Neuropilin-1 attenuates autoreactivity in experimental autoimmune encephalomyelitis

Benjamin D. Solomon[a], Cynthia Mueller[a], Wook-Jin Chae[b], Leah M. Alabanza[a], and Margaret S. Bynoe[a,1]

[a]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853; and [b]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520

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Neuropilin-1 (*Nrp1*) is a cell surface molecule originally identified for its role in neuronal development. Recently, *Nrp1* has been implicated in several aspects of immune function including maintenance of the immune synapse and development of regulatory T (T_{reg}) cells. In this study, we provide evidence for a central role of *Nrp1* in the regulation of CD4 T-cell immune responses in experimental autoimmune encephalitis (EAE). EAE serves as an animal model for the central nervous system (CNS) inflammatory disorder multiple sclerosis (MS). EAE is mediated primarily by $CD4^+$ T cells that migrate to the CNS and mount an inflammatory attack against myelin components, resulting in CNS pathology. Using a tissue-specific deletion system, we observed that the lack of *Nrp1* on $CD4^+$ T cells results in increased EAE severity. These conditional knockout mice exhibit preferential T_H-17 lineage commitment and decreased T_{reg}-cell functionality. Conversely, $CD4^+$ T cells expressing *Nrp1* suppress effector T-cell proliferation and cytokine production both in vivo and in vitro independent of T_{reg} cells. *Nrp1*-mediated suppression can be inhibited by TGF-β blockade but not by IL-10 blockade. These results suggest that *Nrp1* is essential for proper maintenance of peripheral tolerance and its absence can result in unchecked autoreactive responses, leading to diseases like EAE and potentially MS.

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by progressive demyelination of the brain and spinal cord (1). MS patients develop paralysis because of immune-mediated axonal damage. MS is generally considered to be an autoimmune disease orchestrated by T_H-1 and T_H-17 lymphocytes, although various genetic and environmental factors also play a part in disease etiology (2, 3). Evidence for the role of immune cells in MS pathogenesis is provided by studies using the mouse model experimental autoimmune encephalomyelitis (EAE). In EAE, myelin-specific $CD4^+$ T lymphocytes migrate into the CNS and mediate neuronal demyelination and destruction similar to that seen in MS patients (4), leading to loss of motor function and paralysis.

Comparisons between the immune system and the CNS began with the naming of dendritic cells (5). For example, the term immunological synapse describes the junction formed between T cells and antigen-presenting cells (APCs), which resembles the synapse between neurons in both formation and architecture (6). In the nervous system, chemorepulsive factors, such as semaphorins, are required for guiding the formation of neuronal synapses. Several reports have also suggested important roles for semaphorins in the immune system (7, 8). Neuropilin-1 (*Nrp1*) is a type 1 transmembrane protein, originally identified for its role in the development of growing neurons, which can serve as a receptor for semaphorin-3A in combination with plexin molecules to regulate growth cone collapse (9–11). In addition, *Nrp1* is involved in the process of angiogenesis through interactions with vascular endothelial growth factor (VEGF) (12). *Nrp1* has been recently implicated to play a role in the immunological synapse (13) and has been reported to be constitutively expressed on murine $CD4^+CD25^+$ regulatory T (T_{reg}) cells, suggesting a potential role for *Nrp1* in the attenuation of autoreactive immune responses (14).

We have shown that mice epicutaneously immunized (ECi) with myelin peptide before induction of EAE show a significant degree of protection compared with non-ECi mice (15). Myelin-specific $CD4^+$ T cells from these ECi mice are able to confer protection from EAE to naïve recipient mice upon adoptive transfer (15). Through gene analysis, we observed that *Nrp1* is highly expressed on $CD4^+$ T suppressor cells from mice protected from EAE development by ECi with myelin antigen. We therefore examined the role of *Nrp1* in the immune response in EAE, because we hypothesized that *Nrp1* may have a protective function in EAE development.

Here, we show that overexpression of *Nrp1* attenuates EAE progression and, conversely, the lack of *Nrp1* results in disease aggravation. This increase in disease severity occurs in a $CD4^+$ T-cell–dependent manner (that skews the balance of helper T cells away from regulatory subtypes toward inflammatory T_H-17 subtypes). We demonstrate that the suppressive effect of $CD4^+$ T cells from myelin antigen-ECi mice appears to be independent of *Foxp3*, because the lack of *Nrp1* impairs immune suppression without altering *Foxp3* expression. Because of the complex relationship among *Foxp3*, *Nrp1*, and T_{reg} cells in general, $CD4^+Nrp1^+$ cells, such as those studied in the ECi model, are hereafter referred to as simply "suppressor T cells" so as not to erroneously place them into a specific area of the current T_{reg} cell paradigm. These results demonstrate a specific role for *Nrp1* in $CD4^+$ T-cell immune response.

Results

***Nrp1* Expression Is Protective Against EAE.** We have shown that mice with T-cell receptor transgenic for the peptide Ac1-11 of myelin basic protein, when epicutaneously immunized (ECi) with the same peptide, are protected from EAE (15). Further, C57BL/6 mice ECi with myelin oligodendrocyte glycoprotein peptide ($MOG_{35–55}$, referred to as MOG) are resistant to EAE pathogenesis (Fig. 1*A*). $CD4^+$ T cells from these mice can confer dominant suppression against EAE (ref. 15 and Fig. S1*A*). To determine the basis of this protection, we performed microarray gene analysis to assess the gene expression profile of $CD4^+$ T cells from MOG ECi mice compared with PBS control or unimmunized control mice. One of the most up-regulated genes in this study was *Nrp1*, exhibiting greater than fivefold induction in the MOG ECi mice compared with control mice. Because *Nrp1* has been proposed to be a constitutive marker of T_{reg} cells (14), we compared *Nrp1* mRNA expression in $CD4^+$ T cells of both naïve and MOG ECi mice to naïve $CD4^+CD25^+$ T cells. As expected, *Nrp1* expression was substantially higher (≥7-fold) in naive $CD4^+CD25^+$ T cells compared with naïve WT $CD4^+$ T cells (Fig. S1*B*). Con-

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: msb76@cornell.edu.

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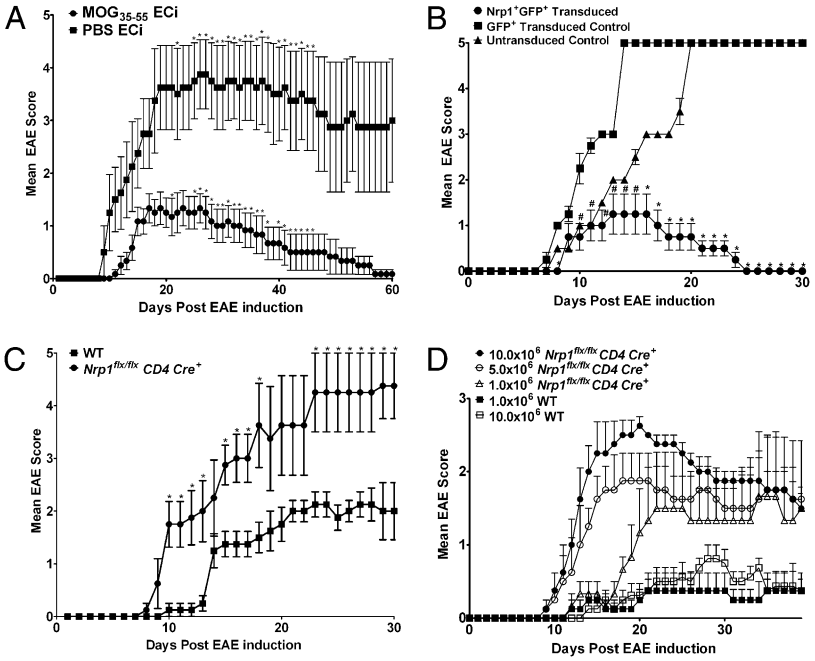

Fig. 1. *Nrp1* expression is protective against EAE, whereas the lack of *Nrp1* increases disease severity. (*A*) WT mice were ECi with 100 μg of $MOG_{35–55}$ ($n = 6$) or PBS ($n = 4$) and immunized with $MOG_{35–55}$/CFA plus pertussis toxin to induce EAE. Representative (1 of 6) results are expressed as mean EAE score (±SEM, $*P < 0.05$). (*B*) $CD4^+CD25^-$ T cells were isolated from Ac1-11–activated MBP-TCR-Tg mice, transduced with a retroviral *GFP* construct containing *Nrp1* (circle, $n = 3$) or an empty vector (square, $n = 3$), and 10^6 cells were adoptively transferred into B10.PI $TCR\alpha^{-/-}$ recipient mice concomitant with 10^6 (untransduced) Ac1-11–activated $CD4^+CD25^-$ cells. Untransduced cells served as a control (triangle, $n = 3$). Results from one experiment are expressed as mean EAE score (±SEM, $\#P < 0.05$ for $Nrp1^+GFP^+$ transduced vs. GFP^+ transduced controls; $*P < 0.05$ for both $Nrp1^+GFP^+$ transduced vs. GFP^+ transduced and $Nrp1^+GFP^+$ transduced vs. untransduced controls). (*C*) EAE was induced by using $MOG_{35–55}$/CFA plus pertussis toxin in $Nrp1^{flx/flx}CD4Cre^+$ ($n = 5$) and WT mice ($n = 5$). Representative (1 of 4) results are expressed as mean EAE score (±SEM, $*P < 0.05$). (*D*) $CD4^+$ cells from $Nrp1^{flx/flx}CD4Cre^+$ ($n = 20$) and WT mice ($n = 5$) primed s.c. with a $MOG_{35–55}$/CFA emulsion were isolated and transferred into C57BL/6-$TCR\alpha^{-/-}$ recipient mice ($n = 4$ mice each) followed by immunization using $MOG_{35–55}$/CFA plus pertussis toxin to induce EAE. Results from one experiment are displayed as mean (±SEM) EAE score.

sistent with increased mRNA expression, *Nrp1* expression was almost threefold higher in $CD4^+$ T cells from MOG ECi mice compared with naïve $CD4^+$ $CD25^+$ T cells or PBS control (Fig. S1*C*). These results demonstrate higher expression of *Nrp1* on MOG ECi $CD4^+$ T "suppressor" cells compared with traditional T_{reg} cells.

To determine whether the protection seen in MOG or Ac1-11 ECi mice could be explained solely by the up-regulation of *Nrp1*, we overexpressed *Nrp1* in vivo and followed EAE progression. We first constructed a retroviral GFP vector containing mouse *Nrp1* cDNA. We then isolated $CD4^+$ T cells from naïve myelin basic protein (MBP)-T-cell receptor (TCR)-Transgenic (Tg) mice (MBP-TCR-Tg) (15), activated them in vitro with Ac1-11 peptide of MBP, and transduced them with either the *Nrp1* construct or an empty vector. Successfully transduced $CD4^+$ T cells were then cotransferred with untransduced, Ac1-11 activated, MBP-TCR-Tg, $CD4^+$ T cells into syngeneic recipient (B10.PL-$TCR\alpha^{-/-}$) mice. Mice receiving T cells transduced with the *Nrp1* expression vector exhibited significant resistance to EAE pathogenesis compared with those receiving the GFP null vector or the naïve $CD4^+$ T cells alone (Fig. 1*B* and Table S1). This result suggests that increased expression of *Nrp1* alone is sufficient to recapitulate the EAE protection previously observed in mice ECi with myelin peptide.

***Nrp1* Conditional Knockout Mice Exhibit Severe EAE.** We next asked whether the lack of *Nrp1* would result in increased susceptibility to EAE by using a T-cell specific *Nrp1* knockout mouse ($Nrp1^{flx/flx}CD4Cre^+$). We compared the development of EAE in WT and $Nrp1^{flx/flx}CD4Cre^+$ mice and demonstrated that $Nrp1^{flx/flx}CD4Cre^+$ mice developed significantly more severe disease than WT mice (Fig. 1*C*). Disease onset was more rapid in the $Nrp1^{flx/flx}CD4Cre^+$ mice, appearing as early as 8 d after immunization (Fig. 1*C*) compared with 10 d in WT mice (Fig. 1*C* and Table S2). Examination of brains on day 30 of EAE and enumeration of $CD4^+$ T-cell infiltration in the brain parenchyma showed considerably more $CD4^+$ T cells in brains of $Nrp1^{flx/flx}CD4Cre^+$ mice compared with WT mice (Fig. S2).

To determine whether the aggravated disease in $Nrp1^{flx/flx}CD4Cre^+$ mice is due specifically to the effects of $CD4^+$ T cells, we isolated $CD4^+$ T cells from both WT and $Nrp1^{flx/flx}CD4Cre^+$ mice primed with MOG and adoptively transferred them into $TCR\alpha^{-/-}$ recipients. After transfer, we induced EAE in recipients and recorded disease progression. Indeed, mice receiving cells from $Nrp1^{flx/flx}CD4Cre^+$ donors exhibited greater disease severity than mice receiving cells from WT donors (Fig. 1*D*). Moreover, by injecting recipient mice with increasing amounts of $CD4^+$ T cells from $Nrp1^{flx/flx}CD4Cre^+$ mice, we observed that EAE severity is directly correlated with the total number of *Nrp1*-deficient $CD4^+$ T cells (Fig. 1*D* and Table S3).

$CD4^+$ T Cells from $Nrp1^{flx/flx}CD4Cre^+$ Mice Display a Skewed T_H-17 Response and Are More Proliferative than Wild-Type $CD4^+$ T Cells. $CD4^+$ T cells can increase EAE severity in two general ways. First, $CD4^+$ T cells may perpetuate increased autoreactivity if suppressive subtypes become functionally impaired. Second, $CD4^+$ T cells may lead to more severe pathogenesis if they possess enhanced inflammatory capacity.

To investigate whether $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+$ T cells possess greater inflammatory capability than WT $CD4^+$ T cells, we assessed proliferation and T_H-17 cell differentiation. Under all culture conditions, $CD4^+$ T cells from $Nrp1^{flx/flx}CD4Cre^+$ mice were more proliferative in response to antigen-specific and non-antigen-specific stimuli. Naive WT $CD4^+$ T cells and $Nrp1^{flx/flx}CD4Cre^+$ cells skewed to T_H-17 and stimulated with MOG, or anti-CD3/anti-CD28 proliferated more than WT cells (Fig. 2*A*). Similarly, $CD4^+$ T cells obtained from $Nrp1^{flx/flx}CD4Cre^+$ mice on day 30 after EAE induction also proliferated significantly more than day 30 $CD4^+$ T cells from WT mice (Fig. 2*B*). Because IL-23 is required for expansion of T_H-17 cells, we cultured $Nrp1^{flx/flx}CD4Cre^+$ and WT cells under T_H-17 conditions in the absence of IL-23. Notably, $Nrp1^{flx/flx}CD4Cre^+$ cells skewed to T_H-17 proliferated more than WT cells even in the absence of IL-23 (Fig. 2*B*). Further, the frequency of T_H-17 cells was significantly greater in $Nrp1^{flx/flx}CD4Cre^+$ mice compared with WT mice under T_H-17 polarizing conditions (Fig. 2*C*) (with IL-23) ($P = 0.035$), as well as under neutral (no skewing) conditions ($P = 0.0077$) (Fig. 2*D*). Consistent with these observations, $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+$ T cells secrete significantly more IL-17 than their WT counterparts under both T_H-17 polarizing ($P = 0.0017$) and neutral ($P = 0.0003$) conditions (Fig. 2*E*). Furthermore, $CD4^+$ T cells from $Nrp1^{flx/flx}CD4Cre^+$ mice expressed increased levels of the transcription

factor *RORγt*, which is important for T_H-17 differentiation (Fig. S3). Because a subset of T_H-17 cells have been identified that can produce IL-10 or IFN-γ in addition to IL-17 (16), we next determined whether altered IL-10 production by T_H-17 may be the cause for increased EAE pathogenicity. IL-10 production by $CD4^+IL\text{-}17^+$ T cells from *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ mice is significantly less than that from $CD4^+IL\text{-}17^+$ T cells from WT mice ($P = 0.044$) (Fig. S4). Together, these data indicate that cells lacking *Nrp1* are biased toward a T_H-17 phenotype and that *Nrp1* regulates $CD4^+$ T-cell expansion.

In Vivo Blockade of T_H-17 Cell Development Ameliorates EAE. To further pinpoint the role of T_H-17 cells in EAE, we treated *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ or WT mice with antibodies to cytokines involved in T_H-17 cell polarization and expansion. Because T_H-17 cells play a critical role in EAE pathogenesis, we reasoned that blocking T_H-17 cell development at the initiation phase of disease might protect both WT and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ mice from EAE. Both *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ and WT mice treated with the T_H-17 anti-cytokine antibody regimen show significant disease amelioration compared with controls (Fig. 2*F* and Table S4). These results confirm T_H-17 involvement in EAE pathogenesis in *Nrp1*$^{flx/flx}$*CD4 Cre*$^+$ mice.

***Nrp1*-Deficient T_{reg} Cells Are Impaired in Their Ability to Suppress CD4 Autoreactive Cells.** $CD4^+$ T cells could lead to increased EAE pathogenesis through reduced T_{reg} cell function. The lack of *Nrp1* might impair the ability of the immune system to suppress autoreactive cells, indirectly leading to increased autoinflammatory cell proliferation. Previous findings support this hypothesis because *Nrp1* has been reported to be constitutively expressed on T_{reg} cells (14). Therefore, we asked whether immune suppression in *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ mice is altered, in addition to the predisposition toward inflammatory subtypes, as demonstrated earlier (Fig. 2). We assessed the ability of WT $CD4^+$ T cells expressing *Nrp1* ($CD4^+Nrp1^+$) to suppress the proliferation of target cells in vitro compared with $CD4^+CD25^+$ T cells. We also compared the suppressive capabilities of these WT $CD4^+Nrp1^+$ T cells to $CD4^+CD25^+$ T cells from *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ mice. Responder $CD4^+$ T cells were isolated from MOG-TCR-transgenic mice (2D2-Tg) (17) and primed with MOG. WT $CD4^+CD25^+$ T or $CD4^+Nrp1^+$ T suppressor cells, or *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ $CD4^+CD25^+$ T suppressor cells were cultured with responder cells, APCs, and MOG. We observed a statistically significant decrease in the proliferation of target cells cultured with $CD4^+CD25^+Nrp1^+$ T cells or with $CD4^+CD25^+$ T cells from WT mice at all ratios (Fig. 3*A*). Interestingly, at all suppressor to responder cell ratios, $CD4^+Nrp1^+$ T cells suppressed effector cell proliferation more efficiently than $CD4^+CD25^+$ T cells (Fig. 3*A*). In contrast, $CD4^+CD25^+$ T cells from *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ mice were not suppressive at the same cell ratios as WT $CD4^+CD25^+$ T or $CD4^+Nrp1^+$ T cells (Fig. 3*A*). These results indicate that the corresponding *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ $CD4^+$ T cells are impaired in their ability to curb immune proliferation.

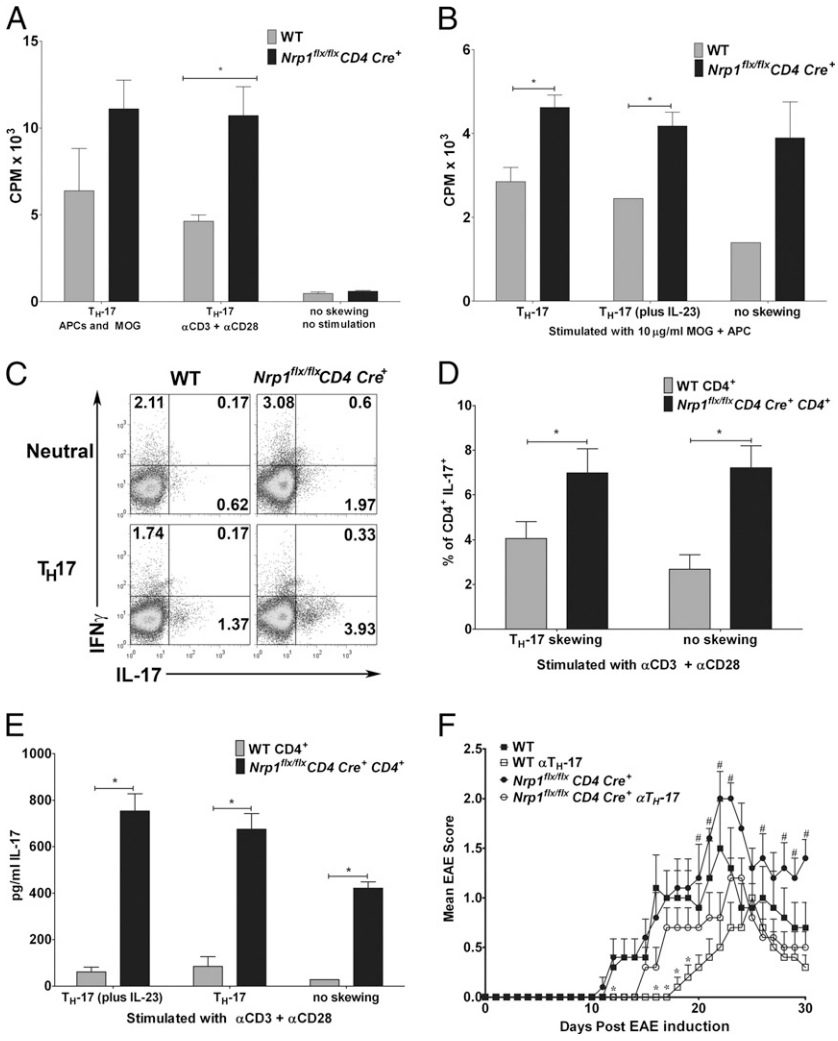

Fig. 2. *Nrp1*-deficient $CD4^+$ T cells display an increased T_H-17 phenotype and are more proliferative than WT. (*A*) Naïve $CD4^+$ cells [WT (gray bars) and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ (black bars), $n = 5$ each] were skewed toward T_H-17. After 7 d, skewed cells were stimulated with either APC and MOG, or with anti-CD3 and anti-CD28, or were left unskewed and unstimulated. Representative (1 of 5) results are displayed as mean (±SEM) CPM × 10^3 (*$P <$ 0.05). (*B*) EAE was induced by using $MOG_{35–55}$/CFA plus pertussis toxin in WT (gray bars) and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ (black bars) mice ($n \geq 3$ each). On day 30, $CD4^+$ cells were stimulated with 10 μg/mL MOG and cultured under neutral, T_H-17 polarizing, or T_H-17 polarizing with supplemental IL-23 conditions. Representative results (1 of 3) are displayed as mean (±SEM) cpm × 10^3. (*$P < 0.05$). (*C*) Naïve $CD4^+$ T cells (WT and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$, $n = 4$ mice each), were cultured under neutral or T_H-17 polarizing conditions. Cells were stained for CD4, IFNγ, and IL-17 and analyzed by flow cytometry. Data are representative of three experiments. (*D*) Naïve $CD4^+$ cells [WT (gray bars) and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ (black bars)] were cultured under neutral or T_H-17 polarizing conditions, stained for CD4 and IL-17 expression, and analyzed by flow cytometry. Representative (1 of 5) results are presented as mean (±SEM) percent of total $CD4^+$ cells, which are $IL\text{-}17^+$ (*$P < 0.05$). (*E*) Naïve $CD4^+$ cells [WT (gray bars) and *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ (black bars), $n = 5$ each] were cultured under neutral or T_H-17 polarizing conditions, and cell culture supernatant was analyzed by ELISA for IL-17. Representative (1 of 5) results are displayed as mean (±SEM) IL-17 pg/mL (*$P \leq 0.005$). (*F*) WT (squares) or *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ (circles) mice ($n = 5$ each) immunized for EAE by using $MOG_{35–55}$/CFA plus pertussis toxin were treated with (open symbols) or without (filled symbols) anti–T_H-17 antibodies (anti–IL-6, anti–IL-23, anti–TGF-β). Representative (1 of 2) results are presented as mean EAE score ± SEM (#$P < 0.05$ for the *Nrp1*$^{flx/flx}$*CD4Cre*$^+$ versus the *Nrp1*$^{flx/flx}$*CD4Cre*$^+$-anti–T_H-17 treated group. *$P <$ 0.05 for the WT versus WT anti–T_H-17 group.)

To determine whether this difference in suppression is significant in vivo and to more specifically define the role of *Nrp1* in immune suppression, four different populations of $CD4^+$ T cells from WT or *Nrp1* conditional knockout mice were adoptively transferred into naïve TCR-$\alpha^{-/-}$ recipients with concomitant adoptive transfer of MOG-stimulated, T_H-17–polarized $CD4^+$ responder cells. WT $CD4^+CD25^+Nrp1^+$ and $CD4^+CD25^-Nrp1^+$ T cells exhibited similar disease profiles and significantly suppressed EAE (Fig. 3*B* and Table S5). Recipients of $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+CD25^+$ T cells exhibited a much more severe EAE disease profile than their WT $CD4^+CD25^+Nrp1^+$ counterparts (Fig. 3*B* and Table S5). From these observations, we conclude that *Nrp1*-expressing $CD4^+$ T cells are capable of suppressing EAE inflammatory response independent of naturally occurring T_{reg} cell involvement. We also conclude that naturally occurring T_{reg} cells expressing *Nrp1* are more efficient suppressors than T_{reg} cells lacking *Nrp-1* expression. These findings suggest that a major role of *Nrp1* in the immune response is in regulating inflammatory responses by $CD4^+$ effector T cells.

***Foxp3* Expression Is Unaffected by the Lack of *Nrp1* in $Nrp1^{flx/flx}CD4Cre^+$ Mice.** Because *Nrp1* appears to play an important role in T-cell–mediated immune suppression, we examined the effect of *Nrp1* on the expression of another gene associated with the archetypal T_{reg} cell, *Foxp3*. $CD4^+$ T cells from $Nrp1^{flx/flx}CD4Cre^+$ mice express *Foxp3* at a similar frequency to WT mice (Fig. 3*C*). Consistent with these data, the dominant immune suppression induced in $CD4^+$ T cells in mice ECi with myelin antigen does not correspond to an increase in *Foxp3* expression. These results suggest that *Nrp1* suppression is independent of *Foxp3*.

TGF-β, but Not IL-10, Is Important for *Nrp1*-Mediated Suppression. One of the mechanisms by which suppressor T cells suppress autoimmunity is by the induction of anti-inflammatory cytokines such as IL-10 or TGF-β. Because IL-10 is decreased in T_H-17–skewed $CD4^+$ T cells from $Nrp1^{flx/flx}CD4Cre^+$ mice (Fig. S4), we asked whether IL-10 is involved in *Nrp1* suppression of target cell proliferation by neutralizing IL-10 in vitro (18). $CD4^+CD25^+$ T cells or $CD4^+CD25^-Nrp1^+$ cells from WT mice were used as suppressors in the presence or absence of anti–IL-10 (Fig. 4*A*). Interestingly, anti–IL-10 abrogated WT $CD4^+CD25^+$ cell suppression of target cell proliferation (Fig. 4*A*), but had little effect on WT $CD4^+CD25^-Nrp1^+$ cell suppression (Fig. 4*A*). These results suggest that *Nrp1* suppressor function does not depend on IL-10. We next asked whether TGF-β is involved in *Nrp1*-mediated suppression by evaluating it in the presence or absence of anti–TGF-β (18). As shown in Fig. 4*B*, anti–TGF-β significantly inhibited WT $CD4^+CD25^-Nrp1^+$ T-cell suppression of effector cell proliferation but had little effect on WT $CD4^+CD25^+$ T-cell suppression. These data strongly indicate that *Nrp1* suppression of $CD4^+$ T-cell effector function depends on TGF-β.

Discussion

The goal of this study was to elucidate the role of *Nrp1* in EAE pathogenesis. As shown in Fig. 1*A*, initial ECi with MOG before induction of EAE results in complete protection against disease progression. Through global gene expression analysis, we found that *Nrp1* is one of the most highly expressed genes in this protective response. These results, along with previous data suggesting a role for *Nrp1* in immune suppression, led us to hypothesize that *Nrp1* is important for preventing autoinflammatory conditions such as EAE. Our hypothesis is supported by data showing that overexpression of *Nrp1* is sufficient to protect mice from EAE pathogenesis as well as the converse finding that the lack of *Nrp1* results in increased disease severity. Protection and disease aggravation are $CD4^+$ T-cell–dependent, because these disease states can be recapitulated in T-cell–deficient recipients when exogenous T cells overexpressing or lacking *Nrp1*, respectively, are transferred. Furthermore, our data strongly demonstrate that *Nrp1* plays a critical role in regulating the expansion and cytokine production of T_H-17 cells both in vitro and in vivo.

Because of the discovery of the T_H-17 lineage of $CD4^+$ T cells, many autoimmune disorders previously described as T_H-1–mediated, including MS and EAE, have been reattributed to T_H-17 cells (19). Accordingly, we demonstrate that *Nrp1*-deficient $CD4^+$ T cells are poised to differentiate into the T_H-17 lineage. Furthermore, blockade of T_H-17 cell development with an anti–

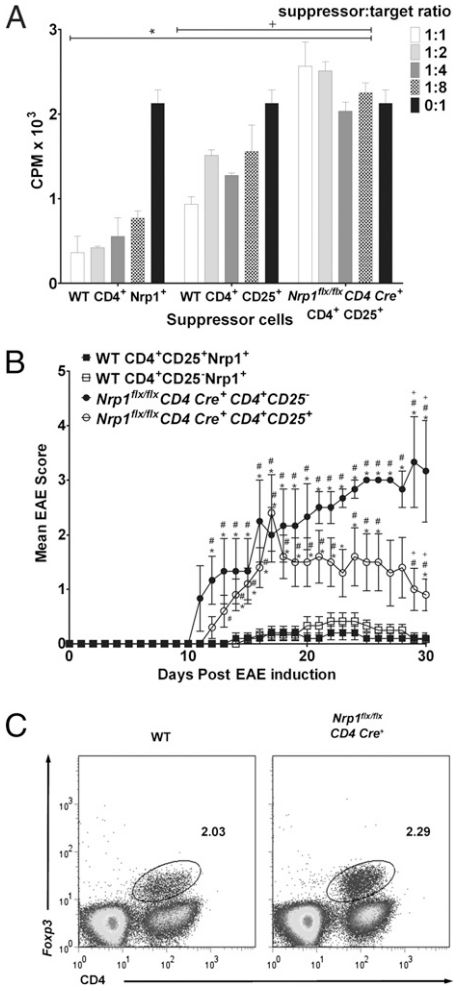

Fig. 3. *Nrp1* deficiency impairs T_{reg} cell function. (*A*) The 2D2-Tg (17) $CD4^+$ cells ($n \geq 3$ mice) were used as target cells. Naïve $Nrp1^{flx/flx}CD4Cre^+$ or WT $CD4^+CD25^+$ cells ($n \geq 7$ each), as well as WT $CD4^+CD25^-Nrp1^+$ cells (all purified by using magnetic beads), were used as suppressor T cells. Cells were stimulated with 10 μg/mL MOG and APC (5:1 APC:target ratio), cultured for 48 h, then pulsed with 0.5 μCi/well Td-^{3}H for 18 h. *P* values compare either WT $CD4^+Nrp1^+$ (*$P < 0.05$) or WT $CD4^+CD25^+$ (+$P < 0.05$) suppressor cells to $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+CD25^+$ cells of the same ratio. Representative (1 of 4) results are expressed in mean (±SEM) CPM × 10^3. (*B*) The 2D2-Tg $CD4^+$ T cells ($n = 5$ mice) were skewed to T_H-17 in vitro, and 10^7 cells were transferred into C57BL/6-TCR-$\alpha^{-/-}$ recipients along with: 10^6 naïve WT $Nrp1^+CD4^+$ $CD25^+$, or $Nrp1^+CD4^+CD25^-$; or, naïve $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+CD25^+$ or $CD4^+CD25^-$ cells ($n = 10$ mice each). EAE was then induced in recipients by using MOG_{35-55}/CFA plus pertussis toxin. Results from one experiment are expressed as mean (± SEM) EAE score. *P* values compare: WT $CD4^+CD25^-Nrp1^+$ versus either $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^+$ or $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^-$, #$P < 0.05$; WT $CD4^+CD25^+Nrp1^+$ versus either $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^+$ or $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^-$, *$P < 0.05$; $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^+$ versus $Nrp1^{flx/flx}CD4Cre^+CD4^+CD25^-$, +$P < 0.05$). (*C*) $CD4^+$ T cells from WT and $Nrp1^{flx/flx}CD4Cre^+$ mice ($n \geq 3$) were isolated, stained for CD4 and *Foxp3*, and analyzed by FACS. Results are representative of three experiments.

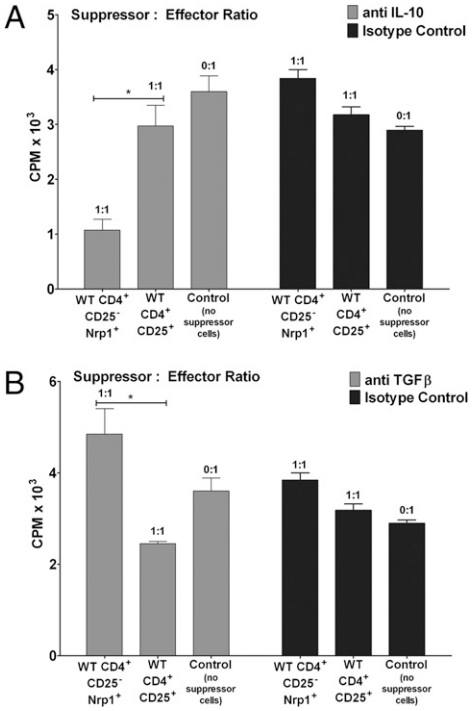

Fig. 4. Suppression by $CD4^+Nrp1^+$ cells is abrogated in the presence of anti–TGF-β but not anti–IL-10. (*A*) The 2D2-Tg $CD4^+$ T cells ($n \geq 3$ mice) were primed in vivo, isolated by using magnetic beads, and used as target cells. Naïve WT and $Nrp1^{flx/flx}CD4Cre^+$ $CD4^+CD25^+$ T cells ($n = 10$) and naïve WT $CD4^+CD25^-Nrp1^+$ T cells ($n = 10$) were isolated by using magnetic beads and used as suppressor T cells. Suppressors and targets were combined at a 1:1 ratio. Cells were treated with anti–IL-10 (10 μg/mL) or an isotype control (10 μg/mL) and stimulated with 10 μg/mL MOG and APC (5:1 APC:target ratio). Representative (1 of 3) results are expressed as mean (±SEM) CPM × 10^3. (WT $CD4^+CD25^+$ versus WT $CD4^+CD25^-Nrp1^+$, $^*P < 0.05$.) (*B*) Cell populations were purified, combined, and cultured as in Fig. 4*A*, except cells were treated with anti–TGF-β (10 μg/mL) or an isotype control (10 μg/mL). Representative (1 of 3) results are expressed as mean (±SEM) cpm × 10^3. (WT $CD4^+CD25^+$ versus WT $CD4^+CD25^-Nrp1^+$, $^*P < 0.05$.)

T_H-17 antibody regimen suppressed EAE in $Nrp1^{flx/flx}CD4Cre^+$ mice. These results further support our hypothesis that one mechanism by which *Nrp1* controls autoreactivity is by regulating T_H-17 cell expansion and cytokine production.

As of yet, no signaling pathway has been attributed specifically to *Nrp1*. Although *Nrp1* comprises one chain of the semaphorin-3A receptor, the second chain, plexin-A, is responsible for initiating cell signaling as a result of receptor ligation (20). Studies show that dendritic cells produce large amounts of semaphorin-3A (8). Moreover, the side chain of plexin-A is expressed on $CD4^+$ T cells and plexin-$A4^{-/-}$ mice exhibit increased EAE pathogenicity (21). Together with our findings, these results suggest that secretion of semaphorin-3A by DCs may represent a negative feedback loop to reduce the duration of T-cell–APC interaction and, consequently, reduce the expression of inflammatory molecules such as IL-17. In neuronal cells, semaphorin-3 signaling through the *Nrp1*:plexin-A complex acts through a rho/rac-dependent pathway leading to actin depolymerization and growth cone collapse (10). Similarly, *Nrp1*:plexin-A signaling in T cells could lead to similar actin depolymerization, causing the disassembly of molecular scaffolding supporting cellular polarization and the supramolecular activation complex at the immune synapse.

$Nrp1^{flx/flx}CD4Cre^+$ mice may be susceptible to increased EAE severity because the lack of *Nrp1* impairs suppression of autoreactive cells by the immune system, indirectly leading to increased autoinflammatory cell proliferation. Such a hypothesis is supported by previous findings that *Nrp1* is constitutively expressed on T_{reg} cells (14). Indeed, we found that suppressor cells from $Nrp1^{flx/flx}CD4Cre^+$ mice have an impaired ability to suppress effector cells both in vitro and in vivo. Moreover, we found that WT $CD4^+$ T cells sorted specifically for *Nrp1* expression were as capable of or more effective at suppressing target cells than suppressors sorted specifically as $CD4^+CD25^+$, indicating that *Nrp1* plays an important role in immune suppression.

Our result is consistent with findings that show that *Nrp1* expression allows T_{reg} cells to supplant effector T cells for the limited space available on primed APCs (22). Along with our data showing that antigen and APCs are required for successful T_{reg} cell function (as opposed to contact independent PMA/ionomycin stimulation), this result supports the notion of antigen-dependent regulatory T-cell suppression. As described by Sarris et al. (22), *Nrp1* greatly contributes to the fidelity of the immune synapse, thus favoring APC interactions with T cells that express *Nrp1*.

Alternatively, *Nrp1* may function at the level of T_{reg} cell stimulation. Several reports have shown that *Nrp1* is important for T_{reg} cell development (23, 24), and *Nrp1* has been proposed as a receptor for TGF-β (25). Whether this interaction actually contributes to signaling, the functional activity of T_{reg} cells is enhanced by *Nrp1*:TGF-β ligation. Because of the shared requirement of TGF-β for both T_{reg} and T_H-17 cells (26, 27), *Nrp1* may function to sequester TGF-β, simultaneously preventing the development of inflammatory T_H-17 cells and promoting the differentiation of peripheral T_{reg} cells. Consistent with this idea, neutralization of TGF-β inhibits *Nrp1* suppressor capacity, whereas blockade of IL-10 had little or no impact.

In addition to naturally occurring T_{reg} (nT_{reg}) cells, additional T_{reg} subtypes emerge from the thymus as $CD4^+CD25^-Foxp3^-$ cells. Although appearing phenotypically similar to a naïve $CD4^+$ T cell, these inducible T_{reg} (iT_{reg}) cells can be induced to express *Foxp3* and CD25 in the periphery (28). Such a notion of T_{reg} cell heterogeneity is consistent with our results. We demonstrate that EAE-tolerant $CD4^+$ cells from MOG ECi mice, with highly up-regulated *Nrp1* levels, have only modestly up-regulated *Foxp3* levels. Moreover, $Nrp1^{flx/flx}CD4Cre^+$ mice display virtually no variation in the $Foxp3^+$ cell population. Despite this lack of fluctuation in *Foxp3* expression, *Nrp1*-deficient suppressor T cells still have an impaired ability to attenuate immune proliferation. These results suggest that *Nrp1* may simply be a mechanism used by cells capable of immune suppression and not specific to any regulatory cell lineage in particular. A cell capable of immune suppression would alter *Nrp1* expression depending on inflammatory cues. As a result, any suppressive cell incapable of expressing *Nrp1,* whether a $Foxp3^+$ nT_{reg} cell or a type of iT_{reg} cell, would have an impaired ability to curb autoinflammation.

In summary, we demonstrate that *Nrp1* plays a critical role in the pathogenesis of EAE. Mice that lack *Nrp1*-expressing $CD4^+$ T cells exhibit increased degenerative signs and CNS infiltration associated with EAE, preferential T_H-17 cell commitment, enhanced proliferation and cytokine production, and impaired suppressor capacity. Our study provides evidence for a direct role of *Nrp1* in an immune-mediated disease. This phenomenon is the combined result of increased inflammatory lineage commitment and impaired regulatory T-cell function. This study supports previous findings that *Nrp1* may be essential for proper immune suppression. As the body of knowledge surrounding *Nrp1* and T_{reg} cell function expands, this molecule may prove to be a novel target for new treatments and therapies for diseases like MS.

Materials and Methods

Mice. *Nrp1* conditional knockout mice were generated by crossing $Nrp1^{flx/flx}$ mice [graciously provided by David Ginty, (The Solomon H. Snyder Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD)] (12) on the C57BL/6 background with $CD4Cre^+$ mice

(Taconic), generating *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ mice (*SI Materials and Methods*). MBP-TCR-Tg mice on the B10.PL background were described (29). C57BL/6-Tg (Tcra2D2,Tcrb2D2)1Kuch/J mice, which have a transgenic T-cell receptor recognizing the $MOG_{35–55}$ peptide (MOG-TCR-Tg), were kindly provided by Vijay Kuchroo (Harvard Medical School, Center for Neurologic Diseases, Brigham & Women's Hospital, Boston, MA) (17). C57BL/6 mice and TCR$\alpha^{-/-}$ (B6.129S2-Tcrα^{tm1Mom}/J or B10.PL-TCR$\alpha^{-/-}$) mice were purchased from Jackson Laboratory. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Cornell University.

EAE Induction and Scoring. EAE was induced by injecting 50 μL s.c. in each flank a 1:1 CFA (Thermo Scientific): MOG (Anaspec) [3 mg/mL in PBS (Mediatech)] emulsion on day one, in parallel with i.v. pertussis toxin (List Biological Laboratories) (200 ng) on days 0 and 2. Mice were scored daily for EAE based on a numerical score of disease sign severity: 0 = no disease, 0.5–1 = weak/limp tail, 2 = limp tail and partial hind limb paralysis, 3 = total hind limb paralysis, 4 = both hind limb and fore limb paralysis, and 5 = death.

Adoptive Transfer and in Vivo Suppression. Mice were primed with s.c. CFA: MOG peptide. After 1 wk, CD4^{+} T cells were isolated from spleen and lymph nodes and negatively selected for using magnetic separation (*SI Materials and Methods*). CD4^{+} cells were transferred to *TCR*$\alpha^{-/-}$ mice at the indicated dose in a total of 200 μL of sterile PBS. For in vivo suppression, CD4^{+}CD25^{+} cells from unimmunized mice were sorted by using a magnetic regulatory T-cell separation kit (Miltenyi Biotech). Either 10^{7} WT or *Nrp1*$^{flx/flx}$*CD4Cre*$^{+}$ CD4^{+}CD25^{+} cells were injected i.v. into WT recipients in a total dose of 200 μL of sterile PBS.

T-Cell Polarization. Purified naïve CD4^{+} T cells (*SI Materials and Methods*) were cultured in Bruff's media (Invitrogen) and stimulated with immobilized mouse anti-CD3 and soluble anti-mouse CD28 (BD Biosciences) in the presence of T_H-17–polarizing (with IL-23 where indicated) or neutral conditions (*SI Materials and Methods*). At day 3 after stimulation, cells were expanded for an additional 4 d in fresh media containing 25 U/mL mouse IL-2. At day 7, cells were washed and restimulated with either anti-mouse CD3/CD28 (1 μg/mL each) plus mouse IL-2 (25 U/mL), or with $MOG_{35–55}$ and APC plus mouse IL-2 (25 U/mL) for 48 h. Cell culture supernatant was collected for ELISA, and differentiated T cells were collected for either proliferation or intracellular cytokine staining.

Flow Cytometry. Cell suspensions were stained with fluorochrome-conjugated antibodies for CD4, IFN-γ, *Foxp3*, IL-10, IL-4, and IL-17 (BD Biosciences and eBioscience) or with rabbit anti-Nrp1 (AbCam) and then with anti-rabbit AF488 (Invitrogen) (*SI Materials and Methods*). Samples were acquired on a FACSCalibur (BD Biosciences) by using CellQuest (BD Biosciences) software and analyzed with FlowJo software (Tree Star).

T-Cell Suppression Assay. For a full description, please refer to *SI Materials and Methods*. Briefly, CD4^{+} responder cells were primed in vivo by immunization of WT mice with a 1:1 CFA:MOG (3 mg/mL in PBS) emulsion (50 μg in both flanks of the mouse) on day 0 and day 5 and then isolated on day 7. CD4^{+} responder cells from 2D2-Tg mice were also used in certain assays (17). For suppressor cells, CD4^{+} cells were first isolated from naïve WT or *Nrp1*$^{flx/flx}$ *CD4Cre*$^{+}$ mice. Then, positive magnetic selection was used to isolate CD25^{+} suppressor cells, and WT CD4^{+}CD25^{-}Nrp1^{+} cells were finally selected from the CD4^{+}CD25^{-} population. Primed CD4^{+} responder cells (10^{5}) were cultured with suppressor cells and irradiated APCs (1:5 T-cell:APC) in the presence of 10 μg/mL MOG. In certain experiments, anti–IL-10 (eBioscience), sIL-10 receptor (R&D Systems) or anti–TGF-β (eBioscience) were used. Proliferation was measured by ^{3}H-thymidine incorporation.

Epicutaneous Immunization, Retroviral Overexpression, ELISA, and RT-PCR. A description of the methods used for epicutaneous immunization, retroviral overexpression, ELISA, and RT-PCR can be found in *SI Materials and Methods*.

Statistics. *P* values are calculated by using the Student's *t* test.

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