学 位 論 文

Doctoral Thesis

The immunoregulatory effect of
tumor necrosis factor-related apoptosis inducing ligand (TRAIL)

(免疫抑制分子 TRAIL による自己免疫疾患の制御機構に関する研究)

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2011年3月

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２０１１年３月

1. **Contents**

2. Summary

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is known to play a pivotal role in the inhibition of autoimmune disease. We previously reported a disease-preventive effect of embryonic stem cell-derived dendritic cells (ES-DC) genetically engineered to express TRAIL along with a myelin antigen, MOG, on experimental autoimmune encephalomyelitis (EAE), and also suggested that $CD4^+CD25^+$ regulatory T (Treg) cells were involved in mediating this preventive effect. In the current study, we investigated the effect of TRAIL on Treg cells as well as conventional T cells, using TRAIL-deficient mice. Upon induction of EAE, TRAIL-deficient mice showed more severe clinical symptoms, a higher frequency of IFN-γ-producing $CD4^+$ T (Th1) cells, and a lower frequency of $CD4^+Foxp3^+$ Treg cells than wild type mice. *In vitro,* conventional T cells stimulated by bone marrow-derived DC (BM-DC) from TRAIL-deficient mice showed a higher magnitude of proliferation than those stimulated by BM-DC from wild type mice. In contrast, TRAIL expressed on the stimulator BM-DC enhanced the proliferative response of $CD4^+CD25^+$ Treg cells in the culture. The functional TRAIL-receptor, mDR5, was expressed in both conventional T cells and Treg cells upon stimulation. On the other hand, the decoy receptor, mDc-TRAIL-R1 was slightly expressed only on $CD4^+CD25^+$ Treg cells. Therefore, the distinct effects of TRAIL may be due to differences in the mDc-TRAIL-R1-expreesion or the signaling pathways down-stream of mDR5 between the two T cell subsets. Our data suggests that TRAIL suppresses autoimmunity by two mechanisms; one is the inhibition of Th1 cells and the other is the promotion of Treg cells**.**

3. Publication list

Ikeda T, Hirata S, Fukushima S, Matsunaga Y, Ito T, Uchino M, Nishimura Y, and Senju S. 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, 2010

Senju S, Haruta M, Matsunaga Y, Fukushima S, Ikeda T, Takahashi K, Okita K, Yamanaka S, Nishimura Y. Characterization of Dendritic Cells and Macrophages Generated by Directed Differentiation from Mouse Induced Pluripotent Stem Cells. ***Stem cells*** 27:1021-1031, 2009

Matsunaga Y, Fukuma D, Hirata S, Fukushima S, Haruta M, Ikeda T, Negishi I, Nishimura Y, and Senju S. Activation of antigen-specific cytotoxic T lymphocytes by β2-microglobulin or TAP1 gene disruption and the introduction of recipient-matched MHC class I gene in allogeneic ES cell-derived dendritic cells. ***J.Immunol***. 181: 6635-6643, 2008

4. Acknowledgments

These series of investigations were performed from 2007 to 2010, in the Department of Immunogenetics and the Department of Neurology, Graduate School of Medical Sciences, Kumamoto University.

I wish to extend my warmest thanks to Professor Yasuharu Nishimura and Associate Professor Satoru Senju, the department of Immunogenetics, and Professor Uchino Makoto, the department of Neurology, Graduate School of Medical Sciences, Kumamoto University. They generously gave me advices and suggestions. I would also like to express my gratitude for their spending valuable time to correct my paper.

I am grateful to all staffs and students in the department of Immunogenetics and the department of Neurology, Graduate School of Medical Sciences, Kumamoto University. They provided me with their valuable time and gave me much advice.

This work was supported in part by Grants-in-Aid Nos. 18014023, 19591172, and 19059012 from the Ministry of Education, Culture, Sports, Science and Technology, Japan; the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, launched as a project commissioned by the Ministry of Education, Culture, Sports, Science and Technology, Japan; a Research Grant for Intractable Diseases from the Ministry of Health and Welfare, Japan; and grants from the Japan Science and Technology Agency, the Uehara Memorial Foundation, and the Takeda Science Foundation, Advanced Education Program for Integrated Clinical, Basic and Social Medicine, Graduate School of Medical Sciences, Kumamoto University (Program for Enhancing Systematic Education in Graduate Schools, Ministry of Education, Culture, Sports, Science and Technology, Japan).

Finally, I would like to thank my wife, Mika for her support, encouragement, and unshakable faith in my abilities during the course of my studies. Without her support, I may not have persevered in my studies.

5. Abbreviations

DC, Dendritic cell;

APC, Antigen presenting cell;

MHC, Major histocompatibility complex;

TNF, Tumor necrosis factor;

TRAIL, Tumor necrosis factor-related apoptosis inducing ligand;

Treg cell, Regulatory T cell;

WT, Wild-type;

ES cell, Embryonic stem cell;

ES-DC, Embryonic stem cell-derived dendritic cell;

BM-DC, Bone marrow-derived dendritic cell;

DN, Double negative;

DP, Double positive;

mDR5, Mouse death receptor 5;

EAE, Experimental autoimmune encephalomyelitis;

EAT, Experimental autoimmune thyroiditis;

ILN, Inguinal lymph node;

rTRAIL, Recombinant mouse TRAIL;

SP CD4, $CD4^+$ single positive;

SP CD8, $CD8^+$ single positive;

MOG, Myelin oligodendrocyte glycoprotein;

MBP, Myelin basic protein;

PLP, Proteolipid protein;

PD-L1, Programmed Death-1 ligand;

i.p., Intraperitoneally;

s.c., Subcutaneous

iPS cell, Induced pluripotent stem cell;

6. Introduction

6.1. Experimental autoimmune encephalomyelitis (EAE), as a model of organspecific autoimmune disease

Experimental autoimmune encephalomyelitis (EAE), an animal model for human multiple sclerosis, is characterized by neurological impairment resulting from demyelination in the CNS. This EAE system is induced in female mice by subcutaneous (s.c.) immunization with myelin antigen and adjuvant. Myelin antigens, for example, are myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and myelin proteolipid protein (PLP). After immunization, myelin antigen-specific CD4 positive T cells are induced and this more lead to induce B cells and a part of CD8 positive T cells. These cells invade central nerves system, mainly spinal cord and attack myelin sheaths. In the results, clinical symptoms in these mice manifest first as an ascending paralysis, initially affecting the tail and hind limbs, and subsequently affecting the forelimbs and brain. In addition, it is known that appropriate antigen used for EAE induction is different between the species and strains of animals.

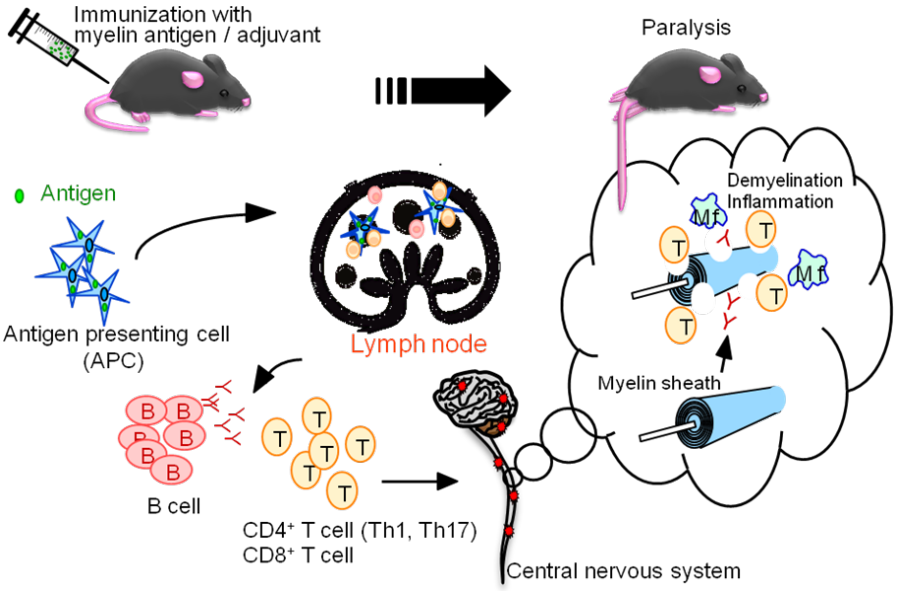

Figure 1. Experimental autoimmune encephalomyelitis (EAE).

This disease is produced in experimental animals by injecting them with isolated spinal cord (such as myelin antigen) homogenized in complete Freunds' adjuvant. EAE is due to an inflammatory reaction in the central nervous system that causes a progressive paralysis affecting first the tail and hind limbs before progressing to forelimb paralysis and eventual death. Plaques active disease show infiltration of nervous

tissue by lymphocytes, plasma cells, and macrophages, which cause destruction of the myelin sheaths that surround nerve cell axons in the brain and spinal cord.

6.2. Tumor necrosis factor apoptosis inducing ligand (TRAIL)

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a type II membrane protein belonging to the TNF superfamily (1, 2). TRAIL is expressed in a variety of cell types, including lymphocytes, NK cells, NKT cells, dendritic cells (DC), macrophages and virus-infected APCs (3-6). In humans, TRAIL can bind two apoptosis-inducing receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and two decoy receptors, TRAIL-R3 (LIT, DcR1) and TRAIL-R4 (TRUNDD, DcR2). A soluble receptor called osteoprotegerin (OPG) is also capable of binding TRAIL (Figure 2A) (1, 7, 8). In contrast, mice have only one apoptosis-inducing receptor, called murine TRAIL-R (MK, mDR5) and two decoy receptors, mDc-TRAIL-R1 and mDc-TRAIL-R2 (Figure 2B) (9).

TRAIL induces apoptosis and activates the transcription factor NF-κB (1, 10). The TRAIL/TRAIL receptor system is implicated in immune-regulation and anti-tumor immunity (11, 12). Indeed, TRAIL-deficient mice are highly susceptible to autoimmune arthritis, diabetes and experimental autoimmune encephalomyelitis (EAE) (13-15). In addition, TRAIL is involved in the regulation of the Th1/Th2 balance (16). TRAIL on $CD4^+CD25^+$ regulatory T (Treg) cells has been reported to induce death of $CD4^+CD25^-$ conventional T cells (17).

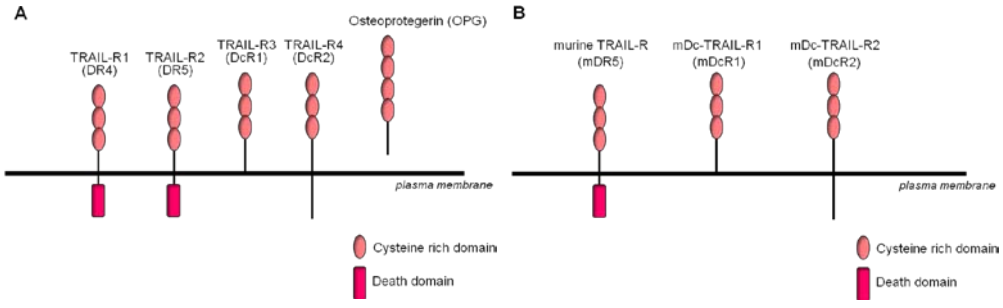

Figure 2. Schematic of the five TRAIL receptors in human (A) and the three TRAIL receptors in mice (B).

6.3. ES cell-derived dendritic cell (ES-DC)

DC are antigen-presenting cells with the unique capacity to take up and process antigens in the peripheral blood and tissues. They subsequently migrate to draining lymph nodes, where they present antigen to resting lymphocytes. Although immature DC are particularly good at antigen ingestion and processing, for a fully productive T cell response, they need to differentiate into mature DC, which express high levels of cell surface major histocompatibility complex (MHC) antigens complexes and costimulatory molecules. DC of various phenotypes serve as sentinel cells in virtually all tissues- including the peripheral blood, where they are continuously exposed to antigens. Very small numbers of mature DC are highly efficient at generating immune responses against viruses, other pathogens and endogenous tumors. In addition, it was reported that DC are also involved in the maintenance of immunological selftolerance, promoting T cells with regulatory functions or inducing anergy of T cells. Recently, culture procedures to generate DC from mouse ES cells were established (Figure 3) (18, 19). ES cell derived DC (ES-DC) have the capacity to present antigens for T cells and to stimulate naïve T cells, being comparable to bone marrow-derived DC (BM-DC) *in vivo* (20, 21). Moreover, because ES cells can readily be genetically moditied, we performed ES cell-mediated genetic engineering of DC to generate DC with specific functions. Then, we generated genetically modified ES-DC, which expressed the product of introduced gene.

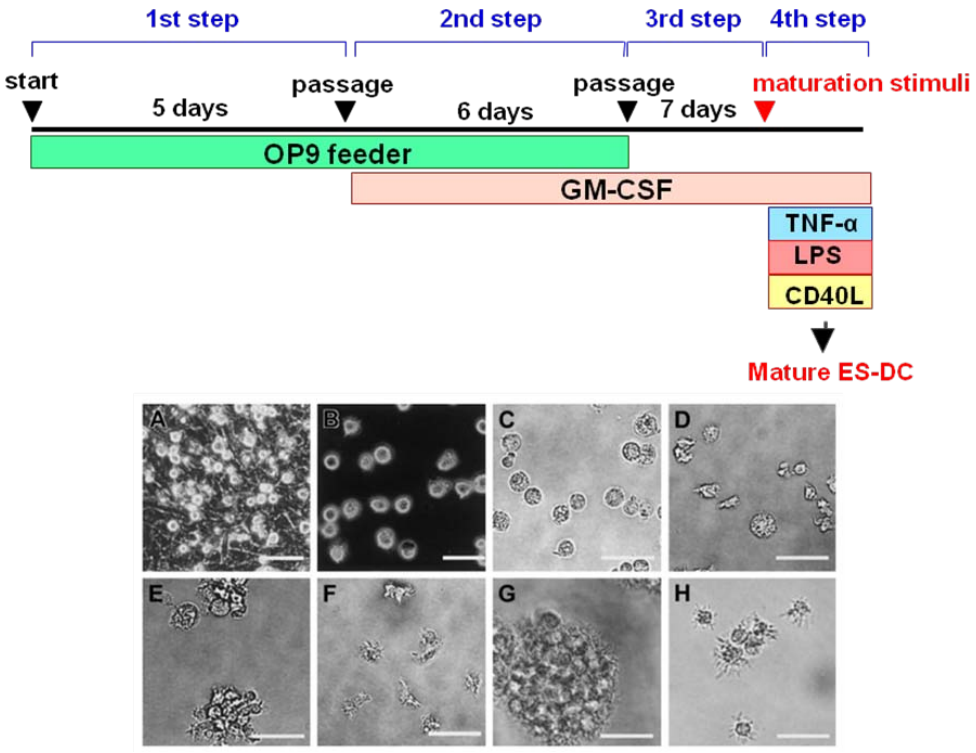

Figure 3. Differentiation and morphology of DC from ES cells.

Overview of the culture protocol for generation of DC from ES cells (A-H). ES-DC on day 8 (A), day12 (B, C), day17 (D, E) and day 27 (F) of differentiation culture are shown. Cells on day 24 were recovered and stimulated for 2 days with IL-4, TNF-α plus agonistic anti-CD40 mAb (G) or with IL-4, TNF-α plus LPS (H). Panel (A) and (B) are phase contrast micrographs. Scale bars represent 20 μm.

6.4. Enhanced capacity of ES-DC expressing TRAIL to induce the *in vitro* proliferation of $CD4^+CD25^+$ regulatory T cells

As previously indicated, genetically modified ES-DC was established. the protection from MOG-induced EAE by the adoptive transfer of genetically modified ES-DC presenting MOG peptide in the context of MHC class II molecules and simultaneously expressing TRAIL (ES-DC-TRAIL/MOG) have been reported. Moreover, it has been reported that the severity of EAE induced by another myelin autoantigen, myelin basic protein (MBP), was also decreased after treatment with ES-DC-TRAIL/MOG (Figure 4) (22, 23). In addition, adoptive transfer of $CD4^+CD25^+$ Treg cells isolated from donor mice treated with ES-DC-TRAIL/MOG protected the recipient mice from subsequently induced EAE (23). Furthermore, TRAIL on ES-DC enhanced the proliferation of $CD4^+CD25^+$ Treg cells *in vitro* (Figure 5) (23). As for the

relationship between TRAIL and Treg cells, TRAIL was shown to expand $CD4^+CD25^+$ Treg cells in a study of experimental autoimmune thyroiditis (EAT) (24). With regards to the ability of TRAIL to induce cell-proliferation, it was recently reported that TRAIL promotes the proliferation of vascular smooth muscle cells and carcinoma cells (25, 26).

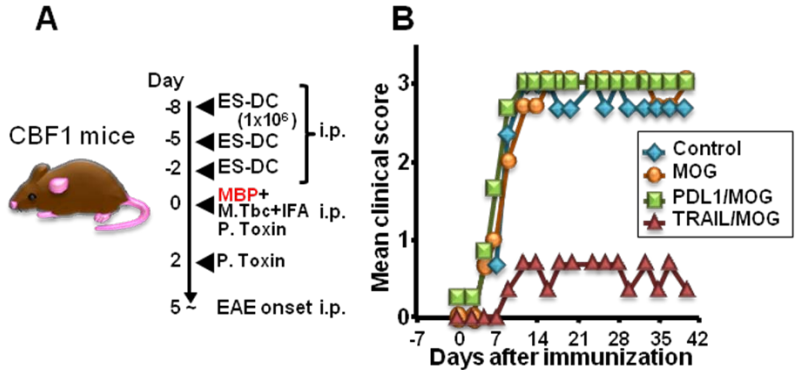

Figure 4. ES-DC expressing TRAIL along with MOG peptide decreased the severity of MBP-induced EAE.

Prevention of MBP-induced EAE by treating the mice with ES-DC-TRAIL/MOG. A, The schedule for the pretreatment and induction of EAE is shown. CBF1 mice (three to four mice per group) were i.p. injected with ES-DC (1×10^6 cells/mouse/injection) on days -8, -5, and -2. EAE was induced by the immunization of MBP peptide on day 0, and the injection of *B. pertussis* toxin on days 0 and 2. B, The disease severity of mice immunized with MBP peptide is shown.

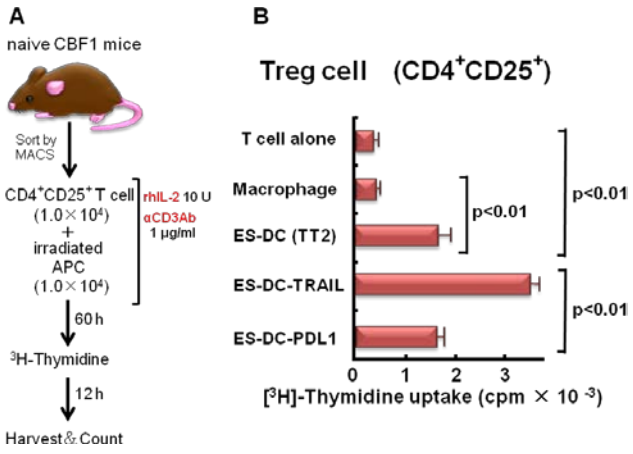

Figure 5. Enhanced capacity of APC expressing TRAIL to induce the proliferation of $CD4^+CD25^+$ Tregs *in vitro*.

$CD4^+CD25^+$ T cells (1×10^4) isolated from the spleen of the naive CBF1 mice were cultured alone or cocultured with irradiated stimulators (1×10^4), syngeneic splenic macrophages, nontransfectant ES-DC

(TT2), ES-DC-TRAIL, or ES-DC-PDL1, for 3 days in the presence of anti-CD3 mAb (0.1 μg/ml) and human IL-2 (10 U/ml). The proliferation of responder T cells was quantified by measuring the [3H]thymidine incorporation in the last 12 h of the culture. *, Statistical significance ($p < 0.05$). The results were expressed as the mean of a triplicate assay ± SD. The data are each representative of more than three independent experiments with similar results.

6.5. Aims of this work

We hypothesized that TRAIL promotes the proliferation of Treg cells in certain situations. To evaluate this hypothesis, we investigated the effect of TRAIL on Treg cells using TRAIL-deficient mice.

7. Materials and methods

7.1. Mice

Six- to eight-week-old wild-type (WT) C57BL/6 mice were purchased from Clea Animal (Tokyo, Japan), and TRAIL-deficient ($TRAIL^{-/-}$) C57BL/6 mice were kindly provided by Amgen (Thousand Oaks, CA). The two mouse strains were crossed to obtain heterozygotes, which were used as the parental generation. The offspring of the heterozygotes were genotyped, and the WT and $TRAIL^{-/-}$ littermates were used in the experiments. The TRAIL allele was examined by PCR of genomic DNA prepared from tail biopsies, using primer pairs specific to the WT TRAIL allele (59-GGT TAT CAT CAG CTT CAT GG-39 and 59-GAA ATG GTG TCC TGA AAG GTT C-39) and specific to neomycin phosphotransferase gene (59-AGC GAG CAC GTA CTC GGA TGG AA-39 and 59-CCC ATT CGC CGC CAA GCT CTT CAG CAA TAT-39). Mice were housed in a specific pathogen-free barrier facility. All experiments were approved by the Animal Research Committee of Kumamoto University.C57BL/6 mice were obtained from SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions. All studies were done with C57BL/6 mice syngeneic to the mouse ES cell line B6 at 6-8 weeks of age. The mouse experiments were approved by the Animal Research Committee of Kumamoto University.

7.2. Peptides and cytokines

Mouse MOG peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK) and soluble recombinant mouse TRAIL (rTRAIL) were purchased from AnyGen (Gwangju, Korea) and Alexis Biochemicals (San Diego, CA), respectively. Rat anti-mouse CD25 mAb (clone PC61.5.3) was produced, as described previously (19).

7.3. Antibodies

The following Abs and secondary reagents were purchased from eBioscience (San Diego, CA), unless otherwise stated: FcR blocking Ab (anti-mouse CD16/CD32, clone 2.4G2; BD Pharmingen, San Diego, CA), PE-conjugated anti-mouse TRAIL (clone N2B2), FITC- and PE-conjugated anti-mouse CD4 (clone RM4-5),

PE-conjugated anti-mouse CD8a (clone 53-6.7), FITC-conjugated anti-mouse/human B220 (clone RA3-6B2), FITC-conjugated anti-mouse/rat Foxp3 (clone FJK-16s), FITC-conjugated antimouse IL-17A (clone eBio17B7), FITC-conjugated anti-mouse IFN-γ (clone XMG1.2), biotin-conjugated anti-mouse DR5 (clone MD5-1), anti-mouse DcTRAIL-R1 (clone mDcR1-3; BioLegend, San Diego, CA), antimouse DcTRAIL-R2 (clone mDcR2-1; BioLegend), FITC-conjugated streptavidin, FITC-conjugated anti-hamster (Armenian) IgG (BioLegend), Armenian Hamster IgG isotype-matched control (BioLegend), FITC- and PE-conjugated rat IgG2a isotype-matched control, FITC-conjugated rat IgG1 isotype-matched control, and biotin-conjugated Armenian Hamster IgG isotype-matched control.

7.4. Flow-cytometry analysis

Spleen and inguinal lymph node (ILN) cells were incubated with FcR blocking Ab on ice for 15 min and subsequently labeled with specific Abs at 4°C before analysis using a FACScan flow cytometer (BD Biosciences, San Jose, CA). In some experiments, intracellular cytokine analysis was performed, as described previously (23, 24). Briefly, spleen or ILN cells were resuspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS and 2-ME. PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 mg/ml; all from Sigma-Aldrich, St. Louis, MO) were added to the cells. After incubation for 5 h, cells were washed and stained with PE-conjugated anti-mouse CD4. The fixation and permeabilization of cells were performed using IntraPrep reagent (Beckman Coulter, Fullerton, CA), according to the manufacturer's instructions, and intracellular cytokines were stained with FITC-conjugated anti-mouse IFN-γ or IL-17A. To demonstrate the specificity of staining, isotype-matched control mAbs were also used. The stained cells were analyzed by flow cytometry.

7.5. Induction of EAE

EAE was induced in 6–8-wk-old female mice by s.c. immunization with 200 mg MOG 35–55 peptide emulsified in CFA containing 2 mg/ml heat-killed Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI) at the base of the tail on day 0. Additionally, mice received 350 ng Bordetella pertussis toxin (Calbiochem, San Diego, CA) i.p. in 0.5 ml PBS on days 0 and 2. In some experiments,

to deplete $CD25^+$ T cells, anti-mouse CD25 mAb (clone PC61.5.3, 400 mg/mouse) or rat IgG (Chemicon International, Temecula, CA) as a control was injected i.p. at 14 d before the immunization. Residual $CD4^+Foxp3^+$ T cells in spleen cells were examined by flow cytometry after 12 d (2 d before the immunization). Clinical signs of EAE were assessed with a 0–6-point scoring system: 0, normal; 1, weakness of the tail and/or paralysis of the distal half of the tail; 2, loss of tail tonicity and abnormal gait; 3, partial hindlimb paralysis; 4, complete hindlimb paralysis; 5, forelimb paralysis or moribundity; and 6, death.

7.6. Generation of bone marrow-derived dendritic cells (BM-DC)

The generation of BM-DC was carried out as described previously (18). In brief, BM cells from WT or $TRAIL^{-/-}$ mice were cultured in RPMI-1640 supplemented with 10 % fetal bovine serum, GM-CSF (500 U/ml) and 2-ME for 9 days. To induce maturation of DC, immature BM-DC were stimulated with 1 μg/ml LPS during the final 48 h.

7.7. T cell proliferation assay

Twenty-one days after immunization with MOG peptide, spleen or ILN cells were isolated and cultured (2.5×10^5 cells/well) with MOG peptide (0, 1, 3.2 μM) or Con A from Sigma (5 μg/ml) as a positive control in 96-well flat-bottomed plates. The cells were cultured for 3 days, and [^{3}H]-thymidine (248 GBq/mM) was added to the culture (37 kBq/well) for the final 16 h. At the end of culture, cells were harvested onto glass fiber filters (Wallac) and the incorporation of [^{3}H]-thymidine was measured by scintillation counting.

7.8. Proliferation assay of $CD4^+CD25^-$ conventional T and $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells from the spleens of unprimed WT or $TRAIL^{-/-}$ mice were purified using the MACS cell sorting system (Miltenyi Biotec) as described previously (23). Cell purity was confirmed to be more than 95% by FACS analysis. The proliferation of $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells was analyzed as described previously (27) with some

modifications. In brief, $CD4^+CD25^-$ conventional T cells or $CD4^+CD25^+$ Treg cells (4.0×10^4 cells) were cocultured with LPS-stimulated and x-ray irradiated (45 Gy) BM-DC (2.0×10^4 cells) in the presence of 1 μg/ml anti-CD3 mAb (Clone: 145-2C11, BD Bioscience) and 10 U/mL human IL-2 in 96-well round-bottomed plates for 3 days. In some experiments, $CD4^+CD25^-$ conventional T or $CD4^+CD25^+$ Treg cells (1.0×10^5 cells / well) from $TRAIL^{-/-}$ mice were stimulated with 3 μg/ml plate-bound anti-CD3 and anti-CD28 mAb (Clone: 37.51, BD Bioscience) and 20 U/mL human IL-2 in the presence or absence of soluble rTRAIL for 3 days. [^{3}H]-thymidine was added to the culture for the final 12 h, and the proliferation of T cells was quantified by measuring [^{3}H]-thymidine incorporation.

7.9. The expression of murine TRAIL-R, mDR5 and two decoy receptors, mDc-TRAIL-R1 and mDc-TRAIL-R2 on $CD4^+CD25^-$ conventional T and $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^-$ conventional T cells or $CD4^+CD25^+$ Treg cells (1.0×10^5 cells) isolated from the spleens of $TRAIL^{-/-}$ mice were stimulated with 3.0 μg/ml plate-bound anti-CD3 and anti-CD28 mAb and 20 U/mL human IL-2 for 72 h. These T cells were stained with biotin-conjugated anti-mouse DR5, and subsequently stained with FITC-conjugated Streptavidin. They were also stained with anti-mouse DcTRAIL-R1 or anti-mouse DcTRAIL-R2, and then stained with FITC-conjugated anti-hamster (Armenian) IgG and analyzed on a flow cytometer.

7.10. Immunohistochemical analysis

Seventeen days after immunization with MOG peptide, animals were sacrificed and the spinal cords were flushed out with PBS. The spinal cords were fixed in 4 % paraformaldehyde for 72 h, paraffin-embedded, and cross-sectioned using standard protocols. Sections were stained with H&E.

7.11. Statistical analysis

The two-tailed Student's t test was used to determine any statistically significant differences. A value of $p < 0.05$ was considered as statistically significant.

8. Results

8.1. Increased numbers of IFN-γ-producing CD4$^+$ T (Th1) cells in TRAIL$^{-/-}$ mice.

It has been reported that TRAIL$^{-/-}$ mice have enlarged lymph nodes, spleens and thymuses (15). As shown in Fig. 6, A-C, the frequencies of CD4$^+$, CD8$^+$ and CD4$^+$Foxp3$^+$ T cells and B cells in TRAIL$^{-/-}$ mice were similar to those of WT mice. On the other hand, the frequency of IFN-γ-producing CD4$^+$ T (Th1) cells in the spleen and ILNs in TRAIL$^{-/-}$ mice was significantly higher than that in WT mice (Fig. 6D). The frequency of IL-17-producing CD4$^+$ T cell (Th17) cells was similar in WT and TRAIL$^{-/-}$ mice (Fig. 6E). Spleen and ILNs of 6- to 8- weeks old TRAIL$^{-/-}$ mice were slightly larger than those of WT mice, and total number of cells of these tissues in TRAIL$^{-/-}$ mice was slightly higher than that of WT mice (Table I). The difference in the absolute number of Th1 cells between WT and TRAIL$^{-/-}$ mice were thus significant. These results suggest that TRAIL is involved in the regulation of the number of Th1 cells in the steady state.

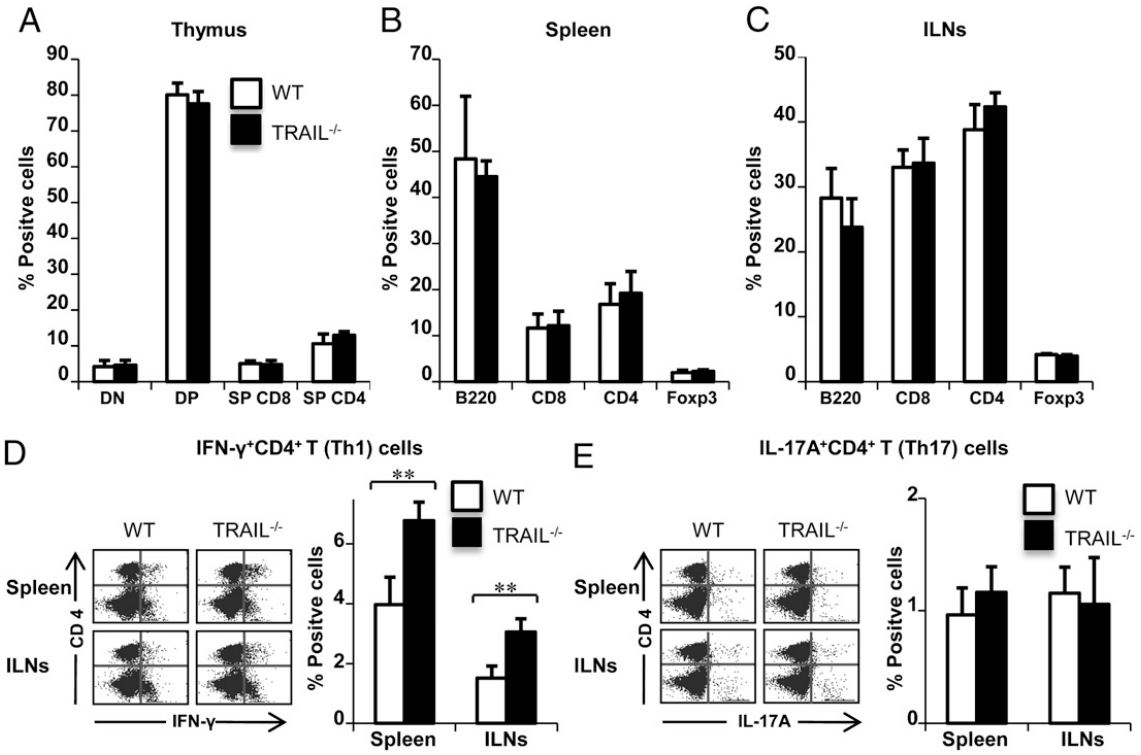

Figure 6. Increased frequency of Th1 cells in TRAIL$^{-/-}$ mice.

The frequencies of specific lymphocytes in the thymus (A), spleen (B) and ILNs (C) of WT (open bars) or

TRAIL$^{-/-}$ (closed bars) mice were assessed by flow cytometry. A, The frequencies of double negative (DN), double positive (DP), CD8^{+} single-positive (SP CD8) and CD4^{+} single-positive (SP CD4) cells among thymocytes are shown. B-C, The frequencies of B220^{+} B cells, CD8^{+} T cells and CD4^{+} T cells in the spleen (B) and ILNs (C) are shown. The frequency of CD4^{+} Foxp3^{+} T cells detected by intracellular staining with anti Foxp3 mAb is also shown. D-E, Whole spleen and ILN cells were stimulated with PMA plus ionomycin in the presence of brefeldin A for 5 h, followed by intracellular cytokine staining of IFN-γ and IL-17A, and analysis by flow cytometry. Dot plots (left) and bar graphs (right) represent the frequencies of IFN-γ^{+} (D) and IL-17A^{+} (E) CD4^{+} T cells within the total CD4^{+} T cells in the whole spleen or ILNs. Bar graphs indicate the mean frequency ± SD of five mice per group. The asterisks indicate that the differences are statistically significant between two values indicated by lines (*, $p < 0.05$; **, $p < 0.01$).

Table I. Cell numbers of total, $CD4^+$, $CD4^+Foxp3^+$, IFN-γ^+CD4^+ and IL-$17A^+CD4^+$ T cells in the thymus, spleen and ILNs in naïve WT and $TRAIL^{-/-}$ mice[a]

Organ	Cell subsets	Group	Total cell numbers
Thymus	Total cells	WT	$9.7 \pm 2.9 \times 10^7$
		$TRAIL^{-/-}$	$11.1 \pm 1.0 \times 10^7$
	DN cells	WT	$4.0 \pm 1.6 \times 10^6$
		$TRAIL^{-/-}$	$5.2 \pm 1.1 \times 10^6$
	DP cells	WT	$7.8 \pm 2.6 \times 10^7$
		$TRAIL^{-/-}$	$8.9 \pm 1.1 \times 10^7$
	SP $CD8^+$ T cells	WT	$4.8 \pm 1.2 \times 10^6$
		$TRAIL^{-/-}$	$5.4 \pm 0.8 \times 10^6$
	SP $CD4^+$ T cells	WT	$9.9 \pm 2.5 \times 10^6$
		$TRAIL^{-/-}$	$14.7 \pm 0.6 \times 10^6$**
Spleen	Total cells	WT	$8.6 \pm 1.6 \times 10^7$
		$TRAIL^{-/-}$	$10.2 \pm 1.7 \times 10^7$
	B cells	WT	$4.1 \pm 1.1 \times 10^7$
		$TRAIL^{-/-}$	$4.5 \pm 0.6 \times 10^7$
	$CD8^+$ T cells	WT	$1.0 \pm 0.3 \times 10^7$
		$TRAIL^{-/-}$	$1.2 \pm 0.3 \times 10^7$
	$CD4^+$ T cells	WT	$1.4 \pm 0.4 \times 10^7$
		$TRAIL^{-/-}$	$1.9 \pm 0.4 \times 10^7$
	$CD4^+Foxp3^+$ T cells	WT	$1.7 \pm 0.6 \times 10^6$
		$TRAIL^{-/-}$	$2.3 \pm 0.5 \times 10^6$
	IFN-γ^+CD4^+ T cells	WT	$0.6 \pm 0.2 \times 10^6$
		$TRAIL^{-/-}$	$1.3 \pm 0.2 \times 10^6$**
	IL-$17A^+CD4^+$ T cells	WT	$0.3 \pm 0.1 \times 10^6$
		$TRAIL^{-/-}$	$0.5 \pm 0.2 \times 10^6$
ILNs	Total cells	WT	$2.3 \pm 0.8 \times 10^6$
		$TRAIL^{-/-}$	$3.3 \pm 0.5 \times 10^6$
	B cells	WT	$0.7 \pm 0.3 \times 10^6$
		$TRAIL^{-/-}$	$0.8 \pm 0.3 \times 10^6$
	$CD8^+$ T cells	WT	$0.8 \pm 0.3 \times 10^6$
		$TRAIL^{-/-}$	$1.1 \pm 0.1 \times 10^6$*
	$CD4^+$ T cells	WT	$0.9 \pm 0.2 \times 10^6$
		$TRAIL^{-/-}$	$1.4 \pm 0.2 \times 10^6$*
	$CD4^+Foxp3^+$ T cells	WT	$1.0 \pm 0.4 \times 10^5$
		$TRAIL^{-/-}$	$1.3 \pm 0.2 \times 10^5$
	IFN-γ^+CD4^+ T cells	WT	$1.4 \pm 0.7 \times 10^4$
		$TRAIL^{-/-}$	$4.3 \pm 1.3 \times 10^4$**
	IL-$17A^+CD4^+$ T cells	WT	$2.1 \pm 0.9 \times 10^4$
		$TRAIL^{-/-}$	$3.0 \pm 1.6 \times 10^4$

[a] Mean absolute cell numbers are calculated from total cell numbers for the whole organ. The values are rounded off to the first decimal place. [b] Asterisks indicate the values of TRAIL$^{-/-}$ mice significantly different from those of WT mice (*, $p < 0.05$; **, $p < 0.01$). [c] WT and TRAIL$^{-/-}$ mice were analyzed (five mice per group).

8.2. Increased severity and protracted disease course of MOG-induced EAE in TRAIL$^{-/-}$ mice.

To investigate the influence of TRAIL in vivo, we used a MOG-induced EAE disease model. Previously, it has been reported that TRAIL$^{-/-}$ mice have exacerbated EAE disease (13). In agreement with this report, we observed earlier onset of EAE and more severe disease in TRAIL$^{-/-}$ mice in comparison to WT mice (Fig. 7A and Table II).

We also analyzed the response of T cells to MOG isolated from MOG immunized mice. Twenty-one days after immunization, spleen and ILN cells isolated from the mice were cultured with MOG peptide. As shown in Fig. 7B, the proliferative response of spleen and ILN cells isolated from TRAIL$^{-/-}$ mice to MOG peptide was much higher than that of WT mice. On the other hand, there was no significant difference in the response to Con A between WT and TRAIL$^{-/-}$ mice. Histological examination of the spinal cords was performed to determine whether or not the difference in clinical symptoms observed between WT and TRAIL$^{-/-}$ mice correlated with the degree of inflammatory infiltration in the central nervous system. Consistent with the data shown in Fig. 7B, more intensive infiltration of inflammatory cells into the spinal cord was observed in TRAIL$^{-/-}$ mice than WT mice (Fig. 7, C-D).

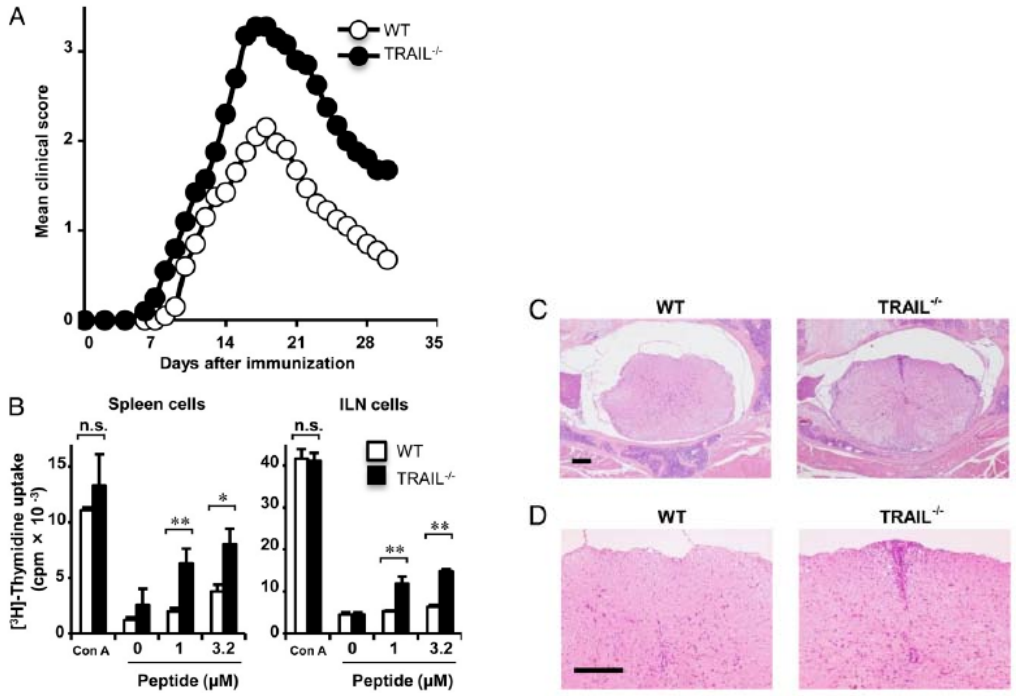

Figure 7. Increased severity and protracted course of MOG-induced EAE in TRAIL$^{-/-}$ mice.

A, WT and TRAIL$^{-/-}$ mice (ten mice per group) were immunized with MOG 35-55 peptide on day 0, and pertussis toxin on day 0 and 2, as described in the materials and methods. Values represent the mean clinical score for each group. The data are summarized in Table II.

B, Isolated spleen cells (2.5×10^5) (left) or ILN cells (right) from WT (open bars) or TRAIL$^{-/-}$ mice (closed bars) at 21 days after immunization were cultured with MOG peptide (0, 1, 3.2 μM) for 3 days. As a positive control, respective cells were stimulated also with Con A (5 μg/ml). The proliferative response was quantified by measuring [^{3}H]-thymidine incorporation in the final 12 h of the culture. The asterisks indicate that the differences in responses are statistically significant between the two values indicated by lines (*, $p < 0.05$; **, $p < 0.01$). The results were expressed as the mean of a triplicate assay ± SD. The data are each representative of three independent and reproducible experiments with similar results. C-D, Spinal cords isolated at day 17 after immunization from WT or TRAIL$^{-/-}$ mice were examined histologically. Cross sections of paraffin-embedded spinal cord samples were stained with H&E. All slides shown are representative of sections taken from 3 mice per group. Lower (C) and higher (D) magnification views are shown and bars indicate 200 μm in (C-D).

Table II. Exacerbation of MOG-induced EAE in TRAIL$^{-/-}$ mice[a]

Group	Disease incidence	Death	Mean day of the onset	Mean peak clinical score
WT mice	10/10	0/10	9.6 ± 0.6	2.2 ± 0.4
TRAIL$^{-/-}$ mice	10/10	0/10	7.3 ± 0.8	3.4 ± 0.3

[a] The data are combined from a total of 2 separate experiments, including those shown in Fig. 2*A*. The values of onset day and mean peak clinical score are rounded off to the first decimal place.

8.3. Increased Th1 cells and decreased Treg cells in EAE-induced TRAIL$^{-/-}$ mice.

Because the frequency of Th1 cells in TRAIL$^{-/-}$ mice was higher than that in WT mice in a non-immunized state (Fig. 6D), we considered the possibility that the more severe EAE disease developed in TRAIL$^{-/-}$ mice was caused by the higher frequency of Th1 cells. To assess this possibility, we analyzed the frequency of CD4^{+} T, Th1, Th17 and Treg cells in whole spleen and ILN cells on days 7, 13, 17 and 21 in EAE-induced WT and TRAIL$^{-/-}$ mice. Increase of the frequency of total CD4^{+} T cells in the spleen was observed on days 7 and 13 in TRAIL$^{-/-}$ mice but not in WT mice (Fig. 8A, left). The frequency of Treg cells in TRAIL$^{-/-}$ mice was lower than that of WT mice at all the assay points in both spleen and ILNs (Fig. 8B). In contrast, the frequency of Th1 cells in TRAIL$^{-/-}$ mice was higher than that of WT mice (Fig. 8C). On the other hand, no significant difference in the frequency of Th17 cells was observed between TRAIL$^{-/-}$ mice and WT mice (Fig. 8D). We also calculated the absolute cell numbers of respective cells in the spleen (Table III) and ILNs (Table IV). The difference in the absolute cell numbers of Treg cells between TRAIL$^{-/-}$ mice and WT mice was significant on day 7 in the spleen (Table III). These results indicate that TRAIL functions to increase Treg cells and decrease Th1 cells upon induction of EAE. The increased proliferative response of the TRAIL$^{-/-}$ mice-derived cells to MOG (Fig. 7B) may be attributed to both the higher population of Th1 and lower population of Treg cells in comparison to WT mice.

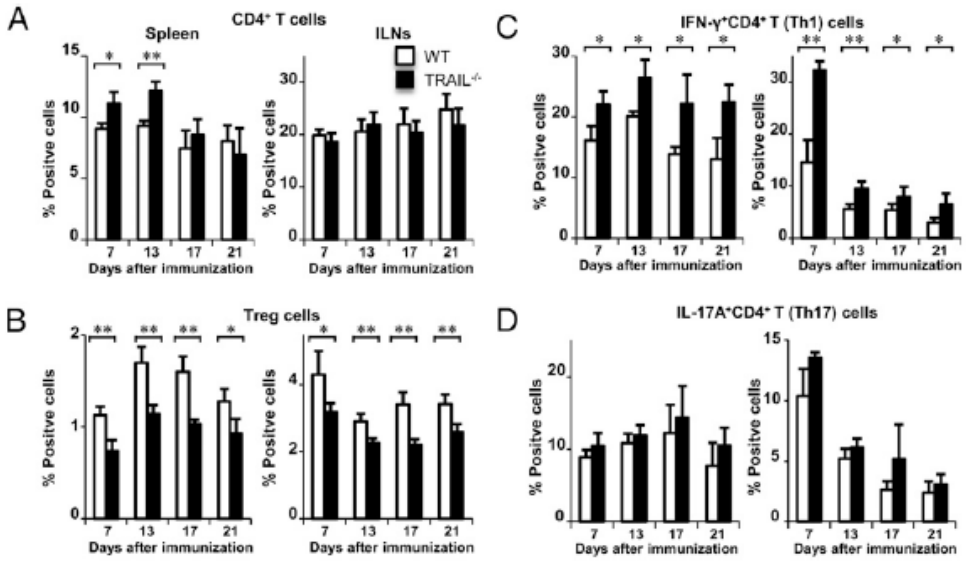

Figure 8. Increased Th1 cells and decreased Treg cells in TRAIL$^{-/-}$ mice upon EAE induction.

The spleen (left) and ILN (right) cells from WT or TRAIL$^{-/-}$ mice were analyzed by flow cytometry at day 7, 13, 17, 21 after immunization. The frequencies of cells positive for the specific indicated markers are plotted. A, Frequency of whole CD4^{+} T cells is shown. B, Frequency of CD4^{+} Foxp3^{+} T cells in whole cells is shown. C-D, Cells were stimulated with PMA plus ionomycin in the presence of brefeldin A for 5 hours, and subsequently analyzed for the production of IFN-γ and IL-17A by intracellular staining and flow cytometry. Frequencies of IFN-γ^{+} CD4^{+} T cells (C) and IL-17A^{+} CD4^{+} T cells (D) in total CD4^{+} T cells are shown. Bar graphs indicate the mean frequency ± SD of four-five mice per group. The asterisks indicate that the differences are statistically significant between the two values indicated by lines (*, $p < 0.05$; **, $p < 0.01$).

Table III. Cell numbers of total, $CD4^+$, $CD4^+Foxp3^+$, IFN-γ^+CD4^+ and IL-$17A^+CD4^+$ T cells in the spleen in EAE-induced WT and $TRAIL^{-/-}$ mice[a]

Cell subsets	Group	Days after immunization			
		7	13	17	21
Total cells	WT	$1.7 \pm 0.1 \times 10^8$	$2.1 \pm 0.6 \times 10^8$	$1.8 \pm 0.2 \times 10^8$	$1.8 \pm 0.4 \times 10^8$
	$TRAIL^{-/-}$	$1.8 \pm 0.3 \times 10^8$	$2.0 \pm 0.3 \times 10^8$	$2.7 \pm 0.2 \times 10^{8**}$	$2.2 \pm 0.3 \times 10^8$
$CD4^+$ T cells	WT	$1.5 \pm 0.2 \times 10^7$	$1.9 \pm 0.5 \times 10^7$	$1.3 \pm 0.3 \times 10^7$	$1.5 \pm 0.5 \times 10^7$
	$TRAIL^{-/-}$	$2.0 \pm 0.3 \times 10^{7*}$	$2.4 \pm 0.3 \times 10^7$	$2.3 \pm 0.2 \times 10^{7**}$	$1.5 \pm 0.7 \times 10^7$
$CD4^+Foxp3^+$ T cells	WT	$1.9 \pm 0.2 \times 10^6$	$3.3 \pm 0.7 \times 10^6$	$2.8 \pm 0.1 \times 10^6$	$2.4 \pm 0.7 \times 10^6$
	$TRAIL^{-/-}$	$1.3 \pm 0.2 \times 10^{6*}$	$2.0 \pm 0.3 \times 10^{6*}$	$2.8 \pm 0.3 \times 10^6$	$2.0 \pm 0.6 \times 10^6$
IFN-γ^+CD4^+ T cells	WT	$2.4 \pm 0.2 \times 10^6$	$3.8 \pm 1.3 \times 10^6$	$1.8 \pm 0.4 \times 10^6$	$2.0 \pm 1.1 \times 10^6$
	$TRAIL^{-/-}$	$4.4 \pm 0.6 \times 10^{6**}$	$6.5 \pm 1.5 \times 10^6$	$5.1 \pm 1.5 \times 10^{6*}$	$3.4 \pm 1.4 \times 10^6$
IL-$17A^+CD4^+$ T cells	WT	$1.3 \pm 0.2 \times 10^6$	$2.1 \pm 0.9 \times 10^6$	$1.7 \pm 0.8 \times 10^6$	$1.2 \pm 0.6 \times 10^6$
	$TRAIL^{-/-}$	$2.1 \pm 0.3 \times 10^{6*}$	$3.0 \pm 0.6 \times 10^6$	$3.3 \pm 1.2 \times 10^6$	$1.7 \pm 1.0 \times 10^6$

[a] Mean absolute cell numbers are calculated from total cell numbers for the whole spleen. The values are rounded off to the first decimal place. [b] Asterisks indicate the values of $TRAIL^{-/-}$ mice significantly different from those of WT mice (*, $p < 0.05$; **, $p < 0.01$). [c] WT and $TRAIL^{-/-}$ mice were analyzed (four-five mice per group).

Table IV. Cell numbers of total, $CD4^+$, $CD4^+Foxp3^+$, IFN-γ^+CD4^+ and IL-$17A^+CD4^+$ T cells in the spleen in EAE-induced WT and $TRAIL^{-/-}$ mice[a]

Cell subsets	Group	Days after immunization			
		7	13	17	21
Total cells	WT	$2.4 \pm 1.1 \times 10^6$	$12.9 \pm 3.5 \times 10^6$	$7.7 \pm 4.1 \times 10^6$	$7.5 \pm 3.7 \times 10^6$
	$TRAIL^{-/-}$	$4.5 \pm 0.7 \times 10^{6*}$	$11.7 \pm 4.6 \times 10^6$	$10.8 \pm 3.8 \times 10^6$	$9.4 \pm 3.5 \times 10^6$
$CD4^+$ T cells	WT	$0.5 \pm 0.2 \times 10^6$	$2.6 \pm 0.5 \times 10^6$	$1.7 \pm 0.9 \times 10^6$	$1.9 \pm 1.2 \times 10^6$
	$TRAIL^{-/-}$	$0.9 \pm 0.2 \times 10^6$	$2.6 \pm 1.2 \times 10^6$	$2.1 \pm 0.6 \times 10^6$	$2.1 \pm 1.0 \times 10^6$
$CD4^+FoxP3^+$ T cells	WT	$1.0 \pm 0.5 \times 10^5$	$3.8 \pm 1.2 \times 10^5$	$2.5 \pm 1.1 \times 10^5$	$2.5 \pm 1.1 \times 10^5$
	$TRAIL^{-/-}$	$1.5 \pm 0.2 \times 10^5$	$2.6 \pm 1.1 \times 10^5$	$2.4 \pm 1.0 \times 10^5$	$2.4 \pm 0.9 \times 10^5$
IFN-γ^+CD4^+ T cells	WT	$0.7 \pm 0.5 \times 10^5$	$1.4 \pm 0.2 \times 10^5$	$0.9 \pm 0.6 \times 10^5$	$0.5 \pm 0.2 \times 10^5$
	$TRAIL^{-/-}$	$2.8 \pm 0.6 \times 10^{5**}$	$2.4 \pm 0.9 \times 10^5$	$1.8 \pm 0.8 \times 10^5$	$1.2 \pm 0.3 \times 10^{5*}$
IL-$17A^+CD4^+$ T cells	WT	$0.5 \pm 0.2 \times 10^5$	$1.3 \pm 0.2 \times 10^5$	$0.5 \pm 0.4 \times 10^5$	$0.4 \pm 0.2 \times 10^5$
	$TRAIL^{-/-}$	$1.2 \pm 0.2 \times 10^{5**}$	$1.6 \pm 0.7 \times 10^5$	$1.2 \pm 1.0 \times 10^5$	$0.6 \pm 0.2 \times 10^5$

[a] Mean absolute cell numbers are calculated from total cell numbers for the whole spleen. The values are rounded off to the first decimal place. [b] Asterisks indicate the values of $TRAIL^{-/-}$ mice significantly different from those of WT mice (*, $p < 0.05$; **, $p < 0.01$). [c] WT and $TRAIL^{-/-}$ mice were analyzed (four-five mice per group).

8.4. Depletion of $CD4^+CD25^+$ regulatory T cells increases the severity of EAE in both WT and $TRAIL^{-/-}$ mice.

Recently, it has been reported that activated $CD4^+CD25^+$ Treg cells displayed TRAIL-dependent cytotoxicity against $CD4^+CD25^-$ conventional T cells in vivo (17). To investigate the effect of TRAIL on $CD4^+CD25^+$ Treg cells in EAE disease, we depleted $CD4^+CD25^+$ Treg cells in both WT and $TRAIL^{-/-}$ mice by administrating anti-CD25 mAb before induction of EAE.

We first analyzed the degree of reduction of $Foxp3^+$ Treg cells after injection of anti-CD25 mAb. Consistent with previous reports (28), the injection of anti-CD25 mAb reduced $Foxp3^+$ Treg cells to less than half of the control in the spleen in both types of mice (Fig. 9A-B).

Based on the above results, we induced EAE in WT and $TRAIL^{-/-}$ mice after administration of control mAb (rat IgG) or anti-CD25 mAb and investigated clinical features. Consistent with the data shown in Figure 7A, $TRAIL^{-/-}$ mice treated with control mAb developed more severe EAE than control mAb-treated WT mice (Fig. 9C and Table V). In WT and $TRAIL^{-/-}$ mice, the depletion of Treg cells before EAE-induction made the disease more severe. Therefore, Treg cells inhibit the disease regardless of the presence of TRAIL. The exacerbation caused by Treg cell-depletion was more drastic in WT mice than in $TRAIL^{-/-}$ mice, suggesting that the disease-inhibiting effect of Treg cells may be partly mediated by TRAIL.

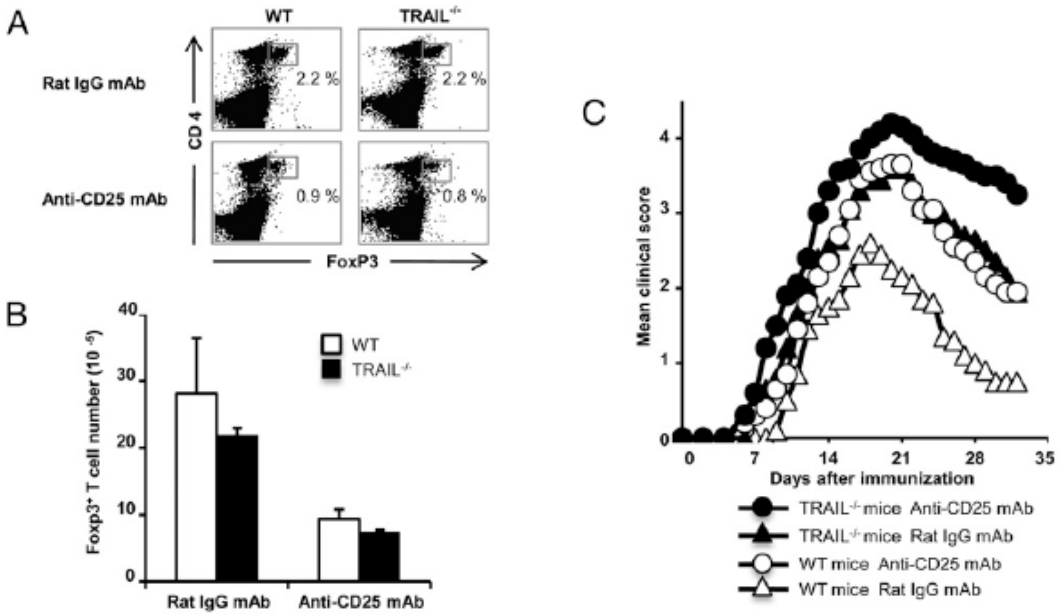

Figure 9. Depletion of $CD4^+CD25^+$ regulatory T cells exacerbates MOG-induced EAE in both WT and $TRAIL^{-/-}$ mice.

A-B, Depletion of $CD4^+Foxp3^+$ T cells was verified by flow cytometric analysis of spleen cells on day 12 after anti-CD25 mAb injection or isotype-matched control mAb (400 μg/mouse). Dot plots represent for CD4 and Foxp3 expression in spleen cells from WT (left) and $TRAIL^{-/-}$ mice (right) and the frequency of $CD4^+$ $Foxp3^+$ T cells is shown (A). Numbers of $CD4^+$ $Foxp3^+$ T cells in the spleen from WT (open bars) and $TRAIL^{-/-}$ mice (closed bars) are shown (B). Bar graphs indicate the mean cell numbers ± SD of three mice per group. C, WT or $TRAIL^{-/-}$ mice (five mice per group) were i.p. injected with anti-mouse CD25 mAb or isotype-matched control mAb (400 μg/mouse) on days 14 days before the immunization. Mice were then immunized with MOG 35-55 peptide and pertussis toxin, as described in the legend for Fig. 2A. Values represent the mean clinical score for each group. The data are summarized in Table V.

Table V. Statistical parameters for MOG-induced EAE in the mice depleted of $CD4^+CD25^+$ regulatory T cells[a]

Group	Disease incidence	Death	Mean day of the onset	Mean peak clinical score
WT mice control	5/5	0/5	9.8 ± 0.4	2.9 ± 0.6
WT mice CD25 depl.[b]	5/5	0/5	7.2 ± 1.2	3.7 ± 0.1
$TRAIL^{-/-}$ mice control	5/5	0/5	7.2 ± 0.4	3.7 ± 0.1
$TRAIL^{-/-}$ mice CD25 depl.[b]	5/5	0/5	6.4 ± 0.5	4.2 ± 0.3

[a] The data are combined from a total of 2 separate experiments, including those shown in Fig. 4. The values of onset day and mean peak clinical score are rounded off to the first decimal place.[b] In these mice, $CD25^+$ T cells were depleted by the treatment with anti-CD25 mAb.

8.5. Effect of TRAIL in the proliferation of conventional T cells and Treg cells

We have so far observed that there is a higher frequency of Th1 cells and lower frequency of Treg cells in EAE-induced TRAIL$^{-/-}$ mice than in WT mice. These observations in vivo suggest that TRAIL negatively regulates Th1 cells and positively regulates Treg cells. We next examined the effect of TRAIL on cell proliferation in vitro.

TRAIL was expressed by LPS-stimulated BM-DC from WT mice (Fig. 10A). Naïve CD4^{+}CD25^{-} conventional T cells and CD4^{+}CD25^{+} Treg cells were isolated from the spleen of WT or TRAIL$^{-/-}$ mice, and cocultured with x-ray irradiated BM-DC derived from WT or TRAIL$^{-/-}$ mice in the presence of anti-CD3 mAb and IL-2. The culture was continued for 3 days and the proliferation of T cells was measured at the end of the culture. As shown in Figure 10B, the proliferation of CD4^{+}CD25^{-} conventional T cells cocultured with BM-DC from TRAIL$^{-/-}$ mice was significantly higher than those cocultured with BM-DC from WT mice. The highest magnitude of proliferation was observed when both the CD4^{+}CD25^{-} conventional T cells and BM-DC were derived from TRAIL$^{-/-}$ mice. Thus, the proliferation of CD4^{+}CD25^{-} conventional T cells was highest in the absolute absence of TRAIL. These results suggest that TRAIL down-modulates the proliferation of CD4^{+}CD25^{-} conventional T cells, and that TRAIL on both BM-DC and T cells exerts this effect.

To investigate the proliferation of CD4^{+}CD25^{+} Treg cells, the experiments were repeated using BM-DC and CD4^{+}CD25^{+} Treg cells derived from WT or TRAIL$^{-/-}$ mice (Fig. 10C). As a result, the proliferation of CD4^{+}CD25^{+} Treg cells in the absolute absence of TRAIL was lower than those observed in the other 3 combinations, suggesting that TRAIL up-regulates the proliferation of CD4^{+}CD25^{+} Treg cells. Collectively, TRAIL may inhibit the growth of CD4^{+}CD25^{-} conventional T cells and enhance the growth of CD4^{+}CD25^{+} Treg cells.

To further analyze the effect of TRAIL on CD4^{+}CD25^{-} conventional T cells and CD4^{+}CD25^{+} Treg cells, both T cells derived from TRAIL$^{-/-}$ mice were stimulated with plate-bound anti-CD3, anti-CD28 mAb, and IL-2 in the presence or absence of soluble rTRAIL (0 - 100 ng/ml). The proliferation of T cells was measured at the end of the 3 day-culture. As shown in Fig. 10D, the proliferation of CD4^{+}CD25^{-} conventional T cells was inhibited by the addition of soluble rTRAIL in a dose-dependent manner.

On the other hand, the proliferation of $CD4^+CD25^+$ Treg cells was not affected by soluble rTRAIL even when 100 ng/ml of soluble rTRAIL was added (Fig. 10E).

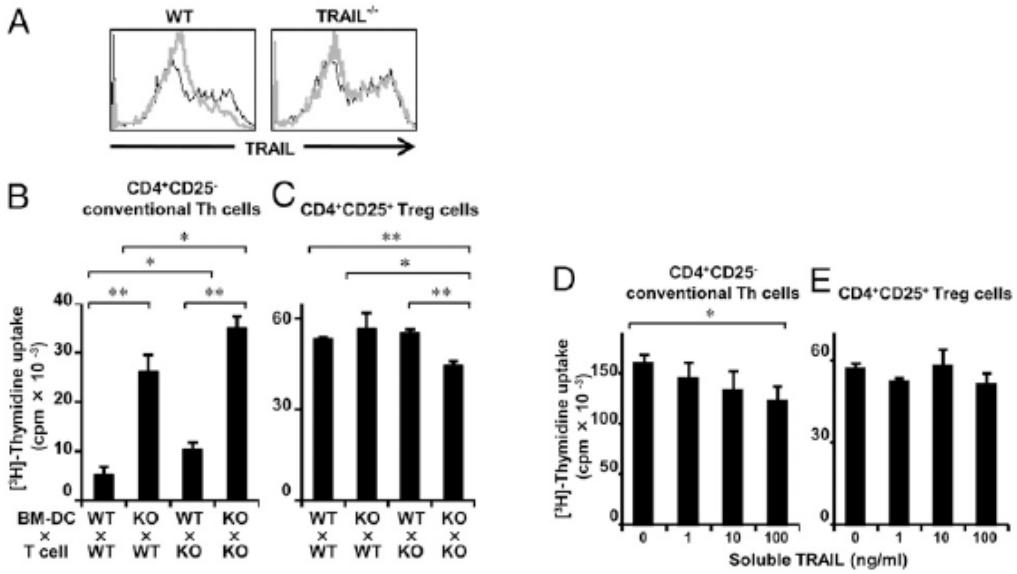

Figure 10. Effects of TRAIL on the proliferation of $CD4^+CD25^-$ conventional Th cells and $CD4^+CD25^+$ Treg cells.

A, BM-DC derived from WT or $TRAIL^{-/-}$ mice was stimulated with 1 μg/ml LPS for 48 h before the expression of TRAIL was analyzed by flow cytometry. Staining patterns of TRAIL (black line) and isotype-matched control (gray line) are shown. B-C, $CD4^+CD25^-$ conventional T cells (4.0×10^4) (B) or $CD4^+CD25^+$ Treg cells (4.0×10^4) (C) isolated from the spleens of unimmunized WT or $TRAIL^{-/-}$ mice were cocultured with irradiated LPS-stimulated BM-DC (2.0×10^4) derived from WT or $TRAIL^{-/-}$ mice for 3 days in the presence of soluble anti-CD3 mAb (1μg/ml) and human IL-2 (10 U/ml). D-E, $CD4^+CD25^-$ conventional T cells(1.0×10^5) (D) or $CD4^+CD25^+$ Treg cells (1.0×10^5) (E) isolated from the spleens of unimmunized $TRAIL^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (3 μg/ml), anti-CD28 mAb (3 μg/ml) and human IL-2 (20 U/mL) in the presence or absence of soluble recombinant mouse TRAIL for 3 days. T cell proliferation was quantified by measuring $[^3H]$-thymidine incorporation in the final 12 h of culture. The asterisks indicate that the differences in responses are statistically significant between the two values indicated by lines (*, $p < 0.05$; **, $p < 0.01$). KO means $TRAIL^{-/-}$. The results were expressed as the mean of a triplicate assay ± SD. The data are each representative of more than three independent experiments with similar results.

8.6. The expression of TRAIL-receptor and two decoy receptors on $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells.

We examined the level of expression of the TRAIL-receptor, mDR5, and decoy receptors, mDc-TRAIL-R1 and mDc-TRAIL-R2 on $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells derived from $TRAIL^{-/-}$ mice. After stimulation with anti-CD3 and CD28 mAb and IL-2, mDR5-expression was up-regulated in both T cell subsets (Fig. 11A-B). As for decoy receptors, mDc-TRAIL-R2 was not expressed in both T cell subsets. On the other hand, mDc-TRAIL-R1 was slightly expressed in $CD4^+CD25^+$ Treg cells but not in $CD4^+CD25^-$ conventional T cells. The difference in the expression of mDc-TRAIL-R1 between the two T cell subsets may affect their responsiveness to TRAIL.

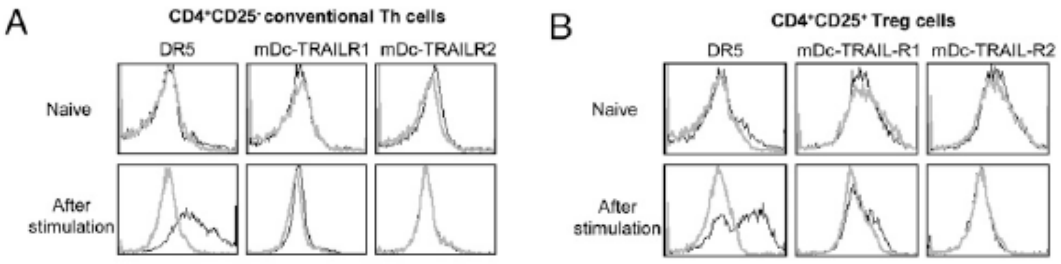

Figure 11. The expression of the functional TRAIL-receptor and two decoy receptors on $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells.

A-B, $CD4^+CD25^-$ conventional T cells (1.0×10^5) or $CD4^+CD25^+$ Treg cells (1.0×10^5) isolated from the spleens of unimmunized $TRAIL^{-/-}$ mice were stimulated with plate-bound anti-CD3 (3 μg/ml) and anti-CD28 mAb (3 μg/ml) and human IL-2 (20 U/mL) for 72 h. Then, mDR5, mDc-TRAIL-R1 and mDcTRAIL-R2 on each T cell subset were analyzed by flow cytometry. Staining patterns of mDR5, mDc-TRAIL-R1 and mDcTRAIL-R2 (black line) and isotype-matched control (gray line) are shown.

9. Discussion

9.1. Summary of the observations in the present study.

In the present study, we observed the following phenomena, 1) IFN-γ-producing $CD4^+$ T (Th1) cells in the spleen and ILNs in $TRAIL^{-/-}$ mice were significantly higher than those of WT mice, 2) $TRAIL^{-/-}$ mice manifested increased severity and prolonged disease course on EAE induction, 3) $TRAIL^{-/-}$ mice manifested increased frequency of Th1 cells and decreased frequency of Treg cells in the spleen and ILNs during EAE induction, 4) the depletion of $CD4^+CD25^+$ Treg cells increases the severity of EAE in both WT and $TRAIL^{-/-}$ mice, 5) both membrane-bound and soluble TRAILs inhibited the proliferative immune response of $CD4^+CD25^-$ conventional T cells, 6) only membrane-bound TRAIL but not soluble TRAIL enhanced the proliferative response of $CD4^+CD25^+$ Treg cells, 7) the functional TRAIL-receptor, mDR5, was up-regulated in both $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells activated by TCR-mediated stimuli, 8) the decoy receptor, mDc-TRAIL-R1, was slightly expressed only in activated $CD4^+CD25^+$ Treg cells.

9.2. Decreased Treg cells in EAE-induced $TRAIL^{-/-}$ mice.

The frequency of Treg cells was similar in naïve WT and $TRAIL^{-/-}$ mice (Fig. 6B-C). However, upon induction of EAE, the frequency of Treg cells in $TRAIL^{-/-}$ mice was significantly lower than that in WT mice at all the assay points in both spleen and ILNs (Fig. 8B). Wang and colleagues (24) reported that the administration of recombinant TRAIL expanded $CD4^+CD25^+$ Treg cells in mice that were immunized with thyroglobulin to develop experimental autoimmune thyroiditis (EAT). These results demonstrated that the proliferation of Treg cells is induced by TRAIL in vivo, when exposed to the risk of autoimmunity. This phenomenon may be induced by on going inflammatory responses including increased productions of various cytokines and their effects on microenvironment where Treg cells exhibit immune response.

9.3. Increased Th1 cells but not Th17 cells in EAE-induced TRAIL$^{-/-}$ mice.

On the other hand, the frequency of Th1 cells was higher in TRAIL$^{-/-}$ mice than that in WT mice, both in the normal condition (Fig. 6D) and upon induction of EAE (Fig. 8C). The difference in the frequency of Th1 cells between WT and TRAIL$^{-/-}$ mice indicates that TRAIL negatively regulates Th1 cells. In fact, a previous study revealed that Th1 cells were sensitive to TRAIL-induced cell death (16, 29). It is also reported that TRAIL functionally inactivated Th1 cells. A recent study has revealed that the administration of recombinant TRAIL decreased the production of IFN-γ in EAT-induced mice (30). Elevated production of IFN-γ was observed in murine cytomegalovirus-infected TRAIL-R-deficient mice in comparison to WT mice (31).

Many studies showed that not only Th1 cells but also Th17 cells are involved in a development of EAE. Therefore, we investigated the status of Th17 cells in TRAIL$^{-/-}$ mice. As a result, in contrast to Th1 cells, there was no difference in the frequency of Th17 cells between WT and TRAIL$^{-/-}$ mice in either steady state or disease conditions (Fig. 6E, 8D). The present study reveals for the first time that TRAIL exerts the inhibitory effect on Th1 cells but not on Th17 cells.

9.4. The effect of TRAIL on CD4^{+}CD25^{+} Treg cells and CD4^{+}CD25^{-} conventional T cells in vitro.

We also investigated the effect of TRAIL in CD4^{+}CD25^{+} Treg cells and CD4^{+}CD25^{-} conventional T cells in vitro. To investigate the effect of TRAIL in CD4^{+}CD25^{+} Treg cells, CD4^{+}CD25^{+} Treg cells isolated from WT or TRAIL$^{-/-}$ mice were cocultured with BM-DC derived from both mice in the presence of anti-CD3 mAb and IL-2. When CD4^{+}CD25^{+} Treg cells isolated from TRAIL$^{-/-}$ mice were stimulated by TRAIL$^{-/-}$-derived BM-DC, the magnitude of cell proliferation was smaller than when stimulated by WT-derived BM-DC. This result suggests that TRAIL positively regulates the proliferation of Treg cells. On the other hand, when CD4^{+}CD25^{+} Treg cells isolated from WT mice were stimulated by WT-derived BM-DC, the proliferation rates were similar when they were stimulated by TRAIL$^{-/-}$-derived BM-DC (Fig. 10C). This result indicated that the proliferative response of CD4^{+}CD25^{+} Treg cells from WT mice was not affected by the expression of TRAIL on the stimulator cells. Ren and colleagues reported (17) that CD4^{+}CD25^{+} Treg cells stimulated with anti-CD3/CD28

mAbs and IL-2 were mostly TRAIL positive. Thus, in our experiments, not only BM-DC but also $CD4^+CD25^+$ Treg cells from WT mice should have expressed TRAIL (Fig. 10C). Therefore, TRAIL was absolutely absent in the culture only when both BM-DC and $CD4^+CD25^+$ Treg cells were derived from $TRAIL^{-/-}$ mice. This may explain why decreased proliferation of Treg cells was observed only when both $CD4^+CD25^+$ Treg cells and BM-DC were TRAIL-deficient.

However, the addition of soluble TRAIL did not elicit positive proliferative responses in $CD4^+CD25^+$ Treg cells stimulated with plate-coated anti-CD 3mAb and anti-CD28 mAb (Fig. 10E). This may be because TRAIL needs to be in a transmembrane form to exert the proliferative effect on $CD4^+CD25^+$ Treg cells. On the other hand, the decrease in the proliferation of $CD4^+CD25^+$ Treg cells was never observed even in the presence of 100 ng/ml of rTRAIL, at which dose affected the proliferation of $CD4^+CD25^-$ conventional T cells (Fig. 10D-E). Other reports showed that Th2 cells, distinct from Th1 cells, were resistant to TRAIL-induced apoptosis (7, 16). Collectively, TRAIL exerts distinct effects on the different T cell subsets.

On the contrary to the observations in $CD4^+CD25^+$ Treg cells, our experiments showed an inhibitory effect of TRAIL on the proliferation of $CD4^+CD25^-$ conventional T cells (Fig. 10B, D). Collectively, these results suggest that $CD4^+CD25^-$ conventional T cells, especially Th1 cells as guessed the results of Fig. 8C, may be down-regulated both qualitatively and quantitatively by TRAIL through multiple pathways, including the induction of cell death, functional inactivation, and inhibition of growth.

9.5. The expression of TRAIL receptors in $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells and TRAIL signaling pathway.

In the regard to this point, TRAIL binds a functional receptor, mDR5, and two decoy receptors, mDc-TRAIL-R1 and mDc-TRAIL-R2 (9). This functional TRAIL receptor, mDR5, was up-regulated in both $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg cells (Fig. 11A-B). On the other hand, one of the decoy receptors, mDc-TRAIL-R1, was expressed in only $CD4^+CD25^+$ Treg cells but not in $CD4^+CD25^-$ conventional T cells (Fig 11A-B). mDc-TRAIL-R1-expression might function in a similar manner as the two human decoy receptors, TRAIL-R3 and TRAIL-R4 (32, 33), to affect the responsiveness of Treg cells to TRAIL.

However, the level of the expression of mDc-TRAIL-R1 on $CD4^+CD25^+$ Treg cells was low. Therefore, the differences in the responsiveness to TRAIL may not be accounted for only by the difference in the expression of mDc-TRAIL-R1. In this regards, in human, it is known that TRAIL binding to death receptors TRAIL-R1 and TRAIL-R2 results in recruitment of Fas-associated death domain (FADD) by cytoplasmic death domain, followed by the recruitment and activation of the caspases-8 and caspases-10 (34). Apoptosis induction is the major outcome of this signaling pathway. In addition, they can also activate survival signals via the transcription factor NF-κB, which induce anti-apoptotic genes (35). Recently, it has been reported that the catalytic activity of caspase-8 is essential for T cell clonal expansion in non-apoptopic cells based on the observation with *casp8*$^{-/-}$ T cells (36). Therefore, although little is known about TRAIL receptor signaling in mice, the existence of differential activated modes of caspase-8 may also affect signaling pathways in mice. There may induce some differences in the downstream of mDR5-mediated signaling pathway between conventional T cells and Treg cells, the former is mainly an apoptotic signal and the latter is a survival signal. As for the cell type-specific signaling pathways downstream of mDR5, up-regulation of cellular FLICE inhibitory protein (c-FLIP) in Th2 cells has been reported (16, 29). Further investigations are required to explore the distinct functions of TRAIL in different T cell subsets.

9.6. TRAIL promotes the proliferative response of Treg cells.

Consistent with a previous study by Cretney and colleagues (13), we observed that $TRAIL^{-/-}$ mice developed more severe EAE than did WT mice (Fig. 8A). Moreover, removal of TRAIL by systemic application of TRAIL receptor led to a more severe course of EAE (37). These observations indicate that TRAIL functions to suppress autoimmunity in EAE. $TRAIL^{-/-}$ mice showed a lower frequency of $Foxp3^+$ Treg cells in the spleen and ILNs after EAE-induction as compared with WT mice (Fig. 8B). In vitro, TRAIL on stimulator cells enhanced the growth of $CD4^+CD25^+$ Treg cells (Fig. 10C). These results indicate that TRAIL positively regulates Treg cells. The effect of Treg cells to suppress severity of EAE has been demonstrated by studies of several groups (38, 39), and this is supported also by the data shown in Fig. 9C. Collectively, it is

suggested that the effect of TRAIL to ameliorate EAE is mediated at least in part by the increase of Treg cells.

9.7. A possible application of TRAIL in human autoimmune disease.

A means to negatively manipulate immune responses is eagerly needed for the treatment of autoimmune, allergic and inflammatory diseases. The regulation of allo-reactive immune responses might be a key for the treatment of the graft rejection and GVHD in transplantation medicine. For these purposes, administration of genetically modified DC with enhanced immune-regulatory functions may be a promising strategy. Using directed differentiation of ES cells or induced pluripotent stem cells into DC (ES-DC or iPS-DC), we have developed efficient methods for genetically engineering mouse and human DC (18, 40, 41). In this study, we demonstrate that TRAIL has dual effects, the inhibition of pathogenic Th1 cells and the promotion of Treg cells. In the therapy of human autoimmunity, this dual effects of TRAIL may be important in the systemic inflammatory responses during the disease course. In clinical autoimmune disease, various auto-antigens are recognized by autoreactive T cells by the mechanism of “epitope spreading” and probably some of such antigens have not been identified as target antigens. The propagation of Treg cells to some tissue specific antigens and the activation of antigen non-specific immunosuppressive effect of Treg cells on T cells autoreactive to various kinds of auto-antigens may be effective to treat autoimmune diseases. Therefore the proliferative effect of TRAIL in Treg cells may be effective. Therapy with DC expressing high level of TRAIL along with tissue-specific antigen may be ideal in this regard. The exploration of TRAIL function may be of considerable significance for not only understanding the physiological mechanism of immune regulation but also the development of novel medical technology to control immunity.

10. Conclusions

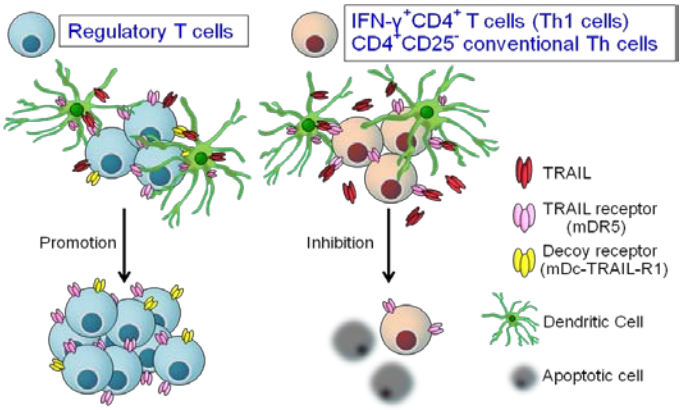

Figure 12. Conclusions of this study.

In this study, our data suggests that TRAIL suppresses autoimmunity by two mechanisms; one is the inhibition of IFN-γ-producing $CD4^+$ T (Th1) cells, conventional $CD4^+$ Th cells and the other is the propagation of Treg cells**.**

11. Refferences

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