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The Plasticity of Regulatory T Cell Function

Meenu R. Pillai,* Lauren W. Collison,†‡ Xiaohua Wang,*‡ David Finkelstein,† Jerold E. Rehg,‡ Kelli Boyd,‡,3 Andrea L. Szymczak-Workman,* Teresa Doggett,*§ Thomas S. Griffith,* Thomas A. Ferguson,§ and Dario A. A. Vignali*

Regulatory T cells (Tregs) can suppress a wide variety of cell types, in diverse organ sites and inflammatory conditions. Whereas Tregs possess multiple suppressive mechanisms, the number required for maximal function is unclear. Furthermore, whether any interrelationship or cross-regulatory mechanisms exist to orchestrate and control their utilization is unknown. In this study, we assessed the functional capacity of Tregs lacking the ability to secrete both IL-10 and IL-35, which individually are required for maximal Treg activity. Surprisingly, IL-10/IL-35 double-deficient Tregs were fully functional in vitro and in vivo. Loss of IL-10 and IL-35 was compensated for by a concurrent increase in cathepsin E (Ctse) expression, enhanced TRAIL (Tnfsf10) expression, and soluble TRAIL release, rendering IL-10/IL-35 double-deficient Tregs functionally dependent on TRAIL in vitro and in vivo. Lastly, whereas C57BL/6 Tregs, are normally IL-10/IL-35 dependent, BALB/c Tregs, which express high levels of cathepsin E and enhanced TRAIL expression, are partially TRAIL dependent by default. These data reveal that cross-regulatory pathways exist that control the utilization of suppressive mechanisms, thereby providing Treg functional plasticity. The Journal of Immunology, 2011, 187: 4987–4997.

R egulatory T cells (Tregs) play a key role in maintaining immune tolerance, preventing autoimmune diseases, and limiting inflammatory conditions (1–3). A unique and important feature of Tregs is the brevity and flexibility of their regulatory capacity. Tregs can suppress an array of different cell types [including CD4+ T cells (Th1/Th2/Th17) (4), CD8+ T cells (5), B cells (4, 6), dendritic cells (7), and osteoclasts (8)] in a variety of inflammatory conditions and in distinct tissue locations. The ability of Tregs to suppress a broad range of targets in a variety of scenarios can be attributed to the numerous mechanisms employed by Tregs, to mediate their function (2, 9). However, it is not clear whether all these mechanisms are equally important or whether they have nonredundant roles under different inflammatory settings. Indeed, it was recently reported that Tregs may have specialized mechanisms for controlling specific cell types, as Treg, appear to require IFN regulatory factor-4, T-bet, and STAT3 to suppress Th2, Th1, and Th17 cells, respectively (10–12). However, it is unclear which Treg mechanisms are used under specific conditions, how many mechanisms are required for maximal Treg function, and whether there is any crosstalk between the various regulatory mechanisms used. Although it is well established that Foxp3 is a key transcription factor critical for the stability of Tregs (13), whether there is stability or plasticity in the regulatory mechanisms used by Tregs is unclear.

Tregs use multiple mechanisms to mediate their function, with the immunosuppressive cytokines TGF-β, IL-10, and IL-35 contributing significantly (14–16). IL-10 is important for Treg function in vitro and in vivo, especially in the gut (17, 18). IL-35 is a recently discovered heterodimeric cytokine composed of Ebi3 (also part of IL-27) and Il12a/p35 (also part of IL-12) that is uniquely expressed by Tregs but not by T conventional (Tconv) cells, and is required for maximal Treg function (16). Whereas the loss of either IL-10 or IL-35 significantly reduces Treg function, they do not become completely dysfunctional and deficient mice do not exhibit the lethal multorgan inflammatory disease seen in Scurfy or Foxp3−/− mice that lack Tregs (19, 20). Thus, in the current study, we speculated that Tregs that lacked both IL-10 and IL-35 might exhibit a more profound functional defect and that this approach could be used to assess the relative contributions of different suppressive mechanisms. Alternatively, given the importance of Tregs in the maintenance of immune homeostasis, as yet unknown compensatory mechanisms may be triggered that attempt to restore immune balance. These possibilities were tested in this study.

Materials and Methods

Mice

Ebi3−/− mice (C57BL/6; now 100% C57BL/6 by microsatellite analysis performed by Charles River) were provided by R. Blumberg and T. Kuo (Brigham and Women’s Hospital, Boston, MA); Il10−/− mice were provided by T. Geiger (St. Jude Children’s Research Hospital, Memphis, TN);

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Address correspondence and reprint requests to Dr. Dario A.A. Vignali, St. Jude Children’s Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105-3678. E-mail address: vignali.lab@stjude.org

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Abbreviations used in this article: CTSE, cathepsin E; DR5, death receptor 5; IBD, inflammatory bowel disease; qPCR, quantitative PCR; Tconv, T conventional; Treg, regulatory T cell.

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Treg FUNCTIONAL PLASTICITY

Preparation of anti-CD3/CD28–coated latex beads

The 4 μM sulfate latex beads (Molecular Probes) were incubated overnight at room temperature with rotation in a 1:4 dilution of anti-CD3 and anti-CD28 Ab mix (13.3 μg/ml anti-CD3 (murine clone 145-2c11 and 26.6 μg/ml anti-CD28 murine clone 37.51); eBioscience). Beads were washed three times with 5 mM phosphate buffer (pH 6.5) and resuspended at 5 × 10^6/ml in sterile phosphate buffer with 2 mM BSA.

In vitro Treg suppression assay and Transwell Treg assay

In vitro Treg suppression assays were performed, as described previously (16, 17, 22). Anti-CD3– and anti-CD28–coated beads used for T cell stimulation in these assays were prepared, as described previously (17, 22, 23). RAG1−/− and Treg−/− cells were suspended in Immunoprecipitation and Western blotting

Immunoprecipitation and immunoblotting for CTSE were performed, as previously described (21). Tconv wild-type, or knockout Tregs were cultured at a 2:1 ratio in the Transwell insert with a pore size of 0.4 μm (Millipore). Target wild-type Tconv or Treg−/− (DR5−/−) Tconv cells were activated in the bottom compartment of the Transwell plate with anti-CD3– and anti-CD28–coated latex beads for 72 h. Where indicated, neutralizing IL-10 mAb (JES5-2A5; BD Biosciences), neutralizing IL-35 mAb (V1.4C2.4), isotype control, or DR5-Fc was added to standard Treg assays and Transwell experiments at the concentrations indicated. Where indicated, Tconv cells were activated at a 1:5 dilution of 20% formaldehyde in culture medium, incubated at room temperature for 20 min, and washed three to five times with medium prior to culture. After 64 h in culture, Transwell inserts were removed and [3H]thymidine was added directly to the responder Tconv cells in the bottom chambers of the Transwell plate for the final 8 h of the 72-h assay. Cultures were harvested with a Packard harvester. The cpm were determined using a Packard Matrix 96 direct counter (Packard Biosciences).

CTSE/TRAIL transfection assay

For in vitro assays with transfected 293T cells cocultured with Tconv cells, 293T cells were transfected with Ctse (murine CTSE in pCIneo [Promega] with an IRES-YFP expression cassette); provided by B. Chain, University College London, London, U.K.) or Trf510 (murine TRAIL, in pCIneo [Promega with IRES-GFP]; provided by T. Griffith, University of Minnesota, Minneapolis, MN) alone or in combination. Posttransfection (48 h), Tconv cells were irradiated (3000 rad) and seeded at a density of 7 × 10^6 cells/well in the 96-well flat-bottom plate. Purified C57BL6 Tconv cells were added to the seeded plate at 8 × 10^6 per well and stimulated with anti-CD3– and anti-CD28–coated beads for 72 h with [3H]thymidine added during the last 8 h of culture. T cell proliferation was calculated by subtracting the basal [3H]thymidine incorporation of irradiated 293T plus unstimulated Tconv cells.

Treg-mediated control of homeostatic expansion

Homeostasis assays were performed, as described previously (16, 23). Briefly, naive Thy1.1+ Tconv cells from B6.PL mice, which were used as target cells, and Thy1.2+ wild-type or knockout Treg cells were purified by FACS. Tconv cells (2 × 10^6) and Treg cells (5 × 10^5) were resuspended in 0.5 ml PBS plus 2% FBS and injected i.v. into Rag1−/− mice. Where indicated, the mice were injected on days 0 and 3 with anti-TRAIL Ab (0.3 μg; provided by T. Griffith, University of Iowa) or isotype control Ab (0.3 μg; R&D Systems). Mice were euthanized 7 d post-transfer, and spleenocytes were counted, stained, and analyzed by flow cytometry using Abs against Cd4, Thy1.1, Thy1.2 (BioLegend), and Foxp3 (BD Biosciences). For each group, six to eight mice were analyzed.

Inflammatory bowel disease model

A recovery model of colitis/inflammatory bowel disease (IBD) was used, with some modifications (14, 23). Briefly, Rag1−/− mice were injected i.v.
with 0.5 × 10^6 wild-type or DR5−/− (CD4+/CD45RB+IbpCD25+) naïve Tconv cells to initiate IBD. Mice were weighed at the time of injection (time 0) and every week on the same day. At the onset of clinical symptoms of colitis (∼4 wk post-Tconv cell transfer), the mice were divided into Treg recipient or no Treg control groups. Purified Tregs from wild-type, Ebi3−/−, Il10−/−, or Ebi3−/−Il10−/− were injected i.p. All mice were weighed weekly and euthanized 32 d after the initial T-cell transfer in experimental mice, the colons were collected and fixed in 10% neutral-buffered formalin 4 wk after T-cell injection. The tissues were further processed, and 4-μm sections were cut and stained with H&E. Pathology of the large intestine was scored in a blinded manner using a semiquantitative scale, as described previously (23). In summary, grade 0 was assigned when no changes were observed; grade 1, minimal inflammatory infiltrates present in the lamina propria without or with mild mucosal hyperplasia; grade 2, marked inflammatory infiltrates in the lamina propria with occasional extension into the submucosa, focal erosions, minimal to mild mucosal hyperplasia, and minimal to moderate mucin depletion; grade 3, mild to moderate inflammation in the lamina propria and submucosa occasionally transmural with ulceration and moderate mucosal hyperplasia and mucin depletion; grade 4, marked inflammatory infiltrates commonly transmural with ulceration, marked mucosal hyperplasia and mucin depletion, and multifocal crypt necrosis; grade 5, marked transmural inflammation with ulceration, widespread crypt necrosis, and loss of intestinal glands.

**Foxp3−/− rescue model**

The Foxp3−/− rescue model was performed, as described previously (23). Briefly, wild-type or knockout Treg purified by FACS were injected (10^6) i.p. into 2- to 3-month-old C57BL/6 mice. Recovery from disease was monitored weekly and reported as a clinical score. Five macroscopic categories were used to generate a 6-point scoring system. Mice were scored on the first four categories based on whether they showed (score of 1) or did not show (score of 0) the following characteristics: body size runted; tail is scaly and/or with lesions; ears small and scaly with or without lesions; and eyelids scaly and/or not fully open. The final scoring parameter was monitoring the activity level of the mouse. A score of 0 was assigned if the mouse was normal. A score of 1 was assigned if the mouse’s activity was moderately impaired, and a score of 2 was assigned if the mouse was immobile. A combined score of 4 or greater was assigned moribund for longevity. Mice were euthanized 25 d posttransfer, spleen cells were counted and stained, and cell numbers were determined by flow cytometry. Lung, liver, and ear pinna were prepared for H&E analysis, and the severity of inflammation was assessed and scored in a blinded manner by an experienced veterinary pathologist. The scoring system used for assessing inflammation was based on a simple algorithm for expressing inflammatory infiltrates in the lungs, liver, and ear. The scores allotted to these three tissues were 0–9, 0–11, and 0–8, respectively, giving a maximum possible total of 28. Scoring criteria for each organ was as follows. The lung score was based upon inflammation in the peribronchiolar region, perivascular region, or interstitium. A score of 0–3 was assigned to each category, with 0 being minimal or no inflammation, and scores of 1, 2, or 3 indicative of <10, 10–50, or >50%, respectively. The liver was scored based on three criteria. First was the degree of portal tract inflammation, with a score of 0 assigned to minimal or no inflammation. A score of 1, 2, or 3 was assigned if inflammation was associated with <25, 25–75, or >75% of the liver portal tracts, respectively. The second criterion related to portal/perisplenic interface hepatitis. A focus of interface hepatitis associated with either a few or most of the portal tracts was scored 1 and 2, respectively. Two or more foci of interface hepatitis surrounding <50% or >50% of the portal tracts or perisplenia were scored 3 and 4, respectively. Third, pericentral and periporal foci of hepatitis, with or without macrovesicular hepatocytes that expand the sinusoid were considered foci of inflammation. The number of inflammatory foci in 10 contiguous original magnification ×10 objective fields was counted and recorded as the average number of foci per ×10 field and given a score of 0–4. A score of 0 was assigned when sinusoidal foci of inflammatory cells were absent. One focus or less per ×10 field, 2–4 foci per ×10 field, 5–10 foci per ×10 field, and >10 foci per ×10 field were scored 1, 2, 3, and 4, respectively. The ear score was similarly scored based on two parameters, as follows: the percentage of the ear dermis with inflammatory infiltrates and the intensity of the dermal inflammation. For percentage analysis, a score of 0 was assigned when the inflammatory cells in all segments were not beyond that of normal background level. A score of 1, 2, 3, or 4 was assigned when the average percentages for the segments were <25, 25–50, 51–75, or >75%, respectively. The intensity of the inflammatory infiltrate in the dermis was similarly assessed as being of a loose or dense nature. A score of 0 was assigned when inflammatory cells in the dermis were not beyond the normal background level. When all of the inflammation was of the loose nature, a score of 1 was assigned. When there was a mixture of loose and dense inflammatory cell infiltrates, a score of 2 was assigned when the loose form was dominant. A score of 3 was assigned when the dense form was dominant. A score of 4 was assigned when all of the inflammation was of a dense nature.

**Affymetrix array and analysis**

Wild-type or knockout Tregs were purified by FACS and mRNA isolated using the Qiagen micro RNA kit (Qiagen). Quality was confirmed by UV spectrophotometry and by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (100 ng) was processed in the Hartwell Center for Biotechnology and Bioinformatics according to the Affymetrix eukaryote two-cycle target-labeling protocol and arrayed on a Mouse-430v2 GeneChip array. The expression data from the Affymetrix U133 plus two arrays were analyzed as MAS 5.0 signal-log-transformed using the following formula: log signal = natural log (signal + 20). This transform improves data dispersion and normality, and stabilizes the variance of the data (24). Statistical tests and batch effect removal were performed using Partek Genomics Suite (St. Louis, MO). The log2 ratio of Ebi3−/−Il10−/− Treg to wild-type Treg was calculated, and the 20 most positively induced named genes were selected. The log2 ratios are calculated in STATA/SE 11.0 (College Station, TX) by the following formula: log ratio A over B = log(exponentiation[mean log signal A]/mean log signal B)). Minimum selected gene had a log ratio of 1.65, which is 3.14-fold induced. Log ratios of the Il10−/− Treg and the Ebi3−/− Treg with respect to wild type were also defined and plotted with the ratio of Ebi3−/−Il10−/− Treg to wild-type Treg as a heat map using Spotfire Decision Site software (Fig. 3A). The t tests were then applied to each probe set to compare the Ebi3−/−Il10−/− Treg with wild-type Treg and single knockout Treg samples and log2 ratios were calculated. The p values from the t tests were then log2 transformed to create the significance score seen in the x-axis of the volcano plot (Fig. 3B). A second series of t tests was performed to compare Treg with Tconv, and to develop a Treg signature. Probe sets that had a p value <10−4, an absolute value log ratio of Treg versus Tconv of at least 3 (log2), and a defined gene name were selected for each category in the signature that the mean was found. If a gene name appeared more than once, it was scored once for that gene. The scores were calculated by finding the maximum and minimum values for each gene and then rescaling them from 0 to 1 by the following formula: score = (observed mean − minimum mean)/ (maximum mean − minimum mean) for each gene g. These gene scores were then sorted in descending order by the Treg/Tconv log ratio that includes activated and resting cells and graphed as a heat map in Spotfire Decision Site (data not shown). The microarray data from this study have been submitted to the Gene Expression Omnibus repository, accession number GSE29262 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29262).

**Statistical analysis**

Unless otherwise stated, a Student t test was used to determine statistical significance. All calculations were done using GraphPad software. A p value <0.05 was considered significant.

**Results**

Treg, that lack IL-10 and IL-35 maintain their suppressive activity

We first assessed the functional capacity of Treg that lacked the ability to secrete IL-10 or IL-35 by generating Ebi3−/−Il10−/− and Il12a−/−Il10−/− mice (note that both Ebi3 and Il12a/p35 are required for IL-35 production) (16, 17). Purified wild-type, Ebi3−/−, Il10−/−, Ebi3−/−Il10−/−, and Il12a−/−Il10−/− Tregs were assessed in a standard Treg assay (note that these double-deficient Treg would not be able to secrete IL-10 or IL-35, and although Ebi3 is also used by IL-27 and Il12a/p35 is also used by IL-12, these cytokines are not produced by Tregs (16)). Surprisingly, Ebi3−/−Il10−/− and Il12a−/−Il10−/− Treg function was comparable or slightly better than wild-type Treg in suppressing their target Tconv (Fig. 1A). We have previously shown that if Tregs are optimally stimulated by anti-CD3– and anti-CD28–coated beads and in contact with Tconv cells in the lower chamber (insert) of a Transwell plate, they can suppress third-party Tconv cells in the lower chamber across a semipermeable membrane (17).
Importantly, this suppression requires, and is limited to, IL-10 and IL-35. Thus, we anticipated that the loss of IL-10 and IL-35 would render Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> unable to suppress across a Transwell. Strikingly, Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> suppressed T<sub>conv</sub> cells across a Transwell comparable to their wild-type counterparts, even though Ebi3<sup>−/−</sup> and Il10<sup>−/−</sup> T<sub>reg</sub> were partially defective (Fig. 1B). This equivalency in function was further supported by experiments with CFSE-labeled target T<sub>conv</sub> cells and Transwell experiments with titrated Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> in presence of fixed or unfixed T<sub>conv</sub> cells (Supplemental Fig. 1A, 1B). These data suggest that Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub>, unlike Ebi3<sup>−/−</sup> and the Il10<sup>−/−</sup> T<sub>reg</sub>, are functionally intact in in vitro suppression assays.

We next asked whether Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> were functionally equivalent in several in vivo models. The adoptive transfer of T<sub>reg</sub> into neonatal Scurfy or Foxp3<sup>−/−</sup> mice has been shown to restore normal immune homeostasis and prevent the lethal, systemic autoimmune disease that develops in these mice (19, 25, 26). Two-day-old neonatal Foxp3<sup>−/−</sup> mice were injected with 10<sup>6</sup> wild-type, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, or Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub>. Clinical symptoms, histological analysis, and CD4<sup>+</sup> T cell numbers were determined when the mice were ~4 wk old. Although no defects were observed with the Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> recipients, increased histological scores were observed with Il10<sup>−/−</sup> T<sub>reg</sub> recipients. In contrast, Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> were clearly capable of fully restoring immune homeostasis despite the loss of these two key regulatory cytokines (Supplemental Fig. 2A–C). We also assessed the ability of these T<sub>reg</sub> populations to rescue immune homeostasis in mixed bone marrow chimeras generated using a 50:50 mixture of bone marrow from Foxp3<sup>−/−</sup> mice and either wild-type, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, or Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> mice transferred into Rag1<sup>−/−</sup> mice. Interestingly, significant defects were observed in the ability of Ebi3<sup>−/−</sup> and Il10<sup>−/−</sup> bone marrow to rescue the Foxp3<sup>−/−</sup> phenotype (Supplemental Fig. 2D, 2E). In contrast, the Foxp3<sup>−/−</sup> bone marrow recipients of Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> were largely intact and comparable to their wild-type T<sub>reg</sub>. Foxp3<sup>−/−</sup> recipient counterparts (Supplemental Fig. 2D, 2E).

T<sub>reg</sub> have been shown to regulate the homeostatic expansion of T<sub>conv</sub> cells in lymphopenic Rag1<sup>−/−</sup> mice (27–29). Purified wild-type Thy1.1 T<sub>conv</sub> cells, either alone or in presence of wild-type, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup>, or Il12a<sup>−/−</sup>/Il10<sup>−/−</sup> Thy1.2<sup>+</sup> T<sub>reg</sub> were adoptively transferred into Rag1<sup>−/−</sup> mice, and splenic Thy1.1 T<sub>conv</sub> and Thy1.2 T<sub>reg</sub> numbers (data not shown) were determined 7 d later. In the presence of wild-type, but not Ebi3<sup>−/−</sup> or Il10<sup>−/−</sup> T<sub>reg</sub>, T<sub>conv</sub> cell expansion was significantly reduced (Fig. 1C). Surprisingly, the capacity of Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> and Il12a<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> to control T<sub>conv</sub> cell expansion was comparable to wild-type T<sub>reg</sub>.

T<sub>reg</sub> cure colitis in mice, a model for inflammatory bowel disease (IBD) in humans, in an IL-10– and IL-35–dependent manner (16, 30). Colitis in mice is induced experimentally by transferring low numbers of naive CD4<sup>+</sup>CD45RB<sup>hi</sup>CD25<sup>−</sup> T<sub>conv</sub> cells into Rag1<sup>−/−</sup> mice (31). Recovery from disease, marked by weight gain and decreased histopathology, is observed only in mice that receive purified T<sub>reg</sub> ~4 wk after the initial T<sub>conv</sub> cell

### FIGURE 1. Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub> are suppressive in vitro and in vivo. A. Wild-type or knockout T<sub>reg</sub> purified by FACS were titrated in a standard T<sub>reg</sub> assay with T<sub>conv</sub> cells and anti-CD3– and anti-CD28–coated latex beads. Proliferation of T<sub>conv</sub> responder cells was determined by <sup>3</sup>Hthymidine incorporation (p value: wild-type T<sub>reg</sub> compared with Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, and Ebi3<sup>−/−</sup>/Il10<sup>−/−</sup> T<sub>reg</sub>). B. Wild-type or knockout T<sub>reg</sub> were cultured with anti-CD3– and anti-CD28–coated latex beads and T<sub>conv</sub> cells in the inserts of a Transwell culture plate. Third-party, wild-type responder T<sub>conv</sub> was activated in the bottom chamber of the plate. Proliferation of responder cells was determined by <sup>3</sup>Hthymidine incorporation. Proliferation ranged from 30,000 to 60,000 cpm. C. Congenically marked wild-type T<sub>conv</sub> cells, wild-type, or knockout T<sub>reg</sub> purified by FACS were injected at a 4:1 ratio into Rag1<sup>−/−</sup> mice. CD4<sup>+</sup> cell numbers in the spleen were analyzed after 7 d by flow cytometry. D. Wild-type T<sub>reg</sub> cells (0.5 × 10<sup>6</sup>) were injected into Rag1<sup>−/−</sup> mice. The weight of the mice was monitored weekly for weight loss. Once the mice had lost 5% of its body weight, wild-type or knockout T<sub>reg</sub> were injected. Mice were monitored for percentage of weight change calculated based on the weight at the time of T<sub>reg</sub> injection. E. Colonie tissue sections stained with H&E stain were scored in a blinded manner. Representative images of sections (original magnification ×4) from three independent experiments are shown with histological score in parentheses. Data represent the mean ± SEM of three (A), three to five (B), and four to nine mice per group (C); D and E, three independent experiments. Mann–Whitney test was used to determine statistical significance (*p < 0.05, **p < 0.01).
transfer (14). We used this recovery model of colitis to assess the functional capacity of Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> in vivo. Approximately 4 wk post-T<sub>conv</sub> cell transfer, recipients developed clinical symptoms of colitis (monitored by weight loss) and were either left untreated or treated with either wild-type, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, or Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub>. As expected, mice that did not receive T<sub>regs</sub> continued to lose weight, and exhibited substantial histiocytopic infiltration and goblet cell destruction during the subsequent 4 wk (Fig. 1D, 1E, Supplemental Fig. 2F). In contrast, the wild-type T<sub>reg</sub> recipients started to gain weight within 1 wk of transfer. Despite previous studies clearly demonstrating the inability of Ebi3<sup>−/−</sup> or Il10<sup>−/−</sup> T<sub>reg</sub>, to cure colitis, weight gain and improved histological parameters were evident in the Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>reg</sub> recipients, suggesting that these double-inhibitory cytokine-deficient T<sub>regs</sub> had regained their regulatory potential (Fig. 1D, 1E, Supplemental Fig. 2F).

To rule out the possibility that this regulatory restoration had occurred as a consequence of their development in the absence of IL-10 and IL-35 and/or due to alternate cell-extrinsic mechanisms, we directly compared the suppressive capacity of wild-type and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> that had developed in the same environment. To address this possibility, we generated mixed bone marrow chimeras with a 1:1 ratio of congenically marked Thy1.1<sup>+</sup> wild-type bone marrow with Thy 1.2<sup>+</sup> wild-type or Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> bone marrow into sublethally irradiated Rag1<sup>−/−</sup> mice. Eight weeks posttransfer, T<sub>regs</sub> were purified by FACS from the mixed bone marrow chimeras and assessed in vitro Transwell and in vivo homeostasis assays. Chimera-derived Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> and wild-type T<sub>regs</sub> suppressed third-party T<sub>conv</sub> cells comparably across a Transwell (Fig. 2A). In contrast, similarly prepared Ebi3<sup>−/−</sup> and Il10<sup>−/−</sup> T<sub>regs</sub> were defective (data not shown). Furthermore, Thy1.2<sup>+</sup> Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> and wild-type T<sub>regs</sub> that had developed in the bone marrow chimeras suppressed T<sub>conv</sub> expansion comparably in homeostasis assay (Fig. 2B). Taken together, these data suggest that a cell-intrinsic modification had occurred in the Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> to render them functionally comparable to wild-type T<sub>regs</sub> to compensate for their inability to secrete IL-10 and IL-35.

**Loss of IL-10/IL-35 results in a compensatory increase in CTSE**

Given that Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> can suppress T<sub>conv</sub> cells across a Transwell, they had clearly acquired a suppressive mechanism that operated via a soluble mediator. Beyond IL-10 and IL-35, TGF-β is the only other known cytokine or soluble factor that would likely function across a Transwell that has been suggested to play a role in T<sub>reg</sub> function (note that cAMP and adenosine are highly labile inhibitors that are only active in very close proximity) (2, 3, 9). We assessed any potential role for TGF-β by comparing the capacity of wild-type and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> to suppress across a Transwell using third-party T<sub>conv</sub> cells from CD4–dominant negativeTGF-βRII transgenic mice that are resistant to TGF-β–mediated suppression (32). The data clearly show that the suppressive capacity of wild-type and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> was comparable when T<sub>conv</sub> cells resistant to TGF-β–mediated suppression were used as target cells (Supplemental Fig. 1C). This suggested that the compensatory suppressive mechanism used by Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> was not TGF-β.

To identify this compensatory suppressive mechanism, we compared the gene expression profile of wild-type, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> using Affymetrix GeneChip arrays. We first generated a list of highly differentially expressed wild-type T<sub>reg</sub> signature genes, by comparison of the array profile with wild-type T<sub>conv</sub> to determine whether there were any notable global changes in gene expression in wild-type versus Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub>. Minimal variations were observed in the expression (up or down) of 47 highly modulated T<sub>reg</sub> signature genes (data not shown). Indeed, global analysis revealed very few differences between wild-type and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> (Fig. 3A, 3B, data not shown). The two notable exceptions were Apis3 (adaptor-related protein complex 1, σ 3 subunit) and Ctse (Fig. 3A, 3B). A Pis3 is the σ subunit of the adaptor protein-1 complex that is a component of the clathrin-coated vesicles associated with the trans-Golgi network that mediate vesicular formation and transport (33). The significance of its upregulation in Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> is unknown and was not selected for further study in this work. CTSE is an intracellular aspartic protease of the endolysosomal pathway that has been primarily implicated as a component of the Ap1S3 subunit of the adaptor protein-1 complex 1, σ 3 subunit) and that is known and was not selected for further study in this work.

![FIGURE 2](http://www.jimmunol.org/ Downloaded from)

**FIGURE 2.** Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> that developed in a mixed bone marrow chimera can function in vitro and in vivo. Congenically labeled wild-type bone marrow and knockout bone marrow were mixed at a 1:1 ratio and injected into sublethally irradiated Rag1<sup>−/−</sup> mice. A, After 8 wk, Thy 1.2<sup>+</sup> wild-type T<sub>reg</sub>, or knockout T<sub>reg</sub>, were purified by FACS from the bone marrow chimeric mice and cultured in the inserts of a Transwell plate in the presence of wild-type T<sub>conv</sub> cells and anti-CD3– and anti-CD28–coated latex beads. Wild-type naive T<sub>conv</sub>, cells were activated in the presence of anti-CD3– and anti-CD28–coated beads in the bottom chamber of a Transwell for 72 h. Proliferation was determined by [H]thyidine incorporation. Data represent the mean ± SEM of two independent experiments. B, Purified wild-type or Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> bone marrow chimeric-derived T<sub>reg</sub> were injected into Rag1<sup>−/−</sup> mice in the presence of congenically marked naive T<sub>conv</sub> cells. The expansion of naive Thy 1.1 CD4<sup>+</sup> T cells was assessed by flow cytometry. Data represent the mean ± SEM of two independent experiments with three to four mice per group. Statistical significance was determined by the Mann–Whitney test (*p = 0.06).
release of soluble TRAIL. To directly examine this possibility, 293T cells were transfected with expression plasmids encoding Ctse and/or Tnfsf10 and used to assess the ability of TRAIL to limit T cell proliferation. TRAIL transfectants limited T cell proliferation, and this was further enhanced in the presence of CTSE (Fig. 4). These data suggest that CTSE may play a role in enhancing the function of TRAIL by either increasing its activity via processing or increasing the generation of soluble TRAIL. These data also raised the possibility that Ebi3^{−/−} Il10^{−/−} Tregs are dependent on TRAIL for their suppressive activity, whereas wild-type Tregs are not.

**IL-10/IL-35−deficient Tregs suppress via TRAIL**

We first assessed whether there were any changes in the level or rate of TRAIL expression during activation of wild-type, Ebi3^{−/−}, Il10^{−/−}, or Ebi3^{−/−} Il10^{−/−} Tregs. Minimal alterations in Tnfsf10 (TRAIL) mRNA expression were observed over time or between the four Treg populations (Supplemental Fig. 3D). Whereas all Treg populations exhibited increased TRAIL surface expression following activation, Ebi3^{−/−} Il10^{−/−} Tregs expressed significantly higher levels of TRAIL after 16 h, but not 24 h, postactivation (Fig. 5A, 5B, Supplemental Fig. 3E). This suggested that the kinetics of TRAIL expression is accelerated in Ebi3^{−/−} Il10^{−/−} Tregs. Interestingly, although IL-10 appeared to influence CTSE expression (Fig. 3C, 3E, Supplemental Fig. 3A–C), IL-35 may influence other parameters that influence TRAIL expression, as Ebi3^{−/−} Tregs expressed slightly higher levels of TRAIL at 16 h compared with wild-type or Il10^{−/−} Tregs (Supplemental Fig. 3E).

We then used various approaches to determine the extent to which this accelerated TRAIL expression meant that the Ebi3^{−/−} Il10^{−/−} Tregs were dependent on TRAIL-mediated suppression. TRAIL mediates its suppression in part via caspase-mediated apoptosis (37). Thus, we asked whether Ebi3^{−/−} Il10^{−/−} Tregs mediated suppression in a caspase-dependent fashion by performing a Transwell suppression assay in the presence of the general caspase inhibitor z-VAD-Fmk or a vehicle control (42). Although wild-type Treg suppression was unaffected by z-VAD-Fmk, Ebi3^{−/−} Il10^{−/−} Tregs were inhibited by z-VAD-Fmk, whereas Ebi3^{−/−} Il10^{−/−} Tregs were not.

**FIGURE 3.** Upregulation of CTSE by Ebi3^{−/−} Il10^{−/−} Tregs. A, mRNA was isolated from wild-type or knockout Tregs purified by FACS and used for Affymetrix analysis. Modulated genes in knockout Treg compared with wild-type Treg are depicted in a heat map. B, Volcano plot comparing wild-type and Ebi3^{−/−} Il10^{−/−} Treg. Highest modulated genes are marked. C, mRNA was isolated from wild-type or knockout Treg purified by FACS and cDNA synthesized, and Ctse expression was assessed by qPCR. Data are the mean of two independent experiments. D, Wild-type or knockout Tregs were stained for intracellular CTSE (gray, second Ab control, open histograms; in green, wild-type Treg, and in blue, Ebi3^{−/−} Il10^{−/−} Treg). E, Equal numbers of FACS-purified wild-type or knockout Treg were lysed, CTSE immunoprecipitated, and analyzed by SDS-PAGE/Western blot. Data are representative (A, B, D, E) of three independent experiments.

**FIGURE 4.** CTSE enhances the suppression of Tconv cells by TRAIL. The 293T cells were transfected either with Ctse and Tnfsf10 alone or together. The cells were irradiated with 3000 rad 48 h posttransfection and seeded at a density of 7000 cells/well in a 96-well flat-bottom plate. Freshly isolated C57BL/6 Tconv cells were added to the seeded plate at 8 × 10^4 per well and stimulated with anti-CD3– and anti-CD28–coated beads for 72 h. Proliferation of responder cells was determined by [3H]thymidine incorporation. Tconv cell proliferation was calculated by subtracting the basal [3H]thymidine incorporation of irradiated 293T plus T cells without anti-CD3 and anti-CD28 stimulation. Data represent the average of three independent experiments. *p < 0.05.
was plotted. Student t test, **p < 0.05. C, Wild-type or knockout T*regs were cultured in the insert of a Transwell culture plate in the presence of wild-type T*conv cells. zVAD or DMSO control was added to the Transwell assay. Freshly purified wild-type responder T*conv cells were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [3H]thymidine incorporation. Data represent the mean ± SEM of two independent experiments. *p = 0.07.

FIGURE 5. TRAIL dependence and modulation in Ebi3−/− Il10−/− T*regs. Wild-type or knockout T*regs, purified by FACS, were activated in presence of anti-CD3- and anti-CD28-coated latex beads with IL-2 for 16 and 24 h. A, Cells were collected and surface TRAIL expression was detected by flow cytometry using an antiserum TRAIL Ab. Data are representative of three independent experiments. B, Mean fluorescence intensity (MFI) of surface TRAIL expression following activation from three to four independent experiments was plotted. Student t test, **p < 0.05. C, Wild-type or knockout T*regs were cultured in the insert of a Transwell culture plate in the presence of wild-type T*conv cells. zVAD or DMSO control was added to the Transwell assay. Freshly purified wild-type responder T*conv cells were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [3H]thymidine incorporation. Data represent the mean ± SEM of two independent experiments. *p = 0.07.

Second, we assessed the extent to which the Ebi3−/− Il10−/− T*regs could cure colitis induced by DR5−/− T*conv cells. The development and severity of colitis induced by wild-type or DR5−/− T*conv cells in Rag−/− mice were comparable (Fig. 6F, 6F, Supplemental Fig. 4B). At the onset of clinical symptoms (5% loss of body weight; ∼4 wk), mice were treated with wild-type or Ebi3−/− Il10−/− T*regs. Wild-type T*reg recipients gained weight and recovered from the clinical symptoms of colitis regardless of whether the disease had been induced by wild-type or DR5−/− T*conv cells (Fig. 6E, 6F). In contrast, Ebi3−/− Il10−/− T*regs could cure colitis caused by wild-type, but not DR5−/− T*conv cells. Histological analysis of the colon 4 wk post-T*reg treatment confirmed that Ebi3−/− Il10−/− T*regs were unable to reverse DR5−/− T*conv cell-induced colitis (Supplemental Fig. 4B).

Third, if TRAIL was essential for Ebi3−/− Il10−/− T*reg-mediated suppression, then its genetic deletion should abrogate their regulatory capacity. Our data suggest that although wild-type and Ebi3−/− Il10−/− T*regs could effectively mediate suppression of T*conv cells across a Transwell, Ebi3−/− Il10−/− T*regs could not inhibit T*conv target cell proliferation (Fig. 6G). Taken together, these data clearly demonstrate that Ebi3−/− Il10−/− T*regs require TRAIL for maximal suppressive function, and that soluble TRAIL appears to be their only mechanism of suppression. In contrast, wild-type T*regs exhibit minimal TRAIL dependence and use IL-35 and IL-10 as their soluble mediators of suppression. Differential utilization of suppressive mechanisms by genetically distinct T*reg.

Loss of IL-10 and IL-35 production by T*regs led to increased CTSE expression and subsequent dependence on TRAIL-mediated suppression. We questioned the extent to which unmanipulated examples of this T*reg functional plasticity might exist. Differential CTSE expression has been reported in different inbred mouse strains (21). In particular, C57BL/6 mice express low levels of CTSE, whereas expression in BALB/c and 129 mice is high. We first confirmed these observations by assessing CTSE expression by qPCR and intracellular staining (Fig. 7A, 7B). The results clearly indicate that BALB/c T*regs express higher levels of CTSE,
consistent with previous observations (21). Next, we assessed the kinetics of TRAIL surface expression on BALB/c Tregs following activation. Interestingly, BALB/c Tregs expressed slightly higher levels of surface TRAIL than C57BL/6 Tregs, particularly at 16 h postactivation (Fig. 7C). Indeed, the pattern of CTSE and TRAIL expression exhibited by BALB/c Tregs was analogous to observations made with Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> Tregs (compare Figs. 3C, 3D, 5A with Fig. 7A–C), and was consistent with previous suggestions (45). We then examined the suppressive capacity of BALB/c and C57BL/6 Tregs in presence or absence of reagents that block IL-10, IL-35, or TRAIL. Whereas anti–IL-10 and the isotype control Ab had little effect on the suppression mediated by either Treg population in a Transwell assay, IL-35 neutralizing mAb blocked suppression mediated by C57BL/6, but not BALB/c, Tregs (Fig. 7D). In contrast, DR5-Fc partially inhibited suppression mediated by BALB/c, but not C57BL/6, Tregs. Thus, C57BL/6 Tregs seem to be more dependent on IL-35, whereas BALB/c Tregs are more dependent on TRAIL-mediated suppression. This raises the

**FIGURE 6.** Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> Treg-mediated suppression is TRAIL dependent. Wild-type or knockout Tregs purified by FACS were titrated in a Treg assay with wild-type or DR5<sup>−/−</sup> Tconv cells and stimulated with anti-CD3– and anti-CD28–coated latex beads (A) or were cultured with wild-type Tconv cells in the insert of a Transwell culture plate (B). Wild-type or DR5<sup>−/−</sup> Tconv cells were activated in the bottom chamber of the plate with anti-CD3– and anti-CD28–coated latex beads. Proliferation of responder wild-type or DR5<sup>−/−</sup> Tconv cells was determined by [3H]thymidine incorporation. The cpm ranged between 30,000 and 65,000. Results shown here are average of four to five independent experiments. Significance was determined by the Mann–Whitney test (A, *p < 0.05; B, ***p < 0.005). C. Wild-type and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> Tregs were stimulated with anti-CD3– and anti-CD28–coated latex beads in the presence of Tconv cells in the insert of a Transwell culture plate. Figs. 3C, 3D, 5A with Fig. 7A–C, and was consistent with previous suggestions (45). We then examined the suppressive capacity of BALB/c and C57BL/6 Tregs in presence or absence of reagents that block IL-10, IL-35, or TRAIL. Whereas anti–IL-10 and the isotype control Ab had little effect on the suppression mediated by either Treg population in a Transwell assay, IL-35 neutralizing mAb blocked suppression mediated by C57BL/6, but not BALB/c, Tregs (Fig. 7D). In contrast, DR5-Fc partially inhibited suppression mediated by BALB/c, but not C57BL/6, Tregs. Thus, C57BL/6 Tregs seem to be more dependent on IL-35, whereas BALB/c Tregs are more dependent on TRAIL-mediated suppression. This raises the
BALB/c Tregs preferentially use TRAIL-mediated pathways compared with C57BL/6 Tregs. A. mRNA was isolated from freshly purified C57BL/6 or BALB/c Tconv cells and Tregs, and cDNA synthesized, and qPCR was performed to assess Ctse expression. B. Intracellular staining for CTSE was performed with purified C57BL/6 or BALB/c Tregs (gray filled, secondary Ab only control; open histogram, C57BL/6 Tregs, and closed histogram, BALB/c Tregs). C. TRAIL staining was performed with Tnfsf10−/−, wild-type C57BL/6, or BALB/c Tregs activated in presence of anti-CD3− and anti-CD28−coated latex beads with IL-2 for 16 h, and surface TRAIL expression was detected by flow cytometry using an anti-mouse TRAIL Ab (mean fluorescence intensity from three independent experiments, p = 0.07). D. Wild-type C57BL/6 or BALB/c Tregs were mixed at 1:2 ratio with naive wild-type Tconv cells in the presence of anti-CD3− and anti-CD28−coated beads in the insert of a Transwell culture plate for 72 h. Neutralizing Abs against IL-10, IL-35, or a DR5-Fc protein were added to the Transwell assay at predetermined concentrations, as described in Materials and Methods. Freshly purified wild-type responder Tconv cells were activated in the bottom chamber of a Transwell culture plate. Proliferation of the responder cells was determined by [3H]thymidine incorporation. Data represent three to four independent experiments.

Discussion

Tregs can function in diverse anatomical locations and in a wide variety of immunological and disease settings (46). Consequently, the large array of suppressive mechanisms that Tregs are reported to possess may help them maintain immune homeostasis under diverse scenarios. Indeed, Tregs may have specialized mechanisms for controlling specific cell types as Treg, appear to require IFN regulatory factor-4, T-bet, and STAT3 to suppress Th2, Th1, and Th17 cells, respectively (10–12). However, this may have a greater influence on their migratory behavior than the mechanisms they use to mediate suppression. Importantly, the relative importance of specific mechanisms of Treg function and whether Treg possess mechanistic flexibility have not been elucidated. Previous studies have reported that deficiency of IL-10 or IL-35 alone results in defective Treg function (16, 18). Thus, our finding that Tregs lacking IL-35 and IL-10 are fully functional, instead of relying on TRAIL-mediated suppression as a primary mechanism of action, was very surprising. This implies that Tregs can exhibit remarkable functional plasticity and possess control mechanisms to compensate for the loss of key regulatory tools.

There is a reciprocal relationship in the expression of IL-10 and CTSE (47). Our data clearly show that Ebi3−/−Il10−/− Tregs are dependent on TRAIL for their regulatory function in vitro and in vivo. Furthermore, our studies suggest that increased expression of CTSE enhances the rate and extent of TRAIL surface expression and TRAIL function in mediating T cell suppression. It is possible that CTSE may process full-length TRAIL to enhance its ligand binding and/or may mediate the cleavage of cell surface TRAIL to generate a soluble version. Soluble TRAIL is thought to be either secreted into microvesicles (48) or cleaved from the cell surface (49). Whereas the precise mechanism by which CTSE enhances TRAIL function requires further elucidation, consistent with our results, previous studies have shown that proteolytic cleavage of TRAIL from the cell surface can be mediated by CTSE (35, 36). Thus, in Ebi3−/−Il10−/− Tregs, CTSE upregulation may play a role in the generation of soluble TRAIL. In contrast, expression of IL-10 by wild-type Tregs may suppress CTSE expression and thus reduce the contribution of TRAIL-mediated killing. These data also support the capacity of activated Tregs to use TRAIL (40, 41), and further highlight the complex interregulatory pathways modulated by inhibitory cytokines. However, TRAIL is clearly not used by Il10−/− Tregs, emphasizing that loss of IL-35 expression also contributes to the ability of Ebi3−/−Il10−/− Tregs, CTSE upregulation and thus reduce the contribution of TRAIL-mediated killing. Although the contribution of IL-35 in minimizing TRAIL-mediated suppression remains to be defined, it is noteworthy that Ebi3−/− Tregs exhibit accelerated TRAIL expression following activation, raising the possibility that IL-35 may suppress a distinct component of the TRAIL-processing machinery.

An important question is whether the extent of the physiological impact of the Treg functional plasticity revealed in our study has applicability. As shown in this study and previous studies, substantial differences in CTSE expression occur in different mouse strains with BALB/c mice expressing high levels of CTSE and C57BL/6 mice expressing low levels (21, 45). Interestingly,
BALB/c T\textsubscript{regs} appeared to phenocopy Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{regs} in terms of their pattern of CTSE and TRAIL expression and, thus, their dependence on TRAIL-mediated suppression. Although there are certainly multiple genetic factors that might underlie differences in the function of T\textsubscript{regs} from distinct genetic backgrounds, our data suggest differential CTSE expression may be one contributing factor. Whether this is related to the necessity of T\textsubscript{regs} to adapt to different Th cell bias exhibited in different mouse strains remains to be determined (50, 51). Given that previous studies have shown that T\textsubscript{regs} can use different transcription factors to tackle different Th environments (10–12), it is possible that these may underlie the differential utilization of T\textsubscript{regs} suppressive mechanisms observed in this study. This remarkable T\textsubscript{reg} functional plasticity may also be important in providing a backup mechanism in scenarios in which IL-10 and IL-35 production and/or signaling may be perturbed, and thus may empower T\textsubscript{regs} with the ability to adjust to different environmental settings. Lastly, the possibility that TRAIL may be a legitimate target for the treatment of diseases impacted by excessive T\textsubscript{reg} function, such as cancer, requires further study.

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Disclosures

D.A.A.V. and L.W.C. have submitted patents that are pending and are entitled to a share in net income generated from licensing of these patent rights for commercial development. The other authors have no financial conflicts of interest.

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