The Plasticity of Regulatory T Cell Function


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The Plasticity of Regulatory T Cell Function


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 adopts to 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 Il12ap35 (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<sup>−/−</sup> 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 Treg 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<sup>−/−</sup> mice (C57BL/6; now 100% C57BL/6 by microsatellite analysis performed by Charles River) were provided by T. Kuo (Brigham and Women’s Hospital, Boston, MA). Il10<sup>−/−</sup> mice were provided by T. Geiger (St. Jude Children’s Research Hospital, Memphis, TN);
**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, 26b). Tconv and Treg cells from wild-type C57BL/6 mice were purified by FACS. Purified Treg were titrated into a 96-well round-bottom plate starting at a 2:1 ratio (Tconv:Tregs) with 5 × 10^4 Tconv cells per well. The cells were cultured with anti-CD3/anti-CD28–coated latex beads for 72 h. Cultures were pulsed with 1 μCi [3H]thymidine for the final 8 h of the 72-h assay and harvested with a Packard harvester. The cpm were determined using a Packard Matrix 96 direct counter (Packard Biosciences).

In vitro Transwell suppression assays were performed, as described previously, to assess the ability of Tregs to suppress via soluble mediators (17, 22). Tconv and Treg cells from wild-type C57BL/6 mice, and Treg cells from Eb1b^−/−, Il10^−/−, Il10r1b^−/−, and Il12a^−/− mice were purified by FACS. Wild-type Tconv and wild-type or knockout Treg 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 (Il12a^−/− or DR5^−/−) 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 (V1.4C4.22), isotype control, or DR5-Fc was added to standard Treg assays and Transwell experiments at the concentrations indicated. Where indicated, Treg 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, the Transwell inserts were removed and [3H]thymidine was added directly to the responder Tconv 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 pPLneo with an IRES-YFP expression cassette; provided by B. Chain, University College London, London, U.K.) or Tnfrsf10b (murine TRAIL in pPLneo [pPLneo with IRES-GFP]; provided by T. Griffith, University of Minnesota, Minneapolis, MN) alone or in combination. Postransfection (48 h), Tconv cells were irradiated (3000 rad) and seeded at a density of 7 × 10^4 cells/well in the 96-well flat-bottom plate. Purified C57BL/6 Tconv cells were added to the seeded plate at 8 × 10^5 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, naïve Thy1.1^+ Tconv cells from B6.PL mice, which were used as target cells, and Thy1.2^+ wild-type or knockout Treg 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.
with 0.5 × 10^6 wild-type or DR5^−/− (CD4^+CD45RB^hiCD25^−) 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 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 Treg 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 inflammatory infiltrates, focal erosion, 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 wild-type 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.

**Results**

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).
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−/− Il10−/− Treg unable to suppress across a Transwell. Strikingly, Ebi3−/− Il10−/− Treg suppressed Tconv cells across a Transwell comparable to their wild-type counterparts, even though Ebi3−/− and Il10−/− Treg were partially defective (Fig. 1B). This equivalency in function was further supported by experiments with CFSE-labeled target Tconv cells and Transwell experiments with titrated Ebi3−/− Il10−/− Treg in presence of fixed or unfixed Tconv cells (Supplemental Fig. 1A, 1B). These data suggest that Ebi3−/− Il10−/− Treg, unlike Ebi3−/− and the Il10−/− Treg, are functionally intact in in vitro suppression assays.

We next asked whether Ebi3−/− Il10−/− Treg were functionally equivalent in several in vivo models. The adoptive transfer of Treg into neonatal Scuffy or Foxp3−/− 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−/− mice were injected with 106 wild-type, Ebi3−/−, Il10−/−, or Ebi3−/− Il10−/− Treg. Clinical symptoms, histological analysis, and CD4+ T cell numbers were determined when the mice were ~4 wk old. Although no defects were observed with the Ebi3−/− Treg recipients, increased histological scores were observed with Il10−/− Treg recipients. In contrast, Ebi3−/− Il10−/− Treg 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 Treg populations to rescue immune homeostasis in mixed bone marrow chimeras generated using a 50:50 mixture of bone marrow from Foxp3−/− mice and either wild-type, Ebi3−/−, Il10−/−, or Ebi3−/− Il10−/− mice transferred into Rag1−/− mice. Interestingly, significant defects were observed in the ability of Ebi3−/− and Il10−/− bone marrow to rescue the Foxp3−/− phenotype (Supplemental Fig. 2D, 2E). In contrast, the Foxp3−/− bone marrow recipients of Ebi3−/− Il10−/− Treg were largely intact and comparable to their wild-type Treg Foxp3−/− recipient counterparts (Supplemental Fig. 2D, 2E).

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

Treg 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+CD45RBhighCD25− Tconv cells into Rag1−/− mice (31). Recovery from disease, marked by weight gain and decreased histopathology, is observed only in mice that receive purified Treg ~4 wk after the initial Tconv cell displacement.
transfer (14). We used this recovery model of colitis to assess the functional capacity of Ebi3^{-/-}I10^{-/-} Tregs in vivo. Approximately 4 wk post-Tconv cell transfer, recipients developed clinical symptoms of colitis (monitored by weight loss) and were either left untreated or treated with either wild-type, Ebi3^{-/-}, I10^{-/-}, or Ebi3^{-/-}I10^{-/-} Tregs. As expected, mice that did not receive Tregs continued to lose weight, and exhibited substantial histiocyte infiltration and goblet cell destruction during the subsequent 4 wk (Fig. 1D, 1E, Supplemental Fig. 2F). In contrast, the wild-type Treg recipients started to gain weight within 1 wk of transfer. Despite previous studies clearly demonstrating the inability of Ebi3^{-/-} or I10^{-/-} Treg, to cure colitis, weight gain and improved histological parameters were evident in the Ebi3^{-/-}I10^{-/-} Treg recipients, suggesting that these double-inhibitory cytokine-deficient Tregs 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^{-/-}I10^{-/-} Tregs 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 wild-type bone marrow with Thy 1.2 wild-type or Ebi3^{-/-}I10^{-/-} bone marrow into sublethally irradiated Rag1^{-/-} mice. Eight weeks posttransfer, Tregs were purified by FACS from the mixed bone marrow chimeras and assessed in vitro Transwell and in vivo homeostasis assays. Chimera-derived Ebi3^{-/-}I10^{-/-} Tregs and wild-type Tregs suppressed third-party Tconv cells comparably across a Transwell (Fig. 2A). In contrast, similarly prepared Ebi3^{-/-} and I10^{-/-} Tregs were defective (data not shown). Furthermore, Thy1.2^{+} Ebi3^{-/-}I10^{-/-} Tregs and wild-type Tregs that had developed in the bone marrow chimeras suppressed Tconv expansion comparably in homeostasis assay (Fig. 2B). Taken together, these data suggest that a cell-intrinsic modification had occurred in the Ebi3^{-/-}I10^{-/-} Tregs, to render them functionally comparable to wild-type Tregs 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^{-/-}I10^{-/-} Tregs can suppress Tconv 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 Treg 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^{-/-}I10^{-/-} Tregs to suppress across a Transwell using third-party Tconv cells from CD4–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^{-/-}I10^{-/-} Tregs was comparable when Tconv 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^{-/-}I10^{-/-} Tregs was not TGF-β.

To identify this compensatory suppressive mechanism, we compared the gene expression profile of wild-type, Ebi3^{-/-}, I10^{-/-}, and Ebi3^{-/-}I10^{-/-} Tregs using Affymetrix GeneChip arrays. We first generated a list of highly differentially expressed wild-type Treg signature genes, by comparison of the array profile with wild-type Tconv to determine whether there were any notable global changes in gene expression in wild-type versus Ebi3^{-/-}I10^{-/-} Tregs. Minimal variations were observed in the expression (up or down) of 47 highly modulated Treg signature genes (data not shown). Indeed, global analysis revealed very few differences between wild-type and Ebi3^{-/-}I10^{-/-} Tregs (Fig. 3A, 3B, data not shown). The two notable exceptions were Api53 (adaptor-related protein complex 1, σ 3 subunit) and Ctse (Fig. 3A, 3B, data not shown). Api53 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^{-/-}I10^{-/-} Tregs 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).

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^{+}Foxp3^{+} Tregs and CD8^{+} Tregs may express and use TRAIL as a suppressive mechanism (40, 41). Thus, we speculated that increased CTSE in Ebi3^{-/-}I10^{-/-} Tregs 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<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub> are dependent on TRAIL for their suppressive activity, whereas wild-type T<sub>reg</sub> are not.

**IL-10/IL-35–deficient T<sub>reg</sub> 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<sup>−/−</sup>, Il10<sup>−/−</sup>, or Ebi3<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub>. Minimal alterations in Tnfsf10 (TRAIL) mRNA expression were observed over time or between the four T<sub>reg</sub> populations (Supplemental Fig. 3D). Whereas all T<sub>reg</sub> populations exhibited increased TRAIL surface expression following activation, Ebi3<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub> 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<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub>. 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<sup>−/−</sup> T<sub>reg</sub> expressed slightly higher levels of TRAIL at 16 h compared with wild-type or Il10<sup>−/−</sup> T<sub>reg</sub> (Supplemental Fig. 3E).

We then used various approaches to determine the extent to which this accelerated TRAIL expression meant that the Ebi3<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub> were dependent on TRAIL-mediated suppression. TRAIL mediates its suppression in part via caspase-mediated apoptosis (37). Thus, we asked whether Ebi3<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>reg</sub> 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 T<sub>reg</sub> suppression was unaffected by z-VAD-
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 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\(^{-/-}\) Treg. Wild-type or knockout Treg, purified by FACSs 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 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\(^{-/-}\) 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). At the onset of clinical symptoms (5% loss of body weight; ~4 wk), mice were treated with wild-type or Ebi3\(^{-/-}\) Il10\(^{-/-}\) 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\(^{-/-}\) Il10\(^{-/-}\) 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\(^{-/-}\) Il10\(^{-/-}\) Tregs were unable to reverse DR5\(^{-/-}\) Tconv cell-induced colitis (Supplemental Fig. 4B).

Third, if TRAIL was essential for Ebi3\(^{-/-}\) Il10\(^{-/-}\) Treg-mediated suppression, then its genetic deletion should abrogate their regulatory capacity. Our data suggest that although wild-type and Ebi3\(^{-/-}\) Il10\(^{-/-}\) Tregs could effectively mediate 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\(^{-/-}\) Il10\(^{-/-}\) Tregs in a dose-dependent manner (Fig. 6C). Similarly, anti-TRAIL, but not an isotype control Ab, reduced the suppressive capacity of Ebi3\(^{-/-}\) Il10\(^{-/-}\), 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).

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 first 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,
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>I10<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...
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 Tnf10−/−, 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.

The possibility that genetic variations predispose Tregs to preferential modes of immunosuppression.

**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 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−/− 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 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−/− 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,
BAFLc Treggs appeared to phenocopy Ebi3\(^{-/-}\)Il10\(^{-/-}\) Treggs 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 Treggs from distinct genetic backgrounds, our data suggest differential CTSE expression may be one contributing factor. Whether this is related to the necessity of Treggs 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 Treggs can use different transcription factors to target different Th environments (10–12), it is possible that these may underlie the differential utilization of Treggs-suppressive mechanisms observed in this study. This remarkable Treg 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 Treggs 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 Treg 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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