



Expression of $\alpha\upsilon\beta 8$ integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice

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Th17 cells promote a variety of autoimmune diseases, including psoriasis, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. TGF- β is required for conversion of naive T cells to Th17 cells, but the mechanisms regulating this process are unknown. Integrin $\alpha\upsilon\beta 8$ on DCs can activate TGF- β , and this process contributes to the development of induced Tregs. Here, we have now shown that integrin $\alpha\upsilon\beta 8$ expression on DCs plays a critical role in the differentiation of Th17 cells. Th17 cells were nearly absent in the colons of mice lacking $\alpha\upsilon\beta 8$ expression on DCs. In addition, these mice and the DCs harvested from them had an impaired ability to convert naive T cells into Th17 cells *in vivo* and *in vitro*, respectively. Importantly, mice lacking $\alpha\upsilon\beta 8$ on DCs showed near-complete protection from experimental autoimmune encephalomyelitis. Our results therefore suggest that the integrin $\alpha\upsilon\beta 8$ pathway is biologically important and that $\alpha\upsilon\beta 8$ expression on DCs could be a therapeutic target for the treatment of Th17-driven autoimmune disease.

Introduction

For the adaptive immune system to elicit an effective response to different types of pathogens, naive T cells must undergo a series of epigenetic changes that specialize them for specific responses. Th17 cells develop from naive T cells and aid in the destruction of certain bacteria and fungi. The cytokines IL-17A, IL-17F, and IL-22, produced by these cells, support the recruitment and activation of neutrophils and drive the production of antimicrobial peptides by epithelial cells (1). Because of the prominent role these cells play in host defense and autoimmune disease, the mechanisms that regulate Th17 development are a topic of intensive investigation.

IL-6 and TGF- β are sufficient to differentiate Th17 cells from naive CD4⁺ T cells (2–4). Other cytokines, including IL-21 and IL-23, augment Th17 conversion and expand the population (5, 6). TGF- β directs the transcriptional program of Th17 cells by inducing expression of the Th17 lineage-specific transcription factor ROR γ t (7). TGF- β also stimulates expression of Foxp3, a transcription factor that is required for Treg function, and IL-6 is required to mediate the stabilization of ROR γ t and repression of Foxp3 (8). TGF- β is also necessary for expression of the IL-23 receptor on T cells, which allows the Th17 population to expand in response to IL-23 (4). While TGF- β is known to be a critical factor for Th17 differentiation and function, the mechanisms that regulate TGF- β activity in Th17 development have not been described.

TGF- β is produced in a latent form and must be activated to exert a biological effect. Each of the 3 TGF- β mammalian genes encode a translation product consisting of an N-terminal propeptide

(termed “latency-associated peptide” [LAP]) and bioactive TGF- β . This product is cleaved intracellularly by furin, and LAP associates with TGF- β in a noncovalent fashion to form the small latent complex. In most cells, LAP is covalently attached to 1 of 3 latent TGF- β binding proteins prior to secretion to form a large latent complex (9). TGF- β is generally inactive when it is incorporated into a small latent complex or large latent complex and is unable to bind TGF- β receptors. Latent TGF- β has been reported to be activated by proteases, thrombospondin-1, reactive oxygen species, and, at least for TGF- β 1 and - β 3, the integrins $\alpha\upsilon\beta 6$ and $\alpha\upsilon\beta 8$ (10, 11). A prominent role for integrin-mediated TGF- β activation is suggested by recent evidence that mice expressing a mutant form of TGF- β 1 that cannot be activated by integrins phenocopy mice completely lacking TGF- β 1 (12), and mice lacking $\alpha\upsilon\beta 8$ treated at birth with antibodies blocking $\alpha\upsilon\beta 6$ express all of the phenotypic features of mice lacking TGF- β 1 and TGF- β 3 (13).

We have recently shown that mice lacking $\alpha\upsilon\beta 8$ on DCs develop colitis and autoimmunity and have a quantitative defect in development of CD4⁺ Foxp3⁺ Tregs in the colon (14), demonstrating a critical role for this pathway in maintenance of immune homeostasis. However, the importance of this pathway in the induction of effector Th17-mediated immune responses is completely unknown. Because of the prominent role of TGF- β in Th17 cell induction and the absence of any data describing how TGF- β gets activated to induce these cells, we sought to determine what role integrin $\alpha\upsilon\beta 8$ on DCs plays in the development of Th17 cells.

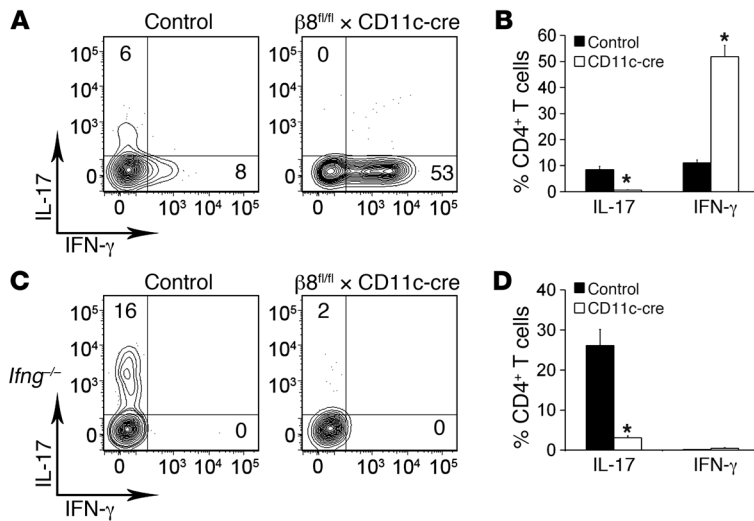
Results

We examined the colons of mice lacking $\alpha\upsilon\beta 8$ on DCs for the presence of IL-17–producing CD4⁺ T cells. Intracellular flow cytometry analysis of cells isolated from the colonic lamina propria of $\beta 8^{\text{fl/fl}} \times \text{CD11c-cre}$ mice showed a near absence of IL-17–expressing CD4⁺

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**Figure 1**

Impaired Th17 development in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (A and C) Flow cytometry plots of control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ (A) or *Ifng*^{-/-} control and *Ifng*^{-/-} $\beta 8^{fl/fl} \times CD11c\text{-cre}$ (C) colon lamina propria CD4⁺ T cells stained for IL-17 and IFN- γ after activation with PMA/ionomycin. Numbers in quadrants indicate the percentage of positive cells. (B and D) Percentage of IL-17- and IFN- γ -producing CD4⁺ T cells isolated from the colon lamina propria of control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ (B) or *Ifng*^{-/-} control and *Ifng*^{-/-} $\beta 8^{fl/fl} \times CD11c\text{-cre}$ (D) mice after activation with PMA/ionomycin. Data represent the mean \pm SEM of 3 independent experiments and more than 6 mice per group. **P* < 0.005.

T cells ($0.6\% \pm 0.2\%$), whereas $8.5\% \pm 1.3\%$ of colonic CD4⁺ T cells in control mice expressed IL-17 (Figure 1, A and B). In contrast, in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice a majority ($52\% \pm 4.4\%$) of CD4⁺ T cells expressed IFN- γ (Figure 1B). Since IFN- γ impedes development of Th17 cells in vitro, we examined whether increased production of IFN- γ was responsible for impaired Th17 development in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice by crossing them to mice deficient in IFN- γ . The colons of *Ifng*^{-/-} $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice also showed a dramatic reduction in the number of CD4⁺ IL-17⁺ cells, with only $3.1\% \pm 0.5\%$ of the CD4⁺ cells expressing IL-17 compared with $26\% \pm 4.0\%$ in control *Ifng*^{-/-} mice (Figure 1, C and D). Interestingly, *Ifng*^{-/-} $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice exhibited colitis and enlarged mesenteric lymph nodes that closely resembled $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice, suggesting that the colitis seen in the absence of $\alpha\beta 8$ on DCs occurs independently of IFN- γ (data not shown). We previously reported that $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice have a decreased frequency of Tregs in the colon but a normal proportion in the spleen (14). Since Tregs are significant producers of TGF- β , we questioned whether a decreased frequency of Tregs in the colon could decrease the amount of total TGF- β available for Th17 development in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. We found no significant difference in the amount of TGF- β in the colons from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (417 ± 29 and 384 ± 13 pg/ml; *P* = 0.16). These data indicate that excess production of IFN- γ and total TGF- β concentrations were not responsible for the absence of Th17 cells in the colons of $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice.

To determine the potential clinical relevance of impaired IL-17 production by T cells stimulated with $\alpha\beta 8$ -deficient DCs, we employed the EAE disease model. The EAE model has been extensively studied and depends on the formation of pathogenic IL-17-producing T cells. We immunized control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice for EAE and scored them based on paralysis severity. $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice were almost completely protected from paralysis (3.1 ± 0.2 mean peak EAE clinical score in control mice vs. 0.2 ± 0.1 in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice; Figure 2A). We then examined the CNS of mice at peak disease for the presence of Th17 cells. As expected, induction of EAE caused a marked accumulation of CD4⁺ and CD8⁺ T cells in the CNS of control mice, but the numbers of T cells were dramatically reduced in the CNS of $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (Figure 2B). Of the CD4⁺ T cells that were present in the CNS

of in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice, $1.4\% \pm 0.4\%$ expressed IL-17 (Figure 2, C and D). In contrast, $23\% \pm 2\%$ of CD4⁺ T cells produced IL-17 in the CNS of control mice (Figure 2, C and D).

Since Th17 cells were nearly absent in the CNS of $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice and these mice also exhibited a marked reduction in the total number of CNS T lymphocytes, we examined the periphery of these mice for Th17 cells before onset of EAE. At day 8 after immunization for EAE, we observed $9.2\% \pm 3\%$ of CD4⁺ T cells producing IL-17 in the draining lymph nodes of control mice, but only $0.6\% \pm 0.1\%$ in the draining lymph nodes of $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (Figure 2, E and F). To determine whether mice lacking integrin $\alpha\beta 8$ on DCs could develop EAE if the T cell priming phase was bypassed, we transferred activated 2D2 TCR transgenic T cells (transgenic for a T cell receptor to the MOG₃₅₋₅₅ peptide derived from myelin oligodendrocyte protein) into control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. Control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice developed similar EAE after passive immunization with activated 2D2 T cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43786DS1). We also checked whether $\beta 8$ expression on microglia, an important antigen-presenting cell in the CNS, was affected in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. We found no difference in microglia $\beta 8$ mRNA expression from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice ($1.3 \times 10^{-3} \pm 4.2 \times 10^{-4}$ and $1.1 \times 10^{-3} \pm 2.5 \times 10^{-4}$; *P* = 0.35). These data support a critical role for induction of Th17 cells by $\alpha\beta 8$ on DCs in periphery during the development of EAE.

To assess whether integrin $\alpha\beta 8$ on DCs participates in the conversion of naive CD4⁺ T cells to Th17 cells, we transferred Thy1.1-labeled naive CD4⁺ 2D2 TCR transgenic T cells into control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice immunized with an emulsion of MOG₃₅₋₅₅ and CFA. The Thy1.1 label allowed us to identify the transferred cells among endogenous CD4⁺ T cells upon re-isolation from the draining lymph nodes and spleen. When transferred Thy1.1⁺ 2D2 T cells were re-stimulated and analyzed 8 days after immunization, $60\% \pm 1.1\%$ of the cells produced IL-17 (Figure 3, A and B). However, only $22\% \pm 1.4\%$ Thy1.1⁺ 2D2 T cells produced IL-17 after transfer into $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (Figure 3, A and B). Additionally, IL-17-expressing 2D2 T cells recovered from $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice produced significantly less IL-17 as measured by MFI of IL-17 staining (Figure 3C). We also determined

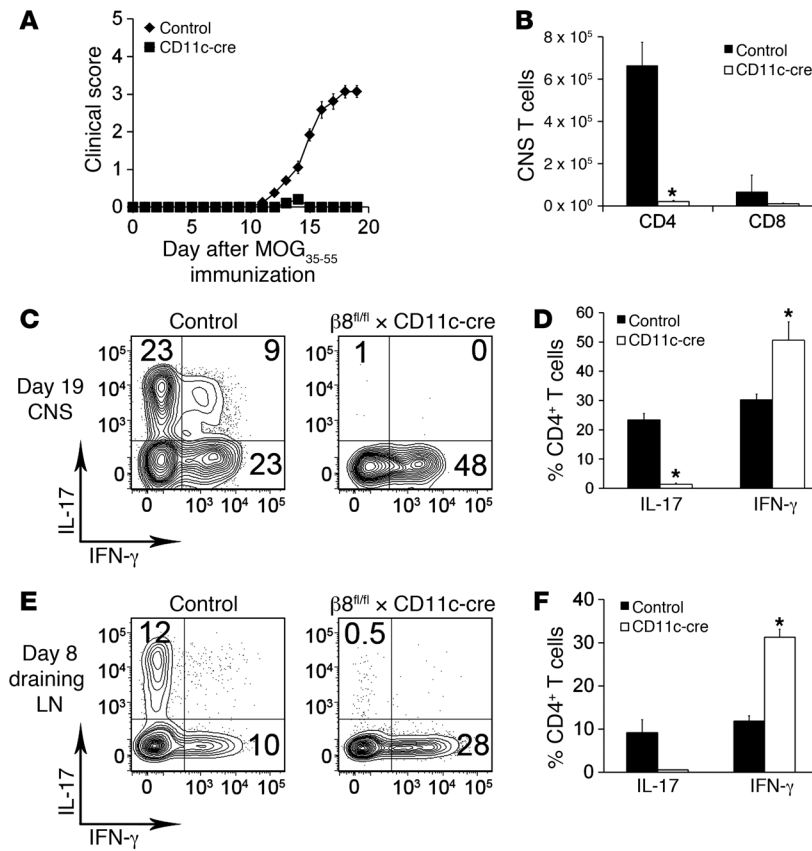


Figure 2

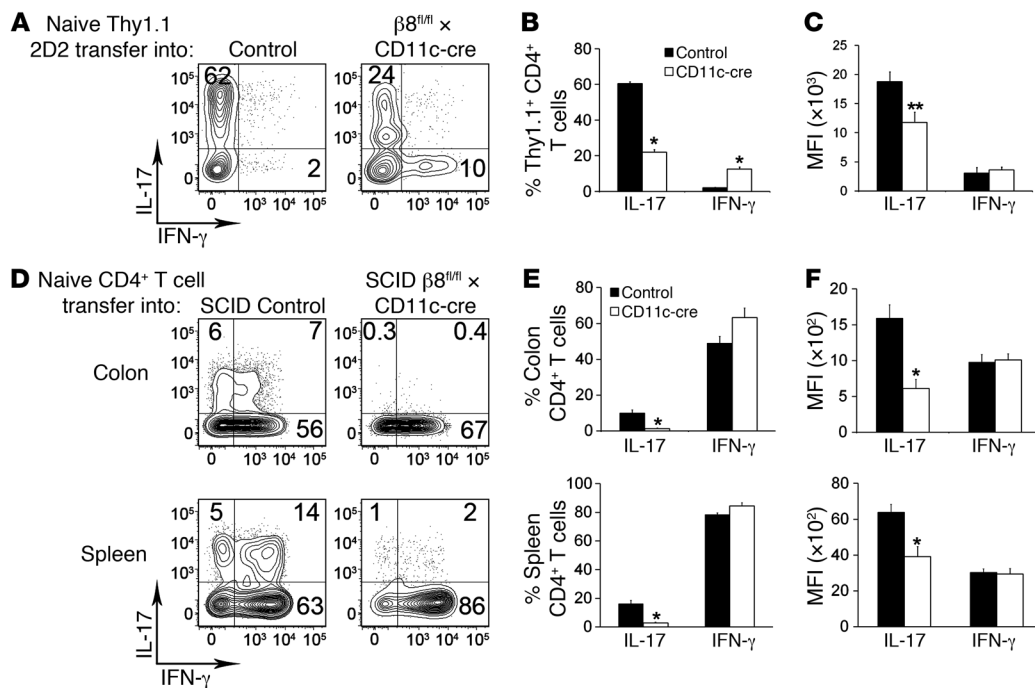
$\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice are protected from EAE. (A) Clinical scores of control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice after EAE immunization. (B) Total number of CD4 and CD8 T cells isolated from the CNS of control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice on day 19 after EAE immunization. (C) Flow cytometry plots of CD4⁺ T cells from the CNS of control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice on day 19 after EAE immunization. Cells were activated with PMA/ionomycin and then stained for IL-17 and IFN- γ . (D) Percentage of IL-17- and IFN- γ -producing CD4⁺ T cells from the CNS of control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (E) Flow cytometry plots of CD4⁺ T cells from the draining lymph nodes of control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice on day 8 after EAE immunization. Cells were activated with PMA/ionomycin and then stained for IL-17 and IFN- γ . (F) Percentage of IL-17- and IFN- γ -producing CD4⁺ T cells from the draining lymph nodes of control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. Data represent the means \pm SEM of 3 independent experiments and at least 10 mice in each group. * $P < 0.01$.

the role of integrin $\alpha\beta 8$ on DCs in the conversion of naive CD4⁺ T cells to Th17 cells by transferring polyclonal naive CD4⁺ T cells into $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice crossed onto the SCID background. SCID mice lack functional T and B cells and therefore allowed us to study the role of integrin $\alpha\beta 8$ on DCs in Th17 differentiation in the absence of endogenous lymphocytes. When naive CD4⁺ T cells were transferred into SCID control mice and recovered from the colonic lamina propria and spleen 12 days later, $10\% \pm 1.2\%$ and $16.1\% \pm 2.4\%$, respectively, produced IL-17 upon restimulation (Figure 3, D and E). However, only $1.2\% \pm 0.3\%$ and $2.8\% \pm 0.6\%$ of CD4⁺ T cells recovered from the colonic lamina propria and spleen, respectively, of SCID $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice produced IL-17. Furthermore, the few IL-17-expressing CD4⁺ T cells isolated from SCID $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice produced significantly less IL-17 as measured by MFI of IL-17 staining (Figure 3F). These data indicate that integrin $\alpha\beta 8$ on DCs is critical for the development of Th17 cells in response to antigen challenge in vivo.

To examine the mechanism by which loss of $\alpha\beta 8$ on DCs protected mice from EAE and impaired Th17 differentiation, we characterized DCs from the draining lymph nodes of $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. DCs have been categorized into the lymphoid, myeloid, and plasmacytoid groups based on their developmental origin and function. The presence of these DC populations in the draining lymph nodes of control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice was determined 7 days after immunization for EAE. We observed $31.4\% \pm 1.7\%$ lymphoid, $66.8\% \pm 1.7\%$ myeloid, and $3.9\% \pm 0.6\%$ plasmacytoid DCs in control mice and $35.1\% \pm 3.4\%$ lymphoid, $62.6\% \pm 3.4\%$ myeloid, and $4.5\% \pm 0.6\%$ plasmacytoid DCs in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (Figure 4, A and B). Next, we checked the ability of DCs from $\beta 8^{fl/fl} \times$

CD11c-cre mice to stimulate the proliferation of naive T cells after immunization for EAE. At day 7 after immunization, there were $6.0\% \pm 1.5\%$ myelin oligodendrocyte glycoprotein-specific (MOG-specific) T cells after enrichment for tetramer-labeled cells in control mice and $8.1\% \pm 0.8\%$ in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice (Figure 4, C and D). Furthermore, we saw that MOG-specific TCR transgenic T cells (clone 2D2) adoptively transferred into control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice underwent a similar number of cell divisions following EAE immunization (Figure 4, E and F). We then analyzed the expression of costimulatory and coinhibitory molecules on DCs from $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. As shown in Table 1, no significant differences were found in the expression of CD40, CD80, CD86, PD-L1, PD-L2, ICAM-1, OX40L, ICOSL, or MHCII. Finally, we examined the ability of DCs from $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice to produce cytokines related to the development of Th17 cells. As shown in Table 2, we found no significant differences in the levels of *Il1b*, *Il6*, *Il23*, *Tgfb*, or *Tnfa* mRNA or secreted protein. We also measured the effects of CFA immunization on DC $\beta 8$ integrin expression in control mice and found that CFA slightly reduced $\beta 8$ mRNA, from $5.0 \times 10^{-5} \pm 7.3 \times 10^{-6}$ to $2.4 \times 10^{-5} \pm 3.2 \times 10^{-6}$ ($P = 0.037$). Collectively, these data suggest that DCs from $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice are similar to control mice in their composition, ability to stimulate naive CD4 T cell proliferation, and capacity to make cytokines that promote Th17 differentiation.

Integrin $\alpha\beta 8$ controls the activation of TGF- $\beta 1$ and - $\beta 3$ through interaction with an Arg-Gly-Asp sequence on the LAP of the latent TGF- β complex (11). To determine whether integrin $\alpha\beta 8$ on DCs regulates Th17 development through activation of latent TGF- β , we used an in vitro assay to monitor

**Figure 3**

Blunted Th17 conversion of naive CD4⁺ T cells adoptively transferred into $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (A) Flow cytometry plots of Thy1.1 2D2 TCR transgenic T cells isolated from the spleen and draining lymph nodes 8 days after transfer into control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice immunized with MOG plus CFA. Isolated cells were activated with PMA/ionomycin and stained for IL-17 and IFN- γ . Numbers in quadrants indicate the percentage of positive cells. (B) Percentage of IL-17- and IFN- γ -producing Thy1.1⁺CD4⁺ T cells isolated from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (C) MFI of IL-17 and IFN- γ staining in Thy1.1⁺CD4⁺ T cells isolated from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (D) Flow cytometry plots of polyclonal CD4⁺ T cells isolated from the colonic lamina propria (top) or spleen (bottom) 12 days after transfer into SCID control or SCID $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. Isolated cells were activated with PMA/ionomycin and stained for IL-17 and IFN- γ . Numbers in quadrants indicate the percentage of positive cells. (E) Percentage of IL-17- and IFN- γ -producing CD4⁺ T cells isolated from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. (F) MFI of IL-17 and IFN- γ staining in CD4⁺ T cells isolated from control and $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. Data represent the mean \pm SEM of 2 independent experiments and 3 to 5 mice per group. * $P < 0.005$; ** $P < 0.01$.

Th17 development in the presence of control or $\alpha\beta 8$ -deficient DCs. As shown in Figure 5, $\alpha\beta 8$ -deficient DCs cultured with naive CD4⁺ 2D2 T cells under conditions that drive Th17 development (i.e., IL-1 β and IL-6), but in the absence of exogenous TGF- β , showed a reduced ability to promote IL-17 production by T cells. However, IL-17 production after culture with $\alpha\beta 8$ -deficient DCs was restored by the addition of exogenous TGF- β (Figure 5, A-C). Furthermore, the addition of a TGF- β -neutralizing antibody significantly reduced IL-17 production by T cells cultured with control DCs but had a minimal effect on $\alpha\beta 8$ -deficient DCs (Figure 5, A-C). To confirm that IL-17 produced by T cells in this assay was from bona fide Th17 cells, we measured IL-17F, another canonical Th17 cytokine. Indeed, IL-17F was detected in the supernatant of cultures grown without the addition of exogenous TGF- β and was significantly reduced in cultures with $\alpha\beta 8$ -deficient DCs (Figure 5D). Furthermore, we measured mRNA expression of 2 genes that have been shown previously to be regulated by TGF- β – *Rorc*, which encodes the Th17-specific transcription factor ROR γ t, and *Il23r*, which encodes the IL-23 receptor, a receptor required for clonal expansion of Th17 cells. We detected transcripts of these genes in T cells from cultures with control DCs but found a dramatic reduction in expression of these genes in T cells from cultures with $\alpha\beta 8$ -deficient DCs (Figure 5E).

The mechanism by which integrin $\alpha\beta 8$ on DCs presents active TGF- β to induce IL-17 expression in T cells is unknown. One possibility is that latent TGF- β is constitutively activated by $\alpha\beta 8$ on DCs to create a surrounding milieu of active TGF- β (i.e., a field effect). Alternatively, $\alpha\beta 8$ might only induce IL-17 expression in T cells if it activates TGF- β in the context of antigen presentation. This would be a teleologically attractive mechanism to tightly regulate, in space and time, this important immunologic decision. To distinguish between these mechanisms, we cultured naive CD4⁺ 2D2 T cells under Th17-polarizing conditions (in the absence of exogenous TGF- β) with mixtures of $\alpha\beta 8$ -deficient DCs and $\alpha\beta 8$ -containing DCs that express either matched or mismatched MHCII. Thus, MHCII-mismatched DCs would be unable to present MOG peptides to 2D2 T cells. We first established that a 1:1 mixture of control and $\alpha\beta 8$ -deficient DCs was able to completely rescue the defect in IL-17 induction seen with $\alpha\beta 8$ -deficient DCs alone (Figure 6). We then cultured $\alpha\beta 8$ -expressing, MHCII-mismatched (H2q MHC haplotype from FVB mice) DCs at a 4:1 mixture with $\alpha\beta 8$ -deficient DCs (H2b MHC haplotype from C57BL/6 mice). MHCII-mismatched DCs were unable to rescue the impaired IL-17 production seen with $\alpha\beta 8$ -deficient DCs (Figure 6). These data suggest that Th17 cell induction requires simultaneous presentation of antigen (by MHCII) and activation of TGF- β by integrin $\alpha\beta 8$ on the same DCs.

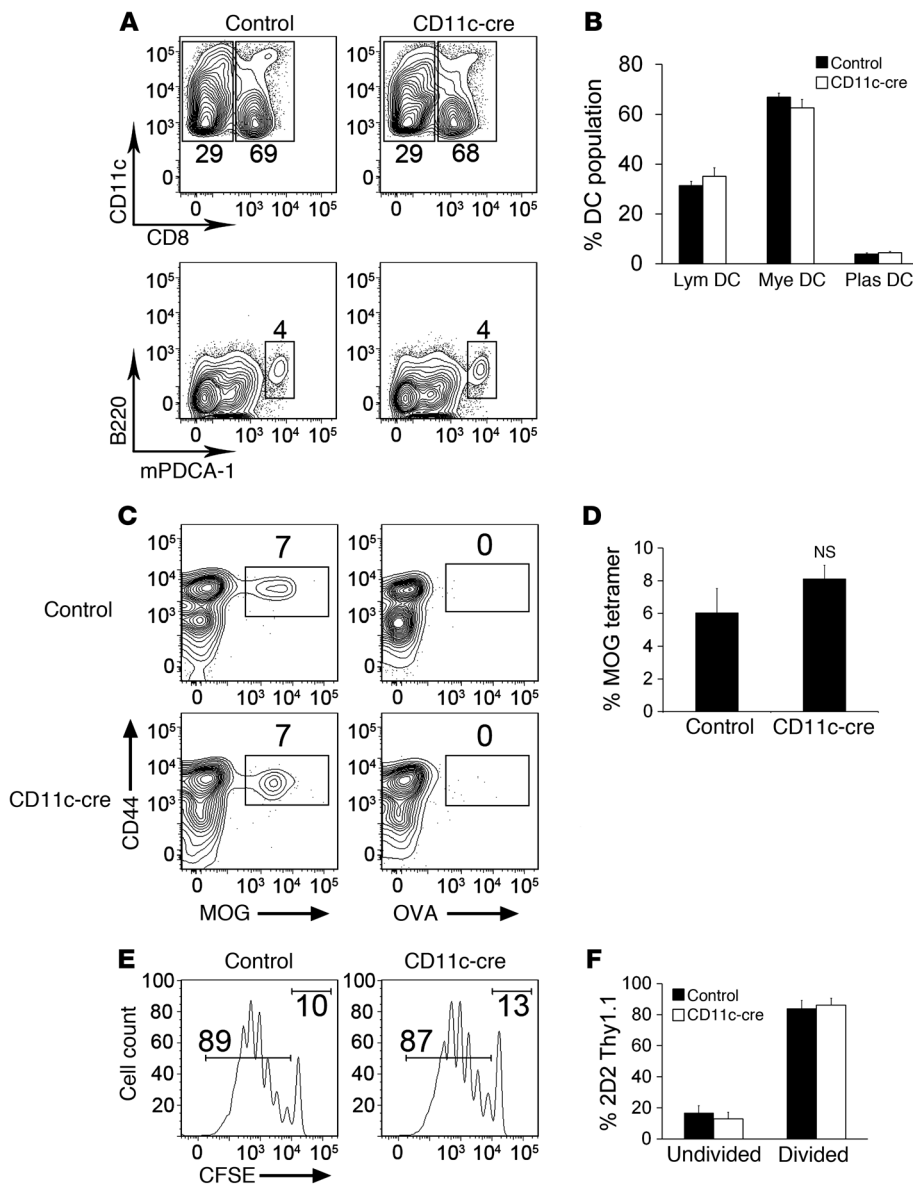


Figure 4

Characterization of draining lymph node DCs in $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. **(A)** Flow cytometry plots of MHCII⁺CD11c⁺ DCs isolated from the draining lymph nodes of control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice 7 days after in vivo stimulation with CFA. DCs within plots are gated on CD8⁺ (lymphoid) and CD8⁻ (myeloid) DCs (top) or B220⁺mPDCA-1⁺ (plasmacytoid; bottom). Numbers next to the gates indicate the percentage of CD8⁺ or CD8⁻ MHCII⁺CD11c⁺ cells (top) or B220⁺mPDCA-1⁺MHCII⁺CD11c⁺ cells (bottom). **(B)** Percentage of lymphoid (Lym), myeloid (Mye), and plasmacytoid (Plas) DCs isolated from control $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. **(C)** Flow cytometry plots of CD4⁺ T cells isolated from control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice 7 days after immunization with MOG plus CFA and stained with MHCII tetramers loaded with MOG or OVA peptide. Numbers above the gates indicate the percentage of cells labeled with tetramer after enrichment for tetramer-positive cells. **(D)** Percentage of CD4⁺ T cells labeled with MOG tetramer after enrichment for tetramer-positive cells isolated from control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice. **(E)** Flow cytometry histograms of CFSE-stained Thy1.1⁺2D2⁺ TCR transgenic T cells isolated from control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice 7 days after immunization with MOG plus CFA. Numbers above gates indicate the percentage of divided (left gate) and undivided (right gate) cells. **(F)** Percentage of undivided and divided 2D2⁺ Thy1.1⁺ cells isolated from control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice.

Discussion

How TGF- β is regulated in the extracellular space and presented to naive T cells during Th17 development has been unknown. Our results strongly suggest that the integrin $\alpha v\beta 8$ on DCs is required for maximal Th17 development. This effect appears to be due to TGF- β activation by this integrin, since the defect in Th17 induction by DCs lacking $\alpha v\beta 8$ can be completely rescued by the addition of exogenous TGF- β . Furthermore, these data suggest that activation of TGF- β by integrin $\alpha v\beta 8$ on DCs may be a novel target in the development of therapeutics for autoimmune diseases.

We tested the in vivo relevance of TGF- β activation by integrin $\alpha v\beta 8$ on DCs in the EAE disease model. The EAE model is widely used to study the formation of pathogenic T cells to self-molecules and has been utilized as a model of multiple sclerosis. Our result that mice lacking $\alpha v\beta 8$ on DCs are almost completely protected from EAE suggests that TGF- β activation by $\alpha v\beta 8$ on DCs is a required event in the formation of pathogenic self-reactive T cells. The nearly complete absence of CD4⁺ T cells in the CNS of EAE-immunized

mice lacking $\alpha v\beta 8$ on DCs suggests that these mice may be protected from EAE in part due to impaired formation of MOG-reactive Th17 cells. This concept is also supported by our observation that at day 8 following EAE immunization, a time point before the onset of symptomatic EAE, Th17 cells failed to develop in the draining lymph nodes of mice lacking in $\alpha v\beta 8$ on DCs. These data agree with a previous report that mice engineered to express a dominant-negative TGF- β receptor-II in T cells are protected from EAE through a defect in Th17 cell induction (15). Interestingly, transgenic mice that overexpress TGF- β within T cells develop more severe EAE than wild-type mice and also exhibit greater numbers of Th17 cells in the CNS (2). While Th17 cells are regarded as essential for EAE, several recent studies have called into question which type or types of CD4⁺ T cells are encephalitogenic in this model (16, 17). In the present study, the scarcity of Th17 cells found in the CNS and periphery of EAE-primed mice lacking $\alpha v\beta 8$ on DCs, and their dramatic protection from EAE, indicates that TGF- β activated via $\alpha v\beta 8$ on DCs is an important step in the generation of encephalitogenic CD4⁺ T cells.



Table 1
Costimulatory and coinhibitory protein expression on DCs from $\beta 8^{fl/fl} \times CD11c$ -cre mice

Protein	Control	CD11c-cre	P value
CD40	10,539 ± 287	11,158 ± 288	0.09
CD80	407 ± 7	722 ± 221	0.10
PD-L1	49,069 ± 3,690	48,896 ± 2,318	0.48
CD86	6,048 ± 231	5,894 ± 195	0.31
ICAM-1	6,144 ± 29	5,901 ± 143	0.07
OX40L	2,463 ± 420	2,570 ± 516	0.44
PD-L2	1,022 ± 71	1,136 ± 41	0.11
ICOSL	1,054 ± 89	965 ± 54	0.22
MHCII	3,824 ± 446	3,895 ± 236	0.45

DCs were isolated from the draining lymph nodes of control or $\beta 8^{fl/fl} \times CD11c$ -cre mice 7 days after immunization for EAE and stained with antibodies for the costimulatory or coinhibitory proteins listed above. Stained cells were analyzed by flow cytometry, and the MFI of each label was determined. Data represent the MFI ± SEM of labeled DCs from at least 4 mice in each group.

An absolute requirement for TGF- β in Th17 development has been controversial. A combination of TGF- β and IL-6 was first reported to be sufficient to induce Th17 differentiation in mice (2–4). Subsequently, 2 groups reported that TGF- β was not required for the conversion of naive T cells to Th17 cells in humans (18, 19). However, the human naive T cells used in those studies were isolated using protocols that may have introduced TGF- β contamination and were cultured with serum containing TGF- β . Subsequently, 3 groups used rigorous methods to exclude TGF- β contamination and showed that TGF- β is required for Th17 differentiation by human T cells (20–22). However, a recent study that used mice lacking the transcription factors required to drive Th1 and Th2 differentiation suggested that TGF- β may function indirectly in Th17 conversion by suppressing Th1 and Th2 factors, which in turn suppress ROR γ t (23). Nonetheless, TGF- β appears to be crucial for Th17 induction in vivo and at the site of T cell priming, as administration of a TGF- β -blocking antibody delivered with antigen and CFA abrogates Th17 induction and EAE (15). The data presented in this study are also in line with previous findings on a requirement for TGF- β in the generation of intestinal lamina propria Th17 cells. Lamina propria Th17 cells are absent in *Tgfb1*^{C33S/C33S} knockin mice, which have impaired TGF- β activation through inhibition of covalent TGF- β attachment to latent TGF- β binding protein 1 (24). Furthermore, Atarashi et al. demonstrated that an α v β 8-expressing CD11c⁺ DC lamina propria subset preferentially induces Th17 generation from naive T cells in vitro (25).

We were concerned that the increased production of IFN- γ by T cells from mice lacking α v β 8 on DCs might contribute to the markedly impaired Th17 cell induction in these animals. Our finding that mice deficient in both IFN- γ and α v β 8 on DCs still have a dramatic defect in Th17 cell induction excludes that explanation. We also saw that some MOG-specific TCR transgenic T cells adoptively transferred into mice lacking α v β 8 on DCs were able to express IL-17, albeit at lower levels than those transferred into control mice. This finding suggests that the presence α v β 8 on DCs may not be an absolute requirement for the generation of Th17 cells, or alternatively, that a small number of memory cells were present in the naive transferred cells.

The present study did not examine which cell type or types are responsible for producing the latent TGF- β required for Th17 development. A previous report from Li et al. showed that deletion of the *Tgfb* gene in T cells protects mice from EAE and Th17 development (26). In context with the findings presented here, these data suggest a model whereby T cells provide their own latent TGF- β , which then gets activated by α v β 8 on DCs. However, DCs also produce significant amounts of TGF- β , and it has been reported that a subset of DCs in the CNS that preferentially induce Th17 cells also produce elevated levels of TGF- β (27). These data suggest that TGF- β signaling within the immune system may be regulated by both expression and activation of the latent form.

The spatial and temporal regulation of TGF- β in the extracellular space during Th17 development has been unknown. In the current study, we provide a partial answer to this question by showing that α v β 8 integrin-mediated TGF- β activation by DCs can only induce antigen-specific T cells to become Th17 cells if the same cell that is expressing the integrin is also actively presenting antigen in the context of MHCII. Since TGF- β signaling in T cells has the potential to direct the formation of a pro-inflammatory (i.e., Th17) or immunosuppressive (i.e., Treg) response to antigen, it makes teleologic sense for this process to be tightly regulated in space and time. Our results suggest that one important factor is precise spatial regulation of delivery of the active TGF- β , potentially allowing the same DCs to provide active TGF- β and other polarizing cytokines (for example, IL-6 in the context of Th17 cell induction). Presentation of active TGF- β when T cells are in close proximity to DCs would allow for precise control of T cell fate at a time when T cells are receiving signals from costimulatory and coinhibitory molecules as well as secreted cytokines.

Collectively, the data presented here indicate that integrin α v β 8 on DCs facilitates the development of Th17 cells, and consequently contributes to IL-17-mediated CNS inflammation, through activation of TGF- β . This finding suggests that the α v β 8 integrin, or the steps upstream and downstream of α v β 8-mediated TGF- β activation, could be novel targets for treatment of multiple sclerosis and other autoimmune diseases.

Table 2
Production of Th17-promoting cytokines by DCs from $\beta 8^{fl/fl} \times CD11c$ -cre mice

Gene/protein	Control	CD11c-cre	P value
Relative mRNA expression			
<i>Il1b</i>	$1.6 \times 10^{-2} \pm 1.5 \times 10^{-3}$	$9.6 \times 10^{-3} \pm 8.3 \times 10^{-4}$	0.17
<i>Il6</i>	$3.7 \times 10^{-4} \pm 1.6 \times 10^{-5}$	$2.9 \times 10^{-4} \pm 2.2 \times 10^{-5}$	0.14
<i>Il23</i>	$1.5 \times 10^{-5} \pm 5.1 \times 10^{-6}$	$1.3 \times 10^{-5} \pm 4.6 \times 10^{-6}$	0.31
<i>Tgfb</i>	$1.2 \times 10^{-3} \pm 9.0 \times 10^{-5}$	$8.8 \times 10^{-4} \pm 2.0 \times 10^{-4}$	0.23
<i>Tnfa</i>	$5.5 \times 10^{-4} \pm 1.1 \times 10^{-5}$	$8.5 \times 10^{-4} \pm 4.8 \times 10^{-5}$	0.19
Secreted protein (pg/ml)			
IL-1 β	104 ± 7	122 ± 5	0.07
IL-6	9,022 ± 1,182	9,767 ± 135	0.30
IL-23	1,272 ± 33	1,302 ± 25	0.12
TGF- β	543 ± 17	542 ± 10	0.49
TNF- α	356 ± 45	380 ± 10	0.33

Relative mRNA expression was determined by qRT-PCR of RNA isolated from draining lymph node DCs of control or $\beta 8^{fl/fl} \times CD11c$ -cre mice 7 days after immunization for EAE. Th17-promoting cytokines were measured in the supernatants of draining lymph node DCs cultured in the presence of LPS (1 ng/ml) for 3 days.

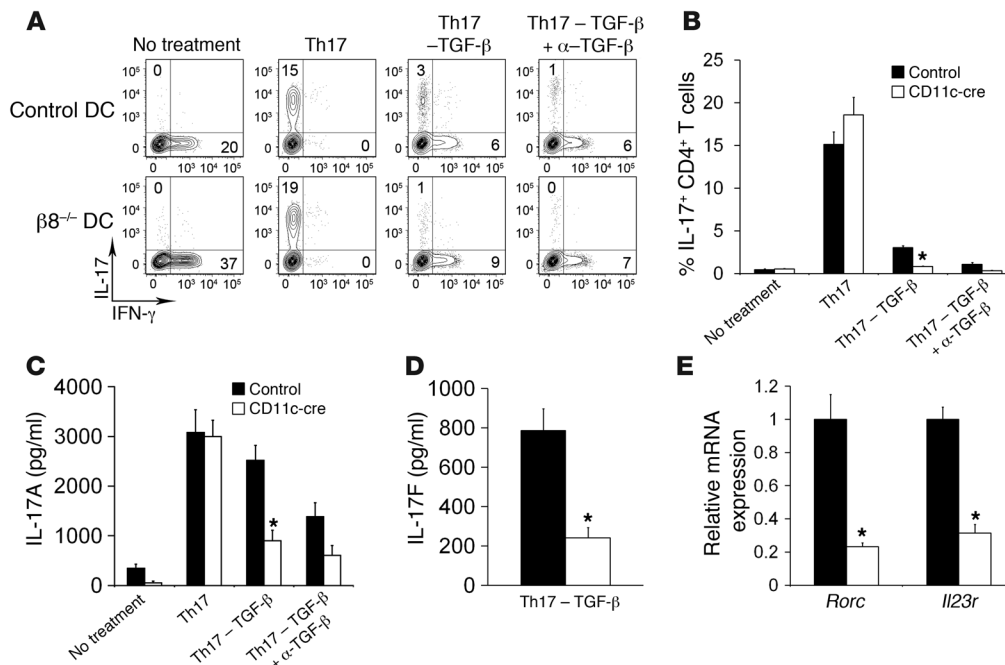


Figure 5

DCs from $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice fail to drive Th17 development through a defect in TGF- β activation. **(A)** Flow cytometry plots of naive CD4⁺ 2D2 TCR transgenic T cells after culture with DCs from control or $\beta 8^{fl/fl} \times CD11c\text{-cre}$ mice for 5 days. Cells were activated with PMA/ionomycin for 4 hours and then stained for IL-17 and IFN- γ . Cultures were grown under the following conditions: no treatment, Th17 (see Methods), Th17 without TGF- β , and Th17 with TGF- β -neutralizing antibody. **(B)** Percentage of IL-17-producing CD4⁺ 2D2 T cells after cultured as described in **A**. **(C)** Measurement of IL-17A in the supernatants of the cultures described in **A** using ELISA. **(D)** Measurement of IL-17F in the supernatant of the Th17 without the TGF- β condition described in **A**. **(E)** qRT-PCR analysis of the *Rorc* and *Il23r* genes from T cells after culture under the Th17 without the TGF- β condition described in **A**. Data represent the means \pm SEM of at least 3 independent experiments with 8 mice in each group. **P* < 0.01.

Methods

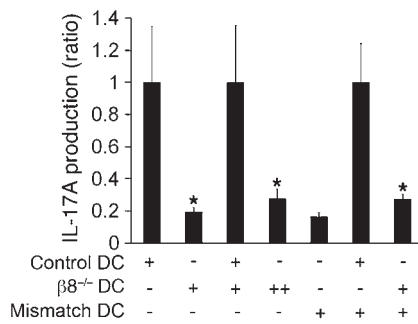
Mice. C57BL/6 $\beta 8^{fl/fl} \times CD11c\text{-cre}$ (14) and C57BL/6 2D2 TCR transgenic (28) mice have been previously described. C57BL/6 SCID mice (strain B6.CB17-Prkdc^{scid}/SzJ) were purchased from The Jackson Laboratory and crossed to C57BL/6 $\beta 8^{fl/fl} \times CD11c\text{-cre}$. FVB/NJ mice were purchased from The Jackson Laboratory. Mice used for all experiments were 8–12 weeks old and were housed under specific pathogen-free conditions in the Animal Barrier Facility of the University of California, San Francisco. All experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Flow cytometry. For intracellular cytokine analysis, cells were incubated for 4 hours at 37°C in complete RPMI containing 50 ng/ml PMA (Sigma-Aldrich), 1 μ M ionomycin (Sigma-Aldrich), and 2 μ M monensin (eBioscience). Cells were then washed with ice-cold PBS and blocked for 20 minutes at 4°C with PBS containing 2 μ g/ml α -CD16/32 (UCSF hybridoma core) and 10% heat-inactivated normal rat serum (Sigma-Aldrich). After washing with PBS, cells were incubated for 30 minutes in PBS with cell-surface antibodies and Aqua Live/Dead Fixable Dead cell stain (Invitrogen). Cells were then fixed and permeabilized with eBioscience Fix/Perm solution according to the manufacturer’s instructions. Intracellular cytokines were labeled with antibodies for 45 minutes at 4°C in permeabilization buffer (eBioscience). Cells were analyzed on an LSRII (BD Biosciences), and post-analysis of flow cytometry data was performed with FlowJo software (Tree Star Inc.). The following antibodies were used in experiments and were purchased from BD Biosciences – Pharmingen or eBioscience: anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD11c (clone N418), anti-MHCII (clone M5/114.15.2), anti-B220 (clone RA3-6B2), anti-mPDCA1 (clone

eBio927), anti-CD44 (clone IM7), anti-Thy1.1 (clone HIS51), anti-IL-17 (clone eBio17B7), anti-IFN- γ (clone XMG1.2), anti-PD-L1 (clone MIH5), anti-PD-L2 (clone 122), anti-ICOSL (clone HK5.3), anti-CD40 (clone 1C10), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), anti-ICAM-1 (clone YN1/1.7.4), and anti-OX40L (clone RM134L).

MOG tetramer staining. CD4 T cells expressing a MOG-specific TCR were stained with PE-labeled I-A(b) GWYRSPFSRVVH tetramer (NIH Tetramer Core Facility at Emory University) as previously described with modifications (29). Briefly, cells (1 \times 10⁸/ml) from the draining lymph nodes and spleen of mice at day 7 after EAE immunization were incubated with a 1:100 dilution of tetramer in HEPES-buffered DMEM plus 2% FBS for 1 hour at room temperature. Cells were then washed and incubated with anti-PE microbeads (Miltenyi Biotec) in HEPES-buffered DMEM plus 2% FBS for 30 minutes at 4°C. After washing, tetramer-labeled cells were enriched by passing over a magnetic column (LS column; Miltenyi Biotec) according to the manufacturer’s instructions. Eluted cells were stained for cell surface markers and analyzed on an LSRII as described above.

Lymphocyte isolation from colonic lamina propria. Lymphocytes were isolated from the colonic lamina propria as previously described with slight modification (30). Briefly, colons were removed, washed with ice-cold PBS, and cut into 2- to 3-cm-long pieces. Pieces were predigested in 5 ml of HBSS containing 5 mM EDTA and 1 mM DTT and rotated (180 rotations/min) for 20 minutes at 37°C to remove debris and intraepithelial lymphocytes. Pieces were then washed with PBS and predigested a second time. Pieces were next cut to 0.5 cm and digested in serum-free RPMI containing 0.1 U/ml liberase blendzyme 3 (Roche) and 10 U/ml DNase I (Roche) at 37°C for 20 minutes with rotation. After vortexing, the supernatants were

**Figure 6**

Th17 development through integrin $\alpha\beta 8$ on DCs is dependent on MHCII-TCR engagement. DCs were isolated from control, $\beta 8^{\text{fl/fl}}$ \times CD11c-cre, and MHCII-mismatched (H2q MHC haplotype from FVB strain) mice and cultured in different combinations with naive CD4⁺ 2D2 TCR transgenic T cells under Th17-driving conditions without exogenous TGF- β . Supernatants were collected and analyzed by ELISA for the presence of IL-17. Data are means \pm SEM of the ratio of IL-17 produced between control and $\beta 8^{\text{fl/fl}}$ \times CD11c-cre wells under each condition. Twice as many $\beta 8^{\text{fl/fl}}$ \times CD11c-cre DCs were present in 1 condition (indicated by ++) to account for a doubling in the number of DCs in the control plus $\beta 8^{-/-}$ group. MHCII-mismatched DCs were present in cultures at a 4:1 ratio, compared with control or $\beta 8^{-/-}$ DCs. * $P < 0.05$.

collected and placed on ice while the pieces were digested 2 more times. Supernatants from all 3 digestions were combined and washed with complete RPMI. Cells were then separated on a 40%/80% Percoll (Sigma-Aldrich) gradient and analyzed by flow cytometry.

EAE induction. Mice were given s.c. injections into the flanks and nape with 100 μ l of an emulsion containing 200 μ g MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK; Genemed Synthesis Inc.) and 200 μ g *Mycobacterium tuberculosis* H37 RA (Difco) in CFA. Mice also received 200 ng pertussis toxin (List Biologicals) i.p. on the day of immunization and 2 days later. Mice were scored for clinical disease in a blinded fashion as follows: 1, tail paralysis; 2, hindlimb weakness; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, moribund.

Lymphocyte isolation from the CNS. Lymphocytes were isolated from the CNS of mice as previously described (27). Briefly, mice were perfused with PBS and spinal cords removed. Spinal cords were cut into 2- to 3-cm-long pieces and digested in 0.2 U/ml liberase blendzyme 3 (Roche) and 50 U/ml DNase I (Roche) at 37°C for 30 minutes. Lymphocytes were then enriched by separation on a 30%/70% percoll gradient and analyzed by flow cytometry.

Adoptive transfer of naive CD4 T cells. Naive Thy1.1⁺ 2D2 T cells were isolated as described below and 7.5×10^4 were transferred i.v. into control or $\beta 8^{\text{fl/fl}}$ \times CD11c-cre mice. In some cases cells were labeled with CFSE (Invitrogen) before transfer by incubation with 5 μ M CFSE for 5 minutes at room temperature. Mice were then immunized for EAE as described above. Lymph nodes and spleens were removed 8 days after transfer, and Thy1.1⁺ 2D2 cells were analyzed by intracellular flow cytometry. Polyclonal naive CD4 T cells were isolated from the spleen and lymph nodes of a wild-type sex-matched C57BL/6 mouse using the CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec), and 2.0×10^6 cells were transferred i.v. into SCID control or SCID $\beta 8^{\text{fl/fl}}$ \times CD11c-cre mice.

Activation of 2D2 T cells for EAE passive immunization. CD4 T cells from C57BL/6 2D2 TCR transgenic mice were activated for EAE passive immunization as previously described (31). Briefly, 3×10^7 naive 2D2 T cells and 3×10^6 DCs were cultured on 10-cm dishes in the presence of MOG (10 μ g/ml), IL-12 (20 ng/ml), IL-18 (25 ng/ml), and IL-2 (100 U/ml) for 4 days. Cells were then activated on plates coated with anti-CD3 (1 μ g/ml)/anti-CD28 (1 μ g/ml) in the presence

of IL-12 (2 ng/ml), IL-18 (2.5 ng/ml), and IL-2 (10 U/ml) for 24 hours. Cells were then washed 3 times with PBS and transferred i.v. into mice.

DC isolation. DCs were isolated from the draining lymph nodes of $\beta 8^{\text{fl/fl}}$ \times CD11c-cre or sex-matched littermate control mice 7–8 days after receiving s.c. injections into the flanks and nape with 100 μ l of an emulsion containing equal parts PBS and 200 μ g *M. tuberculosis* H37 RA (Difco) in CFA. Lymph nodes were digested at 37°C for 30 minutes in serum-free RPMI containing 0.14 U/ml liberase (Roche) and 50 U/ml DNase I (Roche). CD11c⁺ cells were selected with anti-CD11c magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. CD11c⁺ DCs used were typically 90%–95% pure.

In vitro Th17 differentiation assay and cytokine measurement. DCs were isolated as described above and plated at 20,000 cells/well in complete Iscove modified Dulbecco medium (IMDM; Sigma-Aldrich). Naive 2D2 T cells were isolated from the lymph nodes and spleen of sex-matched 2D2 TCR transgenic mice using the CD4⁺ CD62L⁺ T cell isolation kit (Miltenyi Biotec), and 50,000 cells were added to each well along with MOG₃₅₋₅₅ (10 μ g/ml), IL-6 (50 ng/ml; Peprotech), IL-1 β (10 ng/ml; Peprotech), FICZ (6-formylindolo [3,2-b] carbazole; 300 nM; Biomol), and TGF- β (1 ng/ml; R&D Systems) or TGF- β -neutralizing antibody (clone 1d11; 50 μ g/ml). 2D2 T cells were harvested 5 days later and analyzed by flow cytometry or quantitative RT-PCR (qRT-PCR). Cytokines in the culture supernatants were measured with IL-17A (eBioscience) and IL-17F (eBioscience) ELISA reagent sets according to the manufacturer's instructions. Measurement of TGF- β in colon tissue and IL-1 β , IL-6, IL-23, TGF- β , and TNF- α production by DCs was performed using the Mouse FlowCytomix Kit (eBioscience) according to the manufacturer's instructions.

qRT-PCR. RNA was isolated from T cells and DC using an RNeasy Mini Kit (Qiagen). cDNA transcription and qPCR were performed using a SYBR-Greener Two-Step qRT-PCR kit (Invitrogen). Samples were amplified on an ABI 7900HT thermocycler and normalized to β -actin expression. Primers used were as follows: *B-actin* forward: TGTTACCAACTGGGACGACA, reverse: GGGGTGTTGAAGGTCTCAA; *Rorc* forward: GACAGGGAGCCAAGT-TCTCA, reverse: GGTGATAACCCCGTAGTGGA; *Il23r* forward: TTCAGAT-GGGCATGAATGTTTCT, reverse: CCAAATCCGAGCTGTTGTTCTAT; *Il1b* forward: GCAACTGTTCTGAACCAACT, reverse: ATCTTTTGGGGTCC-GTCAACT; *Il6* forward: TAGTCCTTCTACCCCAATTTC, reverse: TTG-GTCCTTAGCCACTCCTTC; *Il23* forward: ATGCTGGATTGCAGAG-CAGTA, reverse: ACGGGGCACATTATTTTAGTCT; *Tgfb* forward: CTCCCGTGGCTTCTAGTGC, reverse: GCCTTAGTTTGGACAGGATCTG; *Tnf* forward: CCCTCACACTCAGATCATCTTCT, reverse: GCTACGAC-GTGGGCTACAG; *Itgb8* forward: CTGAAGAAATACCCCGTGGA, reverse: ATGGGGAGGCATACAGTCT.

Statistics. The statistical significance of differences between groups was calculated with a 2-tailed Student's *t* test. Differences with a *P* value of less than 0.05 were considered statistically significant.

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