Normal Thymocyte Negative Selection in TRAIL-deficient Mice

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Abstract

The molecular basis of thymocyte negative selection, which plays a critical role in establishing and maintaining immunological tolerance, is not yet resolved. In particular, the importance of the death receptor subgroup of the tumor necrosis factor (TNF)-family has been the subject of many investigations, with equivocal results. A recent report suggested that TRAIL was a critical factor in this process, a result that does not fit well with previous studies that excluded a role for the FADD-caspase 8 pathway, which is essential for TRAIL and Fas ligand (FasL) signaling, in negative selection. We have investigated intrathymic negative selection of TRAIL-deficient thymocytes, using four well-established models, including antibody-mediated TCR/CD3 ligation in vitro, stimulation with endogenous superantigen in vitro and in vivo, and treatment with exogenous superantigen in vitro. We were unable to demonstrate a role for TRAIL signaling in any of these models, suggesting that this pathway is not a critical factor for thymocyte negative selection.

Key words: T cell selection • thymus • apoptosis • tumor necrosis factor • antigen

Introduction

Intrathymic T cell development involves the differentiation and selection of T cells that carry a TCR that is self-MHC restricted but does not bind with high affinity to self-antigen derived peptides presented by self-MHC molecules. The processes that produce such a selected T cell repertoire are called positive selection and negative selection respectively (1, 2). Negative selection is central to the generation of a self-tolerant T cell repertoire, and recent studies have reiterated the importance of intrathymic negative selection in the prevention of autoimmune disease (3–5). The phenomenon of negative selection has always presented a paradox to immunologists, as it is mediated by the same TCR/CD3 complex that governs positive selection and CD4/CD8 lineage commitment and later in the life of a T cell mediates activation and clonal expansion. Negative selection is a differentiation stage-specific phenomenon, and depending on the model system, may operate at the immature CD4+CD8+ double-positive stage of thymocyte dif-

ferentiation in the thymic cortex and/or at the later CD3+CD4+CD8− or CD3+CD4−CD8+ stage when thymocytes transit through the cortico-medullary junction (2). Medullary thymocytes continue to differentiate and eventually reach a stage where they are beyond the window of negative selection and are ready to migrate from the thymus to peripheral lymphoid tissues. This transition is thought to occur as these cells down-regulate the maturation marker CD24 (heat stable antigen [HSA]), a very late event in intrathymic T cell development (6).

The clearly distinct outcomes (deletion versus activation) after TCR ligation in immature versus mature T cells may reflect a change in the way these cells interpret signals from their TCR, or alternatively, other possibilities are that apoptotic death may result from additional signals received at the immature stage or absence of survival-promoting signals that are received only later in more differentiated cells. The search for the apoptosis signaling pathway(s) in thymocyte negative selection has been a long-term quest for T cell immunologists. Mammals have two distinct apoptosis signaling pathways (7), one regulated by pro- and antiapoptotic members of the Bcl-2 protein family and the other activated by 'death receptors' members of the TNF-R family

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with an intracellular death domain, and both have been implicated in thymocyte negative selection. Three intracellular signaling factors have been clearly shown to be critical for negative selection, being Nur-77/Nurr1/Nor1 transcription factor family (8, 9) and the pro-apoptotic Bcl-2 family members BIM (4), and Bax plus Bak (10). Many studies have investigated whether death receptors, including: FAS, TNF, DR3, LT, LIGHT, and CD30 (for reviews, see references 11–15), play a role in thymocyte negative selection. Evidence has been provided suggesting a role for FAS at high but not low intensity TCR/CD3-stimulation. This is, however, not supported by studies showing that negative selection is intact in transgenic mice lacking FADD or caspase-8 function due to expression of a dominant-interfering mutant of FADD or the viral serpin CrmA (16, 17). Recently, a critical role for the TNF family member TNF-related apoptosis–inducing ligand (TRAIL) was proposed, based upon studies using several in vitro and in vivo models where negative selection was at least partially impaired in TRAIL-deficient mice or in the presence of blocking soluble TRAIL receptor, DR5 (18). This result is also at odds with early results from FADD dominant negative and CrmA transgenic mice, as FADD and caspase-8 are essential for TRAIL-induced apoptosis. Even more puzzling, it clearly contrasts with a previous study using TRAIL inhibitors that indicated that negative selection of mouse and human thymocytes was TRAIL-independent (19). The reasons for these discrepancies are unclear, although it was suggested that TRAIL might utilize an alternative (undefined) apoptosis signaling pathway that does not involve FADD-caspase 8 but may activate Bim and Bax/Bak (20).

We sought to investigate this problem using a range of well-established negative selection models, including in vitro TCR/CD3 ligation, in vivo and in vitro deletion of endogenous superantigen reactive T cells, and in vitro stimulation with the superantigen staphylococcal enterotoxin B (SEB).

Materials and Methods

Mice. Inbred BALB/c and C57BL/6 (B6) WT mice were purchased from the Walter and Eliza Hall Institute. The TRAIL gene-targeted mice were originally derived at Immunex Corporation as described (18, 21) and bred at the Peter MacCallum Cancer Centre: BALB/c TRAIL-deficient mice (BALB/c TRAIL$^{-/-}$) were 10 generations backcrossed to BALB/c and C57BL/6 TRAIL-deficient (B6 TRAIL$^{-/-}$) mice (5 generations backcrossed to C57BL/6). Mice were used in all experiments under specific pathogen-free conditions according to animal experimental ethics committee guidelines. Mice were culled and their thymus, spleen, axial, brachial, and inguinal lymph nodes removed for analysis of cellularity and composition.

Fetal Thymic Organ Culture. Fetal thymic lobes were isolated from day 15 BALB/c WT and BALB/c TRAIL$^{-/-}$ embryos and cultured for 12 d in RPMI-1640 (Invitrogen/Life Technologies) medium supplemented with 10% FCS (Commonwealth Serum Laboratories [CSL]), 2 mM GlutaMAX, 100 IU/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES buffer (Invitrogen),

and 1 mM sodium pyruvate (Invitrogen) with or without 20 µg/ml anti-mTRAIL mAb (N2B2; reference 22) and 10 µg/ml SEB (Toxin Technology Co.). Lobes were cultured in groups of 2 per well of a 6-well plate and medium was changed after 6 d. At the end of culture, thymocytes were harvested from lobes, counted and analyzed by flow cytometry.

Anti-CD3 Ligation In Vitro. For in vitro analysis, thymocyte suspensions were produced from E18 thymic lobes of BALB/c and BALB/c TRAIL$^{-/-}$ mice. Cells were cultured at a density of 10^6 cells/ml, in DMEM medium supplemented with 10% FCS in wells of a 96-well plate that had been coated overnight with hamster anti–mouse CD3ε (145–2C11), anti-CD28 (37.51) mAb (both from BD Biosciences), or PBS. 20 h later, proportions of live and apoptotic cells were identified by staining with propidium iodide (Sigma Aldrich) and annexin V-FITC (Roche). Live cells were considered to be PI$^-$ and annexin V-FITC$^-$.

Immunofluorescence Staining and Flow Cytometric Analysis. Thymocytes, splenocytes, and lymph node cells were stained with surface marker-specific mAbs including: anti-CD3-FITC (17A2), anti-CD4-PerCP-Cy5.5 (RM4–5), anti-CD8α-PE (53–6.7), anti-TCRVβ3-biotin (KJ25), anti-TCRVβ5.1/5.2-biotin (MR9–4), anti-TCRVβ6-biotin (RR4–7), anti-TCRVβ8.1–8.2-biotin (MR5–2), and anti-TCRVβ11-biotin (RR3–15) (all BD Biosciences), in the presence of anti-Fcγ receptor block CD16/32 (clone 2.4G2). Biotinylated antibodies were detected with streptavidin-APC (BD Biosciences). Analysis was performed on a FACSCalibur™ (Becton Dickinson).

Results

We first examined anti-CD3 plus anti-CD28 mAb-induced apoptosis in vitro. E18 mice are a rich source of DP immature thymocytes (90–95%), and these importantly lack mature T cells that might upon TCR/CD3 ligation release soluble factors that kill DP thymocytes indirectly. Stimulation with anti-CD3 plus anti-CD28 mAbs killed thymocytes from E18 WT BALB/c mice in a dose-dependent manner as determined by staining with the vital dye PI and FITC-coupled annexin V (Fig. 1), which detects externalized phosphatidyl-serine, a hallmark of apoptotic
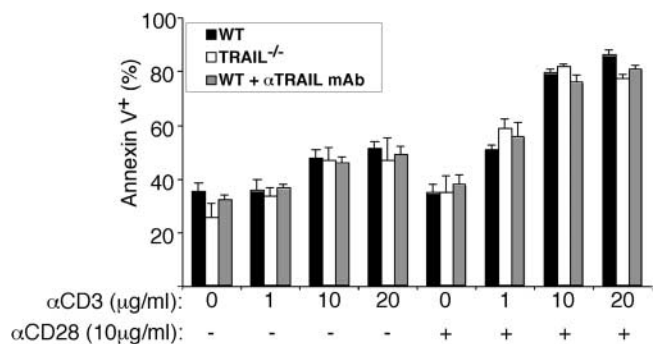

Figure 1. Thymocyte apoptosis in vitro. Thymocytes from BALB/c and BALB/c TRAIL$^{-/-}$ E18 mice were cultured in plates coated with anti-CD3ε (145–2C11) plus anti-CD28 (37.51) mAbs or PBS. Neutralizing anti-TRAIL antibodies were added to some of the cultures. After 20 h, live and apoptotic cells were discriminated by staining with annexin V-FITC plus propidium iodide and flow cytometric analysis. Data represent mean and standard error of the mean for six BALB/c mice plus or minus the addition of anti-mTRAIL mAb) or four BALB/c TRAIL$^{-/-}$ mice.

cells. TRAIL-deficient (TRAIL$^{-/-}$) BALB/c thymocytes or blocking of TRAIL with a neutralizing antibody in cultures of WT thymocytes afforded no protection at any of the concentrations of anti-CD3/anti-CD28 mAbs used (Fig. 1). The spontaneous death of thymocytes in culture was also equivalent between WT and TRAIL$^{-/-}$ mice. Similar results were obtained in vitro when analyzing DP thymocytes from adult BALB/c or C57BL/6 WT and TRAIL$^{-/-}$ mice (unpublished data).

The effect of TRAIL-deficiency was also examined in fetal thymus organ culture (FTOC) (Fig. 2). Consistent with previous reports, addition of SEB induced complete deletion of CD3^{high} TCR Vβ8$^+$ T cells in fetal thymus lobes derived from BALB/c WT mice whereas TCRVβ6$^+$ T cells, which do not respond to SEB, remained unaffected (Fig. 2). Loss of TRAIL or addition of neutralizing anti-TRAIL antibodies had no effect on SEB-induced deletion of TCRVβ8$^+$ T cells (Fig. 2). T cells developing in FTOC were also examined for the deletion of Vβ5$^+$ T cells that is

caused by the endogenous superantigen mouse mammary tumor virus (MMTV)9 presented by I-E$^+$ class II MHC. Also in this depletion process, lack of TRAIL or addition of neutralizing anti-TRAIL antibodies afforded no protection against negative selection (Fig. 2).

Intrathymic deletion of developing T cells caused by endogenous superantigens MMTV6 and MMTV9, was further assessed in adult BALB/c mice. These endogenous superantigens delete TCRVβ3$^+$ and TCRVβ5$^+$ T cells, respectively, in mice that express the class II MHC antigen (I-E). We examined the frequencies of TCRVβ3$^+$ and TCRVβ5$^+$ T cells amongst CD4$^+$ and CD8$^+$ subsets in thymus, spleens and peripheral lymph nodes (Fig. 3) in BALB/c WT or BALB/c TRAIL$^{-/-}$ mice by immunofluorescent staining with TCRVβ-specific mAbs and flow cytometry. Similar deletion of T cells with these specificities was observed in BALB/c WT and TRAIL$^{-/-}$ mice, compared with C57BL/6 mice (I-E$^-$) where cells with these specificities were not deleted (Fig. 3). As a control, we also
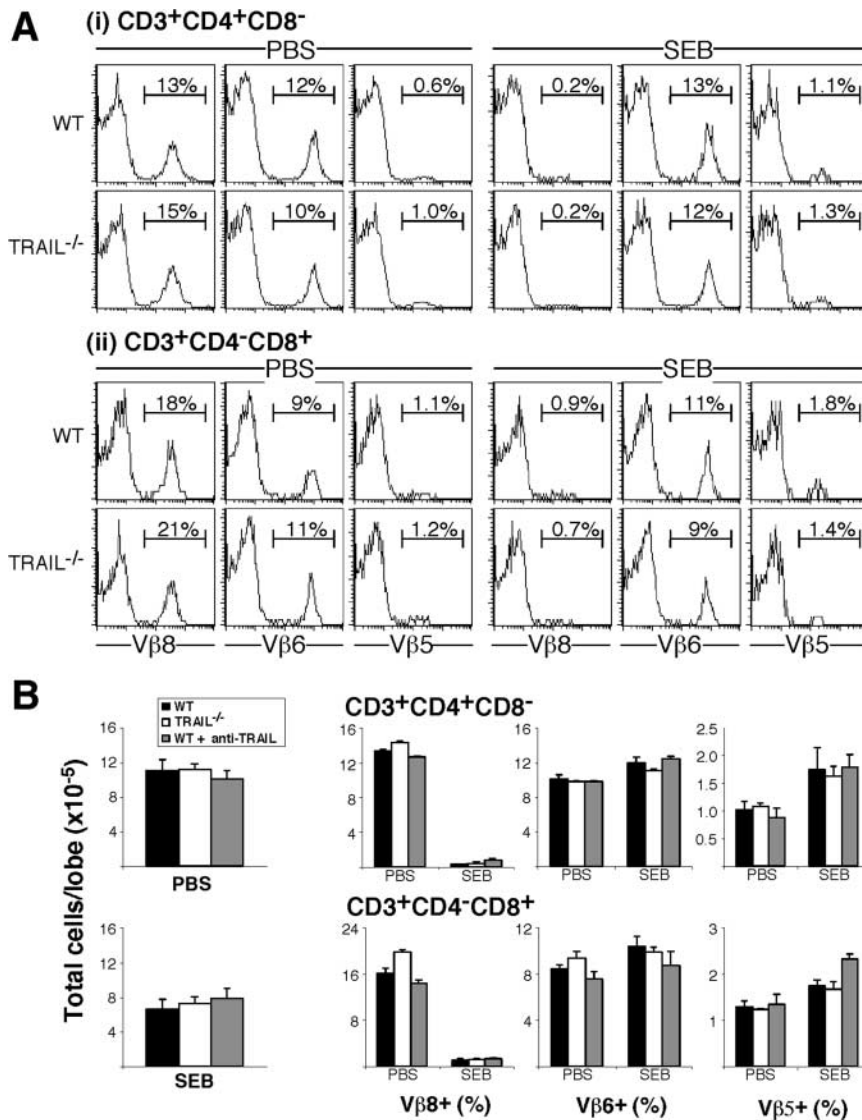

Figure 2. SEB-induced deletion of TCR Vβ8$^+$ cells in FTOC. Thymic lobes from E15 mice were cultured for 12 d in the presence of 10 μg/ml SEB plus or minus 20 μg/ml anti-mTRAIL mAb or an equivalent volume of PBS. Thymocyte suspensions were isolated, counted, and stained with antibodies to CD4, CD8, CD3, and TCR Vβ8, Vβ6, or Vβ5. (A) Histograms demonstrate expression of TCR Vβ8, TCR Vβ6, or TCR Vβ5 on CD3^{high} thymocytes (CD4$^+$ or CD8$^+$) after incubation with SEB or PBS; and (B) total cells per lobe and TCR Vβ8$^+$, Vβ6$^+$, or Vβ5$^+$ cells as a percentage of CD3$^+$CD4$^+$CD8$^-$ and CD3$^+$CD4$^-$CD8$^+$ thymocyte subsets. Mean and standard error of mean were calculated from analysis of six BALB/c TRAIL$^{-/-}$ and eight BALB/c WT thymic lobes each for both PBS and SEB treatment.
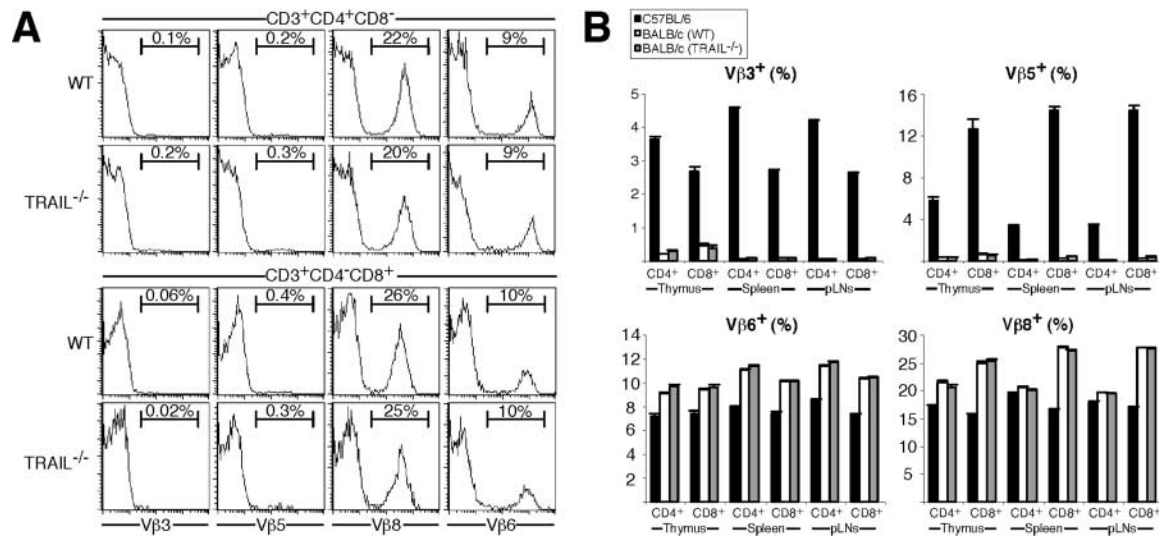
Figure 3. MMTV-6 and MMTV-9 induced deletion of TCR Vβ3 and Vβ5 T cells. Thymocytes, splenocytes, and lymph node cells were isolated from 8-wk-old C57BL/6 ($n = 3$), BALB/c ($n = 12$), and BALB/c TRAIL$^{-/-}$ mice ($n = 11$). Cell suspensions were stained with antibodies to CD4, CD8, CD3, and TCR Vβ3, Vβ5, Vβ6, or Vβ8. (A) Histograms demonstrate expression of TCR Vβ3, TCR Vβ5, TCR Vβ6, or TCR Vβ8 on CD4$^+$ CD3^{high} or CD8$^+$ CD3^{high} thymocytes. (B) TCR Vβ3, TCR Vβ5, TCR Vβ6, or TCR Vβ8 cells as a percentage of CD3$^+$CD4$^+$CD8$^-$ and CD3$^+$CD4$^-$CD8$^+$ subsets of thymocytes, splenocytes, and peripheral lymph node cells.

showed that TCRVβ6$^+$ and TCRVβ8$^+$ T cells are not deleted in either BALB/c WT or TRAIL$^{-/-}$ mice. Consistent with an earlier report (18), the thymus of adult TRAIL$^{-/-}$ mice ($2.19 \pm 0.14 \times 10^8$) was slightly enlarged compared with WT mice ($1.77 \pm 0.04 \times 10^8$; $P = 0.0127$, Mann Whitney), however there was no significant difference in spleen or lymph node cellularity between the strains (unpublished data). The basis for this subtle difference is unclear, but does not appear to be due to impaired negative selection.

Discussion

The role of TRAIL signaling in intrathymic negative selection is controversial. Our data, using four separate models of thymocyte negative selection, has fallen clearly on the side arguing that TRAIL is not required for this process. Thus, we agree with earlier results by Simon et al. (19). Moreover, our data fits well with results from Smith et al. (17) and Newton et al. (16) demonstrating normal negative selection of thymocytes lacking caspase-8 or FADD function. It is difficult to explain why our results are so distinct from those recently published (18), which use TRAIL$^{-/-}$ mice from the same source and similar models for studying negative selection. Lamhamedi-Cherradi et al. additionally examined thymocyte apoptosis after anti-CD3 treatment in vivo; however this form of apoptosis is known to be largely TCR-independent, with a major role for TNF and glucocorticoid hormones (23, 24). Nevertheless, we also performed such experiments and TRAIL was not critical for deletion (unpublished data).

Careful analysis of at least some of the data in the Lamhamedi-Cherradi et al. study reveals some uncertainties—in particular, the proportions of TCRVβ5$^+$ and

TCRVβ11$^+$ T cells in peripheral lymphoid organs in TRAIL-deficient mice are only slightly (albeit significantly) increased. These T cells are deleted due to the presence of MMTV9 presented by I-E as occurs in BALB/c mice. In MMTV9$^+$ and I-E negative mice, such as C57BL/6, the proportion of these cells is at least 10 times that detected in BALB/c mice. The percentage of these cells in TRAIL-deficient BALB/c mice is barely above the percentage in control BALB/c mice, which indicates that at least for that model, the impact of TRAIL in negative selection is rather subtle at best. Perhaps the best results on MMTV9-mediated deletion would have been obtained from SP thymocytes, rather than DP thymocytes as presented (18). Analysis of DP thymocytes is complicated by the fact that most of these cells express only very low levels of cell surface TCRαβ, and only the very latest stages of DP thymocyte development are susceptible to MMTV-mediated negative selection (25, 26). Thus, changes in TCRVβ representation in this population are far more difficult to detect and less reliable than in SP thymocytes. Indeed, our findings were that in BALB/c mice TCRVβ5$^+$ T cells were not dramatically under-represented in the DP thymocyte population, in contrast to their clear deficiency at the SP stage, which is consistent with the earlier study by Hugo et al. (25). Furthermore, MMTV9 is able to mediate significant deletion of TCRVβ5$^+$ cells in C57BL/6 mice (I-A$^+$, I-E$^-$) and the proportion and number of these cells is increased in BIM-deficient C57BL/6 mice (4) that are known to have impaired negative selection. By contrast, as no difference in the percentage of TCRVβ5$^+$ cells was reported in C57BL/6 TRAIL-deficient mice (18), this again raises some concern over the extent to which TRAIL regulates negative selection. It will be interesting to further assess intrathymic negative selection in mice lacking the

TRAIL death receptor (DR5) or TRAIL and additional TNF family members.

BIM mutation leads to impaired negative selection of T cells in mice and spontaneous development of autoimmune glomerulonephritis (3). Mutation of autoimmune regulator (AIRE) in mice also leads to a failure to delete organ-specific cells in the thymus and predisposes to a spectrum of organ-specific autoimmune diseases in mice (5, 27) and humans (28). We have failed to reveal any signs of spontaneous autoimmunity, even in very old B6 TRAIL$^{-/-}$ mice (>550 d) and BALB/c TRAIL$^{-/-}$ (>300 d) mice, compared with similarly aged syngeneic WT mice (unpublished data). Similar peripheral lymph node and spleen cellularity and composition, serum Ig levels, and pancreas, kidney, and spleen architecture were detected in these aged mice. While TRAIL-deficient mice may be more susceptible to the induction of autoimmunity in certain experimental models (29, 30), it remains unclear by what mechanism TRAIL suppresses the induction of autoimmunity. Clearly, disease induction in these models is complex and often involves adjuvants that stimulate dendritic cells and innate immune cell networks. The lack of any signs of autoimmunity in aged TRAIL-deficient mice is further evidence that TRAIL is not critical for maintaining self-tolerance, and is dispensable for intrathymic negative selection.

In summary, while TRAIL may be functional on activated mature T cells and NK cells and may be capable of suppressing induction of autoimmune disease, we have shown in four different models that TRAIL is not critical for intrathymic negative selection. Using both TRAIL-deficient mice, and a powerful neutralizing anti–mouse TRAIL mAb, we failed to detect an essential role for TRAIL in T cell deletion in these systems. We are unable to easily explain the basis of the discrepancies between our results and those recently published, but in light of our data and other studies supporting our results (19), we suggest that the importance of TRAIL in intrathymic negative selection is, at best, unclear and requires further investigation.

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