

Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL^{-/-} mice

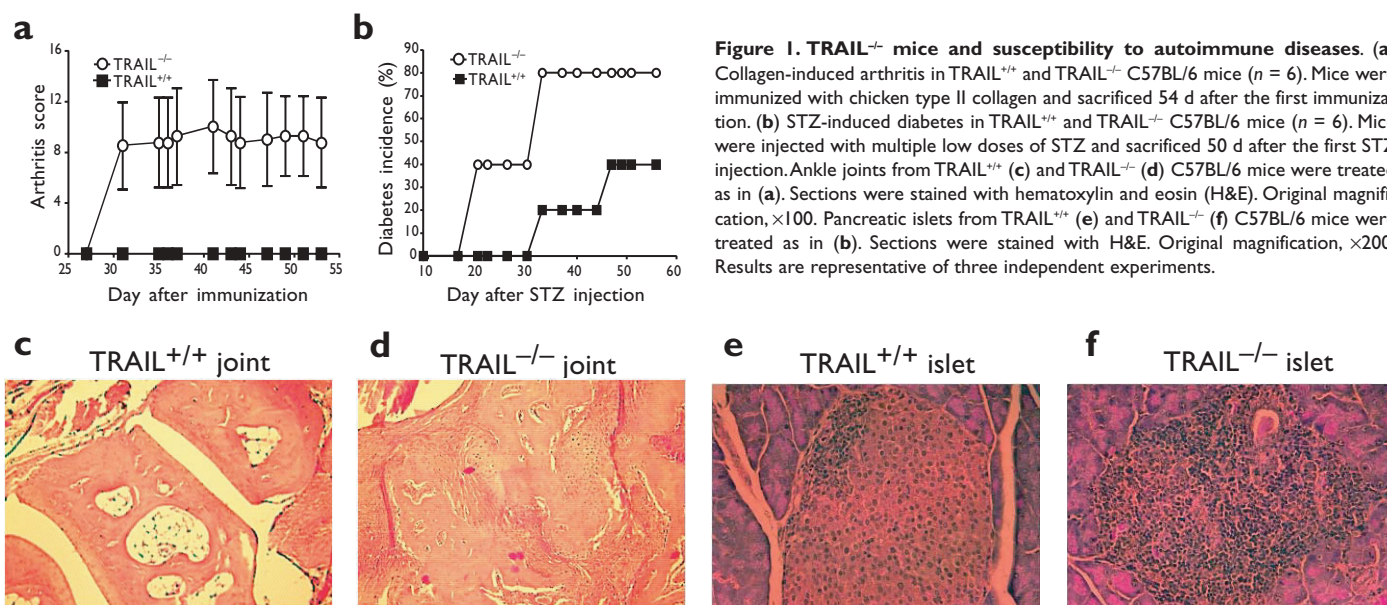
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TRAIL, the tumor necrosis factor-related apoptosis-inducing ligand, selectively induces apoptosis of tumor cells, but not most normal cells. Its role in normal, nontransformed tissues is not clear. We report here that mice deficient in TRAIL have a severe defect in thymocyte apoptosis—thus, thymic deletion induced by T cell receptor ligation is severely impaired. TRAIL-deficient mice are also hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes and develop heightened autoimmune responses. Thus, TRAIL mediates thymocyte apoptosis and is important in the induction of autoimmune diseases.

Immunological tolerance to self is essential for maintaining the integrity of organ systems, and its breakdown may lead to the development of autoimmune diseases. Tolerance to self is maintained through several mechanisms, which include negative selection, functional inactivation (anergy) and suppression of autoreactive lymphocytes. However, only negative selection permanently removes autoreactive cells through apoptosis. Although it has long been known that negative selection requires a T cell receptor (TCR) signal, it is unclear whether a death ligand and signal is also involved^{1,2}.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family³. Unlike other members of the TNF family, TRAIL may interact with at least two death receptors (death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2)) and two decoy receptors (decoy receptor 1 (DcR1, TRAIL-R3, TRID) and decoy receptor 2 (DcR2, TRAIL-R4, TRUND))⁴⁻¹⁰. *In vitro*, TRAIL induces apoptosis of many, but not all, tumor cell lines^{4,8}. This seems to be mediated by DR4 and DR5, which possess intracellular death domains similar to those of TNF receptor I



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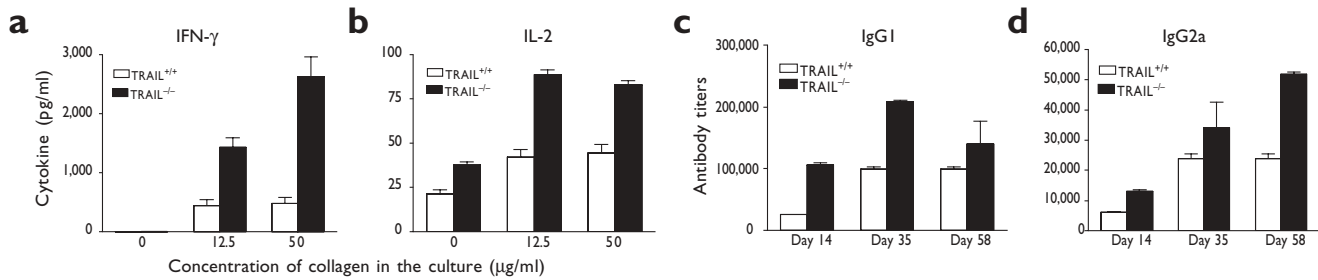


Figure 2. Anti-collagen immune responses. TRAIL^{+/+} and TRAIL^{-/-} mice were immunized with chicken type II collagen and anti-collagen immune responses were determined. IFN- γ (a) and IL-2 (b) concentrations are shown for cultures with or without chicken type II collagen. (c,d) Anti-collagen IgG1 and IgG2a titers, respectively, at different time points after the first immunization. Each data point represents a mean \pm s.d. from six mice. The experiments were repeated twice with similar results.

and CD95 (Fas or Apo-1). The DR4 and DR5 death domains activate both mitochondria-dependent and mitochondria-independent pathways of apoptosis through FADD-caspase 8, leading to the activation of the caspase cascade^{11–14}. The decoy receptors DcR1 and DcR2, which do not contain functional death domains, can block TRAIL-induced apoptosis^{4,8}. Although both TRAIL and TRAIL receptors are constitutively expressed in various tissues^{3,4,15–17} and are up-regulated upon cell activation^{7,18,19}, TRAIL may not induce apoptosis of most nontransformed cells^{4,8}. *In vivo* administration of recombinant TRAIL selectively kills tumor cells, but not normal cells, leaving most organ systems unharmed^{20,21}. However, recent *in vitro* studies suggest that unlike most normal cells, thymocytes, neurons and human hepatocytes may be sensitive to TRAIL-induced apoptosis^{22–24}. The biological relevance of these findings *in vivo* is not clear. Using mice deficient in TRAIL, we have now discovered an essential role for TRAIL in thymocyte apoptosis and autoimmunity.

Results

TRAIL^{-/-} mice and the induction of autoimmune diseases

To determine the roles of TRAIL in autoimmunity, we immunized TRAIL^{+/+} and TRAIL^{-/-} C57BL/6 mice^{25,26} with chicken type II collagen (Fig. 1). TRAIL^{+/+} C57BL/6 mice were not susceptible to collagen-induced arthritis due to their genetic background (Fig. 1a). In contrast,

most TRAIL^{-/-} C57BL/6 mice developed severe arthritis 4 weeks after the immunization. Arthritis was characterized by footpad swelling, synovitis, pannus formation, and bone and cartilage destruction of paw joints (Fig. 1c,d), which was indistinguishable from arthritis in DBA1 mice²⁷. To determine whether this increased disease susceptibility could be generalized to other experimental models, we also treated animals with a low dose of streptozotocin (STZ) to induce diabetes. Diabetes induced by a low dose of STZ is an animal model for human type-I diabetes. Although the exact autoantigens involved are not clear, autoimmune responses against self-islet antigens are crucial in the pathogenesis of the disease^{28–30}. STZ treatment induced diabetes in both TRAIL^{+/+} and TRAIL^{-/-} C57BL/6 mice, but the onset of the disease was accelerated and the incidence increased in the TRAIL^{-/-} group (Fig. 1b). Most pancreatic islets of TRAIL^{+/+} mice were either normal or mildly infiltrated by leukocytes. In contrast, severe insulinitis and massive islet destruction were observed in TRAIL^{-/-} mice (Fig. 1e,f). Thus, TRAIL deficiency increased the susceptibility of mice to both autoimmune arthritis and diabetes.

TRAIL^{-/-} mice and anti-collagen immune responses

Collagen-induced arthritis is initiated by collagen-specific lymphocytes. To determine whether susceptibility to arthritis in TRAIL^{-/-} mice was associated with alterations of collagen-specific lymphocytes, we

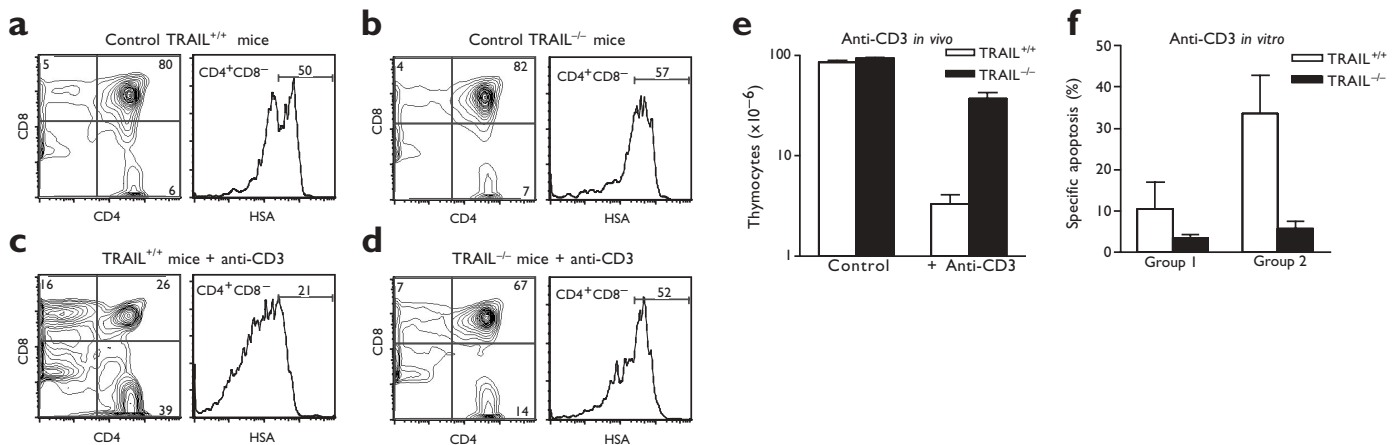
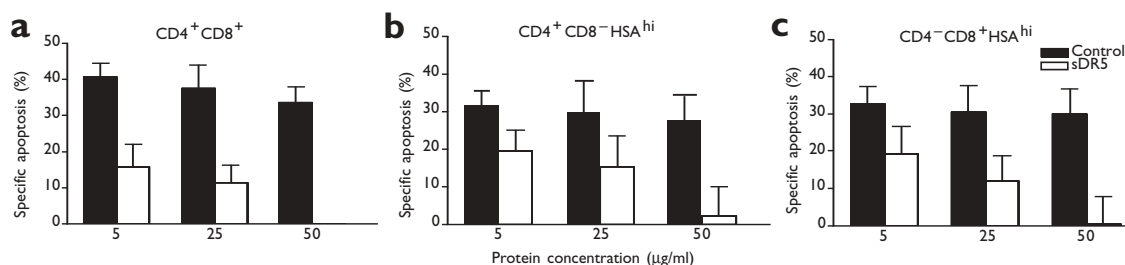


Figure 3. Thymocyte apoptosis *in vivo* and *in vitro*. Seven-week-old TRAIL^{+/+} and TRAIL^{-/-} C57BL/6 mice ($n = 4$) were injected i.p. with 25 μ g of 145C11 CD3 mAb at 0 and 24 h. Twenty-four hours after the last injection, mice were sacrificed and their thymocytes were collected and analyzed by flow cytometry. Both the percentages of thymocytes (a–d) and total numbers of immature CD4⁺CD8⁺ thymocytes (e) are shown. (f) Thymocytes from TRAIL^{+/+} and TRAIL^{-/-} mice were cultured in plates coated with either 1 μ g/ml CD3 mAb plus 2 μ g/ml CD28 mAb (group 1), or 1 μ g/ml CD3 mAb plus 20 μ g/ml CD28 mAb (group 2) for 24 h. The specific apoptosis shown represents the percentages of apoptotic CD4⁺CD8⁺ cells in test cultures minus the percentages of apoptotic CD4⁺CD8⁺ T cells in control cultures not containing CD3 or CD28 mAb, which were 22% and 34% for TRAIL^{-/-} and TRAIL^{+/+} cultures, respectively. Results are representative of four independent experiments. The differences between anti-CD3-treated TRAIL^{-/-} and TRAIL^{+/+} groups are statistically significant ($P < 0.01$) for all the parameters shown.

Figure 4. Effects of TRAIL blockade on thymocyte apoptosis. Thymocytes from 6- to 7-week-old C57BL/6 mice were cultured in plates coated with 1 $\mu\text{g/ml}$ of CD3 mAb plus 20 $\mu\text{g/ml}$ of CD28 mAb in the presence of different amounts (5, 25 and 50 $\mu\text{g/ml}$) of sDR5²⁷ (open bars) or control protein human serum albumin (filled bars).

After 24 h, cells were harvested, stained with CD4 mAb, CD8 mAb, anti-HSA and annexin V5, and analyzed by flow cytometry. The specific apoptosis shown represents the percentages of apoptotic cells in test cultures minus the percentages of apoptotic cells in control cultures not containing CD3 or CD28 mAb, which were 25.3%, 25.2% and 35.4% for (a), (b) and (c), respectively. Human sDR5 did not promote or inhibit apoptosis of thymocytes in cultures not treated with CD3 mAb (data not shown). The differences between the control protein- and sDR5-treated cultures are statistically significant ($P < 0.01$) as determined by ANOVA.



examined both cellular and humoral anti-collagen immune responses. Mice were treated as in **Fig. 1a**, and their blood and lymphoid tissues were collected 2–8 weeks after immunization. Splenocytes from control mice responded moderately to collagen stimulation by producing interleukin (IL)-2 and interferon (IFN)- γ (**Fig. 2a,b**). This response was greatly enhanced in TRAIL^{-/-} cultures. Collagen antibody titers in the sera were determined by collagen-specific ELISA. Both anti-collagen IgG1 and IgG2a were substantially increased in TRAIL^{-/-} mice (**Fig. 2c,d**). These results indicate that mice deficient in TRAIL have increased cellular and humoral immune responses against self-antigens.

TRAIL^{-/-} mice and thymus size

Although mice deficient in TRAIL developed normally^{25,26}, they had moderately enlarged thymuses compared with normal TRAIL^{+/+} littermates. Thus, the total number of thymocytes from 6- to 7-week-old C57BL/6 mice was $80 \times 10^6 \pm 30 \times 10^6$. This was increased to $90 \times 10^6 \pm 40 \times 10^6$ in TRAIL^{-/-} C57BL/6 mice ($n = 10$, $P < 0.01$). Similarly, the total number of thymocytes in 6- to 7-week-old BALB/c mice was

$116 \times 10^6 \pm 21 \times 10^6$, which was increased to $132 \times 10^6 \pm 24 \times 10^6$ in TRAIL^{-/-} littermates ($n = 12$, $P < 0.003$). Multi-color flow cytometry analysis of TRAIL^{-/-} thymocytes revealed no abnormal changes in the percentages of CD4⁺CD8⁻, CD4⁺CD8⁺ and CD4⁺CD8⁺ thymocytes, but the total number of each cell subset was substantially increased compared with TRAIL^{+/+} thymus. Unlike mice deficient in Fas ligand (FasL) or Fas, TRAIL^{-/-} C57BL/6 or BALB/c mice had only slightly enlarged spleens and lymph nodes, and did not develop overt systemic autoimmune diseases during the first few months of their lives (data not shown).

TRAIL^{-/-} mice and thymic negative selection

The enlarged thymus prompted us to investigate thymic negative selection in TRAIL^{-/-} mice. First, we treated mice with CD3 monoclonal antibody (mAb) and evaluated thymocyte deletion *in vivo*. CD3 mAb induced loss of immature CD4⁺CD8⁺ thymocytes and semi-mature HSA^{hi} thymocytes in TRAIL^{+/+} mice (**Fig. 3**). This effect was reduced in TRAIL^{-/-} mice. The total number of thymocytes and the total number of CD4⁺CD8⁺ immature thymocytes in TRAIL^{-/-} mice were substantially greater than those in the control mice following CD3 treatment (**Fig. 3e**). Because cytokines and hormones released in the periphery may mediate some of the thymocyte deletion in this *in vivo* model, we next investigated CD3 mAb-induced thymocyte apoptosis in better-defined *in vitro* systems. Coculturing TRAIL^{+/+} thymocytes with CD3 mAb induced apoptosis of CD4⁺CD8⁺ cells, as determined by annexin-V5 staining (**Fig. 3f**). In contrast, the same CD3 treatment failed to induce substantial apoptosis of TRAIL^{-/-} thymocytes, even in the presence of a high dose of CD28 mAb. Addition of soluble DR5 (sDR5) protein to TRAIL^{+/+} cultures blocked the apoptosis of immature and semi-mature thymocytes in a dose-dependent manner (**Fig. 4**). The blocking of apoptosis by sDR5 was also apparent in the fetal thymus organ culture (FTOC) treated with CD3 mAb. Thus, CD3 mAb induced deletion of CD4⁺CD8⁺ thymocytes in the FTOC. However, addition of sDR5 inhibited thymocyte deletion in this system (**Fig. 5**). When TRAIL^{-/-} thymus was tested in the same FTOC system, thymocyte deletion was reduced compared with TRAIL^{+/+} thymus (data not shown). These results indicate that TRAIL is required for CD3 mAb-induced thymocyte apoptosis both *in vivo* and *in vitro*.

To investigate whether the TRAIL effect on negative selection can be extended to other antigen systems and genetic backgrounds, we studied ovalbumin (OVA)-induced negative selection of MHC class-II restricted T cells in BALB/c mice. Systemic administration of OVA into OVA-specific TCR transgenic DO11.10 mice reduced both the percentage and the absolute number of immature transgenic thymocytes (**Fig. 6**). These effects were blocked in TRAIL^{-/-} mice. In contrast to OVA-specific TCR

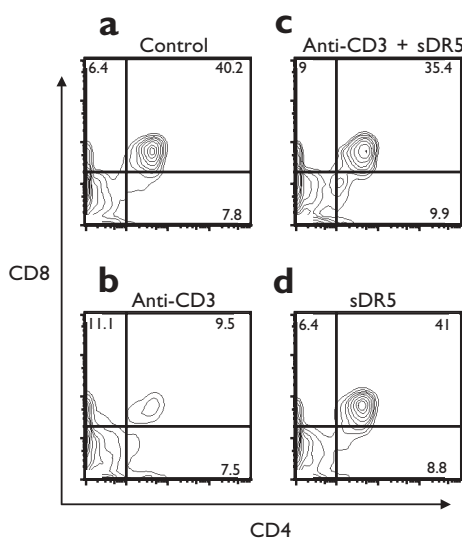


Figure 5. Roles of TRAIL in fetal thymus organ culture. Fetal thymuses were collected from day 15 embryos of C57BL/6 mice and cultured, with or without CD3 mAb, CD28 mAb and sDR5. Twelve days later, single thymocyte suspensions were prepared, stained with CD4 mAb and CD8 mAb, and analyzed by flow cytometry. (a) Control thymuses not treated with antibody or sDR5. (b) Thymuses were treated with CD3 mAb and CD28 mAb. (c) Thymuses were treated with CD3 mAb, CD28 mAb and sDR5. (d) Thymuses were treated with sDR5 alone. The experiment was repeated twice with similar results.

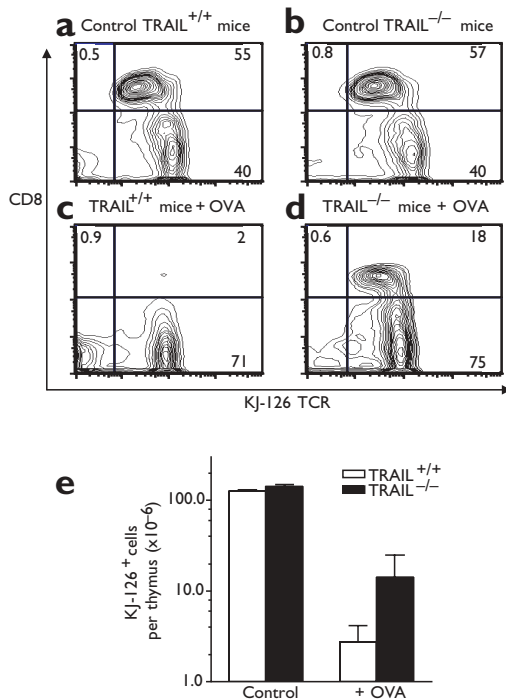


Figure 6. Ovalbumin-induced thymocyte apoptosis *in vivo*. Groups of TRAIL^{+/+} and TRAIL^{-/-} OVA-specific DO11.10 TCR transgenic littermates ($n = 4$) were injected i.p. with 10 mg OVA at 0, 24 and 48 h. Twenty-four hours after the last injection, mice were sacrificed and their thymocytes stained with CD4, CD8 and KJ-126 mAb and analyzed by flow cytometry. (a–d) The percentages of KJ-126⁺ cells in each group. Because all KJ-126⁺ cells express CD4, the CD8⁺KJ-126⁺ cells are CD4⁺CD8⁺ thymocytes. (e) The total numbers of transgenic thymocytes in each group. Results are representative of three independent experiments. The differences between OVA-treated TRAIL^{+/+} and TRAIL^{-/-} groups are statistically significant ($P < 0.01$) for all the parameters shown.

transgenic cells, thymocytes that express endogenous TCR were not significantly affected by TRAIL gene mutation after systemic OVA administration. The total numbers of nontransgenic T cells in thymus of TRAIL^{+/+} and TRAIL^{-/-} mice as treated in Fig. 6 were $1.5 \times 10^6 \pm 0.4 \times 10^6$ and $2.2 \times 10^6 \pm 1.1 \times 10^6$, respectively ($P > 0.5$).

All models of negative selection described above depend on the addition of exogenous TCR ligands to the system. To determine whether negative selection induced by endogenous antigens is also affected by TRAIL gene mutation, we studied mammary tumor virus

Table 1. The percentages of different V β T cells in BALB/c mice

T cell subsets	Mean	s.d.	P value ^a ($n = 5$)
CD4 ⁺ CD8 ⁺ thymocytes			
V β 11 ⁺ TRAIL ^{+/+}	2.98	0.18	0.018
V β 11 ⁺ TRAIL ^{-/-}	3.6	0.32	
V β 5 ⁺ TRAIL ^{+/+}	2.66	0.09	
V β 5 ⁺ TRAIL ^{-/-}	3.14	0.23	0.008
CD4 ⁺ or CD8 ⁺ splenocytes			
V β 11 ⁺ CD4 ⁺ TRAIL ^{+/+}	0.52	0.11	0.015
V β 11 ⁺ CD4 ⁺ TRAIL ^{-/-}	1.78	0.65	
V β 5 ⁺ CD4 ⁺ TRAIL ^{+/+}	0.17	0.05	0.022
V β 5 ⁺ CD4 ⁺ TRAIL ^{-/-}	0.35	0.07	
V β 11 ⁺ CD8 ⁺ TRAIL ^{+/+}	1.1	0.17	0.006
V β 11 ⁺ CD8 ⁺ TRAIL ^{-/-}	3.1	0.35	
V β 5 ⁺ CD8 ⁺ TRAIL ^{+/+}	0.39	0.09	0.004
V β 5 ⁺ CD8 ⁺ TRAIL ^{-/-}	1.12	0.36	
CD4 ⁺ or CD8 ⁺ mesenteric lymph node cells			
V β 11 ⁺ CD4 ⁺ TRAIL ^{+/+}	1.1	0.83	0.012
V β 11 ⁺ CD4 ⁺ TRAIL ^{-/-}	1.96	0.54	
V β 5 ⁺ CD4 ⁺ TRAIL ^{+/+}	0.46	0.32	0.012
V β 5 ⁺ CD4 ⁺ TRAIL ^{-/-}	0.96	0.56	
V β 11 ⁺ CD8 ⁺ TRAIL ^{+/+}	1.8	1.1	0.01
V β 11 ⁺ CD8 ⁺ TRAIL ^{-/-}	3.5	0.88	
V β 5 ⁺ CD8 ⁺ TRAIL ^{+/+}	2.42	2.8	0.027
V β 5 ⁺ CD8 ⁺ TRAIL ^{-/-}	5.6	4.3	

^aStatistical analyses were performed for each cell subset by comparing the percentages in TRAIL^{+/+} and TRAIL^{-/-} mice using analysis of variance (ANOVA). In contrast to these results, the percentages of V β 8, V β 14 and V β 6 T cells were not significantly different between TRAIL^{+/+} and TRAIL^{-/-} BALB/c mice. Specifically, in the thymus, the percentages of CD4⁺CD8⁺V β 8⁺ cells of TRAIL^{+/+} and TRAIL^{-/-} mice were 9.8 ± 1.5 and 9.9 ± 1.5 , respectively ($n = 4$, $P = 0.8$). In the spleen, the percentages of CD4⁺V β 8⁺ cells of TRAIL^{+/+} and TRAIL^{-/-} mice were 8.9 ± 0.3 and 8.8 ± 0.4 , respectively ($n = 4$, $P = 0.6$), and the percentages of CD8⁺V β 8⁺ cells of TRAIL^{+/+} and TRAIL^{-/-} mice were 3.9 ± 0.4 and 3.9 ± 0.4 , respectively ($n = 4$, $P = 0.6$). Similarly, in the mesenteric lymph nodes, the percentages of CD4⁺V β 8⁺ cells of TRAIL^{+/+} and TRAIL^{-/-} mice were 17.1 ± 1.8 and 17.8 ± 2.0 , respectively ($n = 4$, $P = 0.3$), and the percentages of CD8⁺V β 8⁺ cells of TRAIL^{+/+} and TRAIL^{-/-} mice were 6.1 ± 0.8 and 6.1 ± 0.8 , respectively ($n = 4$, $P = 1.0$).

(Mtv) 9-induced negative selection *in vivo*. Mtv-9 encodes an endogenous superantigen, which selectively deletes V β 11, V β 5 and V β 3 T cells in mice that express major histocompatibility antigen (I-E)^{31,32}. We examined the frequencies of these T cells in the thymus, spleen and mesenteric lymph node of BALB/c (I-E⁺) and C57BL/6 (I-E⁻) mice that do or do not carry the TRAIL gene mutation, by flow cytometry. In C57BL/6 mice that do not express I-E, the frequencies and the total numbers of these T cell subpopulations were not affected by the TRAIL gene mutation. Specifically, in both TRAIL^{+/+} and TRAIL^{-/-}

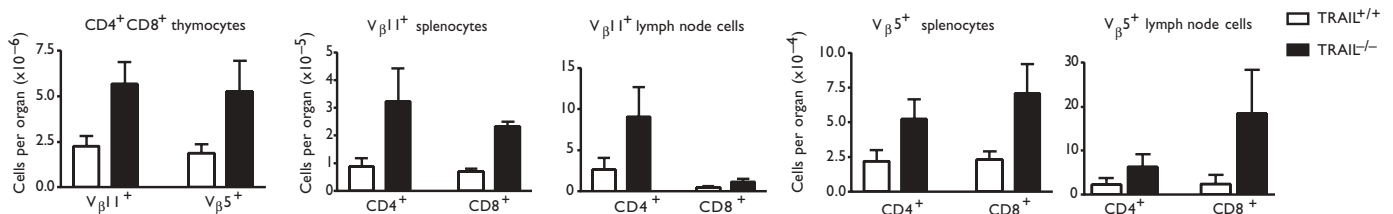


Figure 7. Mtv-9-induced T cell deletion *in vivo*. TRAIL^{+/+} and TRAIL^{-/-} BALB/c littermates, 4–5 mice per group, were sacrificed at the age of 6–7 weeks. Single cell suspensions of thymus, spleen and mesenteric lymph node were prepared and the total number of cells in each organ was determined. The frequencies of CD4⁺, CD8⁺, V β 11⁺ and V β 5⁺ cells in each organ were determined by flow cytometry, and the total number of each T cell subpopulation was calculated. Data presented are means \pm s.d. of total numbers of cells of each subset in different organs. The percentages of each cell subset are shown in Table 1. The differences between TRAIL^{+/+} (open bars) and TRAIL^{-/-} (filled bars) groups are statistically significant for all cell subsets ($P < 0.01$ as determined by ANOVA). Similar differences exist for CD4⁺CD8⁻ and CD4⁻CD8⁺ thymocytes that express V β 11 or V β 5 (data not shown). The experiments were repeated twice with similar results.

C57BL/6 mice, the total numbers of $V_{\beta}11$, $V_{\beta}5$ and $V_{\beta}3$ immature thymocytes ($CD4^+CD8^+$) per organ were approximately 5×10^6 , 3×10^6 and 1×10^6 , respectively. In the spleen, the total numbers of $V_{\beta}11$, $V_{\beta}5$ and $V_{\beta}3$ $CD4^+$ cells were approximately 6.5×10^5 , 3.5×10^5 and 8×10^5 , respectively, whereas in the mesenteric lymph nodes, the total numbers of $V_{\beta}11$ and $V_{\beta}5$ $CD4^+$ cells were 2×10^5 and 1.5×10^5 , respectively. In contrast to these observations, TRAIL gene mutation in BALB/c mice substantially altered the frequencies and total numbers of these T cells in both thymus and peripheral lymphoid organs (Table 1 and Fig. 7). Thus, the numbers of $V_{\beta}11$ and $V_{\beta}5$ cells in TRAIL^{+/+} BALB/c mice were extremely low due to negative selection^{31,32}. In contrast, the numbers of these T cells in TRAIL^{-/-} BALB/c mice were increased, approaching the numbers in C57BL/6 mice (Fig. 7). Similarly, the numbers of $V_{\beta}3^+$ T cells were low in TRAIL^{+/+} BALB/c mice, but substantial increases were also observed in TRAIL^{-/-} animals. Specifically, the total numbers of $CD4^+CD8^+ V_{\beta}3^+$ cells in the thymuses of TRAIL^{+/+} and TRAIL^{-/-} mice were $7 \times 10^5 \pm 2 \times 10^5$ and $21 \times 10^5 \pm 8 \times 10^5$, respectively ($n = 5$, $P < 0.01$), whereas the total numbers of $CD4^+ V_{\beta}3^+$ T cells in the spleens of TRAIL^{+/+} and TRAIL^{-/-} mice were $2.3 \times 10^4 \pm 1.0 \times 10^4$ and $5.1 \times 10^4 \pm 0.8 \times 10^4$, respectively ($n = 5$, $P < 0.001$). In contrast to these observations, the frequencies of $V_{\beta}8^+$ T cells, which do not respond to Mtv-9, were not affected by TRAIL gene mutation (Table 1). To determine whether the $V_{\beta}11$ T cells of TRAIL^{-/-} mice could respond to TCR stimulation, we cultured these cells in plates that were pre-coated with 0.1–10 $\mu\text{g/ml}$ of $V_{\beta}11$ mAb. After 48 h and 72 h, proliferation, IL-2, IL-4, IL-10 and IFN- γ secretions by cultured cells were determined by [³H]thymidine incorporation and ELISA. We found that TRAIL^{-/-} splenocytes responded vigorously to $V_{\beta}11$ mAb stimulation and secreted much higher amounts of cytokines than TRAIL^{+/+} splenocytes under the same culture conditions (data not shown). These results indicate that TRAIL mediates negative selection against both exogenous and endogenous antigens.

Discussion

Although it has long been suspected that a death ligand signal may be required for negative selection, recent studies of other death ligands and receptors have cast doubt on this theory. Thus, in Fas- or FasL-deficient mice, negative selection was normal for immature $CD4^+CD8^+$ T cells, although negative selection of semi-mature thymocytes was reduced when a strong TCR signal was present^{1,2,33–35}. Similarly, no overt defect in negative selection was observed in TNF or TNF receptor mutant mice^{36,37}. However, unlike FasL and TNF, which induce apoptosis of a variety of normal cells, TRAIL does not seem to induce apoptosis of most normal cells, except thymocytes, neural cells and human hepatocytes^{22,23,38}. Data reported here indicate that unlike FasL and TNF, which mediate activation-induced cell death of mature T cells, TRAIL is essential for the death of immature and semi-mature thymocytes during negative selection. Although it is known that thymocytes constitutively express TRAIL and DR5^{3,9,17,23} and that CD3 stimulation up-regulates DR5 expression²³, it remains to be determined which thymic cell expresses TRAIL that is used for negative selection.

If TRAIL mediates thymic negative selection, what type of intracellular death signals might it generate? Previous studies suggest that apoptosis can be mediated by at least two molecular pathways: the mitochondria-dependent (intrinsic, stress-induced) and the mitochondria-independent (extrinsic, death receptor-induced) pathways^{13,14,39}. TRAIL is able to activate the extrinsic pathway through interaction with FADD and caspase 8, although it can also activate the intrinsic pathway through Bax and Smac (DIABLO)^{11–14}. By western blot analyses, we found that both

caspase 8 and caspase 9 are cleaved in thymocytes 3 h after CD3 mAb stimulation, suggesting that both enzymes may be involved (data not shown). However, studies using mice deficient for a single gene suggest that neither caspase 8 nor caspase 9 may be sufficient to mediate negative selection, and that deficiency in FADD alone does not abrogate negative selection^{40–42}, although FADD deficiency unexpectedly diminished the proliferative ability of thymocytes^{40,43}. Similarly, CrmA, Bcl-2 and Bcl-x transgene expression have little effect on thymic negative selection^{42,44}. In contrast, deficiency in the BH3-only Bcl-2 family member Bim or the transcription factor Nur77 substantially blocks thymic negative selection^{45–47}. Therefore, TRAIL-mediated activation of the extrinsic pathway alone may not fully explain its effect on negative selection. In this regard, it has been recently reported that, in addition to activating caspases, TRAIL can induce cell cycle arrest of T cells through inhibiting cyclin-dependent kinase 4 (refs. 27,48). Additionally, TRAIL also activates NF- κ B and c-Jun pathways of signal transduction through interaction with TRAF-2 and/or RIP^{49–51}. Using gene microarray technology, it was found that TRAIL is able to activate a large number of genes that are important for regulating apoptosis⁵². Whether any of these TRAIL-mediated effects has a role in thymic deletion needs to be investigated. Regardless of the downstream molecular mechanisms, our observation that TRAIL is required for negative selection of thymocytes indicates that the death receptor pathway is involved in thymic cell death. If TRAIL, Bim and Nur77 are all involved in negative selection, how do they relate to each other? Because there are no established connections among these molecules and deficiency in any one of them does not completely abrogate negative selection, it is likely that they represent players of different molecular pathways. This may explain why in TRAIL^{-/-} mice no overt spontaneous autoimmune diseases occur—despite a defect in negative selection. Therefore, both death-receptor-dependent and death-receptor-independent pathways of cell death may be required for thymic negative selection, and maintenance of self-tolerance is likely dependent on multiple molecular mechanisms.

The results reported here indicate that TRAIL-deficient mice not only have a defect in negative selection, but also have an increased susceptibility to autoimmune diseases. However, it is unclear whether the defect in negative selection is directly responsible for the increase in disease susceptibility. We have previously reported that TRAIL may also regulate lymphocyte functions in the periphery by inhibiting cell cycle progression of T cells²⁷. Therefore, further studies are required to determine whether and to what degree a defect in negative selection may increase the susceptibility to autoimmune diseases.

Methods

Mice. TRAIL^{-/-} mice were generated by gene targeting as described²⁶, and have been backcrossed to C57BL/6 or BALB/c mice for more than 10 generations. All mice used in this study were housed in the University of Pennsylvania Animal Care Facilities under pathogen-free conditions. All procedures used were preapproved by the Institutional Animal Care and Use Committee.

Induction and evaluation of autoimmune diseases. For arthritis experiments, C57BL/6 mice were immunized by multiple intradermal injections of 200 μg chicken type II collagen (Sigma, St. Louis, MO) in 100 μl PBS emulsified in an equal volume of complete Freund's adjuvant containing 2 mg/ml mycobacterium tuberculosis H37 RA (Difco, St. Louis, MO). Mice were rechallenged with the same antigen preparation subcutaneously on the flanks after 21 d. Mice were examined daily for signs of joint inflammation, and scored as follows: 0, normal; 1, erythema and mild swelling confined to the ankle joint or mid-foot; 2, erythema and mild swelling extending from the ankle to the mid-foot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling extending from the ankle to the digits. The maximal disease score per foot is 4 and the maximal disease score per mouse is 16. The mean disease score per group is calculated as follows: total disease scores from all animals in the group / the number of animals in the group.

For diabetes experiments, C57BL/6 mice were injected intraperitoneally with 40 mg/kg of STZ on days 1, 2, 3 and 4. Urine glucose levels were measured three times a week using

Keto-Diastix (Bayer, Birmingham, NJ). Diabetes was defined as the elevation of glucose levels above 300 mg/dl for two consecutive tests.

Measurement of anti-collagen immune responses. To test cellular immune responses, mice were sacrificed 58 d after the first immunization and their spleens collected. Splenocytes, 1.5×10^6 per well, were cultured in 0.2 ml of DMEM with or without 12.5–50 $\mu\text{g/ml}$ of chicken type II collagen. Culture supernatants were collected 40 h later, and IL-2/IFN- γ concentrations were determined by sandwich ELISA as described⁵³. To test humoral immune responses, mice were bled retroorbitally 14, 35 and 58 d after the first immunization, and collagen IgG1 and IgG2a antibodies in the sera were determined by ELISA using chicken type II collagen as the detecting antigen.

In vivo and in vitro models of negative selection. To study negative selection *in vivo*, mice were injected intraperitoneally (i.p.) with either CD3 mAb (for C57BL/6 mice) or purified ovalbumin (for OVA-specific TCR transgenic BALB/c mice) at 0 and 24 h. Twenty-four hours after the last injection, mice were sacrificed and their thymuses collected. Thymocytes were stained with CD4 mAb, CD8 mAb, heat stable antigen (HSA, CD24) mAb and annexin V5, and analyzed by flow cytometry.

To study negative selection *in vitro*, both adult thymocyte cultures and FTOC were used. For adult thymocyte cultures, thymocytes from 6- to 8-week-old C57BL/6 mice were cultured at $5 \times 10^6/\text{ml}$ in plates coated with CD3 mAb and CD28 mAb. Cells were harvested 24 h later and analyzed by flow cytometry. For FTOC, fetal thymuses were collected from day 15 embryos of C57BL/6 mice and cultured in DMEM supplemented with 50 μM β -mercaptoethanol and 10% FCS with or without 1 $\mu\text{g/ml}$ CD3 mAb, 20 $\mu\text{g/ml}$ CD28 mAb, and 50 $\mu\text{g/ml}$ sDR5. Twelve days later, thymocytes were collected, counted and analyzed by flow cytometry as described above.

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Competing interests statement

The authors declare that they have no competing financial interests.

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