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Id1 Expression Promotes T Regulatory Cell Differentiation by Facilitating TCR Costimulation

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T regulatory (Treg) cells play crucial roles in the regulation of cellular immunity. The development of Treg cells depends on signals from TCRs and IL-2Rs and is influenced by a variety of transcription factors. The basic helix-loop-helix proteins are known to influence TCR signaling thresholds. Whether this property impacts Treg differentiation is not understood. In this study, we interrogated the role of basic helix-loop-helix proteins in the production of Treg cells using the CD4 promoter-driven Id1 transgene. We found that Treg cells continued to accumulate as Id1 transgenic mice aged, resulting in a significant increase in Treg cell counts in the thymus as well as in the periphery compared with wild-type controls. Data from mixed bone marrow assays suggest that Id1 acts intrinsically on developing Treg cells. We made a connection between Id1 expression and CD28 costimulatory signaling because Id1 transgene expression facilitated the formation of Treg precursors in CD28−/− mice and the in vitro differentiation of Treg cells on thymic dendritic cells despite the blockade of costimulation by anti-CD80/CD86. Id1 expression also allowed in vitro Treg differentiation without anti-CD28 costimulation, which was at least in part due to enhanced production of IL-2. Notably, with full strength of costimulatory signals, however, Id1 expression caused modest but significant suppression of Treg induction. Finally, we demonstrate that Id1 transgenic mice were less susceptible to the induction of experimental autoimmune encephalomyelitis, thus illustrating the impact of Id1-mediated augmentation of Treg cell levels on cellular immunity. The Journal of Immunology, 2014, 193: 663–672.

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Abbreviations used in this article: bHLH, basic helix-loop-helix; EAE, experimental autoimmune encephalomyelitis; iTreg, inducible Treg; MOG, myelin oligodendrocyte glycoprotein; Treg, T regulatory; WT, wild-type.

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(33–38). The function of these proteins in the production and maintenance of Treg cells is less well understood. Maruyama et al. (39) showed that Id3-deficient mice possess fewer Treg cells at 3 wk of age and naïve T cells from these mice exhibited impaired Treg induction in vitro. As the mice age, Treg cells accumulate to a normal or higher than normal level. However, this phenotype may be complicated by the fact that Id3−/− mice develop autoimmune diseases and exhibit abnormal γδ T cell differentiation at later stages of adult life (40, 41). Intriguingly, these authors have demonstrated that E2A proteins promote the transcription of the Foxp3 gene. To reconcile the disconnect between a potential increase in E protein activity in Id3-deficient cells and a reduction in Treg differentiation, they proposed that elevation of IL-4 and GATA3 expression in Id3−/− mice has a dominant suppressive effect on Foxp3 expression and Treg differentiation (39). Notably, these studies were mostly based on models of iTreg differentiation. The mechanisms remain to be defined how Id3 deficiency impairs the generation of Treg cells in vivo.

To further illustrate the role of E proteins and Id molecules in Treg differentiation, we made use of our transgenic mice in which Id1 cDNA is driven by the CD4 promoter and examined Treg differentiation under the condition of gain-of-Id function (32). Because Id1 and Id3 share extensive homology and have been shown to be functionally redundant (42), overexpression of Id1 should mimic the situation of Id3 upregulation upon TCR signaling. Indeed, we observed opposite effects of Id1 overexpression to that of Id3 deficiency, namely, an overall increase of Treg cell counts in the Id1 transgenic animals in a T cell–intrinsic manner. Accordingly, these mice developed experimental autoimmune encephalomyelitis (EAE) with less severity. We showed that Id1 transgenic mice have a higher frequency of CD4+CD25+CD122+ GITR−Foxp3− cells in the thymus, which are thought to be enriched in Treg precursors and whose formation depends on CD28 signaling (7, 16). Id1 expression in CD28−/− mice partially corrected the deficit of these cells. Moreover, we show that Id1-expressing naïve T cells have a higher propensity to differentiate into iTreg cells in the absence of CD28-mediated TCR costimulation. On the contrary, Treg induction with robust costimulation was found to be suppressed by the Id1 transgene. Because Id3 is upregulated by TCR and CD28 signaling, Id proteins play a distinct role during Treg differentiation.

**Materials and Methods**

**Mice**

The CD4-Id1 transgenic strain was as previously described and backcrossed onto the C57BL/6J background for six to eight generations, and littermates were used as wild-type (WT) controls (32). CD28−/− mice (stock number 2666) and OT-II mice [B6.Cg-Tg(TcraTcrb)425cbn/J; stock number 004194] on the C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbor, ME) and crossed with Id1 transgenic mice. 004194] on the C57BL/6J background were purchased from The Jackson

**Culture medium, Abs, and reagents**

RPMI 1640 medium containing 10% FCS was used for CD4 naïve T cell cultures. The following Abs were purchased from BD Biosciences (San Jose, CA): anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), anti-mouse IL-2, anti-CD4 PerCP, anti-CD4 PE/Cy7, anti-CD8 FITC, anti-CD25 allophycocyanin, anti-CD25 PE, anti-CD44 FITC, anti-CD62L PE, and anti-BrdU FITC. Anti-Foxp3 allophycocyanin, anti–IFN−γ allophycocyanin, and anti–IL-17A PE were from eBioscience (San Diego, CA). Anti-Helios, anti-CD122, and anti-GITR were from BioLegend (San Diego, CA). Mouse rIL-2 and rIL-7 were purchased from R&D Systems (Minneapolis, MN), and OVA was from Sigma-Aldrich (St. Louis, MO).

**Isolation of naïve CD4 cells and in vitro Treg induction**

Lymphocytes from lymph nodes were stained with fluorochrome-conjugated Abs for 30 min at 4°C. CD4+CD62LhighCD44lowCD25− cells were sorted using a BD FACS Aria II. The anti-CD3 Ab was coated onto 96-well flat-bottom plates at 1 μg/ml in PBS overnight and washed once with PBS. Naive CD4 cells in 100 μl complete medium were then added into the well at a density of 2 × 10^6 cells/ml. The culture medium was supplemented with TGF−β (1 ng/ml) plus or minus anti-CD28 (2 μg/ml or as indicated otherwise). For some experiments, exogenous IL−2 or anti–IL−2 was added into the culture. The cells were incubated at 37°C in an atmosphere containing 5% CO2 for 72 h, followed by intracellular staining for Foxp3 expression.

**FACS analysis**

Lymphocytes were usually stained for the expression of surface markers for 30 min in PBS containing anti-CD4 PerCP, anti-CD8 FITC, and anti-CD25 PE. Cells were then washed twice with PBS, and intracellular Foxp3 staining was performed with the Foxp3 staining buffer set (eBioscience), according to the manufacturer’s instructions. After fixation and permeabilization, anti–Foxp3 allophycocyanin was added and incubated for 30 min. For detection of thymus-derived Treg cells, anti-Helios PE was also included.

To detect cytokine production by lymphocytes from mice immunized with peptides derived from myelin oligodendrocyte glycoprotein (MOG), cells were cultured with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h with brefeldin A (3 μg/ml; Sigma-Aldrich) in complete medium. After staining for surface markers, cells were fixed with intracellular fixation buffer (eBioscience) for 1 h, washed twice with permeabilization buffer (eBioscience), and then incubated with anti–IFN−γ allophycocyanin and anti–IL−17A PE Abs. Samples were analyzed on a FACS Calibur using CellQuest software (BD Biosciences) or LSRII with FlowJo software.

**BrdU labeling and detection**

Mice were provided with drinking water containing 1 mg/ml BrdU for 7 d, and the water was changed daily. Thymocytes were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 10 min at 25°C, washed with PBS, treated with 4 N hydrochloric acid for 15 min, and then neutralized with 0.1 M sodium borate (pH 8.5) for 20 min. Cells were incubated with anti-BrdU FITC and anti-Foxp3 allophycocyanin in PBS containing 0.2% Triton X-100 for 30 min at 25°C. Cells were then washed and resuspended into PBS. BrdU incorporation was quantified using a BD FACSCalibur.

**In vitro differentiation of Treg cells**

To generate Treg cells in vitro, 2 × 10^5 CD4+CD25−CD69− thymocytes sorted from OT-II/Id1+ or OT-II mice were cocultured with 2 × 10^4 sorted thymic dendritic cells in the presence of 40 μg/ml OVA, 10 ng/ml rIL-2, and 1 ng/ml IL-7 with or without anti-CD80 and anti-CD86 (5 μg/ml each) for 4 d. Foxp3 expression of the cells was then measured, as described above.

To obtain thymic dendritic cells, thymuses (3, 4) were chopped into small fragments with scissors and washed. The fragments were then digested with collagenase IV (2 mg/ml) and DNase I (0.05 mg/ml) in 7.5 ml RPMI 1640 with FCS for 30 min at 37°C with continuous agitation. Cells were then washed and resuspended in Ficol-Paque (p = 1.077) and centrifuged for 10 min at 1700 × g. A low-density cell fraction was collected, and the cells were stained with CD45 and CD11c Abs for 30 min at 4°C. CD45+CD11c+CD4+CD25+ cells were sorted using a BD FACS Aria II.

**Generation of mixed bone marrow chimera**

Bone marrow cells from CD45.1+ C57BL/6 (competitor) mice were mixed at a 1:1 ratio with CD45.2+ tester bone marrow cells from WT or Id1+ mice. A total of 1 × 10^7 bone marrow cells was injected i.v. into lethally irradiated (900 rad) CD45.1+ recipients. The spleen and thymus of the mixed bone marrow chimera were analyzed by flow cytometry 12 wk after the bone marrow transplantation.

**EAE mouse model**

MOG35−55 peptide (2 mg/ml) was emulsified with an equal volume of CFA (Sigma-Aldrich, St. Louis, MO). The mixture was injected s.c. into each of the four areas near the draining lymph nodes and at 50 μl per site, followed by i.p. injection of 200 ng pertussis toxin on days 1 and 2. Disease progression was monitored daily. The scoring criteria were as follows: 1) weakness of the tail; 2) tail paralysis and hind limb weakness; 3) partial paralysis of hind limbs; 4) complete paralysis of hind limbs; 5) complete paralysis of hind limbs with partial or complete paralysis of fore limbs; and 6) death.

**ELISA**

Culture supernatants were collected 2 d after Treg induction, and IL-2 concentration was measured by using the Mouse IL-2 ELISA Ready-
SET-Go! Kit (eBioscience), according to the manufacturer’s instruction. Each sample was assayed in triplicates. The concentration of IL-2 was calculated using a standard curve.

**Statistical analysis**

Statistical analysis of the data between WT and transgenic samples was carried out using Student’s t-test. Two-way ANOVA was used for multiple variant comparisons. All analyses were carried out using the Prism software.

**Results**

*Increased representation of CD4+ Foxp3+ cells in Id118 mice*

To evaluate the effects of Id upregulation on Treg development, we made use of our CD4-Id118 mice, in which the Id1 cDNA is driven by the CD4 promoter (32). These mice have been shown to exhibit largely normal steady-state T cell development in the thymus and periphery and live a healthy life. We examined Treg cells in the thymus of 8-wk-old Id1 transgenic mice and their WT littermates by staining the cells with Abs against CD4, CD8, and CD25, followed by intracellular staining for Foxp3. We found that both the percentage and absolute number of Foxp3+ Treg cells were increased by ~2-fold in Id1 transgenic mice (Fig. 1A). Likewise, the percentage and number of Treg cells in the spleen were also elevated, albeit not as dramatically as in the thymus (Fig. 1B).

We next monitored the kinetics of Treg cell production as the WT and transgenic littermate aged. Whereas the percentages of Treg cells in the thymus of WT and transgenic mice were in similar ranges in neonates and young animals, transgenic mice accumulated significantly more Treg cells as they aged from 6 to 18 wk (Fig. 1C). In contrast to a relatively stable level of thymic Treg cells in WT mice, the percentages of transgenic Treg cells continued to increase in adulthood. Similarly, Id1 transgenic mice exhibited significant increases in the splenic Treg cell population from 4 wk of age, and the gap widened thereafter (Fig. 1C).

*Augmented production of Treg cells in Id1 transgenic thymus*

Expression of the Helios transcription factor has been shown to distinguish Treg cells derived from the thymus and periphery (43). Treg cells derived from the thymus express Helios, whereas those originating in the periphery or in vitro do not. We therefore analyzed CD4+ thymocytes and splenocytes for Foxp3 and Helios expression (Fig. 2). In the thymus, the percentage and number of Foxp3+Helios+ cells were 2.4- and 2.7-fold higher in Id1 transgenic mice compared with WT controls (Fig. 2A). In contrast, Foxp3+Helios− cells were similar between the two strains. In the spleen, the abundance of Foxp3+Helios+ cells was also increased in Id1 transgenic mice. The Foxp3+Helios− cells, in contrast, were even found to be underrepresented in the transgenic spleen (Fig. 2B). These data suggest that the elevated level of peripheral Treg cells is mainly due to increased thymic output of Treg cells.

To monitor Treg production in the thymus, we performed a BrdU labeling assay by providing the mice with BrdU in drinking water for 7 d. CD4 single-positive thymocytes were then analyzed for BrdU incorporation by providing the mice with BrdU in drinking water (Fig. 3). We determined the percentages of BrdU+ cells in the Foxp3+ and Foxp3− populations separately. As shown in the bar graphs, BrdU uptake by Id1-expressing Foxp3+ cells was twice as high as the WT counterparts, whereas BrdU labeling in the Foxp3− fraction was comparable between WT and transgenic mice (Fig. 3). As Treg cells are thought not to proliferate in thymus (44), the BrdU-labeled cells most likely represent newly made Treg cells. The 2-fold increase in BrdU uptake in Id1 transgenic thymuses also corresponded to the increases in total Foxp3+ cells or Foxp3+Helios+ cells shown in Figs. 1 and 2.

**Id1 potentiates Treg differentiation in a cell-intrinsic manner**

To determine whether the effect of Id1 in augmenting Treg production is intrinsic to developing Treg cells themselves, we generated mixed bone marrow chimera by cotransplanting CD45.2+ WT or Id1 transgenic bone marrows (tester) with an equal number of competitor cells isolated from CD45.1+ C57BL/6 mice. Twelve weeks later, Treg cells were scored by staining thymocytes and splenocytes with Abs against CD4, CD25, and Foxp3 (Fig. 4). As expected, WT tester cells produced similar percentages of Treg cells as the competitors. In contrast, Id1 transgenic testers generated twice as many Treg cells compared with the competitors in both the thymus and spleen. Importantly, the competitors in the WT and Id1 transgenic chimera behaved similarly, suggesting that they were not influenced by any potential extrinsic factors secreted by Id1 transgenic T cells. Rather, cell-intrinsic Id1 expression was required to endow T cells with enhanced potential to differentiate into Treg cells.
Id1 expression promotes the generation of thymic Treg precursors by partially substituting for CD28 signaling

It is shown that the differentiation of Treg cells in the thymus can be divided into TCR-dependent and independent steps (7, 9). First, newly formed CD4 single-positive T cells upregulate CD25 upon TCR signaling to become Treg precursors, which are characterized as CD4+CD25+CD25-Foxp3−. These precursors then differentiate into Foxp3+ cells, and this step is facilitated by signaling from the IL-2R in vivo or in vitro. Vang et al. (17) has used a more refined definition of Treg precursors and Treg cells by including two additional markers, CD122 and GITR, and demonstrated that CD28-mediated signaling is important for the formation of Treg precursors. We have previously shown that Id1 expression could substitute for exogenous CD28 stimulation and allow CD4+ T cells to proliferate when treated with anti-CD3 Ab alone (31, 32). Therefore, we used the scheme described by Vang et al. to determine whether Id1 expression promotes the generation of Treg precursors and whether it can rescue the defect in CD28−/− mice.

Cohorts of 7-wk-old mice of the genotypes of Id1+/+, CD28+/+, and CD28−/−Id1+/+ were analyzed along with negative controls (Fig. 6). Thymocytes were stained with Abs against CD4, CD8, CD3, CD25, CD122, and GITR, followed by intracellular staining for Foxp3. CD4+CD3+ cells were then analyzed for CD25 and Foxp3 expression. The expression of CD122 and GITR was further examined in cells within the CD25−Foxp3− or CD25+Foxp3+ gate. CD122+GITR+ cells in each of these two populations were considered Treg precursors and Treg cells, respectively.

Compared with controls, the frequency of both Treg precursors and Treg cells was found to be higher in Id1+/+ mice (Fig. 6). CD28 deficiency led to dramatic reduction in both populations. Expression of Id1 in CD28−/− mice resulted in a 60% increase in the percentage of Treg precursors compared with CD28−/− mice, whereas the generation of phenotypically mature Treg cells was only increased by 20%. These results suggest that Id1 expression created effects that can partially substitute for the loss of CD28 in promoting the formation of Treg precursors. However, differentiation into Treg cells most likely demands stronger CD28 signaling or additional downstream events, which Id1 expression is not able to provide.

Id1 promotes in vitro Treg induction in the absence of CD28-mediated costimulation

To further examine the effect of Id1 expression in TCR-mediated differentiation, we used an in vitro differentiation system to induce iTreg formation from naive CD4+ T cells in the periphery. In this system, naive T cells are cultured in the presence of anti-CD3 and anti-CD28 plus TGF-β. We first tested the induction of Id3 by culturing CD4+CD62LhighCD44lowCD25− naive T cells in vitro with Abs against CD80 and CD86 to block TCR signaling or additional downstream events, which Id1 expression is not able to provide.

Id1 expression facilitates Treg differentiation in coculture with thymic dendritic cells

To mimic thymic Treg differentiation in vitro, we examined Treg differentiation from CD4+CD25− thymocytes from OTII or OTII/Id1+/− mice on WT thymic dendritic cells pulsed with OVA. As observed in vivo, OTII/Id1+/− thymocytes exhibited a greater differentiation potential than OTII thymocytes (30.5 versus 16.5%; Fig. 5). This result verifies that Id1 expression facilitates thymocyte differentiation into the Treg lineage and provides further support for the notion that the effect of Id1 is T cell intrinsic.

Moreover, we carried out the coculture experiments in the absence or presence of Abs against CD80 and CD86 to block TCR costimulatory signals from the dendritic cells. These Abs markedly diminished Treg production from OTII thymocytes. Treg production from OTII/Id1+/− thymocytes, in contrast, is less severely affected, remaining at a level similar to cultures of OTII thymocytes without Ab treatment (Fig. 5). Apart from confirming a crucial role of TCR costimulation for Treg differentiation, these results provide a clue that Id1 may exert its effect by regulating the costimulatory signal.
from the lymph nodes of WT mice for different lengths of time and found Id3 expression was dramatically stimulated not by anti-CD3 alone but by anti-CD3 plus anti-CD28 (Fig. 7A). This result suggests that Id3 upregulation by TCR costimulation occurs during Treg differentiation.

We then cultured naive T cells on plate-bound anti-CD3 Abs in medium containing TGF-β plus or minus anti-CD28. Foxp3 expression was scored as an indication of Treg differentiation. When stimulated with anti-CD3 alone, WT cells differentiated poorly, but Id1-expressing cells generated a significantly higher percentage and number of Foxp3+ cells (Fig. 7B). In contrast, when both anti-CD3 and anti-CD28 were provided, WT and transgenic naive T cells both exhibited robust differentiation (Fig. 7B). These results suggest that Id1 expression could substitute at least in part the signaling events triggered by anti-CD28 stimulation in promoting Treg differentiation.

To further illustrate the effect of Id1 expression on Treg induction under the influence of TCR costimulation, we titrated the amount of anti-CD28 added to the culture, ranging from 0.002 to 2 μg/ml (Fig. 7C). Like the result from the culture without anti-CD28, in the presence of 0.002 μg/ml Ab, Id1 transgenic cells differentiated more efficiently than WT controls. Interestingly, when the concentration of anti-CD28 reached 0.2 or 2 μg/ml, the transgenic cells displayed a slightly impaired capacity for Treg induction. Therefore, Id1 expression imposed a differential influence on Treg induction dependent on the strength of costimulatory signals. The inhibitory effect at the high end is probably because of the direct counteraction of Id1 on E protein–mediated transcription of Foxp3 gene expression (39). Indeed, we found lower levels of Foxp3 mRNA and mean fluorescence intensity of Foxp3 staining in Id1-expressing cells in the Treg-inducing condition with optimal concentrations of anti-CD3 and anti-CD28 (Fig. 7D).
gave rise to very few Foxp3+ cells either in the presence or absence of costimulation. In contrast, CD282/2 Id1tg cells were as efficient as Id1tg cells to develop into Treg cells in cultures with anti-CD3 alone. Moreover, expression of Id1 transgene partially restored the impaired Treg induction from CD282/2 cells under stimulation with both anti-CD3 and anti-CD28 (Fig. 7E). Therefore, these results suggest that Id1 expression exerts effects similar to those delivered by CD28-mediated signaling to promote Treg differentiation.

Enhanced IL-2 production by Id1-expressing cells contributes to Treg induction in the absence of costimulation

It is well established that Treg differentiation depends on signaling from IL-2Rs. As one of the downstream events elicited by CD28 signaling is the production of IL-2, we determined the effect of Id1 on IL-2 production when naive T cells were stimulated with TGF-β along with anti-CD3 alone or with anti-CD3 plus anti-CD28. We collected the culture supernatants 48 h after the cells were plated and detected the level of IL-2 secreted by WT and Id1 transgenic cells. Although the levels of IL-2 produced by WT and transgenic cells were similar in the presence of anti-CD3 and anti-CD28, Id1-expressing cells generated a much higher level of IL-2 compared with WT controls when stimulated with anti-CD3 alone (Fig. 8A).

To test whether this elevated IL-2 production by Id1-expressing cells contributed to their increased differentiation capacity, we cultured naive T cells on plate-bound anti-CD3 in medium containing TGF-β plus or minus a neutralizing Ab against IL-2. WT cells differentiated poorly under this condition, and addition of the IL-2 Ab had little effects (Fig. 8B). However, the augmented differentiation from Id1-expressing cells was dramatically diminished by the anti–IL-2 Ab (Fig. 8B), which suggests that Id1-mediated elevation of IL-2 secretion facilitates Treg differentiation in the absence of anti-CD28 costimulation.

Furthermore, we examined the response of WT and transgenic T cells to exogenous IL-2 of various concentrations in cultures stimulated with anti-CD3 and TGF-β (Fig. 8C). At a low concentration of IL-2 (2 ng/ml), Id1-expressing cells exhibited su-

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[FIGURE 6.](#) Id1 expression augments the frequency of Treg precursors in WT and CD282/2 mice. Thymocytes from mice of indicated genotypes were stained with Abs against CD3, CD4, CD8, CD25, CD122, and GITR, followed by intracellular staining for Foxp3. CD4+CD3+ cells were analyzed for expression of CD25 and Foxp3. CD25−Foxp3− or CD25+Foxp3+ cells (defining Treg precursor or Treg) were then separately examined for CD122 and GITR levels. Representative plots are shown on the top. Data shown at the bottom are the average percentages from control (WT or CD282/2, n = 11), Id1tg (n = 11), CD282/2 (n = 6), and CD282/2Id1tg (n = 14) analyzed in four independent experiments. Error bar shows SD. Unpaired Student t test was performed to determine the significance between two indicated groups, ***p < 0.001, **p < 0.01, *p < 0.05. In addition, the one-way ANOVA analysis indicates a statistical significance among the four groups (p < 0.0001).
perior differentiation potential over WT cells, similarly to that seen without IL-2 supplementation (Fig. 8B). Such a difference disappeared when IL-2 concentration was raised to 5 ng/ml. In fact, the effect was even reversed with further increase of IL-2 concentration. This was analogous to what was observed with different concentrations of anti-CD28 (Fig. 7C). Collectively, we have obtained several lines of evidence to suggest that elevation of IL-2 production resulting from Id1 expression contributes to the increased capacity for Treg differentiation of Id1 transgenic mice.

Reduced susceptibility of Id1 transgenic mice to EAE induction

To evaluate the biological significance of augmented Treg production in Id1 transgenic mice, we used the EAE model. WT and Id1 transgenic littermates were immunized s.c. with the MOG peptide, followed by administration of pertussis toxin. Disease progression was monitored daily for 36 d. As shown in Fig. 9A, Id1 transgenic mice exhibited a considerably attenuated response to the immunization compared with WT littermates. Fourteen days after immunization, we examined lymphocyte infiltration in the
spinal cord and found significantly lower cell counts in Id1 transgenic mice (Fig. 9B). In the draining lymph nodes of the transgenic mice, we detected a significant increase in Foxp3⁺ cells (Fig. 9C). However, the frequency of Foxp3⁺ cells in spinal cord was not significantly different in WT and transgenic mice (Fig. 9C). Furthermore, we analyzed CD4⁺ effector T cells in the lymph nodes and spinal cord by staining for IL-17 and IFN-γ production at this time point. Interestingly, the frequency of IL-17⁺ Th cells was dramatically reduced in Id1 transgenic mice, suggesting that Th17 cell differentiation was suppressed (Fig. 9D). Of particular note, the frequency of IL-17⁺IFN-γ⁺ Th cells, which are considered encephalitogenic (45, 46), was markedly reduced in both the lymph nodes and spinal cord. In contrast, the percentages of IFN-γ⁺IL-17⁺ Th cells, which are most likely Th1 cells, were similar or slightly higher in the lymph nodes and spinal cord of the transgenic mice, respectively (Fig. 9D). Overall, these results suggest that Id1 expression leads to increased levels of Treg cells in the animals, which may be responsible for an elevated immune suppressive function and reduced susceptibility to EAE, possibly through inhibition of Th17 production in vivo.

Discussion
In this study, we demonstrate that Id1 expression in CD4⁺ cells promotes Treg differentiation in vivo and in vitro by augmenting events that are in common with those triggered by CD28-mediated signaling. As CD28-mediated costimulation plays an important role in upregulating Id3 gene expression (Fig. 7A), our data have significant implications in the biogenesis of Treg cells. Although Id1 itself is not stimulated by TCR signaling, it shares extensive structural and functional similarities with Id3 (42). Hence, the Id1 gain-of-function studies described in this work can shed light on the role of bHLH proteins in Treg differentiation. This is analogous to the investigation into the role of these proteins in TCR-β selection, in which both ablation of the E2A gene and over-expression of Id1 lower the threshold of TCR signaling and enable RAG1-deficient thymocytes to progress to the CD4 and CD8 double-positive stage (30, 47). With regard to the role of these bHLH proteins in Treg differentiation, Id1 transgenic and Id3-deficient mice appeared to display the opposite phenotypes (39). Namely, the former have increased Treg counts, whereas the latter show a deficit of Treg cells at ages before the onset of autoimmune diseases. In vitro, Id1 transgenic naive T cells can differentiate without anti-CD28 costimulation, but Id3-deficient cells fail to produce Foxp3⁺ cells even when stimulated with both anti-CD3 and anti-CD28.
Different underlying mechanisms have been invoked to ascribe the roles of bHLH proteins in Treg development. For example, E2A proteins are found to bind to the promoter of the Foxp3 gene (39), and Id1 expression inhibits Foxp3 expression in vitro when naive T cells were cultured in the presence of anti-CD3 and anti-CD28 as well as TGF-β (Fig. 7D). In this study, we present data to highlight the impact of bHLH proteins on TCR-mediated effects on Treg differentiation. According to the two-step model, the generation of Treg precursors depends on TCR/CD28 signaling (7). Id1 transgenic mice possess a larger number of CD122+GITR+ CD25+Foxp3+ Treg precursors, and expression of Id1 transgene in CD28−/− mice partially rescues the deficit of this population of cells due to CD28 deficiency (16, 17). In vitro, Id1 expression also promoted Treg differentiation without events triggered by anti-CD28. The ability of Id1 to substitute for or mimic CD28 signaling could be highly significant in the animals. Numerous studies have demonstrated that the strength of TCR signaling is critical for developing T cells to commit to the Treg lineage as opposed to undergoing negative or positive selection (8). Id1 expression may contribute to the elevation of net TCR signaling strength by augmenting unknown downstream effects, which could then lead to increases in Treg production and/or homeostasis. Similarly, Id3 upregulation by TCR/CD28 signaling may be instrumental for Treg differentiation as ablation of the Id3 gene impedes Treg development (39). Our data would support the notion that Id3 is a key effector downstream of TCR/CD28 signaling in Treg cell production.

It should be noted that expression of the Id1 transgene could not fully rescue the defect in Treg production in CD28−/− mice. There could be several explanations. First of all, Id1 expression may not impact all aspects of CD28 signaling required for Treg differentiation. Alternatively, quantitative limitations of the effects of Id1 on CD28 signaling may exist. With physiological strengths of TCR signaling, Id1 expression could partially facilitate the formation of Treg precursors, but the production of Foxp3+ cells might demand stronger costimulation. In the case of artificial stimulation of the TCR with anti-CD3 in vitro, Id1 enabled the generation of Treg cells from naive T cells without anti-CD28, although the production was less robust than that when the cells were stimulated with anti-CD3 and anti-CD28. Considering the broad impacts of CD28 signaling on various cellular processes (48), an incomplete rescue of Treg differentiation by Id1 expression in CD28−/− mice is not surprising.

The molecular mechanism whereby Id1 promotes Treg differentiation is not well understood. In addition to an increased frequency of Treg precursors present in Id1 transgenic mice, we have shown that Id1 expression resulted in elevated production of IL-2 under in vitro Treg differentiation conditions without anti-CD28 stimulation. Signaling from IL-2Rs is known to be essential for the development of Foxp3+ regulatory T cells. From anti-CD3 stimulation, naive T cells undergo negative or positive selection (8). Id1 expression may take place, in which case upregulation of Id genes such as Id3 could dampen Treg differentiation by inhibiting Foxp3 expression and thus enabling the function of effector T cells. This would be reminiscent of the situation we observed that Id1-expressing naive T cells exhibited reduction in Foxp3 expression when induced to differentiate by anti-CD3 and anti-CD28. Overall, our studies on the role of Id proteins in Treg development and induction have shed light on the importance of bHLH proteins in regulating cellular immunity in normal and pathologic situations.

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Disclosures

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