]. Itga4 has also been proposed as a marker to identify T cells with suppressive activity[24], and Itga4$^+$ Treg cells have been reported to be less effective[25]. In this report, we observed that Tregs express similar levels of CD103, GITR and CTLA4, regardless of their expression of Itga4 (Figure 2B and 3D). Tregs were also present in similar numbers in the thymus, spleen, LN and mesenteric LN of Itga4$^{fl/fl}$ and Foxp3^{Cre} Itga4$^{fl/fl}$ mice (Figure 2A). Furthermore, Tregs were capable of suppressing the proliferation of effector T cell responses regardless of their expression of Itga4 (Figure 2C). Together, these data demonstrate that the blunting of Itga4 signaling in Tregs does not compromise their development, distribution, and suppressive function at steady state. However, Tregs with less suppressive function may coincidentally downregulate the expression of Itga4 and explain previous report[25]. In this report, we have established that Itga4-deficient Tregs were capable of suppressing T cell responses *in vivo* during CNS inflammation, since their removal led to the development of exacerbated EAE (Figure 1E). The fact that elimination of Itga4 on Tregs does not impact their suppressive functions under inflammatory conditions supports earlier experiments showing that Natalizumab does not alter the suppressive capacity of Treg cells[24].

While both Th1 and Th17 cells can induce EAE development[2] and are present in active MS plaques, the efficacy of certain disease-modifying therapies has been shown to correlate with the prevalence of each Teff population[26]. Importantly, it is still unclear whether different forms of disease mediated by either Th1 or Th17 cells can be efficiently controlled by Tregs. There was speculation that Th17-mediated diseases might not be controlled by Tregs. We have shown that CD4^{Cre} Itga4$^{fl/fl}$ mice develop a disease dominated by Th17 cells (Figure 1B)[9]. We further addressed whether Th17-mediated EAE in CD4^{Cre} Itga4$^{fl/fl}$ mice could be controlled by Tregs. Depletion of Tregs by injection of anti-CD25 antibody led to exacerbated EAE indicating that Tregs control Th17 cells and EAE severity in these mice (Figure 1E). Therefore, while Tregs from MS patients have been

shown to be defective[22], our data suggest that both Th1 and Th17-mediated forms of MS can be controlled by enhancing the function of Tregs.

Tregs exercise their immunomodulatory role both in lymphoid and non-lymphoid tissue. In lymphoid tissues, they can limit T cell expansion and differentiation[27], but once the effector response is established, they regulate local inflammation in target tissue[28,29]. However, whether Itga4 can control the migration of Tregs in the CNS has not been investigated. Using both $CD4^{Cre}$ $Itga4^{fl/fl}$ mice and $Foxp3^{Cre}$ $Itga4^{fl/fl}$ mice, we determined that regulatory T cells migrate into the CNS independently of Itga4, since both strains had equivalent numbers of Tregs in the CNS (Figure 3C and data not shown). It will be of interest in the future to determine whether similar accumulation of Tregs over effector T cells is observed in MS patients treated with Natalizumab. Indeed, an increase in the number of Tregs over Teff in the CNS of Natalizumab-treated patients could explain in part the efficacy of such treatment. Treg cells secrete inhibitory cytokines, such as IL-10 and TGF-β, that limit effector T cell responses involved in viral clearance. Therefore, the prevalence of Tregs over Teffs in the CNS upon elimination of Itga4 might also have major implications for the surveillance of the CNS and could provide clues for the pathogenesis of CNS infections in Natalizumab-treated patients[30].

The presence of a low but significant number of lymphoid cells in the CSF of MS patients treated with Natalizumab[24,31] in conjunction with our data showing that regulatory T cells can traffic to the CNS independently of Itga4, suggested that there are some compensatory or alternative mechanisms allowing regulatory T cells to enter the CNS. During the comparison of the expression levels of adhesion molecules between effector and regulatory T cells, we observed high expression of ItgaL (which together with Itgb2 form LFA-1) on Treg cells compared to effector T cells. This differential expression prompted us to investigate whether ItgaL could drive the entry of Treg cells into the CNS in the absence of Itga4. Using anti-CD11a neutralizing antibodies after the induction of EAE, we demonstrated that Itga4-deficient Tregs used LFA-1 to migrate into the CNS and control disease development, since $Foxp3^{Cre}$ $Itga4^{fl/fl}$ mice treated with anti-CD11a failed to recover and had exacerbated EAE compared to $Foxp3^{Cre}$ $Itga4^{fl/fl}$ mice treated with isotype control antibody (Figure 4C and D). This differential expression of ItgaL between effector and Treg cells is reminiscent of our observation that Itga4 is differentially regulated between Th1 and Th17 cells[9]. Indeed, Th17 and Treg cells share common homing molecules (i.e.CD11a) for their migration into target organs during the development of autoimmunity. Our observations also extend previous publications showing that genetic ablation of CD11a in mice results in higher susceptibility to EAE than control mice[15] and a decrease in the numbers of Tregs present in the CNS of these mice[15,32]. Finally, these findings expand previous reports suggesting an inhibitory role of CD11a in CNS inflammation through its expression, its activity on other non-effector T cell subsets[33], and with previous reports showing an important role of CD11a in Treg homeostasis[15].

Conclusions

In this study, we have shown that Tregs can control Th17-mediated EAE in mice with specific deletion of Itga4 on T cells. Importantly Itga4 elimination does not modulate the activity of Treg cells and does not limit their migration into the CNS during EAE. We further identify LFA-1 as a key molecule, in the absence of functional Itga4, for Treg entry into the CNS during EAE. Therefore, the efficacy of Itga4 blockage in preventing disease progression might not only result in the inhibition of Th1 cell migration in the CNS, as we and others have previously reported[9,10], but also results in the maintenance of Treg migration into the CNS, which allows for the effective control of pathogenic Th17 cells.

Methods

Mice. All mice are on the C57BL/6 background. $Itga_4^{fl/fl}$ mice and $Foxp3^{Cre\text{-}YFP}$ were described[12,34]. All animals were bred and maintained under specific pathogen-free conditions at the Benaroya Research Institute (Seattle, WA). All experimental protocols and procedures were approved by *the Institutional Animal Care and Use Committee of the Benaroya Research Institute*. Animal care and experimental procedures were carried out in accordance *with the guidelines of the Institutional Animal Care and Use Committee of the Benaroya Research Institute and the National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Isolation of CNS mononuclear cells. Mice were sacrificed at the peak of disease and perfused through the left cardiac ventricle with cold 1× PBS. Brain and spinal cords were isolated and digested for 30 min at 37°C with Collagenase D at a concentration of 2.5 mg/ml (Roche). Mononuclear cells were isolated over a 37%/70% Percoll gradient (VWR), washed twice with complete medium and collected in medium for further analysis.

Flow cytometry. Cell suspensions from CNS and spleen were prepared as previously described[9]. Anti-CD4 (GK1.5), anti-CD103 (2E7), anti-CD25 (PC61), anti-IL17 (TC11-18H10.1), anti-IFNγ (XMG1.2), anti-CD49d (R1-2), anti-GITR (YGTIR765), anti-CTLA4 (UC10-4B9), anti-CD11a (M17/4) and anti-Helios (22F6) antibodies were purchased from Biolegend and eBioscience. The mouse MOG_{38-49} I-Ab tetramer was supplied by the National Institute of Health Tetramer Core Facility to identify the MOG-specific $CD4^+$ T cells. For tetramer staining, cells were stained for 2 hours at 37°C. Labeled cells were acquired on LSRII (BD Biosciences), and data were analyzed with FlowJo software.

EAE induction and antibody treatment. Active EAE was induced by subcutaneous immunization of mice with an emulsion of 10 μg of MOG_{35-55} peptide in CFA supplemented with 40 mg of M. tuberculosis extract H37 Ra (Difco). In addition, the animals received 200 ng of pertussis toxin (List Biological Laboratories) i.p. on day 0 and 2 after immunization. Animals were monitored daily for development of EAE according to the following score: 0, no signs of disease; 1, loss of tail tone; 2, hind limb paresis; 3, hind limb paralysis; 4, front and hind limb paralysis. To compare EAE susceptibility between groups, mean clinical score was calculated and plotted daily for each group of mice. To analyze the severity of EAE, the maximal clinical score was determined, corresponding to the highest score reached by a mouse developing EAE. For the treatment of the animals, anti-CD11a (clone M17/4), anti-CD25 (clone PC61), and isotype antibodies were purchased from BioXcell. ItgaL-treated and control mice were treated with 100 μg of antibody administered i.p. 3 times per week starting at day 8 after immunization. For Treg depletion *in vivo*, mice were treated at day −3 and −1 before immunization with 500 μg of anti-CD25 i.p.

Suppression Assay. Tregs cells ($CD4^+$ $CD25^{high}$) and effector ($CD4^+$ $CD25^-$) were sorted from naïve WT and $CD4^{Cre}Itga4^{Fl/fl}$ mice. 2.5×10^4 effector T cells were co-cultured with different ratios of Tregs and 10^5 irradiated syngenic splenic cells in the presence of anti-CD3 (2.5 μg/ml) for 72 h. Cells were pulsed with 1 μCi of [^{3}H] thymidine during the last 16 h and [^{3}H] thymidine incorporation was measured using a β-counter.

Real-time PCR. RNA was extracted from splenic Treg cells using RNeasy Mini Kit (Qiagen). RNA was quantified using a NanoDrop 2000 spectophotometer (Thermo Sicentific). cDNA was transcribed with the RevertAid First Stand cDNA Synthesi Kit (Thermo Scientific) and qPCR was performed using a 7500 Real time PCR machine (Applied Biosystems). The total amount mRNA was normalized across samples according to endogenous β-actin mRNA. The primers sequence (forward and reverse respectively) were as follows: for Itga1 GCCCAGCGATATAGAGCACATC and TCTTGCTTCTTACTTGGGTTACACA; for Itga2 CTATGATAACCCCTG-TCGGTACTTC and TGAGGAAAATGCTGTCACGATT; for Itga3 GCCTT-CTGCCTCTTAGCTTCATA and ATCATCCTCCTCTTGTGGAAGTG; for Itga4 TGTGCAAATGTACACTCTCTTCCA and CTCCCTCAAGATGATAAGTTG-TTCAA; for Itga5 GCTAAGGTTGATGCAGGACACA and CTGCCAGCGCA-TCTCTCA; for ItgaV GCTGAAGCTGTTCTCTTTCTTGCT and ATTTGTAA-TGTACAGGATGGGCTTT; for ItgaL GCAGGCGACCTTGAAACTGT and GCAGAAACACGGAGTCAAGCT; for ItgaM TGTTCACCAGCTGGCTTAGATG and GGGTCATTCGCTACGTAATTGG; for ItgaE TCCCATCCATGTCGATA-TCCA and TTTGTGCGACGGATAGAAGGA and for β-actin ATGGTGCT-AGGAGCCAGAGC and CATTGCTGACAGGATGCAGAA.

Statistical analysis. The Two-Way Anova was used for statistical comparison of clinical EAE scores. The One-Way Anova was applied for statistical analysis of all the other experiments (*$p < 0.05$; **$p < 0.01$; #<0.005).

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Acknowledgments

This work was funded by grants NMSS RG 4486, NIH R01 NS059996 to E.B and FG2019 to S.G. We would like to thank Drs. Papayannopoulou and Dr. Rudensky for providing $Itga4^{fl/fl}$ and $Foxp3^{Cre\text{-}yfp}$ mice respectively.

Author contributions

S.G. and E.B. designed the research, analyzed the data and drafted the manuscript. S.G. performed experiments. R.D., C.A. and S.K. helped for data acquisition and discussion of the data. S.G. and E.B. analyzed the data and prepared the figures. E.B. supervised the whole project and S.G. and E.B. reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Glatigny, S., Duhen, R., Arbelaez, C., Kumari, S. & Bettelli, E. Integrin alpha L controls the homing of regulatory T cells during CNS autoimmunity in the absence of integrin alpha 4. *Sci. Rep.* **5**, 7834; DOI:10.1038/srep07834 (2015).