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The Plasticity of Regulatory T Cell Function

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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.

The sequences presented in this article have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29262) under accession number GSE29262.

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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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Tregs were purified from wild-type C57BL/6 mice or knockout mice were cultured in the presence of anti-CD3– and anti-CD28–coated beads or culture medium alone. Cells were harvested after 48 h. Where indicated for TRAIL expression, cells were activated in presence of IL-2 (1000 IU/ml) and cells were collected from RNA isolation at indicated time points. Cells were resorted based on their congenic markers, where indicated. RNA was isolated using Qiagen micro or mini RNA kit following manufacturer’s instructions. RNA was quantitated using a nano-drop spectrophotometer, and equal amount of total RNA in each sample was reverse transcribed with the high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer’s guidelines. TaqMan primers and probes were designed with Primer Express software and were synthesized by the St. Jude Hartwell Center for Biotechnology and Informatics. The primers for CTSE were CTSE forward, 5′-CAA-CCTCTGGGTCAGATGGTTG-3′; and CTSE reverse, 5′-TCTCTGGAGGAACGGCATC-3′. The primers for TRAIL-Fc were TRAIL-Fc forward, 5′-GCCATCCAGCAGATGGTTG-3′; and TRAIL-Fc reverse, 5′-CTCCTCGGAGGAACGGCATC-3′. Purified Tregs from wild-type C57BL/6 mice or knockout mice 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 Tregs from wild-type C57BL/6 mice 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.C4.22), isotype control, or DR5-Fc was added to standard Tconv assays and Transwell experiments at the concentrations indicated. Where indicated, Tconv cells were fixed 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, TopTranswell 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 transfectected 293T cells cocultured with Tconv cells, 293T cells were transfection with CTSE cDNA (murine CTSE in pFynco) using a pIRES-YFP expression cassette; provided by B. Chain, University College London, London, U.K.) or Tnfrsf10b (murine TRAIL, in pFynco with pIRES-GFP; provided by T. Griffith, University of Minnesota, Minneapolis, MN) alone or in combination. Posttransfection (48 h), TC cells were irradiated (3000 rad) and seeded at a density of 7 × 10^3 cells/well in the 96-well flat-bottom plate. Purified C57BL6 Tconv cells were added to the seeded plate at 8 × 10^3 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 cells 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 Tregs were purified by FAC. Tconv cells (2 × 10^6) and Tregs (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 mg; provided by T. Griffith, University of Iowa) or isotype control Ab (0.3 mg; R&D Systems). Mice were euthanized 7 d post-transfer, and splenocytes 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−/−/CD25−) naive Tconv cells to induce 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 T0 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 with or without mild mucosal hyperplasia; grade 2, mild inflammation in the lamina propria with or without mucosal hyperplasia, 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 mucus 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-mo-old Treg−/− 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 small and scaly with or without 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 26 d posttransfer, spleen cells were counted and stained, and cell numbers were determined by flow cytometry.

**Results**

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

We first assessed the functional capacity of Tregs 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 Tregs 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 Tregs 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 upper chamber (insert) of a Transwell plate, they can suppress third-party Tconv cells in the lower chamber across a semipermeable membrane (17).

Affymetrix array and analysis

Wild-type or knockout Treg 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 start 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]/exponentiation[mean log signal B])/log (2). Minimum selected gene had a log2 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 log ratio of Ebi3−/−Il10−/− Treg to wild type 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 value from the t tests were then −log10 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 mouse appeared more than once for the same gene, then the score was averaged 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 versus 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 into 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.
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. 1). 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> T<sub>reg</sub> 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>reg</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 transfer.
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 histiocytic 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>regs</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>reg</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 postransfer, T<sub>reg</sub> were purified by FACS from the mixed bone marrow chimeras and assessed in 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<sup>+</sup>-dominant negative TGF-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 Ap1S3 (adaptor-related protein complex 1, σ 3 subunit) and Cte (Fig. 3A, 3B). Ap1S3 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 Ag-processing machinery for the MHC class II pathway (34). qPCR, immunoprecipitation/Western blot analysis, and intracellular staining with purified T<sub>regs</sub> confirmed that CTSE mRNA and protein are highly upregulated in Il10<sup>−/−</sup> and Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> compared with wild-type and Ebi3<sup>−/−</sup> T<sub>regs</sub> (Fig. 3C–E, Supplemental Fig. 3A–C).

Interestingly, CTSE has been implicated in the cleavage and/or processing of TRAIL (Tnfsf10; TNF [ligand] superfamily, member 10) and its release from the cell surface (35, 36). TRAIL is a suppressive molecule of the TNF superfamily that can function in its surface-bound form or as a soluble trimer (37, 38). TRAIL can mediate apoptosis and programmed regulated necrosis (necroptosis) or suppress proliferation (37, 39). Furthermore, activated CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> and CD8<sup>+</sup> T<sub>regs</sub> may express and use TRAIL as a suppressive mechanism (40, 41). Thus, we speculated that increased CTSE in Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> might result in an increase in the functional capacity of surface TRAIL and/or an increase the
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.

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, CTSE–deficient Tconv cells were transfected with expression plasmids encoding Ctse and/or Tnfsf10 and used to assess the ability of TRAIL to limit T cell proliferation. Modulated genes in knockout Tregs compared with wild-type Tregs are depicted in a heat map. B, Volcano plot comparing wild-type and Ebi3−/− Il10−/− Tregs. Highest modulated genes are marked. C, mRNA was isolated from wild-type or knockout Tregs purified by FACS and used for Affymetrix analysis. Modulated genes in knockout Tregs compared with wild-type Tregs are depicted in a heat map. B, Volcano plot comparing wild-type and Ebi3−/− Il10−/− Tregs. Highest modulated genes are marked. C, mRNA was isolated from wild-type or knockout Tregs 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 Tregs; and in blue, Ebi3−/− Il10−/− Tregs). E, Equal numbers of FACS-purified wild-type or knockout Tregs were lysed, CTSE immunoprecipitated, and analyzed by SDS-PAGE/Western blot. Data are representative (A, B, D, E) of three independent experiments.

**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 Tregs compared with wild-type Tregs are depicted in a heat map. B, Volcano plot comparing wild-type and Ebi3−/− Il10−/− Tregs. Highest modulated genes are marked. C, mRNA was isolated from wild-type or knockout Tregs 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 Tregs; and in blue, Ebi3−/− Il10−/− Tregs). E, Equal numbers of FACS-purified wild-type or knockout Tregs 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.
 activation from three to four independent experiments.

We then assessed the contribution of TRAIL-mediated suppression by wild-type Tregs to suppress Tconv cells across a Transwell, it blocked suppression by wild-type, but not DR5−/− Tregs (Fig. 6B). These data suggest that Ebi3−/− Tregs mediate suppression across a Transwell in vitro via TRAIL.

Finally, we assessed the extent to which the Ebi3−/− Tregs could cure colitis induced by DR5−/− Tconv cells. The development and severity of colitis induced by wild-type or DR5−/− Tconv cells in Rag−/− mice were comparable (Fig. 6E, 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−/− Tregs. Wild-type Treg recipients gained weight and recovered from the clinical symptoms of colitis regardless of whether the disease had been induced by wild-type or DR5−/− Tconv cells (Fig. 6E, 6F). In contrast, Ebi3−/− Tregs could cure colitis caused by wild-type, but not DR5−/− Tconv cells. Histological analysis of the colon 4 wk post-Treg treatment confirmed that Ebi3−/− Tregs were unable to reverse DR5−/− Tconv cell-induced colitis (Supplemental Fig. 4B).

Third, if TRAIL was essential for Ebi3−/− Tregs-mediated suppression, then its genetic deletion should abrogate their regulatory capacity. Our data suggest that although wild-type and Ebi3−/− Tregs could effectively mediate suppression of Tconv cells across a Transwell, Ebi3−/− Tregs could not inhibit Tconv target cell proliferation (Fig. 6G). Taken together, these data clearly demonstrate that Ebi3−/− Tregs require TRAIL for maximal suppressive function, and that soluble TRAIL appears to be their only mechanism of suppression. In contrast, wild-type Tregs 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 Treg

Loss of IL-10 and IL-35 production by Treg led to increased CTSE expression and subsequent dependence on TRAIL-mediated suppression. We questioned the extent to which unmanipulated examples of this Treg 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 confirmed these observations by assessing Ctse expression by qPCR and intracellular staining (Fig. 7A, 7B). The results clearly indicate that BALB/c Tregs express higher levels of CTSE,

FNK or its DMSO vehicle control, Ebi3−/− Treg-mediated suppression was blocked (Fig. 5C). These data suggest that Ebi3−/− Tregs suppress Tconv proliferation via a caspase-dependent pathway.

TRAIL signaling in the mouse is mediated through DR5 (Tnfrsf10b; TNFR superfamily, member 10b; also known as TRAIL-R2) (43). Therefore, we first asked whether the Ebi3−/− Tregs were able to suppress Tnfrsf10b−/− Tconv cells (hereafter referred to as DR5−/−) in conventional and Transwell suppression assays. As previously shown, wild-type and Ebi3−/− Tregs suppressed wild-type Tconv cells comparably (Fig. 6A). Furthermore, wild-type Tregs could effectively suppress DR5−/− Tconv cells. However, Ebi3−/− Tregs were less effective at suppressing DR5−/− Tconv cells in a standard Treg assay (Fig. 6A) and completely failed to suppress across a Transwell (Fig. 6B). Secondly, we assessed whether a DR5-Fc fusion protein or an anti-TRAIL blocking Ab was able to inhibit Ebi3−/− Treg-mediated suppression of wild-type Tconv cells. Although DR5-Fc had a minimal effect on the suppression mediated by wild-type Tregs across a Transwell, it blocked suppression by Ebi3−/− Tregs in a dose-dependent manner (Fig. 6C). Similarly, anti-TRAIL, but not an isotype control Ab, reduced the suppressive capacity of Ebi3−/− Tregs, but not wild-type, Tregs [note that this TRAIL mAb is known to block activity weakly in vitro, but very effectively in vivo (44)] (Supplemental Fig. 4A). These results suggest that Ebi3−/− Tregs mediate suppression across a Transwell in vitro via soluble TRAIL.

We then assessed the contribution of DR5-mediated suppression by Ebi3−/− Treg in vivo. First, congenic Thy1.1 wild-type Tconv cells were injected either into Rag−/− mice alone or in the presence of Thy1.2 wild-type or Ebi3−/− Treg. Isotype control Ab or anti-TRAIL was injected on days 0 and 3, and homoeostatic expansion of the Thy1.1 Tconv cells was determined 7 d later. Tconv cell expansion, wild-type Treg-mediated suppression, and Treg numbers were unaffected by the anti-TRAIL treatment (Fig. 6D, data not shown). In striking contrast, TRAIL inhibition blocked the ability of Ebi3−/− Treg to suppress Tconv cell expansion in vivo.

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Differential utilization of suppressive mechanisms by genetically distinct Treg

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FIGURE 5. TRAIL dependence and modulation in Ebi3−/− Treg. Wild-type or knockout Treg, 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 following activation from three to four independent experiments was plotted. Student t test, **p < 0.05. C, Wild-type or knockout Treg were cultured in the insert of a Transwell culture plate in the presence of wild-type Tconv cells. zVAD or DMSO control was added to the Transwell assay. Freshly purified wild-type responder Tconv cells were activated in presence of anti-CD3– and anti-CD28–coated beads. 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 Treg were cultured in the insert of a Transwell culture plate in the presence of wild-type Tconv cells. zVAD or DMSO control was added to the Transwell assay. Freshly purified wild-type responder Tconv cells were activated in presence of anti-CD3– and anti-CD28–coated beads.
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> T<sub>reg</sub> (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 T<sub>reg</sub> purified by FACS were titrated in a T<sub>reg</sub> assay with wild-type or DR5<sup>-/-</sup> T<sub>conv</sub> cells and stimulated with anti-CD3- and anti-CD28-coated latex beads (A) or were cultured with wild-type T<sub>conv</sub> cells in the insert of a Transwell culture plate (B). Wild-type or DR5<sup>-/-</sup> T<sub>conv</sub> 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> T<sub>conv</sub> cells was determined by [<sup>3</sup>H]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> T<sub>reg</sub> were stimulated with anti-CD3– and anti-CD28–coated latex beads in the presence of T<sub>conv</sub> cells in the insert of a Transwell culture plate. Freshly purified wild-type responder T<sub>conv</sub> cells were activated in the bottom wells in the presence of a titrated amount of DR5-Fc. Data are average of two to three independent experiments. One-way analysis of covariance, *p = 0.01. D, Congenically marked wild-type T<sub>conv</sub> cells and wild-type or knockout T<sub>reg</sub> were injected at 4:1 ratio into Rag<sup>-/-</sup> mice. On days 1 and 3, TRAIL-neutralizing mAb or isotype control was injected i.p. CD4, Thy1.1, and Thy1.2 T cell numbers in the spleen were analyzed after 7 d by flow cytometry. Data include three to six mice per group from three independent experiments. Significance was determined by the Mann–Whitney test (*p = 0.05, **p < 0.01). Wild-type littermate control T<sub>conv</sub> cells (E) or DR5<sup>-/-</sup> T<sub>conv</sub> cells (F) (0.5 × 10<sup>6</sup> cells) were injected into Rag<sup>-/-</sup> mice. The weight of the mice was monitored weekly for weight loss. Percentage of weight change was calculated based on the weight at the time of T<sub>reg</sub> injection. G, Wild-type or knockout T<sub>reg</sub> purified by FACS were cultured with wild-type T<sub>conv</sub> cells in the insert of a Transwell culture plate. Wild-type T<sub>conv</sub> cells were activated in the bottom chamber of the plate with anti-CD3– and anti-CD28–coated latex beads. Proliferation of responder T<sub>conv</sub> cells was determined by [<sup>3</sup>H]thymidine incorporation. The cpm ranged between 30,000 and 70,000. Results shown here are mean ± SEM of three independent experiments. E–G, *p < 0.05.
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 Tregs 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<sup>−/−</sup>Il10<sup>−/−</sup> 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<sup>−/−</sup>Il10<sup>−/−</sup> 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 inter-regulatory pathways modulated by inhibitory cytokines. However, TRAIL is clearly not used by IL10<sup>−/−</sup> Tregs, emphasizing that loss of IL-35 expression also contributes to the ability of Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> Tregs to mediate suppression via TRAIL. Although the contribution of IL-35 in minimizing TRAIL-mediated suppression remains to be defined, it is noteworthy that Ebi3<sup>−/−</sup> 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<sub>regs</sub> appeared to phenocopy Ebi3<sup>−/−</sup>Ebi6<sup>−/−</sup>/T<sub>regs</sub> 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<sub>regs</sub> from distinct genetic backgrounds, our data suggest differential CTSE expression may be one contributing factor. Whether this is related to the necessity of T<sub>regs</sub> to adapt to the different Th cell bias exhibited in different mouse strains remains to be determined (50, 51). Given that previous studies have shown that T<sub>regs</sub> can use different transcription factors to tackle different Th environments (10–12), it is possible that these may underlie the differential utilization of T<sub>reg</sub>-suppressive mechanisms observed in this study. This remarkable T<sub>reg</sub> 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<sub>regs</sub> 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<sub>reg</sub> 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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