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Cutting Edge: Loss of α4 Integrin Expression Differentially Affects the Homing of Th1 and Th17 Cells

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The neutralization of α4 integrin is currently used as treatment in several autoimmune diseases and is thought to prevent the entry of most immune cells in target tissues. In this study, we showed that selective deletion of α4 integrin in T cells did not prevent but delayed the development of experimental autoimmune encephalomyelitis. Whereas both Th1 and Th17 cells infiltrate the CNS of wild-type mice, T cells present in the CNS of mice lacking α4 integrin were mainly enriched in Th17 cells, suggesting that this T cell subset uses other integrins to access the CNS. In contrast, α4 integrin expression is important for Th1 cells to enter the CNS and for the stability of their Th1-associated genetic program. Therefore, our data suggest that anti-α4 integrin Ab treatment may be more efficient in the treatment of Th1- rather than Th17-mediated disease. *The Journal of Immunology, 2011, 187: 6176–6179.

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS) characterized by multifocal areas of leukocyte infiltration, demyelination, and axonal damage (1). Two subsets of myelin-specific CD4+ T cells have been implicated in the pathogenicity of MS and EAE: Th1 cells and Th17 cells (2–5). Trafficking of autoreactive CD4+ T cells from the systemic compartment into the CNS are crucial early events in the development of MS and EAE lesions and involve specific adhesion molecules (1). α4 (Itga4) β1 (Itgb1) integrin or VLA-4 has been proposed as the major adhesion molecule allowing the entry of T cells in the CNS. Treatment of mice with an anti-α4 integrin Ab has been shown to dramatically reduce leukocyte adhesion and prevent the development of EAE in most animal models (6, 7), except in C57BL/6 mice MOG-induced EAE, in which it has limited effect on clinical disease (8, 9). Based on these observations, a humanized anti-Itga4 mAb was generated to treat patients with MS. Although clinical trials showed a drastic reduction in MS relapse rate, a significant number of patients developed a life-threatening condition called lethal progressive multifocal leukoencephalopathy (10). Therefore, it is important to determine the effect of Itga4 blockade on the migration of different subsets of immune cells in the CNS.

In this article, we show that mice with selective deletion of Itga4 on CD4+ T cells are still susceptible to EAE development. Whereas the number of CNS-infiltrating Th1 cells was significantly decreased, the number of CNS-infiltrating Th17 cells was not impaired in these mice. Using adoptive transfer experiments, we further show that the lack of Itga4 expression on Th1 cells impaired their phenotypic stability and their capacity to infiltrate the CNS. Together, our results suggest the efficacy of Itga4 neutralization for Th1-mediated EAE, but also distinct and new molecular requirements for Th17 cell entry into the CNS during EAE.

Materials and Methods

Mice

All mice are on the C57BL/6 background and used at 8–12 wk. Wild-type (WT), CD4Cre, TCRβ8-deficient, and 2D2 transgenic mice were purchased from The Jackson Laboratory. Itga4fl/fl mice were provided by Dr. Papayannopoulos (University of Washington) (11). All animals were bred and maintained under specific pathogen-free conditions at the Benaroya Research Institute (Seattle, WA), and all experiments were performed in accordance with the guidelines of the Benaroya Research Institute Animal Care and Use Committee.

EAE induction

Active EAE was induced as previously described (12). For passive transfer of EAE, 2D2 naïve CD62L+CD4+ T cells were cultured with irradiated splenocytes, anti-CD3 Ab (clone 145-2C11; 2.5 µg/ml), and either IL-12 (10 ng/ml) for Th1 cells or IL-6 (30 ng/ml), TGF-β (5 ng/ml), and anti–IFN-γ (10 µg/ml) for Th17 cells. After 3 d, 50 × 10^6 cells were injected i.v. into TCRβ8-deficient recipients with pertussis toxin at days 0 and 2. EAE was scored according to the following criteria: 0, no signs of disease; 1, loss of tail tone; 2, hind limb paraparesis; 3, hind limb paralysis; and 4, front and hind limb paralysis.

Flow cytometry

Cell suspensions from brain and spinal cord were prepared as previously described (12). Abs for CD4 (GK1.5), IFN-γ (XMG1.2), IL17A (TC11-18H10.1), and Itga4 (R1-2) were purchased from eBioscience and BioLegend. For surface cytokine staining, CD4+ T cells were stained with Miltenyi secretion assay following the manufacturer’s instructions (Miltenyi Biotec). Cells were acquired on an LSR II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

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The online version of this article contains supplemental material.

Abbreviations used in this article: dLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; Itga4, α4 integrin; Itgb1, β1 integrin; MS, multiple sclerosis; WT, wild-type.

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Draining lymph nodes (dLN) were collected 8 d after immunization. Cells were cultured at $5 \times 10^6$ cells/ml in the presence of different concentrations of MOG for 72 h. During the last 16 h, cells were pulsed with 1 μCi $[^3H]$thymidine. $[^3H]$thymidine incorporation was measured using a β-counter.

**Results and Discussion**

**Itga4 is expressed at higher levels in Th1 cells compared with Th17 cells**

Th cell subsets express distinct chemokine receptors and adhesion molecules conferring specific homing properties. Analysis of Itga4 expression has been previously performed on T cell clones or in vitro-polarized cells, but culture conditions could modulate Itga4 expression. In this study, we analyzed Itga4 expression on ex vivo effector Th1 and Th17 cell subsets isolated from dLN after MOG 35-55 immunization using a secretion assay for IL-17A and IFN-γ (Fig. 1). Itga4 was expressed at higher levels on Th1 than Th17 cells (Fig. 1). In contrast, there was no difference in the expression of Itgb1 and Itgb7 between Th1 and Th17 cells (Supplemental Fig. 1C, 1D). Therefore, the selective higher expression of Itga4 on Th1 cells raises the possibility that these cells might be more dependent on Itga4 for their entry in the CNS than Th17 cells.

Itga4 deletion on CD4+ T cells does not prevent the development of EAE

To determine whether the differential expression of Itga4 on Th cell subsets could affect the development of EAE, we used mice with specific deletion of Itga4 in T cells (CD4Cre Itga4fl/fl). Cre-mediated deletion of Itga4 in CD4+ T cells was efficient at the genomic level and resulted in >95% of CD4+ T cells lacking Itga4 in these animals (Supplemental Fig. 1C, 1D). The majority of WT control mice (95%) developed a monophasic disease with ascending paralysis between 10 and 14 d after immunization (Fig. 2A, Supplemental Table I). Although the mean clinical EAE score in CD4Cre Itga4fl/fl mice (2.5 ± 0.7) was similar to the one observed in WT control mice (2.7 ± 0.7), fewer CD4Cre Itga4fl/fl mice developed disease (incidence: 50 compared with 95% in WT mice; Fig. 2A, Supplemental Table I) and had a delay in disease onset (19.3 ± 4.3 d in CD4Cre Itga4fl/fl mice versus 12.3 ± 2.3 d in WT mice; Fig. 2A, Supplemental Table I). Thus, CD4Cre Itga4fl/fl mice developed EAE with delayed onset and less incidence than WT control mice. However, in agreement with a previous report showing blockade of leukocyte adhesion but limited effect of anti-Itga4 treatment in C57BL/6 mice MOG-induced EAE (8, 9), specific deletion of Itga4 on T cells did not abolish EAE susceptibility in our model. This suggests that pathological mechanisms including but not limited to the dominant type of CNS-infiltrating effector T cells may differ among mouse strains.

**FIGURE 2.** Deletion of Itga4 in T cells does not prevent the development of EAE. A. EAE was induced in CD4Cre Itga4fl/fl mice and control mice. Shown is the mean clinical score for each group over time (± SEM). One representative experiment out of three independent experiments is shown. B. CD4+ T cells from Itga4fl/fl mice and CD4Cre Itga4fl/fl mice were differentiated in Th1- and Th17-polarizing conditions. Plots are gated on CD4+ T cells and representative of two independent experiments. C. Proliferative response of dLN cells isolated from MOG-immunized Itga4fl/fl (open circles) and CD4Cre Itga4fl/fl (filled circles) mice was measured by $[^3H]$thymidine incorporation. Results are representative of four independent experiments.

VLA-4 has been proposed to play an important role in several immune functions such as lymphopoesis, costimulation, and T cell migration (11, 13–17). Therefore, we compared these parameters between CD4Cre Itga4fl/fl and Itga4fl/fl mice. We did not detect any difference in the number, percentage, and phenotype of CD4+ and CD8+ T cells present in the lym-
phoid organs of CD4Cre Itga4fl/fl mice compared with controls (data not shown). In addition, levels of Itgb1, which pairs with Itga4 to form VLA-4, were not affected in CD4Cre Itga4fl/fl mice (Supplemental Fig. 1E). Next, we investigated whether the lack of Itga4 expression on T cells could interfere with the differentiation of naive T cells into pathogenic Th1 and Th17 cells. We determined that CD4+ T cells from CD4Cre Itga4fl/fl and Itga4fl/fl mice could differentiate equally well in Th1 and Th17 subsets (Fig. 2B) and that Itga4 deletion was equivalent in Th1 and Th17 cells (Supplemental Fig. 1F). MOG-specific T cell proliferation was also similar between CD4Cre Itga4fl/fl and Itga4fl/fl mice (Fig. 2C). Collectively, these results indicate that specific deletion of Itga4 on T cells does not interfere with T cell priming, but instead may affect the homing of pathogenic T cells in the CNS.

Itga4 deletion decreases Th1 cells but not Th17 homing into the CNS

To evaluate this hypothesis, we determined the numbers of CD4+ T cells infiltrating the CNS of CD4Cre Itga4fl/fl mice during EAE. At the peak of the disease, there was a decrease in the number of CNS-infiltrating CD4+ T cells in CD4Cre Itga4fl/fl (52.9 ± 10^3 ± 8.4 ± 10^3) compared with control mice (126.5 ± 10^3 ± 17.2 ± 10^3). Before EAE onset and at the peak of the disease, there was a similar proportion of IL-17A+ IFN-γ+ and IL-17A+CD4+ T cells in the brain of CD4Cre Itga4fl/fl and WT control mice (Fig. 3, Supplemental Fig. 1G). However, we detected a significant decrease in the percentage and absolute number of CD4+ T cells that produced only IFN-γ in the CNS of CD4Cre Itga4fl/fl mice compared with control mice (Fig. 3, Supplemental Fig. 1G). Importantly, deletion of Itga4 was equivalent in Th1 and Th17 cells (Supplemental Fig. 1F), and the residual proportion of CD4+ T cells, which remained Itga4+ (4%), did not increase in CNS-infiltrating CD4+ T cells (Supplemental Fig. 1C). Of note, because a recent report suggests that IFN-γ+ T cells present in the CNS during EAE represent ex-Th17 cells (18), the tracking of T cell subsets by cytokine secretion might underestimate the number of CNS-infiltrating Th17 cells in our model. Despite this possibility, there was still a drastic decreased Th1/Th17 ratio in the CNS of CD4Cre Itga4fl/fl mice compared with WT mice (Fig. 3C). This suggests two important mechanisms of Itga4 action: 1) Itga4

FIGURE 3. CNS-infiltrating T cell subsets in CD4Cre Itga4fl/fl and control mice with EAE. EAE was induced in control and CD4Cre Itga4fl/fl mice. At the peak of the disease for each group (and at clinical score 2 or 3), brain and spinal cords were removed, and intracellular cytokine staining was performed. A. Plots show IL-17A and IFN-γ expression in CD4+ T cells. B. Mean numbers of T cell subsets infiltrating the brain and spinal cords of control (white bars) and CD4Cre Itga4fl/fl mice (black bars) with EAE were calculated from three independent experiments with 11 mice per group (*p < 0.005). The total number of cells recovered from the CNS was 0.67 × 10^6 ± 0.10 × 10^6 for CD4Cre Itga4fl/fl mice and 1.01 × 10^6 ± 0.12 × 10^6 for WT mice. C. Ratio of Th1/Th17 cells infiltrating the brain and spinal cord in each group of mice (*p < 0.01). Data are the mean of three independent experiments with 11 mice per group and were analyzed using Student t test.

FIGURE 4. Itga4-deficient Th1 cells do not induce EAE. A. CD4+ T cells from CD4Cre Itga4fl/fl 2D2 (right panels) and 2D2 (left panels) mice were polarized in vitro into Th17 (top panels) and Th1 cells (bottom panels). A. IL-17 and IFN-γ expression in Th1 and Th17 cells prior to adoptive transfer. B. EAE was induced by transfer of CD4Cre Itga4fl/fl 2D2 and 2D2 Th17 cells (top panel) or Th1 cells (bottom panel). Shown is the mean clinical score for each group over time (n = 6 mice per group ± SEM). In each group, disease incidence was equivalent (68%). C. CNS from transferred mice were removed, and intracellular cytokine staining was performed. The plots show the percentage of CNS-infiltrating Th1 and Th17 and are representative of four mice per group.
costimulation of T cells could promote Th1 cells switching to a Th17 phenotype in the CNS; or 2) fewer Th1 cells entered the CNS because their trafficking is dependent on Itga4.

Loss of Itga4 delays Th1-induced EAE

To address some of these possibilities, we differentiated CD4+ T cells from 2D2 CD4Cre Itga4f/f and 2D2 mice into either Th17 or Th1 cells and transferred them into T cell-deficient recipient mice (Fig. 4A, 4B). Th17 cells derived from either 2D2 or 2D2 CD4Cre Itga4f/f mice induced a severe disease (Fig. 4B), and a significant percentage of Th17-transgenic cells recovered from the CNS had maintained IL-17A expression (Fig. 4C, top panel). The transfer of Th1 cells lacking Itga4, in contrast, resulted in delayed disease onset compared with the disease induced by Itga4-sufficient Th1 cells (Fig. 4B). Surprisingly, cells recovered from the CNS of animals transferred with Itga4-deficient Th1 cells were mainly IL-17A-producing cells (Fig. 4C, bottom panel), indicating that in the absence of Itga4 on CD4+ T cells, the disease was mainly Th17 but not Th1 mediated. These results suggest the intriguing possibility that in the absence of Itga4, Th1 cells become unstable and revert to a Th17-like phenotype. The other possibility is that uncommitted cells from the pool of naive donor cells may have differentiated into Th17 cells and have overgrown Th1 cells in vivo, causing EAE independently of Itga4 and in the absence of Th1 cell entry in the CNS (Fig. 4B, 4C, bottom panel).

Conclusions

In this study, we demonstrate that elimination of Itga4 on CD4+ T cells limited the access of Th1 cells in the CNS and affected their phenotypic characteristics, but had less of an effect on the trafficking of Th17 cells. In accordance with these observations, the selective deletion of Itga4 in CD4+ T cells did not prevent the development of Th17-induced EAE but delayed Th1-induced EAE. These findings suggest that anti-Itga4 might be more efficient at treating Th1- rather than Th17-mediated MS. A differential effect of immunomodulatory drugs on effector T cell subsets and disease development has been reported recently in another study (19). These observations together with ours suggest that the Th1 cells-induced CNS autoimmunity might be fairly well controlled by current available drugs but that more specific drugs are required for Th17-induced and dominated CNS pathologies.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

### Supplementary Table I: Clinical course of EAE in CD4\textsuperscript{Cre} Itga\textsubscript{4}^{fl/fl} mice and control mice.

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Incidence</th>
<th>Day of onset (Mean ± SD.)</th>
<th>Maximum clinical score (Mean ± SD.)</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>19 of 20 (95%)</td>
<td>12.3 ± 2.3</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>CD4\textsuperscript{Cre} Itga\textsubscript{4}^{fl/fl}</td>
<td>11 of 22 (50%)*</td>
<td>19.3 ± 4.3*</td>
<td>2.5 ± 0.7</td>
</tr>
</tbody>
</table>

EAE was induced in control and CD4\textsuperscript{Cre} Itga\textsubscript{4}^{fl/fl} mice. The table shows the mean of 3 independent experiments. *Data were analyzed using Student’s t test (p<0.005).
Supplemental FIGURE 1. Characterization of Itg and cytokine expression on T cell subsets from Itgα4^fl/fl CD4^Cre and wild type mice. (A, B) WT mice were immunized with MOG_{35-55} in CFA. Ten days later, draining LNs (dLN) were collected and single-cell suspension prepared. Enriched CD4^+ T cells were stimulated with PMA and ionomycin for 3 hours and stained for IFN_{γ} and IL17A by intracellular (A, left) or secretion assay (A, right). (B) Itgβ1 (left) and Itgβ7 expression (right) gated on Th1 (IFN_{γ}^+, blue line) and Th17 subsets (IL17A^+, red line) stained by secretion assay. Filled histograms represent the isotype staining. Histograms are representative of three independent experiments. (C, E and F) Control and CD4^Cre Itgα4^fl/fl mice were immunized for EAE development with MOG_{35-55}/CFA. dLN and CNS were collected 8 and 20 days after immunization respectively. Itgα4 expression was evaluated on CD4^+ (top) and CD8^+ T cells (bottom) from control (blue line) and CD4^Cre Itgα4^fl/fl mice (red line) in dLN (left) and CNS (right). Overlays are representative of 4 different experiments. Note equivalent deletion of Itgα4 on CD4^+ and CD8^+ T cells isolated from LN and CNS. (D) CD4^+ T cells from CD4^Cre Itgα4^fl/fl, Itgα4^fl/fl and WT mice were isolated by MACS positive purification (overall purity of CD4^+ T cells: 90%) and stimulated with plate bound anti-CD3/CD28 antibodies (both at 1µg/ml) for 48 hours. Genomic DNA was isolated and deletion of Itgα4 at the genomic level was determined using previously described primers (Priestley GV. and al, Blood 2007, 109(1):109-11). Upper band detects Itgα4 deleted allele (∆), middle band detects floxed allele (floxed) and lower band detects wild type allele (WT). (E) Itgβ1 (left) and Itgβ7 (right) expression gated on CD4^+ T cells from control (blue line) and CD4^Cre Itgα4^fl/fl mice (red line). (F) Itgα4 expression was evaluated on Th1 cells (dark line) and Th17 cells (green line) from dLN of CD4^Cre Itgα4^fl/fl mice using secretion assay. (G) CD4^Cre Itgα4^fl/fl and Itgα4^fl/fl mice were immunized for EAE development with MOG_{35-55} in CFA and pertussis toxin. Eight days later, CNS were collected and single cell suspensions prepared. Cells were stimulated with PMA/ionomycin and analyzed for cytokine secretion by intracellular cytokines staining. Percentages of IFN_{γ} and IL17A infiltrating CD4^+ T cells recovered from the CNS of non sick mice were determined and used to calculate the ratio of Th1/Th17 cells in the CNS of CD4^Cre Itgα4^fl/fl (dark) and Itgα4^fl/fl mice (white)(*p<0.01). Data representative of 2 independent experiments with 6 mice per group.