A Regulatory Role for TRAF1 in Antigen-induced Apoptosis of T Cells

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Summary

Tumor necrosis factor receptor (TNFR)–associated factor 2 (TRAF2) and TRAF1 were found as components of the TNFR2 signaling complex, which exerts multiple biological effects on cells such as cell proliferation, cytokine production, and cell death. In the TNFR2-mediated signaling pathways, TRAF2 works as a mediator for activation signals such as NF-κB, but the role of TRAF1 has not been previously determined. Here we show in transgenic mice that TRAF1 overexpression inhibits antigen-induced apoptosis of CD8+ T lymphocytes. Our results demonstrate a biological role for TRAF1 as a regulator of apoptotic signals and also support the hypothesis that the combination of TRAF proteins in a given cell type determines distinct biological effects triggered by members of the TNF receptor superfamily.

Antigen-induced cell death of T cells plays a critical role in the establishment of T cell tolerance and also in feedback regulatory mechanisms of immune responses (1–6). Members of the TNF receptor (TNFR) family are involved in the regulation of antigen-induced cell death of T cells (7, 8). Experiments with spontaneous mutant or knock out mice have shown that Fas(CD95) and the two TNF receptors (TNFR1 and TNFR2) participate in the induction of apoptosis and subsequent deletion of antigen-specific mature T cells (9–13). Progress has also been made by showing that overexpression of various signal transducers can trigger cell death upon overexpression (14–19). Both Fas(CD95) and TNFR1 contain conserved death domains in their cytoplasmic tails, which mediate defined protein-protein interactions (20, 21), allowing the recruitment of other death domain–containing proteins such as FADD (MORT1), TRADD, or RIP (14–19). The association of FADD to Fas(CD95) or TNFR1 results in the recruitment of FLICE/MACH, the activation of which in turn leads to cell death (22, 23). In contrast with Fas(CD95) or TNFR1, the cytoplasmic tail of TNFR2 does not contain a death domain and does not interact with death domain containing proteins such as FADD, RIP or TRADD, suggesting that other signaling molecules mediate the induction of cell death by this receptor.

The cytoplasmic tail of TNFR2 has been shown to interact with signal transduction molecules known as TRAF proteins (TRAF1 and TRAF2) (24). Six members of the TRAF family have been identified that interact with various members of the TNFR superfamily (24–33). In some cases, TRAF proteins have been shown to mediate various biological effects exerted by their cognate receptors. For example, TRAF2 was shown to mediate NF-κB activation induced by TNFR2 and by several other members of the TNFR superfamily (18, 34–36). This was determined by experiments based on a transient transfection assay system in vitro. However, the biological role of TRAF1 in the TNFR2 signaling complex was not revealed by similar in vitro transfection experiments, although it has been implicated as an adapter protein (37). TRAF1 is associated with various members of the TNFR superfamily that exert multiple effects on cells, and TRAF1 mRNA expression can only be detected in limited tissues such as spleen, lung, and testis (24). Therefore, it is likely that TRAF1 mediates some functions other than NF-κB activation, which cannot be elucidated by experiments based on transient transfection in vitro.

In this report, we describe that antigen-induced deletion of mature CD8+ T cells are hampered in transgenic mice overexpressing TRAF1. Induction of TCR-mediated apoptosis of purified T cells from TRAF1 transgenic mice was also inhibited, suggesting that TRAF1 overexpression affected...
the signals required for induction of apoptosis in mature CD8+ T cells. These results suggest that TRAF1 is involved in the signal transduction pathway for antigen-induced apoptosis of T cells. The data in this report also provide evidence for that different combinations of TRAF proteins may mediate different signals such as cell proliferation or apoptosis.

Materials and Methods

Generation of TRAF1 Tg transgenic Mice. An expression vector (pSH-E-TRAF1) for TRAF1 transgenic mice was generated by cloning the Flu epitope-tagged murine TRAF1 cDNA (32) into 5' SalI-BamHI 3' site of the pH SE-3' expression cassette (38, 39). pH SE-3' expression cassette with an H-2K promoter and an IgH enhancer was previously described to express the transgene both in immature thymocytes and in mature T cells (38, 39). Transgene DNA was isolated by XhoI digestion and microinjected into (C57BL/6 × CBA/J)F2 embryos. Transgenic founders were identified by Southern blot analysis of tail DNA and transgenic lines were established by backcrossing with either C57BL/6 or BALB/c mice to yield vSAG7 mice. TRAF1 transgenic or TCR single-transgenic littermates were cultured in 50 IU/ml IL-2 to predispose to apoptosis as previously described (40, 41). The mice were infected on day 16 and tested on day 24 as previously described (42, 43). [3H]Thymidine was added 3 d later for 8–12 h. The culture was harvested and analyzed as previously described (43).

For induction of apoptosis by LCMV, 10^6 TCR transgenic spleen cells from TRAF1/TCR double-transgenic or TCR single-transgenic littermates were injected intravenously in C57BL/6 mice as previously described (42). At the same time, the mice were infected with LCMV WE strain. For in vivo expansion, the viral dose was 200 PFU and analysis was 7 d after infection; for in vivo deletion, the viral dose was 10^6 PFU and analysis was 4 and 7 d after infection. TCR transgenic T cells were determined by double staining with CD8- and Vα2-specific antibodies and flow cytometry analysis by gating for lymphocytes based on forward/side scatter.

Lymphocyte Proliferation and Cytotoxicity Assays. T cells isolated from lymph nodes using T cell enrichment columns (Biotech Laboratories, Inc., Edmonton, Canada) were also induced to proliferate by titrating them in triplicate into anti-TCR Ab (H-57-597) coated tissue culture wells containing mitomycin C–treated splenocytes (5 × 10^5/well) as previously described (43). [3H]Thymidine was added 3 d later for 8–12 h. The culture was harvested and analyzed as previously described (43).

A responder spleen cells (10^6/well) from TRAF1/TCR double-transgenic or TCR single-transgenic littermates were cultured with 2 × 10^4 20 Gy-irradiated stimulator macrophages previously labeled with 10 nM GP33 in 96-well flat-bottomed microtiter plates at 37°C, 5% CO2 in complete IMDM. After 3 d, the cultures were pulsed for 16 h with 1 μCi of [3H]thymidine, harvested, and counted on a MicroBeta 96 direct β counter (Canberra Packard). The data are expressed as mean cpm of triplicate cultures. Cytotoxicity by TCR transgenic spleen cells was tested in duplicate cultures and serial dilutions in 96-well round-bottomed microtiter plates. The data are expressed as mean cpm of triplicate cultures.

Figure 1. Transgenic mice overexpressing TRAF1. (A) Northern blot analysis of TRAF1 expression. Total RNA was isolated from thymocytes and splenocytes, and analyzed by Northern blot analysis using a TRAF1 cDNA probe as previously described (32). TRAF1 mRNAs detected by an arrow. The amount of total RNA loaded in each lane was similar based on the EtBr-stained rRNAs (data not shown). Upon longer exposure of the autoradiogram, low level of TRAF1 mRNAs was detected in thymocytes and splenocytes from control mice (data not shown). (B) Western blot analysis of TRAF1 protein expression. Cell lysates were transferred from the gels and subjected to Western blot analysis using an anti-epitope mAb (12CA5) as previously described (32). Blotting analysis of TRAF1 protein is indicated by an arrow. Western blot analysis with anti-TRAF1 polyclonal Abs showed similar results (data not shown).
plates in 5-h $^{31}$Cr (NEN DuPont, Boston, MA) release assays using EL4 target cells (H-2b) previously incubated with 10 nM GP33 peptide for 1 h at 37°C. The supernatant of the cytotoxicity cultures was counted by a Cobra II Gamma Counter (Canberra Packard).

Results and Discussion

To examine the potential role of TRAF1 during antigen-induced deletion of mature T cells that are mediated in part by TNFRs, we have generated transgenic mice that overexpress TRAF1 in various subsets of T cells. Levels of TRAF1 expression in thymus or spleen from TRAF1 transgenic mice exceeded those seen in control animals by 5- to 20-fold when determined by Northern or Western blot analysis (Fig. 1, A and B). Two of these transgenic lines (TRAF1.TG-11 and TRAF1.TG-10) were used for further analysis.

The TRAF1 transgenic mice revealed no gross anatomical abnormalities. Flow cytometry analysis of thymocytes from 6- to 8-wk-old TRAF1 transgenic or negative littermates did not indicate any significant difference in the total number of thymocytes (data not shown). Moreover, the percentage of double-negative (CD4$^-$CD8$^-$), double-positive (CD4$^+$CD8$^+$), and single-positive (CD4$^+$ or CD8$^+$) thymocytes in TRAF1 transgenic mice was comparable to that in negative littermates (data not shown). The numbers and ratios of T and B cells or CD4$^+$ and CD8$^+$ T cells from spleen or lymph nodes of TRAF1 transgenic mice were also comparable to those in negative littermates (data not shown), suggesting that TRAF1 overexpression did not dramatically affect lymphocyte development. When purified T cells from TRAF1 transgenic and negative littermates were tested for their ability to divide in response to anti-TCR antibody (H57-597) in vitro, T cells from TRAF1 transgenic and negative littermates responded similarly to anti-TCR Ab (Fig. 2A). These results demonstrate that TRAF1 overexpression did not affect TCR-mediated proliferation of mature T cells in vitro.

To examine whether TCR-mediated mature T cell death from TRAF1 transgenic mice was affected in vitro, resting lymph node cells from TRAF1 transgenic and negative littermates were primed for apoptosis by Con A and IL-2. A representative experiment showing the flow cytometric plots displaying propidium iodide (PI) fluorescence versus forward light scatter for TRAF1 transgenic and negative littermates after 48 h with IL-2 in either uncoated wells (medium) or wells coated with anti-CD3e (10 μg/ml) is shown in B. Viable cells were quantified by PI exclusion using a FACSCaliber® with CELLQUEST software and show low PI fluorescence. Percentages of total cells in each region are indicated. In C, the percentages of cell survival in CD8$^-$ (CD4$^+$) or CD8$^+$ T cells were shown as the mean ± SEM from the triplicate samples of three mice from each group. Cells cultured in either uncoated (medium) or anti-CD3e–coated plates as described in B, were recovered from the plates and stained with anti-CD8 Ab (S3-6; FITC conjugated). The CD8 staining was used to gate the cells into CD8$^+$ and CD8$^-$ pools; control staining showed that virtually all CD8$^+$ cells were CD4$^+$. Specific cell survival (%) was determined from the percentages of viable (PI-negative) cells and is equal to $100 \times (%$ survival after CD3 cross-linking/$%$ survival without CD3 cross-linking).

Figure 2. In vitro proliferation and apoptosis of mature T cells. (A) TRAF1 overexpression does not affect TCR-mediated proliferation of mature T cells in vitro. T cells were isolated from lymph nodes of control (open circle) and TRAF1.TG-10 transgenic (closed circle) littermates using T cell enrichment columns (Biotex Laboratories, Inc.) and cultured in triplicate into anti-TCR Ab (H57-597) coated-tissue culture wells containing mitomycin C–treated splenocytes (5 × 10$^5$/well). $[^3]$H thymidine was added 3 d later for 8–12 h. The culture was harvested and analyzed as described in Materials and Methods. The data show the mean cpm ± SEM from the triplicate samples. The data are from one of several similar experiments using T cells from nontransgenic and TRAF1.TG-10 transgenic mice littermates (H-2k). Similar results were obtained when T cells from TRAF1.TG-11 mice were used (data not shown). (B and C) TRAF1 overexpression inhibits TCR-mediated apoptosis of CD8$^+$ T cells in vitro. A representative experiment showing the flow cytometric plots displaying propidium iodide (PI) fluorescence versus forward light scatter for TRAF1 transgenic and negative littermates after 48 h with IL-2 in either uncoated wells (medium) or wells coated with anti-CD3e (10 μg/ml) is shown in B. Viable cells were quantified by PI exclusion using a FACSCaliber® with CELLQUEST software and show low PI fluorescence. Percent of total cells in each region is indicated. In C, the percentages of cell survival in CD8$^-$ (CD4$^+$) or CD8$^+$ T cells were shown as the mean ± SEM from the triplicate samples of three mice from each group. Cells cultured in either uncoated (medium) or anti-CD3e–coated plates as described in B, were recovered from the plates and stained with anti-CD8 Ab (S3-6; FITC conjugated). The CD8 staining was used to gate the cells into CD8$^+$ and CD8$^-$ pools; control staining showed that virtually all CD8$^+$ cells were CD4$^+$. Specific cell survival (%) was determined from the percentages of viable (PI-negative) cells and is equal to $100 \times (%$ survival after CD3 cross-linking/$%$ survival without CD3 cross-linking).
treatment as previously described (12). When the TCR-CD3 complex was cross-linked, T cells from control mice underwent massive cell death as previously shown (12) (Fig. 2 B). However, the percentage of T cells undergoing anti-CD3-induced death from TRAF1-TG-10 mice was significantly lower than that of T cells from negative littersmates (Fig. 2 B). Percent viable cells of CD8+ or CD8− (CD4+) T cells as determined from PI-negative cells with and without anti-CD3 stimulation showed a protective effect of TRAF1 for CD8+ T cell death (Fig. 2 C). No such effect was seen for CD8− (CD4+) T cell death (Fig. 2 C). Because the death of CD8+ T cells in this in vitro experimental system is mostly mediated by TNFR2 (12), the results strongly suggest that TRAF1 overexpression antagonized the apoptotic signals exerted by TNFR2. The results showing that CD8− (CD4+) T cell death was not affected by TRAF1 overexpression indicates that TRAF1 overexpression specifically inhibited TCR-mediated mature CD8+ T cell death at least in this in vitro deletion system. Unlike the TRAF1-TG-10 mice, the death of T cells from TRAF1-TG-11 mice triggered by the similar TCR-CD3 complex cross-linking in vitro was similar to that of T cells from control mice (data not shown). This is probably because of the difference between these two transgenic lines in the amount of TRAF1 expressed.

To examine further the effect of TRAF1 overexpression on the induction of apoptosis of CD8+ T cells, we have examined the deletion of mature T cells in TRAF1 transgenic mice using a defined LCMV glycoprotein-specific TCR model (38, 39). αβ-TCR transgenic mouse line specific for H-2Db and LCMV GP (residues 33–41, GP33) (38, 39) was bred with TRAF1 transgenic mice. Owing to the high mortality of TRAF1-TG-10 mice with yet to be determined reasons, only mice from the TRAF1-TG-11 line were used in these experiments. Development and selection of LCMV-specific αβ-TCRs in TCR-TRAF1 double-transgenic mice was normal when compared with that in control TCR single-transgenic littersmates (data not shown). When splenocytes from αβ-TCR-TRAF1 double-transgenic mice were stimulated with GP33, the degree of proliferation was comparable to splenocytes from αβ-TCR single-transgenic mice (Fig. 3 A). Moreover, CTLs from αβ-TCR-TRAF1 double-transgenic mice showed cytotoxicity to target cells presenting GP33 peptides comparable to αβ-TCR single-transgenic mice (Fig. 3 B). These results are consistent with earlier experiments (Fig. 2 A) and confirm that TRAF1 overexpression did not affect T cell development, the proliferative response, or the effector function of T cells.

To test whether TRAF1 overexpression affected the antigen-induced apoptosis of mature T cells, 30 μg of GP33 peptides was injected into the hind footpad. This protocol induces cell death of antigen-specific T cells in the draining lymph nodes (13). When transgenic TCR+ T cells were analyzed from the draining lymph nodes of TCR single-transgenic mice, antigen-induced cell death of transgenic TCR+ T cells was clearly observed after peptide treatment as previously described (13) (Fig. 3 C). When T cells were examined from the αβ-TCR-TRAF1 double-transgenic mice injected with GP33 peptide, the death of transgenic TCR+ T cells was significantly reduced compared with αβ-TCR single-transgenic mice (Fig. 3 C). These results suggested that TRAF1 overexpression hampered a signal-
transgenic TCR
ble-transgenic mice, the deletion of transgenic TCR overexpression inhibited the deletion of CD8 was significantly lower (Fig. 3

induction of tolerance, H-2b control ( ). For Figure 4. TRAF1 overexpression inhibits peptide-induced T cell tolerance in vivo also in non-TCR transgenic mice. (A) The LCMV-specific cytotoxic responses are similar in TRAF1 transgenic and negative littersmates. H-2b mice were infected intravenously with 2000 PFU LCMV Armstrong strain. On day 8, spleen cells from TRAF1 transgenic (closed symbols) and TRAF1 negative (open symbols) littersmates were tested on GP33-labeled (squares) and not labeled (circles) EL4 target cells in 5-h 3H Cr-release assays as described in Materials and Methods. The mice were infected on day 16 and tested on day 24 as in (A). One of three representative experiments is shown.

mitigation pathway required for the antigen-induced apoptosis of CD8 T cells. Additional experiments involving adoptive transfers of transgenic TCR + T cells along with antigen were performed to determine the effect of TRAF1 overexpression on the deletion of CD8 + T cells. As previously shown, when transgenic TCR + T cells specific for LCMV GP33 were transferred to C57BL/6 mice with a high dose of LCMV, a vigorous expansion of transgenic T cells was followed by a rapid decline due to the induction of cell death (42) (Fig. 3 D). When the same experiment was performed with transgenic TCR + T cells from TCR -TRAF1 double-transgenic mice, the deletion of transgenic TCR + T cells was significantly lower (Fig. 3 D), indicating that TRAF1 overexpression inhibited the deletion of CD8 + T cells induced by high dose antigen treatment. Consistent with in vitro proliferation experiments, TRAF1 overexpression did not affect expansion of transgenic TCR + T cells by a low dose of LCMV during adoptive transfer (42) (Fig. 3 D).

TRAF1 overexpression also inhibited the induction of tolerance to LCMV glycoprotein by high dose antigen treatment in non-TCR transgenic mice. When mice were infected with LCMV, both TRAF1 transgenic and negative littersmates mounted an efficient CTL response (Fig. 4 A). When control mice were pretreated three times intraperitoneally with 500 μg of GP33 in IFA, the induction of CTL activities against LCMV was completely abrogated as previously described (40, 41) (Fig. 4 B). In contrast, TRAF1 transgenic mice mounted significant LCMV-specific CTL activities even after they were pretreated three times intraperitoneally with 500 μg GP33 in IFA (Fig. 4 C). These results indicate that TRAF1 overexpression did not affect CTL activation but inhibited antigen-induced T cell death.

In contrast with the deletion of mature T cells induced by a peptide antigen, TRAF1 overexpression did not inhibit superantigen-induced deletion of mature T cells. When TRAF1 transgenic mice were treated three times intraperitoneally with 100 μg of staphylococcal enterotoxin B (SEB), the deletion of either CD4 + or CD8 + T cells from TRAF1 transgenic mice was comparable to that of T cells from control mice (data not shown). This suggests that TRAF1 overexpression inhibits only specific signaling cascades required for deletion of mature T cells in vivo, rather than acting as a nonspecific anti-apoptotic signal. Complex and multiple pathways were shown to operate in the induction of apoptosis in mature T cells. For example, the involvement of Fas(CD95) has been shown for deletion of CD4 + T cells in the 2B4 TCR transgenic mice system but both Fas(CD95) and TNFRs for deletion of CD4 + T cells in the HNT TCR transgenic mice system (10, 11). Therefore, it is possible that different experimental systems involving various TCR-MHC-Ag complexes with different affinities/avidities may result in the utilization of multiple pathways to mediate cell death. This may explain why TRAF1 overexpression inhibits the induction of apoptosis in mature CD8 + T cells by a peptide antigen (i.e., GP33) but not by a superantigen (SEB).

In summary, the results in this study showed that TRAF1 overexpression inhibits the antigen-induced deletion of activated CD8 + T cells in vivo and in vitro. Although we have failed to show that TRAF1 overexpression did not affect CD4 + T cells, this does not exclude the possibility that TRAF1 can play a role, which needs to be determined, in CD4 + T cell death in some experimental systems. Nevertheless, the results provide evidence for the biological role of TRAF1 as a regulator of the antigen-induced apoptotic signals in mature T cells.

TRAF1 is a component of the TNFR2 signaling complex (24) and TRAF1 overexpression inhibited antigen-induced apoptosis of mature CD8 + T cells in an in vitro system, in which anti-TCR-induced apoptosis of CD8 + T cells is mediated by the TNFR2 signaling complex (12). This suggests that the signal intermediates for induction of apoptosis by the TNFR2 involves TRAF1. Whether TNFR2-mediated signals can induce cell death has been controversial due to various contradicting results (7, 8). Experiments described here showing that the overexpression of TRAF1, a TNFR 2-proximal signaling component, inhibits the induction of apoptosis support the idea that proximal signals induced by the TNFR2 are directly involved in the TCR-mediated cell death of mature CD8 + T cells.

The precise mechanism that TRAF1 overexpression inhibits the induction of apoptosis is not clear at this point. Because TRAF1 (and also other TRAF proteins) does not contain any known catalytic domain, it is most likely that TRAF1 overexpression inhibited the TNFR 2-mediated apoptosis by altering the constituents of the TNFR2 signaling complex. It has been recently shown that NF-κB activation acts as an anti-apoptotic signal during TNF-induced...
apoptosis (44–47). It is not likely that TRAF1 overexpression affected NF-κB-mediated anti-apoptotic signals because it does not influence the TNFR-mediated NF-κB activation in transient transfection experiments (34–36) and also in activated T cells from TRAF1 transgenic mice (data not shown). One plausible mechanism is that TRAF1 overexpression may increase the recruitment of anti-apoptotic protein(s) such as c-IAPs to the TNFR2 signaling complex (37). Another possibility is that TRAF1 overexpression may inhibit the recruitment or aggregation of pro-apoptotic signal transducers to the TNFR2 signaling complex. It is also possible that TRAF1 may mediate an as yet to be determined anti-apoptotic signal during antigen-induced cell death of mature T cells. This anti-apoptotic property of TRAF1 may contribute to cell transformation by LMP protein of EBV, which triggers aggregation of several TRAF proteins, including TRAF1 (30).

In addition to establishing that TRAF1 is a component of the TNFR1 proximal signaling intermediate involved in the regulation of apoptosis, our results also provide in vivo evidence supporting the postulate that TRAF proteins are likely to exert pleiotropic signals (cell proliferation or apoptosis) depending on the various combination of TRAF proteins in a given cell type (25, 37). Future identification of downstream signal transducers that bridge TRAF proteins to the apoptotic machinery such as caspases will be important to understand the detailed molecular mechanisms of how apoptotic signals are regulated by many members of the TNFR family.

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