The Plasticity of Regulatory T Cell Function


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Regulatory 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 (T conv) 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);
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 were cultured at a 2:1 ratio in the Transwell inserts with a pore size of 0.4 μm (Millipore). Target wild-type Tconv or Trf5sf10b+/− (DR5−/−) Treg 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 (V1.4C4.22), isotype control, or DR5-Fc was added to standard Treg assays and Transwell experiments at the concentrations indicated. Where indicated, Treg cells were cultured 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 Cte (murine CTSE) in pYneo [pCNeo (Promega) with an IRES-YFP expression cassette]; provided by B. Chain, University of Iowa) or isotype control Ab (0.3 mg; R&D Systems). Target wild-type Tconv and wild-type or knockout Tregs were cultured at a 2:1 ratio in the Transwell inserts with a pore size of 0.4 μm (Millipore). Target wild-type Tconv or Trf5sf10b+/− (DR5−/−) Treg 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 (V1.4C4.22), isotype control, or DR5-Fc was added to standard Treg assays and Transwell experiments at the concentrations indicated. Where indicated, Tconv cells were cultured 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).

Immunoprecipitation and Western blotting

Immunoprecipitation and immunoblotting for CTSE were performed, as previously described (21). Tconv wild-type, or knockout Treg cells were lysed with lysis buffer. To all supernatants, lysis buffer containing 0.1% Triton X-100 for 2 min prior to staining with anti-CTSE Ab (R&D Systems) and protein G-Sepharose beads. Immunoprecipitates were resolved by 10% SDS-PAGE (Invitrogen Life Technologies), and blots were probed with an anti-goat HRP secondary Ab (Amersham Biosciences). Blots were developed using ECL (Amersham Biosciences) and autoradiography.
with 0.5 × 10^6 wild-type or DR5^−/− (CD4^+CD45RB^hiCD25^−) naïve 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 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 with or without mild mucosal hyperplasia; grade 2, mild inflammation in the lamina propria with or without mucosal hyperplasia and occasional extension to 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 Tregs purified by FACS were injected (10^6) i.p. into 2- to 3-d-old Il10^−/− 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 or with 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 score was based upon inflammation in the peribronchial 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 criteria related to portal/perisepal 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 perisepalae were scored 3 and 4, respectively. Third was the degree of portal tract fibrosis and/or lymphocytic infiltrates with or without mononuclear 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 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 scored 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.

**Affymetrex 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 log^2 ratio of Ebi3^−/−Il10^−/− Treg to wild-type Treg was calculated, and the 20 most positively induced named genes were selected. The log^2 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))/log (2). Minimum selected gene had a log^2 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^−/− over 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 log^2 ratios were calculated. The p value from the t tests were then log^2 ratios 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^−3, 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 in the signature, then the mean was calculated 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 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.

**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 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 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 upper chamber (insert) of a Transwell plate, they can suppress third-party Tconv cells in the lower chamber across a semipermeable membrane (17).

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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 beneficial (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> are functionally intact in both 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>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>high</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.

**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 [H]thymidine incorporation (p value: wild-type T<sub>reg</sub> compared with Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> and Il12a<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 [H]thymidine 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 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>5</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, Colonic 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 median ± 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−/−Il10−/− 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−/−, Il10−/−, or Ebi3−/−Il10−/− 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 Il10−/− Tregs, to cure colitis, weight gain and improved histological parameters were evident in the Ebi3−/−Il10−/− 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−/−Il10−/− 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−/−Il10−/− bone marrow into sublethally irradiated Rag1−/− mice. Eight weeks posttransfer, Tregs were purified by FACS from the mixed bone marrow chimeras and assessed in vivo Transwell and in vivo homeostasis assays. Chimera-derived Ebi3−/−Il10−/− Tregs and wild-type Tregs suppressed third-party Tconv cells comparably across a Transwell (Fig. 2A). In contrast, similarly prepared Ebi3−/− and Il10−/− Tregs were defective (data not shown). Furthermore, Thy 1.2+ Ebi3−/−Il10−/− 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−/−Il10−/− 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−/−Il10−/− 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−/−Il10−/− Tregs to suppress across a Transwell using third-party Tconv cells from CD4−dominant negative TGFRβRII transgenic mice that are resistant to TGF-β-mediated suppression (32). The data clearly show that the suppressive capacity of wild-type and Ebi3−/−Il10−/− 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−/−Il10−/− Tregs was not TGF-β.

To identify this compensatory suppressive mechanism, we compared the gene expression profile of wild-type, Ebi3−/−Il10−/−, and Ebi3−/−Il10−/− 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−/−Il10−/− 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−/−Il10−/− Tregs (Fig. 3A, 3B, data not shown). The two notable exceptions were Ap1S3 (adapter-related protein complex 1, sigma 3 subunit) and Ctsn (Fig. 3A, 3B). AP1S3 is the sigma 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−/−Il10−/− 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). qPCR, immunoprecipitation/Western blot analysis, and intracellular staining with purified Tregs confirmed that CTSE mRNA and protein are highly upregulated in Il10−/− and Ebi3−/−Il10−/− Tregs compared with wild-type and Ebi3−/− Tregs (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 (necrosis) 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−/−Il10−/− 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 Ebi32/2 Il102/2 Tregs are dependent on TRAIL for their suppressive activity, whereas wild-type Tregs are not.

**II-10/IL-35–deficient Treg suppress via TRAIL**

We first assessed whether there were any changes in the level or rate of TRAIL expression during activation of wild-type, Ebi32/2, Il102/2, or Ebi32/2 Il102/2 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, Ebi32/2 Il102/2 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 in Ebi32/2 Il102/2 Tregs is accelerated in Ebi32/2 Il102/2 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 Ebi32/2 Tregs expressed slightly higher levels of TRAIL at 16 h compared with wild-type or Il102/2 Tregs (Supplemental Fig. 3E).

We then used various approaches to determine the extent to which this accelerated TRAIL expression meant that the Ebi32/2 Il102/2 Tregs were dependent on TRAIL-mediated suppression. TRAIL mediates its suppression in part via caspase-mediated apoptosis (37). Thus, we asked whether Ebi32/2 Il102/2 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, Ebi32/2 Il102/2 Treg suppression was unaffected by z-VAD-Fmk.
FIGURE 5. TRAIL dependence and modulation in Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg}. Wild-type or knockout T\textsubscript{reg} 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 was detected by flow cytometry using an anti-mouse 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\textsubscript{reg} were cultured in the insert of a Transwell culture plate in the presence of wild-type T\textsubscript{conv} cells. zVAD or DMSO control was added to the Transwell assay. Freshly purified wild-type responder T\textsubscript{conv} cells were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [\textsuperscript{3}H]thyminidine incorporation. Data represent the mean ± SEM of two independent experiments. *p = 0.07.

TRAIL signaling in the mouse is mediated through DR5 (Tnfsf10b; TNFR superfamily, member 10b; also known as TRAIL-R2) (43). Therefore, we first asked whether the Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} were able to suppress Tnfsf10b\textsuperscript{−/−} T\textsubscript{conv} cells (hereafter referred to as DR5\textsuperscript{−/−}) in conventional and Transwell suppression assays. As previously shown, wild-type and Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} suppressed wild-type T\textsubscript{conv} cells comparably (Fig. 6A). Furthermore, wild-type T\textsubscript{reg} could effectively suppress DR5\textsuperscript{−/−} T\textsubscript{conv} cells. However, Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} were less effective at suppressing DR5\textsuperscript{−/−} T\textsubscript{conv} cells in a standard T\textsubscript{reg} 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, it blocked suppression by Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} across a Transwell, it blocked suppression by Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} in a dose-dependent manner (Fig. 6C). Similarly, anti-TRAIL mAb, but not an isotype control Ab, reduced the suppressive capacity of Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} [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\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} mediate suppression across a Transwell in vitro via soluble TRAIL.

We then assessed the contribution of TRAIL-mediated suppression by Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} in vivo. First, congenic Thy1.1\textsuperscript{+} wild-type T\textsubscript{conv} cells were injected either into Rag\textsuperscript{−/−} mice alone or in the presence of Thy1.2\textsuperscript{+} wild-type or Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg}. Isotype control Ab or anti-TRAIL was injected on days 0 and 3, and homeostatic expansion of the Thy1.1\textsuperscript{+} T\textsubscript{conv} cells was determined 7 d later. T\textsubscript{conv} cell expansion, wild-type T\textsubscript{reg}–mediated suppression, and T\textsubscript{reg} numbers were unaffected by the anti-TRAIL treatment (Fig. 6D, data not shown). In striking contrast, TRAIL inhibition blocked the ability of Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} to suppress T\textsubscript{conv} cell expansion in vivo.

Second, we assessed the extent to which the Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} could cure colitis induced by DR5\textsuperscript{−/−} T\textsubscript{conv} cells. The development and severity of colitis induced by wild-type or DR5\textsuperscript{−/−} T\textsubscript{conv} cells in Rag\textsuperscript{−/−} 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\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg}. Wild-type T\textsubscript{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\textsuperscript{−/−} T\textsubscript{conv} cells (Fig. 6E, 6F). In contrast, Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} could cure colitis caused by wild-type, but not DR5\textsuperscript{−/−} T\textsubscript{conv} cells. Histological analysis of the colon 4 wk post-T\textsubscript{reg} treatment confirmed that Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} were unable to reverse DR5\textsuperscript{−/−} T\textsubscript{conv} cell–induced colitis (Supplemental Fig. 4B).

Third, if TRAIL was essential for Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg}–mediated suppression, then its genetic deletion should abrogate their regulatory capacity. Our data suggest that although wild-type and Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} could effectively mediate suppression of T\textsubscript{conv} cells across a Transwell, Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} could not inhibit T\textsubscript{conv} target cell proliferation (Fig. 6G). Taken together, these data clearly demonstrate that Ebi3\textsuperscript{−/−}Il10\textsuperscript{−/−} T\textsubscript{reg} require TRAIL for maximal suppressive function, and that soluble TRAIL appears to be their only mechanism of suppression. In contrast, wild-type T\textsubscript{reg} 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\textsubscript{reg}.

Loss of IL-10 and IL-35 production by T\textsubscript{reg} led to increased CTSE expression and subsequent dependence on TRAIL–mediated suppression. We questioned the extent to which unmanipulated examples of this T\textsubscript{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\textsubscript{reg} 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−/−Il10−/− 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−/−Il10−/− 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−/− 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−/− 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−/− 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−/−Il10−/− 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. F resly purified wild-type responder Tconv 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 Tconv cells and wild-type or knockout Tregs were injected at 4:1 ratio into Rag−/− 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 Tconv cells (E) or DR5−/− Tconv cells (F) (0.5 × 10^6 cells) were injected into Rag−/− 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 Treg injection. G. Wild-type or knockout Tregs purified by FACS were cultured with wild-type Tconv cells in the insert of a Transwell culture plate. Wild-type Tconv cells were activated in the bottom chamber of the plate with anti-CD3– and anti-CD28–coated latex beads. Proliferation of responder Tconv cells was determined by [3H]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.
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 Tregs 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,
BALB/c T<sub>reg</sub><sup>+</sup> appeared to phenocopy Ebi3<sup>−/−</sup>III-10<sup>−/−</sup> T<sub>reg</sub><sup>+</sup> 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>reg</sub><sup>+</sup> 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>reg</sub><sup>+</sup> 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>reg</sub><sup>+</sup> 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><sup>+</sup> function to tackle different Th environments.

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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.

References


5. Park, Y. S., J. O. Oh, and D. H. Chung. 2009. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells attenuate hypersensitivity pneumonitis by suppressing IFN-gamma production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Leukoc. Biol. 86: 1427–1437.


Supplementary Figure 1

(A) Wild type or knock out T<sub>reg</sub> were cultured with CFSE-labeled wild type T<sub>conv</sub> cells and anti-CD3 and anti-CD28 coated latex beads. The cells were analyzed 72h later on a FACS calibur and CFSE dilution was assessed. Data is representative of 2 independent experiments. (B) Wild type or knock out Treg were cultured either with fixed or fresh wild type T<sub>conv</sub> cells in the inserts of a Transwell™ culture plate in presence of anti-CD3 and anti-CD28 coated latex beads. Third party, wild type responder T<sub>conv</sub> were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [3H]-thymidine incorporation. Proliferation ranged from 30,000- 60,000 cpm. Data represents the mean ± SEM of 2-4 independent experiments. (C) Wild type or knock out T<sub>reg</sub> purified by FACS were cultured with naive fresh or fixed wild type T<sub>conv</sub> cells in the inserts of a Transwell™ culture plate in presence of anti-CD3 and anti-CD28 coated latex beads. in the presence of anti-CD3 and anti-CD28 coated beads for 72 h. Freshly purified wild type or TGFβ RII dominant negative responder T<sub>conv</sub> cells were activated in the bottom chamber of a Transwell™ culture plate. Proliferation of responder cells was determined by [3H]-thymidine incorporation. Results are mean ± SEM of 3 independent experiments. Statistical analysis was carried out with students t test, NS: Not significant.

Figure S1. Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>reg</sub> are suppressive in vitro. (A) Wild type or knock out T<sub>reg</sub> were cultured with CFSE-labeled wild type T<sub>conv</sub> cells and anti-CD3 and anti-CD28 coated latex beads. The cells were analyzed 72h later on a FACS calibur and CFSE dilution was assessed. Data is representative of 2 independent experiments. (B) Wild type or knock out Treg were cultured either with fixed or fresh wild type T<sub>conv</sub> cells in the inserts of a Transwell™ culture plate in presence of anti-CD3 and anti-CD28 coated latex beads. Third party, wild type responder T<sub>conv</sub> were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [3H]-thymidine incorporation. Proliferation ranged from 30,000- 60,000 cpm. Data represents the mean ± SEM of 2-4 independent experiments. (C) Wild type or knock out T<sub>reg</sub> purified by FACS were cultured with naive fresh or fixed wild type T<sub>conv</sub> cells in the inserts of a Transwell™ culture plate in presence of anti-CD3 and anti-CD28 coated latex beads. in the presence of anti-CD3 and anti-CD28 coated beads for 72 h. Freshly purified wild type or TGFβ RII dominant negative responder T<sub>conv</sub> cells were activated in the bottom chamber of a Transwell™ culture plate. Proliferation of responder cells was determined by [3H]-thymidine incorporation. Results are mean ± SEM of 3 independent experiments. Statistical analysis was carried out with students t test, NS: Not significant.
Supplementary Figure 2

Figure S2. Ebi3−/−Il10−/− Tregs can rescue Foxp3−/− phenotype and alleviate inflammation in an inflammatory bowel disease model. Wild type or knock out Tregs (1x10⁶) were injected into 2-3 day old Foxp3−/− pups. (A) CD4 T cell numbers were analyzed in the spleen 4 weeks after injection. (B) Clinical score of the pups were determined as described in the methods. (C) Lung, liver and ear pinna were collected at the time of analysis, sectioned and stained with H&E. The tissue sections were analyzed in a blinded manner as described in methods and total score is plotted. Data represents the mean of 5-7 mice per group. One way Anova., Dunn’s Multiple comparison test, p value., **<0.005, *<0.05. Bone marrow from wild type or knock out Tregs were mixed in a 1:1 ratio with Foxp3−/− bone marrow and injected into sub lethally irradiated Rag−/− mice. The tissue sections were collected 4-5 weeks following injection and histological scores are plotted. (D) Histological score of the skin and (E) total histological score of lung, liver and colon are plotted. Data represents the mean of 3 mice per group. (F) Wild type Tconv cells (0.5x10⁶) were injected into Rag1−/− mice. The weight of the mice was monitored weekly. Once the mice had lost 5% of its body weight, wild type or knock out Tregs were injected. Colonic tissue was collected and stained with H&E 4 weeks after Treg injection. The tissue sections were analyzed in a blinded manner and the histological scores are plotted. Data represents the mean ±SEM three independent experiments with 6-12 mice per group. Statistical analysis was carried out with students t test, p value: *** <0.0005, ** <0.005
**Figure S3.** *Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>reg</sub> expresses higher levels of Cathepsin E and TRAIL.* Wild type or knock out T<sub>reg</sub> were purified by FACS. (A) RNA was isolated from resting, activated or cocultured T<sub>reg</sub> as described in the methods. cDNA was synthesized and qPCR was carried out for Cathepsin E with specific primers and probe. Relative expression of cathepsin E compared to wild type resting T<sub>reg</sub> is shown. Data represent the mean ± SEM of 2-3 independent experiments. Statistical analysis was carried out with Anova, p=<0.05. (B) Wild type or knock out T<sub>reg</sub> purified by FACS were stained for intracellular cathepsin E. Secondary antibody staining alone is shown in red and cathepsin E in blue. Representative histograms from 2-3 independent experiments are shown. (C) Mean fluorescence intensity of cathepsin E intracellular expression from 2-3 independent experiments is shown. Anova, p=<0.05. (D) Wild type or knock out T<sub>reg</sub> were activated with anti-CD3 and anti-CD28 coated beads in presence of rIL2. mRNA was isolated and qPCR was carried out. Statistical analysis was carried out with Anova, NS (E) *Tnfsf10<sup>−/−</sup>, Ebi3<sup>−/−</sup>, Il10<sup>−/−</sup>, Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>reg</sub> were stained for TRAIL following activation for 16 and 24 h respectively. The cells were analyzed on a FACS calibur. *Tnfsf10<sup>−/−</sup> T<sub>reg</sub> were used as a control for staining. Representative histograms from three independent experiments are given.
Figure S4. Abrogating TRAIL function with an anti-TRAIL mAb or TRAIL-R deficient target cells inhibits the suppressive capacity of Ebi3<sup>−/−</sup> Il10<sup>−/−</sup> T<sub>regs</sub> in <em>vitro</em> and <em>in vivo</em>. (A) Wild type or knock out T<sub>regs</sub> purified by FACS were cultured with wild type T<sub>conv</sub> cells in the top compartment of a Transwell™ culture plate. A neutralizing antibody to TRAIL was added to the wells at 10μg/ml concentration. Freshly purified wild type responder T<sub>conv</sub> were activated in the bottom chamber of the plate. Proliferation of responder cells was determined by [<sup>3</sup>H]-thymidine incorporation. Data represents the mean± SEM of 3 independent experiments. (B) Wild type or DR5<sup>−/−</sup> T<sub>conv</sub> cells (0.5x10<sup>6</sup>) were injected into Rag1<sup>−/−</sup> mice. The mice was monitored weekly for weight loss. Once the mice had lost 5% of its body weight, wild type or Ebi3<sup>−/−</sup>Il10<sup>−/−</sup> T<sub>regs</sub> were injected. Colonic tissue was collected and stained with H&E 4 weeks after T<sub>reg</sub> injection. The tissue sections were analyzed in a blinded manner and the histological scores are plotted. Statistical analysis was carried out with students t test. *<0.05, **<0.005.