Regulatory T Cell–Derived TRAIL Is Not Required for Peripheral Tolerance

Rebekah E. Dadey*,†,‡, Stephanie Grebinoski*,†,‡, Qianxia Zhang*,†,¶, Erin A. Brunazzi*,†, Amanda Burton§, Creg J. Workman*,†,¶, Dario A. A. Vignali*,†,§,¶

* Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261
† Tumor Microenvironment Center, University of Pittsburgh Medical Center Hillman Cancer Center, Pittsburgh, PA 15232
‡ Graduate Program of Microbiology and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213
§ Department of Immunology, St. Jude Children’s Research Hospital, Memphis, TN 38105
¶ Cancer Immunology and Immunotherapy Program, University of Pittsburgh Medical Center Hillman Cancer Center, Pittsburgh, PA 15232

Abstract

TRAIL (Tnfsf10/TRAIL/CD253/Apo2L) is an important immune molecule that mediates apoptosis. TRAIL can play key roles in regulating cell death in the tumor and autoimmune microenvironments. However, dissecting TRAIL function remains difficult because of the lack of optimal models. We have now generated a conditional knockout (Tnfsf10^L/L) for cell type–specific analysis of TRAIL function on C57BL/6, BALB/c, and NOD backgrounds. Previous studies have suggested a role for TRAIL in regulatory T cell (T_{reg})–mediated suppression. We generated mice with a T_{reg}-restricted Tnfsf10 deletion and surprisingly found no impact on tumor growth in C57BL/6 and BALB/c tumor models. Furthermore, we found no difference in the suppressive capacity of Tnfsf10-deficient T_{regs} and no change in function or proliferation of T cells in tumors. We also assessed the role of TRAIL on T_{regs} in two autoimmune mouse models: the NOD mouse model of autoimmune diabetes and the myelin oligodendrocyte glycoprotein (MOG) C57BL/6 model of experimental autoimmune encephalomyelitis. We found that deletion of Tnfsf10 on T_{regs} had no effect on disease progression in either model. We conclude that T_{regs} do not appear to be dependent on TRAIL exclusively as a mechanism of suppression in both the tumor and autoimmune microenvironments, although it remains possible that TRAIL may contribute in combination with other mechanisms and/or in different disease settings. Our Tnfsf10 conditional...
knockout mouse should prove to be a useful tool for the dissection of TRAIL function on different cell populations in multiple mouse models of human disease.

INTRODUCTION

TRAIL (Tnfsf10/CD253/Apo2L) is a homotrimeric type II transmembrane TNF superfamily member (1–3). TRAIL, discovered because of its similarity to Fas, is a molecule that induces extrinsic apoptosis (4, 5). This signal is delivered through TRAIL binding to agonistic murine death receptor (DR)5 (Tnfsf10b) or human DR4/TRAILR1 (Tnfsf10a) and DR5/ TRAILR2 (Tnfsf10b) (6, 7). Receptor–ligand interaction recruits adaptor molecule FADD, which recruits and activates initiator caspases such as caspase 8 and 10 (8, 9). The initiators will then cleave and activate an executioner caspase, such as caspase 3, which will degrade cellular components, ultimately leading to cellular apoptosis (10). TRAIL can induce cell death as either a membrane bound or soluble mediator, as it can be cleaved by intracellular aspartic and/or cysteine proteases (7, 11, 12). TRAIL expression is regulated by cell stimulation and presence of type 1 and type 2 IFNs on multiple cell types including T cells, NK cells, monocytes, macrophages, and dendritic cells (13–22). This upregulation can enable TRAIL-expressing cells to cytotoxically target ligand-expressing cells in various environments.

TRAIL was initially discovered as a molecule that specifically targets malignant cells and spares nonmalignant cells. TRAIL- or DR5-deficient mice are more susceptible to tumor growth and metastasis, implicating an important role for TRAIL in controlling tumor growth (23–31). This tumor-specific killing is primarily mediated by NK cells and CD8+ T cells in the tumor microenvironment (TME), although other cells express TRAIL in the TME (17, 31, 32). Moreover, although TRAIL is a molecule that targets cell death, it can also regulate immune cell function and proliferation (33).

Regulatory T cells (T\textsubscript{regs}) are an immunosuppressive subset of CD4+ T cells that can suppress activated immune cells and limit autoimmunity. For example, T\textsubscript{regs} are critical for limiting multiple models of autoimmunity such as the NOD mouse, a spontaneous model of autoimmune diabetes, and the myelin oligodendrocyte glycoprotein (MOG) C57BL/6 model of experimental autoimmune encephalomyelitis (EAE). T\textsubscript{reg} depletion in these models rapidly results in overt diabetes and exacerbated EAE disease severity, respectively (34–36). Despite this important role, T\textsubscript{regs} can also suppress the antitumor response and therefore are an effective barrier to limiting tumor growth (37, 38). T\textsubscript{regs} have multiple mechanisms of suppression and can use these mechanisms in the TME and autoimmune environment. T\textsubscript{regs} can suppress through production of inhibitory cytokines, targeting of dendritic cell function, metabolic disruption, and direct cytolysis (39–41). Our laboratory has shown that T\textsubscript{regs} from IL-10- and IL-35-deficient C57BL/6 mice upregulated TRAIL to suppress responding T cells and that T\textsubscript{regs} from BALB/c mice express higher levels of TRAIL than T\textsubscript{regs} from C57BL/6 mice (42). In addition, it has been reported that T\textsubscript{regs} produce TRAIL in an allogenic skin graft model to suppress activated T cells (43). Taken together, these observations suggest that T\textsubscript{regs} can use TRAIL to suppress immune cells in various disease environments.
In this study, we had two specific goals: 1) investigate TRAIL function in an inducible, cell type-specific manner by generating Tnfsf10^Δ/Δ^ mice on C57BL/6, BALB/c, and NOD backgrounds, as studies thus far have only used blocking Abs or constitutive Tnfsf10 knockout mice, and 2) assess if Tregs require and/or are dependent on TRAIL as a mechanism of suppression within the tumor or autoimmune microenvironment by use of Tnfsf10^Δ/Δ^-Foxp3^Cre^ mice.

MATERIALS AND METHODS

Mice

Foxp3^Cre-YFP^ mice on a C57BL/6 background were obtained from A. Y. Rudensky (Memorial Sloan-Kettering) (44). Foxp3^Cre^ mice on a BALB/c background were obtained from S. Sakaguchi (Osaka University) (45). Foxp3^Cre-GFP^ NOD mice were obtained from J. A. Bluestone (University of California, San Francisco) (46). All animal experiments were performed in the American Association for the Accreditation of Laboratory Animal Care–accredited, specific pathogen-free facilities in Division of Laboratory Animal Resources, University of Pittsburgh School of Medicine. Female and male mice of 4–6 wk of age were used for B6 and BALB/c experiments. All tumor phenotype and functional experiments were performed at 12 d after tumor inoculation unless otherwise specified. Female and male NOD mice were followed for diabetes incidence up to 30 wk of age. All NOD phenotype and functional experiments were performed with female mice at 10 wk unless otherwise specified. Animal protocols were approved by the Institutional Animal Care and Use of Committees of University of Pittsburgh.

Generation of a Tnfsf10^Δ/Δ^ mouse

The Tnfsf10^Δ/Δ^ targeting construct was generated using standard recombineering methods (47). Initially, 26.7 kb of the Tnfsf10 locus were retrieved from a bacterial artificial chromosome plasmid and an Loxp-Neo-Loxp cassette inserted 313 bp upstream of exon 2. The Neo was removed via Cre-mediated recombination, leaving a single Loxp and an StuI restriction site (inserted into the intron of the retrieved Tnfsf10 locus). An Frt-Neo-Frt-Loxp cassette was then inserted 573 bp downstream of exon 5 to establish an alternate exon 2 containing the following: a SpeI restriction site, the splice acceptor from exon 2, “self-cleaving” T2A peptide sequence, a truncated version (nonfunctioning) of the human nerve growth factor receptor (hNGFR), and the SV40 polyadenylation sequence. The linearized targeting construct was electroporated into JM8A3.N1 embryonic stem cells (C57BL/6N background) and neomycin-resistant clones were screened by Southern blot analysis using StuI and SpeI restriction digests for the 5′ and 3′ ends, respectively. Correctly targeted clones were 100% normal diploid by karyotype analysis and were injected into C57BL/6 blastocysts. Chimeric mice were mated to C57BL/6 mice and transmission of the targeted allele verified by PCR. The mice were crossed with actin flippase mice to remove the Neo cassette. The mice were backcrossed >10 generations onto the BALB/c or NOD background and verified by microsatellite analysis. Genotyping primers are 5′-GCCACCGGTGTAAGAGCAGTTC-3′, 5′-GGTGGAAACGCTGACAGACATGATAAGATA-3′, and 5′-GTCTCCCACTCATACTGCTAC-3′. Primers for detection

*Immunohorizons. Author manuscript; available in PMC 2022 January 22.*
of exon 1 of Tnfsf10 are forward 5′-GCACTCCGCTTCTAACTGT-3′ and reverse 5′-GTGCTGACTGAAGCTGAGGT-3′, exon 2 forward 5′-GACGGATGAGATTTCTGGGAC-3′ and reverse 5′-TTCAATGAGCTGATACTGTTGCC-3′, and exon 5 forward 5′-ATGGAAAGACCTTTAGGAGCCAGA-3′ and reverse 5′-TAGATGTAATACAGGCCCCTCTGTC-3′.

**Measurement of diabetes and insulitis**

Measurement of diabetes and insulitis were performed as previously described (48–50). Briefly, diabetes incidence was monitored weekly through presence of glucose in the urine with Diastix (Bayer). Mice positive for glucose on Diastix were then measured for blood glucose with a Breeze2 glucometer (Bayer). Mice were considered diabetic and were marked for sacrifice when blood glucose was ≥400 mg/dl.

Pancreata for histology were prepared as previously described at the University of Pittsburgh Biospecimen Core (48). Briefly, pancreata were embedded in a paraffin block and cut into 4-μm-thick sections with 150-μm steps between sections and stained with H&E. An average of 60–80 islets per mouse were scored in a blinded manner. Two methods of insulitis measurement were used as previously described (51).

**Islet isolation and lymphocyte preparation**

Islets were prepared as previously described (48, 52). Briefly, 3 ml of collagenase (600 U/ml in complete HBSS with 10% FBS) was perfused through the pancreatic duct. Pancreata were then incubated for 30 min at 37°C. Pancreata were then washed two times and resuspended in clear complete HBSS with 10% FBS, and islets were isolated by hand under a dissecting microscope. Isolated islets were dissociated with 1 ml dissociation buffer (Life Technologies) for 15 min at 37°C with vortexing every 5 min. Cells were washed, resuspended, counted, and used.

**EAE induction**

Induction of EAE was performed as described previously (53, 54). Briefly, IFA (Difco) at was supplemented with 5 mg/ml Mycobacterium tuberculosis (Difco) to make CFA. MOG peptide (AAPPTec) was diluted to 1 mg/ml in PBS, and the CFA and MOG peptide were mixed at a 1:1 ratio. Mice were injected with 100 μl of the emulsion on both flanks s.c. Pertussis toxin (200 ng/200 μl PBS; Sigma-Aldrich) was injected i.p. on day 0 and day 2 of injection. Animals were scored blinded for clinical symptoms as follows: 0, no change; 1, limp tail; 2, partial hind limb paralysis; 3, full hind limb paralysis; 4, full hind limb paralysis and partial front limb paralysis; and 5, moribund or death.

**Cell staining, flow cytometry, and purification**

Single-cell suspensions were stained with Abs for CD4 (GK1.5; BioLegend), CD8a (53–6.7; BioLegend), TCRβ (H57–597; eBioscience), cleaved caspase (Asp175; Cell Signaling Technologies, CST), CD45.2 (104; BioLegend), Foxp3 (FJK-16s; eBioscience), Ki67 (B56; BD Biosciences), TNF-α (MP6-XT22; BioLegend), IFN-γ (XMG1.2; BioLegend), DR5 (MD5–1; BioLegend), LAP-TGF-β (TW7–16B4; BioLegend), IL-10 (JES5–16E3;
BioLegend), CTLA-4 (UC10–4B9; BioLegend), CD73 (TY/11.8; BioLegend), CD39 (24DM51; BioLegend), CD11c (N418; BioLegend), CD19 (ID3; BD Biosciences), F4/80 (BM8; BioLegend), NK1.1 (PK136; eBioscience), CD49b (DX5; BioLegend), and insulin (182410; R&D Systems). Surface staining was performed on ice for 15 min. Dead cells were discriminated by staining with Ghost Viability Dye (Tonbo Biosciences) in PBS prior to surface staining. For cytokine expression analysis, cells were activated with 100 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in complete RPMI 1640 containing 10% FBS and monensin (eBioscience) for 4 h. For intracellular staining of cytokines and transcription factors, cells were stained with surface markers, fixed in Fix/Perm buffer (eBioscience) for 45 min, washed twice in permeabilization buffer (eBioscience), and stained in permeabilization buffer for 30 min on ice. Immunostaining for Ki67 was performed using the BD Cytofix/Cytoperm kit. Samples were acquired on a Fortessa (BD Biosciences) and analyzed by FlowJo (Tree Star) or sorted on an Aria II (BD Biosciences). Identification of various immune cell populations was first subgated on live CD45.2+ cells. From this gate, the following strategy for each population was used: TCRβ+CD4+Foxp3− (in this study referred to as CD4+), TCRβ+CD4+Foxp3+ (Treg), TCRβ+CD8+ (CD8+), TCRβ−CD49b+ or TCRβ−NK1.1+ (NK+), TCRβ−CD11c+ (CD11c+), TCRβ−F4/80+ (F4/80+), and all other TCRβ− cells. Gating for sorting these populations remains the same except for the CD4+ Foxp3− and Treg populations. CD4+ Foxp3− and Treg populations used the following strategy, respectively: TCRβ+CD4+Foxp3(YFP)+ (C57BL/6) or TCRβ+CD4+CD25− (BALB/c) (CD4) and TCRβ+CD4+Foxp3(YFP)+ (C57BL/6) or TCRβ+CD4+CD25+ CD127− (BALB/c) (Treg). NOD Tregs were isolated as TCRβ+CD4+Foxp3(GFP)+, and CD4s were isolated as TCRβ+CD4+Foxp3 (GFP)−.

Tumor models

The B16.F10 were obtained from M. J. Turk (Dartmouth College) (55). The MC38 colon adenocarcinoma cells were obtained from J. P. Allison (MD Anderson Cancer Center) (56). The CT26 cells were obtained from R. Binder (University of Pittsburgh) (57). These cells were cultured as previously described (58). C57BL/6 mice were injected with 1.25 × 10^5 B16 melanoma cells (intradermally [i.d.]) or 5.0 × 10^5 MC38 colon carcinoma cells (s.c.). We treated mice injected with MC38 with isotype (Rat IgG2a; Leinco) or anti–programmed cell death (PD)-1 (Leinco) as previously described (59). Tumors were measured every 3 d with a digital caliper in two dimensions (width and length) and presented as tumor size (square millimeters; defined as w × l). BALB/c were injected with 1.25 × 10^5 CT26 colorectal carcinoma s.c. and measured every 3 d for tumor growth. Tumors were prepared for single-cell suspension with an enzymatic digestion of collagenase IV (200 U/ml) and dispase (1 U/ml) in complete RPMI 1640 and mechanical disruption.

In vitro assays

Microsuppression assays were performed as previously described (59, 60). Briefly, Treg cells were isolated from the spleen of naive mice or nondraining lymph node (NDLN) and tumor-infiltrating lymphocytes (TIL) of mice 12 or 18 d after injection with B16 or CT26. Isolated Treg were cocultured with CellTrace Violet (Life Technologies)–labeled
CD4+Foxp3− responder T cells in the presence of mitomycin C–treated, TCRβ-depleted splenocytes and anti-CD3e (1 μg/ml) for 72 h at 37°C.

mRNA isolation, cDNA synthesis, and quantitative PCR

Cell populations were isolated from naive Foxp3Cre-YFP.B6 or Foxp3Cre.BALB/c mice or from the NDLN and TIL of B16-bearing Foxp3Cre-YFP.B6 and Tnfsf10L/L, Foxp3Cre-YFP.B6 mice. Cells were isolated from NDLN, pancreatic draining lymph node, and islet from 10-wk-old female Foxp3Cre-GFP.NOD. RNA was extracted using the RNAeasy Micro Kit (QIAGEN). cDNA was produced using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer’s instructions. EvaGreen-based quantitative PCR (qPCR) was performed using the following primers: Tnfsf10 forward, 5′-TCTGTGGCTGTGACTTACATG-3′ and reverse, 5′-AAGCAGGGTCTGTTCAAGATC-3′; and HPRT forward, 5′-TCAGTCAACGGGGGACATAA-3′ and reverse, 5′-GGGGCTGTACTGCTTAACCAG-3′. Relative quantification was determined via the ΔΔCT method.

Quantification and statistical analysis

Statistical analysis was performed with Prism version 8.0.0. Student t tests were used when only two experimental groups were involved. Tumor growth and EAE curves were analyzed using two-way ANOVA with multiple comparisons correction with sequential time point measurements. The log-rank (Mantel–Cox) test was used for diabetes incidence statistical analysis. Number of mice used in the experiment is represented by “n,” with number of individual experiments listed in legend. All p values were two sided, and statistical significance assessed at ≤0.05.

RESULTS

TRAIL is expressed on Tregs in the TME

We hypothesized that Tregs use TRAIL to suppress the antitumor response. Therefore, we initially assessed TRAIL expression in multiple cell populations isolated from the TME of B16 tumor–bearing mice, and we found substantial upregulation of Tnfsf10 transcript in the TIL compared with the NDLN (Fig. 1A). Interestingly, Tregs and CD4+Foxp3− were trending to have higher Tnfsf10 levels in the TME compared with other cells in the TME. It is important to note that TRAIL protein expression was difficult to discern, as previously reported, which may be due to its low level of expression (61).

Generation of a Tnfsf10L/L mouse

To directly access the importance of TRAIL expression in distinct cell types in the TME, in particular in Tregs, we generated a novel Tnfsf10L/L mouse. LoxP sites were inserted in the intron between exons 1 and 2 and following exon 5 along with an artificial exon containing a truncated nonfunctional version of the hNGFR (Fig. 1B, 1C). The hNGFR was intended to serve as a reporter for Cre-mediated deletion of Tnfsf10. However, upon validation of the strain, it was found that expression of hNGFR was minimal following Cre-mediated deletion, likely because of the weak transcription strength of the Tnfsf10
promoter consistent with challenges experienced in detected TRAIL expression (data not shown). This may also have been due to inefficient splicing into the artificial exon. To assess the role of TRAIL in T\textsubscript{regs}, we crossed the Tnfsf10\textsuperscript{L/L} mice with Foxp3\textsuperscript{Cre-YFP}.B6 mice, and fidelity of T\textsubscript{reg}-specific deletion was verified by cell specific genotyping (Fig. 1D, 1E). Taken together, we have successfully generated a Tnfsf10\textsuperscript{L/L} murine model, thus enabling us to specifically examine the role of TRAIL in T\textsubscript{regs}.

**T\textsubscript{reg}-restricted deletion of Tnfsf10 does not affect tumor growth or suppression in C57BL/6 mice**

Our laboratory and others have suggested that T\textsubscript{regs} from C57BL/6 mice can use TRAIL to suppress the immune response (42, 43). To assess this, we first examined the suppressive capacity of T\textsubscript{regs} from naive Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice. Surprisingly, the suppressive capacity of Tnfsf10-deficient T\textsubscript{regs} was equivalent to wild-type (WT) T\textsubscript{regs} (Fig. 2A). Next, to assess if T\textsubscript{regs} primarily depend on TRAIL to suppress the antitumor response, we injected Foxp3\textsuperscript{Cre-YFP}.B6 and Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice with B16 melanoma. We chose this model because of studies describing the important role of T\textsubscript{reg} suppression in B16 tumor growth (59, 62). However, we found no difference in B16 tumor growth in Tnfsf10\textsuperscript{L/L}Foxp3\textsuperscript{Cre-YFP}.B6 mice (Fig. 2B).

Furthermore, T\textsubscript{regs} from the NDLN or TIL of Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice with B16-bearing tumors were fully capable of suppressing in vitro (Fig. 2C). Moreover, the suppressive activity of T\textsubscript{regs} from Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice did not change if T\textsubscript{regs} were isolated at a later time point (Supplemental Fig. 1A). We also examined an additional tumor model, MC38 colon adenocarcinoma, which has been shown to be sensitive to TRAIL-induced cytotoxicity, but found no differences in tumor growth between Foxp3\textsuperscript{Cre-YFP}.B6 and Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice (Fig. 2D) (63). In an effort to understand if T\textsubscript{reg}-restricted deletion of Tnfsf10 would impact tumor growth in a model of an active immune response that justifies a strong involvement of T\textsubscript{reg}-mediated negative feedback, we treated Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice with anti–PD-1 therapy and found no change in response to the immunotherapy (Fig. 2D).

T\textsubscript{regs} use TRAIL to suppress through induction of cell death in CD4\textsuperscript{+} Foxp3\textsuperscript{−} T cells (42, 43). However, in Tnfsf10\textsuperscript{L/L}-Foxp3\textsuperscript{Cre-YFP}.B6 mice, we did not find a difference in activation/cleavage of the main downstream executioner caspase 3 in CD4\textsuperscript{+} Foxp3\textsuperscript{−} or CD8\textsuperscript{+} T cells when compared with Foxp3\textsuperscript{Cre-YFP}.B6 mice (Fig. 2E, 2F). We also assessed other immune and nonimmune populations, including tumor cells, but did not find differences in cell death (Supplemental Fig. 1B–E). This indicated that loss of TRAIL in T\textsubscript{regs} did not affect cell death in immune and nonimmune populations in the TME. Interestingly, the low expression of the murine TRAIL agonistic cell DR5 may explain the lack of effect of T\textsubscript{reg}-mediated deletion of TRAIL (Supplemental Fig. 1F).

TRAIL can also suppress responding cells by inhibiting proliferation and T cell activation/function rather than cytotoxicity (64–67). However, the proliferation of CD4\textsuperscript{+} Foxp3\textsuperscript{−} and CD8\textsuperscript{+} T cells, measured by Ki67 expression, was not affected (Fig. 2G, 2H). We also analyzed the functional status of CD4\textsuperscript{+} Foxp3\textsuperscript{−} and CD8\textsuperscript{+} T cells and found no changes in production of proinflammatory cytokines TNF-α and IFN-γ (Fig. 2I–L). We conclude that
Treg-restricted deletion of Tnfsf10 does not affect Treg suppression, tumor growth, cell death, or proliferation and function of T cells.

Next, we hypothesized that Treg-restricted deletion of TRAIL may not lead to a change in tumor growth because Tnfsf10^L/L^Foxp3^Cre-YFP^B6 Tregs still retain other mechanisms of suppression. Thus, we examined the expression of suppressive molecules IL-10, LAP-TGF-β, CTLA4, CD39, and CD73, and indeed, expression was equivalent between WT Tregs and TRAIL-deficient Tregs (Supplemental Fig. 1G–K). Moreover, expression of the proliferation marker, Ki67, and markers of activation/exhaustion, PD-1 and LAG3, remained unchanged in the Tregs in tumors of Tnfsf10^L/L^Foxp3^Cre-YFP^ mice (Supplemental Fig. 1L–P). These results further indicate that the suppressive phenotype of Tnfsf10-deficient Tregs is unaffected.

We also found no change in the proportion of Tregs or proportion of total immune cells in the tumor at day 12 (Supplemental Fig. 1Q and 1R) or day 18 (Supplemental Fig. 1S).

Finally, although others have argued that TRAIL plays a role in Treg apoptosis, we found no change in Treg cell death in the TME (Supplemental Fig. 1T) (68). Taken together, these data suggest that Tregs are not primarily dependent upon TRAIL to suppress in the TME via cell death, inhibition of cell proliferation, or function. This may be due to minimal expression of DR5 and/or the use of other suppressive molecules.

**Treg-restricted deletion of Tnfsf10 does not affect tumor growth or suppression in BALB/c mice**

Although we did not observe a primary role for TRAIL in Tregs in C57BL/6 mice, we hypothesized we may see differences in BALB/c mice given our previous studies in which TRAIL had a more predominant role in BALB/c Tregs compared with Tregs from C57BL/6 mice (42). Moreover, other studies have revealed TRAIL can play a part in regulating the Th1/Th2 balance (69–72). Therefore, we backcrossed the Tnfsf10^L/L^ mice to the Th2-prone BALB/c background and then crossed it to the BALB/c Foxp3^Cre^ mouse (45). Initially, we assessed the function of naive TRAIL-deficient Tregs in a standard in vitro suppression assay, and interestingly, the level of suppression was equivalent to WT Tregs (Fig. 3A). Next, we assessed tumor growth in Foxp3^Cre-YFP^BALB/c, Tnfsf10^L/L^BALB/c, and Tnfsf10^L/L^Foxp3^Cre-YFP^BALB/c mice using the BALB/c CT26 colon carcinoma model in which Tregs suppress the antitumor response (73, 74). Although we did not observe a difference in tumor growth (Fig. 3B), we did see a small decrease in suppression in TRAIL-deficient Tregs isolated from CT26 tumors compared with WT Tregs (Fig. 3C).

However, this was not the case at a later time point (Supplemental Fig. 2A). Next, we determined that cleaved caspase levels in CD4^+^Foxp3^−^, CD8^+^ T cells, tumor cells, and other cell populations were equivalent (Fig. 3D, 3E) (Supplemental Fig. 2B–E), suggesting that Tregs were not dependent upon TRAIL-mediated cytotoxicity in the TME of BALB/c mice, possibly because of low DR5 expression in the TME (Supplemental Fig. 2F).

Furthermore, we did not see any changes in Ki67, TNF-α, and IFN-γ in T cells, suggesting that Tregs do not suppress by limiting proliferation nor function of responding T cells (Fig. 3F–K). We also observed that TRAIL-deficient Tregs in the TME still retained other suppressive molecules, indicating that other molecules may aid in suppression in...
the TME despite loss of TRAIL (Supplemental Fig. 2G–K). Furthermore, we did not see any differences in expression of Ki67, PD-1, LAG3, and cleaved caspase 3 on Tregs (Supplemental Fig. 2L–Q). The proportion of immune cells and Tregs remained unchanged on both days 12 and 18 (Supplemental Fig. 2R–T). Taken together, these data suggest that despite the reported higher levels of TRAIL expression in BALB/c Tregs, they are not primarily dependent upon TRAIL as a means of suppression in the TME (42).

Treg-restricted deletion of Tnfsf10 does not affect autoimmune diabetes

Because Tregs are also critical in limiting autoimmunity, we hypothesized that Tregs may use TRAIL to suppress in the autoimmune microenvironment. Also, it has been reported that TRAIL can regulate cell death of diabetogenic T cells in the pancreatic islet of NOD mice (75). Although it was proposed that this was mediated by TRAIL-expressing pancreatic β cells, we hypothesized that Tregs may also use TRAIL to suppress T cells in this environment (75). Indeed, T cells express the highest levels of Tnfsf10 in the islet (Fig. 4A). We hypothesized that Treg-restricted deletion of Tnfsf10 would limit suppression of diabetogenic T cells and lead to exacerbated autoimmune diabetes.

Interestingly, we found that deletion of Tnfsf10 in Tregs did not significantly alter diabetes incidence or insulitis in female (Fig. 4B–D) or male (Supplemental Fig. 3A) mice, although there was a slight trend toward reduced diabetes incidence. Moreover, we did not find any changes in cell death in CD4+Foxp3− and CD8+ T cells in the islet (Fig. 4E, 4F). As seen with our tumor data, we found that the levels of proliferation and cytokine production in the diabetogenic T cells of the islet were similar in both WT and Tnfsf10−/−Foxp3Cre-GFP, NOD mice (Fig. 4G–L). This would indicate that Tregs do not require TRAIL to suppress diabetogenic T cells in the pancreatic islet of NOD mice.

We also examined DR5 expression on immune and nonimmune cells in the islet and found minimal expression of DR5 on immune cells but higher expression on insulin-positive β cells (Supplemental Fig. 3B). Reports of direct TRAIL-mediated β cell killing have been inconsistent (76–80). However, upon examination of insulin-positive cells, we found no change in cell death (Supplemental Fig. 3C). Interestingly, we did see a reduction in cell death in the CD11c+ population (Supplemental Fig. 3D). TRAIL can have an effect on dendritic cells (81); however, it is unclear what impact this may play in our system, as we did not see a consequence of altered disease. Future studies may elucidate what other impact this has in autoimmune diabetes.

We found that Tnfsf10-deficient Tregs isolated from the TME retained their suppressive phenotype. We questioned if this remained true for Tnfsf10-deficient Tregs isolated from the islet. We found Tregs still expressed functional markers such as LAP-TGF-β, IL-10, and CD39 (Supplemental Fig. 3E–G) and even had an increase in CD73 expression (Supplemental Fig. 3H). This further indicates that Tnfsf10-deficient Tregs retain their suppressive phenotype in the islet. As seen in the tumor, we found no change in Treg proliferation (Supplemental Fig. 3I), as measured by Ki67, and no change in activation/exhaustion markers PD-1 and LAG3 (Supplemental Fig. 3J–M).

**Immunohorizons.** Author manuscript; available in PMC 2022 January 22.
We had demonstrated above that TRAIL had no effect on T_{reg} cell death or the proportion of immune cells and T_{regs} in the TME. Interestingly, although we did not observe a difference in immune cell proportions within the islet (Supplemental Fig. 3N), we did see an increased proportion of intra-islet T_{regs} in Tnfsf10^{L/L}-Foxp3^{Cre-GFP}.NOD mice (Supplemental Fig. 3O). Interestingly, reduced T_{reg} death was only observed in 10-wk-old mice (Supplemental Fig. 3P), as there was no difference in 12-wk-old mice (Supplemental Fig. 3Q). Therefore, we conclude that T_{regs} are not dependent on TRAIL to suppress in the islet.

Finally, we examined if T_{reg}-derived TRAIL had a role in the MOG model of EAE using the Tnfsf10^{L/L}-Foxp3^{Cre-YFP}.B6 mice. As seen with the tumor and NOD models, we did not observe a difference in EAE score and initiation of the disease between WT and Tnfsf10^{L/L}-Foxp3^{Cre-YFP}.B6 mice (Supplemental Fig. 3R). Therefore, we conclude that T_{regs} do not require nor are dependent on TRAIL as a means of suppression in autoimmune microenvironments.

**DISCUSSION**

We report four key developments from our studies. First, we created the first conditional Tnfsf10^{L/L} knockout mouse, that we are aware of, which allows for cell type–specific deletion of TRAIL. Although we focused our efforts on understanding TRAIL biology in T_{regs}, this novel resource could be used to examine the role of TRAIL in other cell populations. Second, we used the Tnfsf10^{L/L} mice and determined that T_{regs} are not primarily dependent upon TRAIL as a means of suppression within the TME. Third, we found that T_{regs} from autoimmune diabetes and EAE are not primarily dependent upon TRAIL as a means of suppression. Finally, these data, along with our previous work in which multiple mechanisms of T_{reg} suppression were deleted, suggest that T_{regs} are capable of using multiple mechanisms of suppression and are able to overcome or compensate when a mechanism is compromised or blocked.

Finally, although we did not determine a primary role of TRAIL in T_{regs} within the tumor and autoimmune environments, we cannot rule out the possibility that TRAIL does play a role in T_{reg} function, either in concert with other mechanisms or in disease models we did not examine. It may be important in future studies to assess different models in which DR5 is more highly expressed. It will also be important to examine the role of TRAIL in the absence of other mechanisms of T_{reg} suppression, such as IL-10 or IL-35, in other cell types, and in other disease models such as infectious or inflammatory diseases.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGMENTS**

We thank everyone in the Vignali Lab (Vignali-lab.com, @Vignali_Lab) for constructive comments and advice during this project. The authors thank A. Rudensky and S. Sakaguchi for mice, M. J. Turk (Dartmouth College, NH) for the B16-F10 cells, J. P. Allison (MD Anderson Cancer Center, TX) for the MC38 cells, and R. Binder (University of Pittsburgh, PA) for the CT26 cell line. We also thank A. McIntyre, D. Falkner, H. Shen, H. Gunzelman, N. Sheng, and T. Surgeon, for cell sorting and flow cytometry help, L. Andrews for mouse breeding, K.
Vignali for help with designing the Tnfsf10^{L/L} mouse, and E. Brunazzi and the staff of the Division of Laboratory Animal Services for the animal husbandry.

This work was supported by the National Institutes of Health (R01 CA203689, P01 AI108545, and P50 CA097190 to D.A.A.V. and T32 CA082084 and F31 CA236337 to R.E.D.) and a National Cancer Institute Comprehensive Cancer Center Support CORE grant (CA047904 to D.A.A.V.). This work also benefited from the Immunology Department Flow Cytometry Core Special BD LSRFortessa funded by National Institutes of Health 1S10OD011925-01 (L. Borghesi, Department of Immunology).

DISCLOSURES

D.A.A.V. is a cofounder and stockholder for Novasenta, Tizona, and Potenza; a stockholder for Oncorus and Werewolf; has patents licensed and royalties from Astellas and Bristol Myers Squibb (BMS); is scientific advisory board member of Tizona, Werewolf, and F-Star; is a consultant for Astellas, BMS, Almirall, Incyte, and Bicara; and received research funding from BMS and Novasenta. The other authors have no financial conflicts of interest.

Abbreviations used in this article:

- **BMS**: Bristol Myers Squibb
- **DR**: death receptor
- **EAE**: experimental autoimmune encephalomyelitis
- **hNGFR**: human nerve growth factor receptor
- **i.d.**: intradermally
- **MOG**: myelin oligodendrocyte glycoprotein
- **NDLN**: nondraining lymph node
- **PD**: programmed cell death
- **qPCR**: quantitative PCR
- **TIL**: tumor-infiltrating lymphocyte
- **TME**: tumor microenvironment
- **Treg**: regulatory T cell
- **WT**: wild-type

REFERENCES

1. Frenkel D 2015. A new TRAIL in Alzheimer’s disease therapy. Brain 138: 8–10. [PubMed: 25564490]
2. Thorburn A 2007. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway signaling. J. Thorac. Oncol 2: 461–465. [PubMed: 17545839]
3. Dubuisson A, and Micheau O. 2017. Antibodies and derivatives targeting DR4 and DR5 for cancer therapy. Antibodies (Basel) 6: 16.
4. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673–682. [PubMed: 8777713]
5. Pitti RM, Marsters SA, Rappert S, Donahue CJ, Moore A, and Ashkenazi A. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem 271: 12687–12690. [PubMed: 8663110]

*Immunohorizons*. Author manuscript; available in PMC 2022 January 22.
6. Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N, and Tschopp J. 1997. Characterization of two receptors for TRAIL. FEBS Lett. 416: 329–334. [PubMed: 9373179]

7. Naval J, de Miguel D, Gallego-Lleyda A, Anel A, and Martinez-Lostao L. 2019. Importance of TRAIL molecular anatomy in receptor oligomerization and signaling. Implications for cancer therapy. Cancers (Basel) 11: 444.

8. Wang S, and El-Deiry WS. 2003. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene 22: 8628–8633. [PubMed: 14634624]

9. Cohen GM 1997. Caspases: the executioners of apoptosis. Biochem. J 326: 1–16. [PubMed: 9337844]

10. Pan G, O’Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, and Dixit VM. 1997. The receptor for the cytotoxic ligand TRAIL. Science 276: 111–113. [PubMed: 9082980]

11. Yasukochi A, Kawakubo T, Nakamura S, and Yamamoto K. 2010. Cathepsin E enhances anticancer activity of doxorubicin on human prostate cancer cells showing resistance to TRAIL-mediated apoptosis. Biol. Chem 391: 947–958. [PubMed: 20482316]

12. Kawakubo T, Okamoto K, Iwata J, Shin M, Okamoto Y, Yasukochi A, Nakayama Ki, Kadowaki T, Tsukuba T, and Yamamoto K. 2007. Cathepsin E prevents tumor growth and metastasis by catalyzing the proteolytic release of soluble TRAIL from tumor cell surface. Cancer Res. 67: 10869–10878. [PubMed: 1806832]

13. Kayagaki N, Yamaguchi N, Nakayama M, Kawasaki A, Akiba H, Okumura K, and Yagita H. 1999. Involvement of TNF-related apoptosis-inducing ligand in human CD4+ T cell-mediated cytotoxicity. J. Immunol 162: 2639–2647. [PubMed: 10072506]

14. Jeremias I, Herr I, Boehler T, and Debatin KM. 1998. TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur. J. Immunol 28: 143–152. [PubMed: 9485194]

15. Falschlehner C, Schaefer U, and Walczak H. 2009. Following TRAIL’s path in the immune system. Immunology 127: 145–154. [PubMed: 19476510]

16. Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okumura H, Nakanishi K, Okumura K, and Yagita H. 1999. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. J. Immunol 163: 1906–1913. [PubMed: 10438925]

17. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, Yagita H, and Okumura K. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med 7: 94–100. [PubMed: 11135622]

18. Kayagaki N, Yamaguchi N, Nakayama M, Eto H, Okumura K, and Yagita H. 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J. Exp. Med 189: 1451–1460. [PubMed: 10224285]

19. Ehrlich S, Infante-Duarte C, Seeger B, and Zipp F. 2003. Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. Cytokine 24: 244–253. [PubMed: 14609566]

20. Halaas O, Vik R, Ashkenazi A, and Espievik T. 2000. Lipopolysaccharide induces expression of APO2 ligand/TRAIL in human monocytes and macrophages. Scand. J. Immunol 51: 244–250. [PubMed: 10736093]

21. Fanger NA, Maliszewski CR, Schooley K, and Griffith TS. 1999. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J. Exp. Med 190: 1155–1164. [PubMed: 10523613]

22. Griffith TS, Wiley SR, Kubin MZ, Sedger LM, Maliszewski CR, and Fanger NA. 1999. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J. Exp. Med 189: 1343–1354. [PubMed: 10209050]

23. Gura T 1997. How TRAIL kills cancer cells, but not normal cells. Science 277: 768. [PubMed: 9273698]

24. Nesterov A, Nikrad M, Johnson T, and Kraft AS. 2004. Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. Cancer Res. 64: 3922–3927. [PubMed: 15173003]
25. Wang Y, Quon KC, Knee DA, Nesterov A, and Kraft AS. 2005. RAS, MYC, and sensitivity to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. Cancer Res. 65: 1615–1617. [PubMed: 15735052]

26. Sedger LM, Glaccum MB, Schuh JC, Kanaly ST, Williamson E, Kayagaki N, Yun T, Smolak P, Le T, Goodwin R, and Glipeakia N. 2002. Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. Eur. J. Immunol 32: 2246–2254. [PubMed: 12209637]

27. Zerafa N, Westwood JA, Cretney E, Mitchell S, Waring P, Iezzi M, and Smyth MJ. 2005. Cutting edge: TRAIL deficiency accelerates hematological malignancies. J. Immunol 175: 5586–5590. [PubMed: 16237043]

28. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ, and Smyth MJ. 2002. Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J. Immunol 168: 1356–1361. [PubMed: 11801676]

29. Finnberg N, Klein-Szanto AJ, and El-Deiry WS. 2008. TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J. Clin. Invest 118: 111–123. [PubMed: 18079962]

30. Grosse-Wilde A, Voloshanenko O, Bailey SL, Longton GM, Schaefer U, Csernok AI, Schütz G, Greiner EF, Kemp CJ, and Walczak H. 2008. TRAIL-R deficiency in mice enhances lymph node metastasis without affecting primary tumor development. J. Clin. Invest 118: 100–110. [PubMed: 18079967]

31. Rossin A, Miloro G, and Hueber AO. 2019. TRAIL and FasL functions in cancer and autoimmune diseases: towards an increasing complexity. Cancers (Basel) 11: 639.

32. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, Yagita H, and Okumura K. 2001. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J. Exp. Med 193: 661–670. [PubMed: 11257133]

33. Sag D, Ayyildiz ZO, Gunalp S, and Wingender G. 2019. The role of TRAIL/DRs in the modulation of immune cells and responses. Cancers (Basel) 11: 1469.

34. Feuerer M, Shen Y, Littman DR, Benoist C, and Mathis D. 2009. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. Immunity 31: 654–664. [PubMed: 19818653]

35. Koutrolos M, Berer K, Kawakami N, Wekerle H, and Krishnamoorthy G. 2014. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. Acta Neuropathol. Commun 2: 163. [PubMed: 25476447]

36. Vandenbark AA, and Offner H. 2008. Critical evaluation of regulatory T cells in autoimmunity: are the most potent regulatory specificities being ignored? Immunology 125: 1–13.

37. Togashi Y, Shitara K, and Nishikawa H. 2019. Regulatory T cells in cancer immunosuppression - implications for anticancer therapy. Nat. Rev. Clin. Oncol 16: 356–371. [PubMed: 30705439]

38. Paluskievicz CM, Cao X, Abdi R, Zheng P, Liu Y, and Bromberg JS. 2019. T regulatory cells and priming the suppressive tumor microenvironment. Front. Immunol 10: 2453. [PubMed: 31681327]

39. Vignali DA 2012. Mechanisms of T(reg) suppression: still a long way to go. Front. Immunol 3: 191. [PubMed: 22783262]

40. Vignali DA, Collison LW, and Workman CJ. 2008. How regulatory T cells work. Nat. Rev. Immunol 8: 523–532. [PubMed: 18566595]

41. Schmidt A, Oberle N, and Krammer PH. 2012. Molecular mechanisms of treg-mediated T cell suppression. Front. Immunol 3: 51. [PubMed: 22566933]

42. Pillai MR, Collison LW, Wang X, Finkelstein D, Regh JE, Boyd K, Szmyczak-Workman AL, Doggett T, Griffith TS, Ferguson TA, and Vignali DA. 2011. The plasticity of regulatory T cell function. J. Immunol 187: 4987–4997. [PubMed: 22013112]

43. Ren X, Ye F, Jiang Z, Chu Y, Xiong S, and Wang Y. 2007. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. Cell Death Differ 14: 2076–2084. [PubMed: 17762882]
44. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Slewe L, Roers A, Henderson WR Jr., et al. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28: 546–558. [PubMed: 18387831]

45. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, and Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. Science 322: 271–275. [PubMed: 18845758]

46. Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, and Bluestone JA. 2008. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. J. Exp. Med 205: 1983–1991. [PubMed: 18725525]

47. Liu P, Jenkins NA, and Copeland NG. 2003. A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res. 13: 476–484. [PubMed: 12618378]

48. Zhang Q, Chikina M, Szymczak-Workman AL, Horne W, Kolls JK, Vignali KM, Normolle D, Bettini M, Workman CJ, and Vignali DAA. 2017. LAG3 limits regulatory T cell proliferation and function in autoimmune diabetes. Sci. Immunol 2: eaah4569. [PubMed: 28783703]

49. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, Drake CG, Korman AJ, and Vignali DA. 2011. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. J. Immunol 187: 3493–3498. [PubMed: 21873518]

50. Leiter EH, Prochazka M, and Coleman DL. 1987. The non-obese diabetic (NOD) mouse. Am. J. Pathol 128: 380–383. [PubMed: 3303953]

51. Leiter EH 2001. The NOD mouse: a model for insulin-dependent diabetes mellitus. Curr. Protoc. Immunol 24: 15.9.1–15.9.23.

52. Lennon GP, Bettini M, Burton AR, Vincent E, Arnold PY, Santamaria P, and Vignali DA. 2009. T cell islet accumulation in type 1 diabetes is a tightly regulated, cell-autonomous event. Immunity 31: 643–653. [PubMed: 19818656]

53. Workman CJ, Collison LW, Bettini M, Pillai MR, Rehg JE, and Vignali DA. 2011. In vivo Treg suppression assays. Methods Mol. Biol 707: 119–156. [PubMed: 21287333]

54. Do J, Kim D, Kim S, Valentin-Torres A, Dvorina N, Jang E, Nagarajavel V, DeSilva TM, Li X, Ting AH, et al. 2017. Treg-specific IL-27Rα deletion uncovers a key role for IL-27 in Treg function to control autoimmunity. Proc. Natl. Acad. Sci. USA 114: 10190–10195. [PubMed: 28874534]

55. Baird JR, Byrne KT, Lizotte PH, Toraya-Brown S, Scarlett UK, Alexander MP, Sheen MR, Fox BA, Bzik DJ, Bosenberg M, et al. 2013. Immune-mediated regression of established B16F10 melanoma by intratumoral injection of attenuated Toxoplasma gondii protects against rechallenge. J. Immunol 190: 469–478. [PubMed: 23225891]

56. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAS, Andrews MC, Sharma P, Wang J, Wargo JA, Pe’er D, and Allison JP. 2017. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. Cell 170: 1120–1133.e17. [PubMed: 28803728]

57. Binder RJ 2009. CD40-independent engagement of mammalian hsp70 by antigen-presenting cells. J. Immunol 182: 6844–6850. [PubMed: 19454680]

58. Liu C, Chikina M, Deshpande R, Menk AV, Wang T, Tabib T, Brunazzi EA, Vignali KM, Sun M, Stolz DB, et al. 2019. Treg cells promote the SREBP1-dependent metabolic fitness of tumor-promoting macrophages via repression of CD8+ T cell-derived interferon-γ. Immunity 51: 381–397.e6. [PubMed: 31350177]

59. Collison LW, and Vignali DA. 2011. In vitro Treg suppression assays. Methods Mol. Biol 707: 21–37. [PubMed: 21287326]

60. Mariani SM, and Krammer PH. 1998. Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. Eur. J. Immunol 28: 1492–1498. [PubMed: 9603453]
63. Haynes NM, Hawkins ED, Li M, McLaughlin NM, Hammerling GJ, Schwendener R, Winoto A, Wensky A, Yagita H, Takeda K, et al. 2010. CD11c+ dendritic cells and B cells contribute to the tumoricidal activity of anti-DR5 antibody therapy in established tumors. J. Immunol 185: 532–541. [PubMed: 20505139]

64. Chyuan IT, Tsai HF, Wu CS, Sung CC, and Hsu PN. 2018. TRAIL-mediated suppression of T cell receptor signaling inhibits T cell activation and inflammation in experimental autoimmune encephalomyelitis. Front. Immunol 9: 15. [PubMed: 29403497]

65. Lehnert C, Weiswange M, Jeremias I, Bayer C, Grunert M, Debatin KM, and Strauss G. 2014. TRAIL-receptor costimulation inhibits proximal TCR signaling and suppresses human T cell activation and proliferation. J. Immunol 193: 4021–4031. [PubMed: 25217163]

66. Chyuan IT, Tsai HF, Wu CS, and Hsu PN. 2019. TRAIL suppresses gut inflammation and inhibits colitogenic T-cell activation in experimental colitis via an apoptosis-independent pathway. Mucosal Immunol. 12: 980–989. [PubMed: 31076664]

67. Lünemann JD, Waiczies S, Ehrlich S, Wendling U, Seeger B, Kamradt T, and Zipp F. 2002. Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells. J. Immunol 168: 4881–4888. [PubMed: 11994437]

68. Diao Z, Shi J, Zhu J, Yuan H, Ru Q, Liu S, Liu Y, and Zheng D. 2013. TRAIL suppresses tumor growth in mice by inducing tumor-infiltrating CD4+CD25+ Treg apoptosis. Cancer Immunol. Immunother 62: 653–663. [PubMed: 23143747]

69. Zhang XR, Zhang LY, Devadas S, Li L, Keegan AD, and Shi YF. 2003. Reciprocal expression of TRAIL and CD95L in Th1 and Th2 cells: role of apoptosis in T helper subset differentiation. Cell Death Differ. 10: 203–210. [PubMed: 12700648]

70. Ikeda T, Hirata S, Fukushima S, Matsunaga Y, Ito T, Uchino M, Nishimura Y, and Senju S. 2010. Dual effects of TRAIL in suppression of autoimmunity: the inhibition of Th1 cells and the promotion of regulatory T cells. J. Immunol 185: 5259–5267. [PubMed: 20921531]

71. Janssen EM, Droin NM, Lemmens EE, Pinkoski MJ, Ensinger SJ, Ehlst BD, Griffith TS, Green DR, and Schoenberger SP. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. Nature 434: 88–93. [PubMed: 15744305]

72. Bossi F, Bernardi S, Zauli G, Secchiero P, and Fabris B. 2015. TRAIL modulates the immune system and protects against the development of diabetes. J. Immunol Res 2015: 680749. [PubMed: 25759846]

73. Liu Z, Kim JH, Falo LD Jr., and You Z. 2009. Tumor regulatory T cells potently abrogate antitumor immunity. J. Immunol 182: 6160–6167. [PubMed: 19414769]

74. Magnuson AM, Kiner E, Ergun A, Park JS, Asinovski N, Ortiz-Lopez A, Kilcoyne A, Paoluzzi-Tomada E, Weissleder R, Mathis D, and Benoist C. 2018. Identification and validation of a tumor-infiltrating Treg transcriptional signature conserved across species and tumor types. Proc. Natl. Acad. Sci. USA 115: E10672–E10681. [PubMed: 30348759]

75. Mi QS, Ly D, Lamhamedi-Cherradi SE, Salojin KV, Zhou L, Grattan M, Meagher C, Zucker P, Chen YH, Nagle J, et al. 2003. Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice. Diabetes 52: 1967–1975. [PubMed: 12882912]

76. Ou D, Metzger DL, Wang X, Huang J, Pozzilli P, and Tingle AJ. 2002. TNF-related apoptosis-inducing ligand death pathway-mediated human beta-cell destruction. Diabetologia 45: 1678–1688. [PubMed: 12488957]

77. Ishizuka N, Yagui K, Tokuyama Y, Yamada K, Suzuki Y, Miyazaki J, Hashimoto N, Makino H, Saito Y, and Kanatsuka A. 1999. Tumor necrosis factor alpha signaling pathway and apoptosis in pancreatic beta cells. Metabolism 48: 1485–1492. [PubMed: 10599977]

78. Knight RR, Kronenberg D, Zhao M, Huang GC, Eichmann M, Bulek A, Wooldridge L, Cole DK, Sewell AK, Peakman M, and Skowera A. 2013. Human β-cell killing by autoactive preproinsulin-specific CD8 T cells is predominantly granule-mediated with the potency dependent upon T-cell receptor avidity. Diabetes 62: 205–213. [PubMed: 22936177]

79. Kang S, Park SY, Lee HJ, and Yoo YH. 2010. TRAIL upregulates decoy receptor 1 and mediates resistance to apoptosis in insulin-secreting INS-1 cells. Biochem. Biophys. Res. Commun 396: 731–735. [PubMed: 20451496]
80. Dirice E, Kahraman S, Elpek GO, Aydin C, Balci MK, Omer A, Sanlioglu S, and Sanlioglu AD. 2011. TRAIL and DcR1 expressions are differentially regulated in the pancreatic islets of STZ-versus CY-applied NOD mice. Exp. Diabetes Res 2011: 625813. [PubMed: 22144989]

81. Iyori M, Zhang T, Pantel H, Gagne BA, and Sentman CL. 2011. TRAIL/DR5 plays a critical role in NK cell-mediated negative regulation of dendritic cell cross-priming of T cells. J. Immunol 187: 3087–3095. [PubMed: 21832159]
FIGURE 1. TRAIL is expressed on Tregs in the TME and generation of a Tnfsf10^L/L mouse. (A) C57BL/6 Foxp3^Cre-YFP^ mice were injected with 125,000 B16 cells i.d. and sacrificed 12 d postinoculation. Cells were sorted, and qPCR was performed for Tnfsf10 and HPRT. (B) Schematic of the Tnfsf10^0^-A mouse. (C) Genotyping PCR of genomic tail DNA of Tnfsf10^0^-A-targeted mice. (D) Cells were sorted from Foxp3^Cre-YFP^ B6 and Tnfsf10^0^-A Foxp3^Cre-YFP^ B6 mice, genomic DNA isolated, and PCR performed using primers specific for exons 1, 2, and 5 of Tnfsf10. (E) Cells were sorted from Foxp3^Cre-YFP^ B6 and Tnfsf10^0^-A Foxp3^Cre-YFP^ B6 mice and qPCR performed for Tnfsf10 and HPRT. Data in (A) are representative of one experiment (n = 4–5 mice per group). Data in (C) and (D) are representative of one experiment (n = 1 mouse per group). (E) is representative of two experiments (n = 1–5 mice per group). Statistical analysis was determined by Student unpaired t test. *p < 0.05, **p < 0.01, ****p < 0.0001. ns, not significant.
FIGURE 2. T_{reg}-restricted deletion of Tnfsf10 does not affect tumor growth or suppression in C57BL/6 mice.

(A) T_{reg}+ were isolated from Foxp3^{Cre-YFP}.B6 and Tnfsf10^{L/L}.Foxp3^{Cre-YFP}.B6 naive mice and cultured with effector CD4+ T cells, APCs, and anti-TCR Ab for 72 h in a classical microsuppression assay. (B) Mice were injected with 125,000 B16 i.d., and tumor size was measured. (C) Mice were injected with 125,000 B16 i.d. and sacrificed at day 12 after tumor inoculation. Microsuppression as previously described in (A) was performed. (D) Foxp3^{Cre-YFP}.B6 and Tnfsf10^{L/L}.Foxp3^{Cre-YFP}.B6 mice were injected with 500,000 MC38 s.c. and treated with isotype or anti–PD-1 on days 6, 9, and 12 and measured for tumor growth. (E) CD4+ Foxp3+ and (F) CD8+ T cells were examined for percentage expression of cleaved-caspase3 (c-casp3). (G) CD4+ Foxp3− and (H) CD8+ T cells were examined for percentage expression of Ki67. (I) CD4+ Foxp3− and (J) CD8+ T cells from the TIL were gated for IFN-γ and TNF-α after 4-h stimulation; representative plots shown. (K and L) Tabulated data for IFN-γ and TNF-α from CD4+ Foxp3− and CD8+ T cells. Data in (A) are representative of one experiment (n = 3–4 mice per group). Data in (B)–(L) are representative of two experiments (n = 6–9 mice per group). Statistics were determined using two-way ANOVA (A–D) and Student unpaired t test (E–H, K, and L). ns, not significant.
FIGURE 3. T<sub>reg</sub>-restricted deletion of Tnfsf10 does not affect tumor growth or suppression in BALB/c mice.

(A) T<sub>regs</sub> (TCR<sup>β</sup>CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>) were sorted from Foxp<sup>Cre</sup>-.BALB/c, Tnfsf10<sup>L/L</sup>-BALB/c, Tnfsf10<sup>L/L</sup> Foxp<sup>Cre</sup>-.BALB/c naive mice and cultured with effector T cells, APCs, and anti-TCR Ab for 72 h in a classical microsuppression assay. (B) Mice were injected with 125,000 CT26 s.c., and tumor size was measured. (C) Mice were injected with 125,000 CT26 s.c. and sacrificed at day 12 after tumor inoculation. Microsuppression as previously described in (A) was performed. (D) CD4<sup>+</sup> Foxp3<sup>+</sup> and (E) CD8<sup>+</sup> T cells from were examined for percent expression of cleaved-caspase3 (c-casp3). (F) CD4<sup>+</sup> Foxp3<sup>+</sup> and (G) CD8<sup>+</sup> T cells were examined for percent expression of Ki67. (H) CD4<sup>+</sup> Foxp3<sup>+</sup> and (I) CD8<sup>+</sup> T cells from the TIL were gated for IFN-γ and TNF-α after 4 h stimulation; representative plots shown. (J and K) Tabulated data for IFN-γ and TNF-α from CD4<sup>+</sup> Foxp3<sup>+</sup> and CD8<sup>+</sup> T cells. Data in (A) are representative of one experiment with two to three mice per group. Data in (B) are representative of four experiments (n = 14–25 mice per group). Data in (C)–(K) are representative of two experiments (n = 3–12 mice per group).
Statistics were determined using two-way ANOVA (A–C) and Student unpaired t test (D–G, J, and K). *p < 0.05. ns, not significant.
FIGURE 4. T<sub>reg</sub>-restricted deletion of Tnfsf10 does not affect diabetes incidence, insulitis, or suppression in NOD mice.

(A) Twelve-week-old female NOD Foxp<sup>3<sup>Cro-GFP</sup> mice were sacrificed. Cells were sorted, and qPCR was performed for Tnfsf10 and HPRT. (B) Diabetes onset monitored in Tnfsf10<sup>Δ/Δ</sup>Foxp<sup>3<sup>Cro-GFP</sup> NOD females and cocaged controls. (C and D) Histological assessment of insulitis performed in female Tnfsf10<sup>Δ/Δ</sup>Foxp<sup>3<sup>Cro-GFP</sup> NOD and cocaged controls at 12 weeks of age. (E) CD4<sup>+</sup>Foxp<sup>−</sup> and (F) CD8<sup>+</sup> T cells from were examined for percentage expression of cleaved-caspase3 (c-casp3). (G) CD4<sup>+</sup>Foxp<sup>−</sup> and (H) CD8<sup>+</sup> T cells were examined for percentage expression of Ki67. (I) CD4<sup>+</sup>Foxp<sup>−</sup> and (J) CD8<sup>+</sup> T cells from the TIL were gated for IFN-γ and TNF-α after 4 h stimulation; representative plots shown. (K and L) Tabulated data for IFN-γ and TNF-α from CD4<sup>+</sup>Foxp<sup>−</sup> and CD8<sup>+</sup> T cells. Data in (A) are representative of one experiment (n = 3 mice per group). Data in (B) are representative of more than three experiments (n = 29–42 mice per group). Data in (C) and (D) are representative of one experiment (n = 4–10 mice per group). Data in (E)–(L) are representative of two experiments (n = 4–21 mice per group). Statistics were determined using log-rank (Mantel–Cox) test (B) and Student unpaired t test (E–H, K, and L). ns, not significant.