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TNFR2-Deficient Memory CD8 T Cells Provide Superior Protection against Tumor Cell Growth

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TNF receptor-2 (TNFR2) plays a critical role in promoting the activation and survival of naive T cells during the primary response. Interestingly, anti-CD3 plus IL-2 activated TNFR2−/− CD8 T cells are highly resistant to activation-induced cell death (AICD), which correlates with high levels of prosurvival molecules such as Bcl-2, survivin, and CD127 (IL-7Rα). We determined whether the resistance of activated TNFR2−/− CD8 T cells to AICD contributes to more effective protection against tumor cell growth. We found that during a primary tumor challenge, despite initial inferiority in controlling tumor cell growth, TNFR2−/− mice were able to more effectively control tumor burden over time compared with wild-type (WT) mice. Furthermore, vaccination of TNFR2−/− mice with recombinant Listeria monocytogenes that express OVA confers better protection against the growth of OVA-expressing E.G7 tumor cells relative to similarly vaccinated WT mice. The enhanced protection against tumor cell growth was not due to more effective activation of OVA-specific memory CD8 T cells in vaccinated TNFR2−/− mice. In vitro studies indicate that optimally activated OVA-specific TNFR2−/− CD8 T cells proliferated to the same extent and possess similar cytotoxicity against E.G7 tumor cells as WT CD8 T cells. However, relative to WT cells, activated OVA-specific TNFR2−/− CD8 T cells were highly resistant to AICD. Thus, the enhanced protection against E.G7 in TNFR2−/− mice is likely due to the recruitment and activation of OVA-specific memory TNFR2−/− CD8 T cells and their prolonged survival at the tumor site. The Journal of Immunology, 2009, 183: 6051–6057.

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ostimulation of T cells during Ag encounter through CD28 and receptors of the TNF receptor (TNFR)5 superfamily provides important activation and survival signals during distinct phases of primary and secondary T cell responses. More than 40 members of the TNF and TNFR superfamilies have been identified (1). Current evidence supports central roles for TNF/ TNFR family members in host defense, inflammation, apoptosis, autoimmunity and organogenesis (1). Consequently, members of the TNF/TNF superfamily are important targets in therapies against human diseases such as autoimmune disorders, inflammatory bowel disease, osteoporosis, and cancer. Several members of the TNF family, particularly OX40 (CD134), 4-1BB (CD137), CD27, and herpes-virus entry mediator, provide costimulatory signals to augment cell division, survival, and effector function. The two main signaling pathways that have been described for costimulatory TNFR family members, i.e., the NFκB and JNK/AP-1 axes, are important points of signaling convergence for inducing differential gene expression associated with effector T cell functions and survival. Furthermore, differential expression of costimulatory TNFR members in naive and activated T cells appear to account for exquisite contextual- and temporally dependent control of T cell responses.

With regard to T cell survival, OX40 and 4-1BB up-regulate the expression of the anti-apoptotic proteins Bcl-xL and Bfl-1, correlating with their suppression of apoptosis in activated T cells (2–4). Costimulatory TNFR members appear to promote T cell survival through protein kinase B (PKB, also known as AKT). Retroviral expression of active AKT in OX40-deficient T cells results in the up-regulation of anti-apoptotic Bcl-2-family protein expression and completely reverses the defect in T cell survival, mimicking the action of ectopic expression of Bcl-xL or Bcl-2 (2). As well as regulating cell survival, PI3K and AKT have been implicated in controlling cell division (5) as intermediary signaling molecules linking costimulatory members of the TNFR and CD28 superfamily to transcription factors, such as c-jun and NFκB (6–9). CD28 also promotes the expression of Bcl-xL (2, 10), which is in parallel with the costimulatory activity of TNFR family members.

TNF-α is a pleiotropic cytokine that regulates multiple biological processes. The biologic activities of TNF-α are mediated by two structurally related, but functionally distinct, receptors: TNFR1 (or p55) and TNFR2 (or p75) (11). TNFR1 is the primary signaling receptor on most cell types through which the majority of inflammatory responses classically attributed to TNF occur (11). In contrast, TNFR2 is important in modulating TNFR1-mediated signaling (12), regulating autoimmune diseases (13), and in host defenses against specific viruses (14). More recently, TNFR2 has been shown to play a critical role for effective priming, proliferation, and recruitment of tumor-specific CD8 T cells (15). We have shown that TNFR2 functions as one of the earliest costimulatory members of the TNFR superfamily. Using either anti-TCR Abs
(16) or cognate Ag (17) for T cell activation, we showed that TNFR2 plays a critical role in lowering the threshold for T cell activation. We also found that TCR proximal signaling events, such as global tyrosine phosphorylation, ZAP-70 phosphorylation, and MAPK activation were unperturbed in TNFR2−/− T cells (18). However, TNFR2 is required for sustained AKT activity and NFκB activation in response to TCR/CD28 stimulation. Moreover, TNFR2−/− T cells possess a defect in survival during the early phase of T cell activation that is correlated with a striking defect in Bcl-xL expression (18). These data reveal the synergistic requirement of TCR, CD28, and TNFR2 for optimal IL-2 induction and T cell survival. The proliferative defect of TNFR2−/− CD8 T cells in response to TCR stimulation is overcome by the addition of exogenous IL-2 (16). Interestingly, we found that anti-CD3 plus IL-2 activated TNFR2−/− CD8 T cells were highly resistant to activation-induced cell death (AICD), relative to wild-type (WT) cells, which emphasizes its role in influencing T cell homeostasis (19). In this study, we found that the resistance of activated TNFR2−/−/CD8 T cells to AICD correlated with more effective protection of vaccinated TNFR2−/− mice against the growth of E.G7 tumor cells (recombinant EL4 tumor cells expressing OVA) (20, 21) relative to similarly vaccinated WT mice. This interesting finding reveals a novel mechanism for generating more effective immune responses against tumor cells.

Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2b) and TNFR2-deficient mice (22) on a B6 background were obtained from The Jackson Laboratory. Breeders for OT-1 mice (23) on a B6 background were provided by Dr. Jan Dutz (University of British Columbia, British Columbia, Canada). OT-1 CD8 T cells express a transgenic TCR that is specific for the OVA peptide (SIINFEKL) presented by Kb (23). OT-1 mice with the TNFR2 null mutation (TNFR2−/−/CD8 T cells were cultured in a flat-bottom 24-well plate containing 20 U/ml IL-2. The next day, the percentage of proliferating cells in these cultures was determined by staining for cytotoxicity against 1 × 10^4 51Cr-labeled EL4 or E.G7 tumor target cells in a 3 h assay at the indicated E:T ratios. Percent-specific lysis was calculated as 100% × [(cpm (experimental well) – cpm (spontaneous release))/cpm (maximum release – cpm (spontaneous release))]. Maximum release was determined by freezing and thawing the target cells three times and counting ^51Cr released into the culture supernatant. Spontaneous release was <10% of the maximum release counts. The CTL assays were performed in triplicates.

The susceptibility of the activated OVA-specific CD8 T cells to AICD was determined by transferring cells that had been cultured for 3 days to 0.2 ml flat-bottom wells that were precoated with 10 μg/ml anti-CD3ε (2C11). The cells were cultured for an additional 16 h in medium containing 20 U/ml IL-2. AICD was determined by staining the cells with 7-aminocoumarin D (7-AAD), fixed with 4% paraformaldehyde, and analyzed by flow cytometry using the FACScan.

Recombinant Listeria monocytogenes (LM) that expressing OVA (rLM-OVA) immunization

Mice were immunized with rLM-OVA (28) by i.v. injection of the tail vein containing the indicated number of colony forming units (CFU) in 0.2 ml of PBS. Bacterial doses were determined by plating the injected stock on brain-heart infusion agar. In memory experiments, the number of OVA-specific memory CD8 T cells at >40 days after rLM-OVA immunization was determined by staining the spleen cells with anti-CD8 PE-Cy5, anti-CD4 FITC, and the OVA peptide (SHINEKEL)-Kb PE tetramer (Beckman Coulter) at 10^6 cells per well for 1 h at 4°C in a volume of 50 μl. At the end of the incubation, the cells were washed twice with staining buffer before analysis using the CellQuest software program (BD Biosciences). For determination of secondary responses to rLM-OVA immunization, mice that were immunized with rLM-OVA for >40 days were re-infected with 3 × 10^5 CFU of rLM-OVA and analyzed in a similar manner 5 days after secondary rLM-OVA challenge.

E.G7 growth assay

Unimmunized or rLM-OVA-immunized B6 or TNFR2−/− mice were inoculated (i.p. route) with 5 × 10^6 E.G7 cells (20). At the indicated time points, the peritoneal cavity was washed multiple times with PBS (a total volume of 15 ml) until the wash was clear of tumor cells. The total number of E.G7 cells recovered from the peritoneal cavity was determined by counting a suitably diluted aliquot under the light microscope. Tumor and nontumor cells were distinguished based on the difference in cell size between E.G7 and normal lymphocytes.

Results

Activated TNFR2−/− CD8 T cells are resistant to AICD and express proapoptotic molecules and receptors

We have previously shown that mitogen-activated TNFR2−/− CD8 T cells are highly resistant to AICD (19). In this study, we show that TNFR2−/− CD8 T cells activated with anti-CD3 plus IL-2 are also highly resistant to AICD relative to similarly activated WT CD8 T cells, as indicated by the much higher proportion of live cells revealed by staining with 7-AAD (Fig. 1). We determined whether this resistance to AICD was due to defects
in the expression and processing of death-inducing caspases. We found that caspase-3 and caspase-8 were expressed and processed in AICD-resistant TNFR2<sup>−/−</sup> CD8 T cells to a similar level as WT CD8 T cells (Fig. 2). However, AICD-resistant TNFR2<sup>−/−</sup> CD8 T cells expressed elevated levels of phosphorylated IκBα, suggesting that prosurvival signaling pathways are preferentially activated in these cells (Fig. 2). The elevated level of phosphorylated IκBα was associated with persistently high expression of Bcl-2, CD25 (IL-2Rα), survivin, and CD127 (IL-7Rα) in AICD-resistant TNFR2<sup>−/−</sup> CD8 T cells (Fig. 3).

Collectively, these data indicate that AICD-resistant TNFR2<sup>−/−</sup> CD8 T cells express molecules and cell surface receptors that favor survival. We speculated that this resistance of activated TNFR2<sup>−/−</sup> CD8 T cells to AICD might contribute to more effective immune protection against tumor cell growth.

**Vaccination of TNFR2<sup>−/−</sup> mice with rLM-OVA protects against growth of E.G7 tumor cells**

To determine whether the resistance of activated TNFR2<sup>−/−</sup> CD8 T cells to AICD confer an advantage in the protection of TNFR2<sup>−/−</sup> mice against the growth of tumor cells, we inoculated naive or mutant mice with E.G7 tumor cells (5 × 10<sup>6</sup> i.p.) and determined the numbers of E.G7 cells recovered from the peritoneal cavity at various time points following tumor challenge. As shown in Fig. 4a, E.G7 grew more rapidly in TNFR2<sup>−/−</sup> mice relative to WT mice on day 8, consistent with the requirement for TNFR2 in the optimal activation of naive T cells. However, on day 15, comparable numbers of E.G7 cells were recovered from the peritoneal cavity of TNFR2<sup>−/−</sup> and WT mice. Interestingly, on day 20, significantly less E.G7 cells were recovered from the peritoneal cavity of TNFR2<sup>−/−</sup> mice relative to WT mice. This result is consistent with the hypothesis that although fewer effector CD8 T cells specific for E.G7 are expected to be produced in TNFR2<sup>−/−</sup> mice due to the dependence of naive CD8 T cells on TNFR2 for initial activation (16–18), persistent antigenic stimulation in the context of tumor challenge overcame this dependence, and thereafter these activated TNFR2<sup>−/−</sup> CD8 T cells, by virtue of their resistance to AICD, are able to more effectively control E.G7 cell growth at later time points.

We next determined whether vaccination of TNFR2<sup>−/−</sup> mice with rLM-OVA confers more effective protection against tumor growth relative to similarly vaccinated WT mice. To determine the resistance of rLM-OVA-immunized mice to growth of E.G7 tumors...
cells, we inoculated the rLM-OVA immunized mice with E.G7 cells (5 × 10^6 i.p.) at 42 days post rLM-OVA infection and determined the growth rate of E.G7 cells in the immunized mice. Interestingly, rLM-OVA-vaccinated TNFR2^−/− mice were much more efficient in resisting the growth of E.G7 cells. We found very few E.G7 cells were recovered from the peritoneal cavity of rLM-OVA-immunized TNFR2^−/− mice on days 10, 15, and 20 after E.G7 inoculation. By contrast, greatly increased numbers of E.G7 cells were recovered from the peritoneal cavity of vaccinated WT mice at all three time points (Fig. 4b). These results indicate that rLM-OVA-vaccinated TNFR2^−/− mice provided more effective protection against E.G7 growth in rLM-OVA-immunized mice.

The more effective protection of TNFR2^−/− mice against the growth of E.G7 tumor cells could be due to one or more of the following mechanisms: 1) more effective secondary expansion of Ag-specific memory TNFR2^−/− CD8 cells, 2) more effective killing of tumor cells by Ag-specific TNFR2^−/− CTLs, 3) higher rate of proliferation, and/or 4) higher resistance to AICD, resulting in the persistence of higher numbers of activated Ag-specific TNFR2^−/− CTLs at the tumor site. We tested the first possibility by vaccinating TNFR2^−/− and WT mice with rLM-OVA (3 × 10^6 CFU) to induce the production of OVA-specific memory CD8 T cells. The number of splenic OVA-specific CD8 memory T cells at 50 days post rLM-OVA immunization was quantified by determining the total number of CD8^+ cells that stained positive with the K^b-OVA peptide tetramer. We found that the spleens of rLM-OVA-immunized TNFR2^−/− mice possess slightly fewer OVA-specific CD8 T cells (7.0 ± 3.3 × 10^5) than similarly vaccinated WT mice (13.3 ± 4.6 × 10^5). However, this difference is not statistically significant (p = 0.075; Student t test). The data shown are representative of three independent experiments.

FIGURE 5. Secondary responses of TNFR2^−/− mice to rLM-OVA immunization. B6 and TNFR2^−/− mice were immunized with rLM-OVA (3 × 10^6 CFU i.v.) on day 0. On day 50 the numbers of OVA-specific memory CD8 T cells in immunized mice were determined by staining with anti-CD4 FITC, anti-CD8-PE-Cy5 and PE-labeled OVA/K^b tetramer. The results indicate that ~1 × 10^6 OVA-specific memory CD8 T cells were recovered from the spleens of immunized B6 and TNFR2^−/− mice on day 50. At this time point, four rLM-OVA immunized B6 and TNFR2^−/− mice were reinfected with rLM-OVA (3 × 10^6 CFU i.v.). The numbers of OVA-specific CD8 T cells present in the spleen as a result of secondary rLM-OVA challenge were determined 5 days later (day 55) using the same staining protocol. The results indicate that rLM-OVA immunized TNFR2^−/− mice possess slightly fewer OVA-specific CD8 T cells (7.0 ± 3.3 × 10^5) than similarly vaccinated WT mice (13.3 ± 4.6 × 10^5). However, this difference is not statistically significant (p = 0.075; Student t test). The data shown are representative of three independent experiments.
We also determine whether activated TNFR2\(^{-/-}\) CD8 cells have a growth advantage over similarly activated WT CD8 T cells. We found that OVA peptide plus IL-2 activated CD8 T cells from OT-I and OT-I TNFR2\(^{-/-}\) mice proliferated at about the same rate as revealed by Ki-67 staining (Fig. 7a). It is therefore unlikely that more effective immunity toward E.G7 cells is due to a higher rate of proliferation by OVA-activated TNFR2\(^{-/-}\) CD8 T cells. However, OVA-activated CD8 T cells are highly resistant to AICD as indicated by the low percentage of cells that stained positive with 7-AAD in the AICD assay (Fig. 7b). Because activated TNFR2\(^{-/-}\) CD8 T cells are more resistant to AICD, it is likely that the OVA-specific TNFR2\(^{-/-}\) CD8 T cells that are recruited to the tumor sites will have an advantage in survival. Because OVA-activated TNFR2\(^{-/-}\) CD8 T cells possess similar cytolytic activity against E.G7 tumor cells as OVA-activated WT CD8 T cells, their prolonged survival likely contributes toward the more effective elimination of E.G7 tumor cells in TNFR2\(^{-/-}\) mice.

Discussion
We have previously shown that TNFR2 plays a critical role in lowering the activation threshold of naive CD8 T cells and few TNFR2\(^{-/-}\) CD8 T cells are recruited into the dividing population after stimulation by either anti-TCR Abs (16) or cognate Ag (17). We analyzed additional contributions by TNFR2 in regulating immune responses by CD8 T cells. Although TNFR2 is required for the optimal activation of CD8 T cells by anti-TCR Abs (16), we found that optimally activated TNFR2\(^{-/-}\) OT-I mice were cultured as described in Fig. 6. a, Ki-67 assay: after 3 days of culture, each well was split into two with medium containing 20 U/ml IL-2. The following day the cultured cells were stained with anti-CD8 FITC, fixed, permeabilized, and stained with PE labeled Ki-67. The dotted histograms indicate base level staining with an isotype control Ab. b, 7-AAD assay: After 3 days of culture, the cultured cells (1 \(\times\) 10\(^5\)) were transferred to 0.2 ml wells that were precoated with anti-CD3 Abs (2C11, 10 \(\mu\)g/ml). The cells were stained with anti-CD8 FITC and 7-AAD after 16 h of culture. The data shown are representative of three independent experiments.
to AICD. In this study, we provide support for the hypothesis that the resistance of activated TNFR2−/− CD8 T cells to AICD confers more effective protection against the growth of syngeneic tumor cells.

We found that TNFR2−/− mice were initially less able to control the growth of E.G7 tumor cells. This is consistent with our earlier studies that TNFR2 is required for the efficient priming of T cells by Ag (17, 18) and also supports the recent observation that TNFR2 plays a critical role for the effective priming, proliferation, and recruitment of tumor-specific CD8 T cells (15). However, we have also observed that sufficient stimulation can overcome the dependence of naive CD8 T cells on TNFR2 for activation and proliferation, reflected in the differences observed between different model Ag systems (e.g., responses by TNFR2−/− mice to rLM-OVA challenge herein vs rLM-SIY challenge; Ref. 17, 18).

When this dependence is overcome, we observed a striking resistance to AICD by activated CD8 T cells. The resistance of Ag-activated TNFR2−/− CD8 T cells to AICD provides an explanation for our observation that TNFR2−/− mice were more effective than WT mice in controlling the growth of E.G7 tumor cells over time. According to this model, although fewer numbers of tumor-specific effector TNFR2−/− CD8 T cells were generated at early time points, correlating with greater tumor growth on day 8 (Fig. 4), the persistence of OVA Ag in the context of tumor challenge eventually overcame the dependence of Ag-specific CD8 T cells on TNFR2. The resistance of the activated OVA-specific TNFR2−/− CD8 T cells to AICD renders them more effective in controlling tumor growth at day 20 (Fig. 4).

We also used a vaccination vehicle system to directly assess whether sufficiently activated Ag-specific TNFR2−/− CD8 T cells that formed a memory pool (i.e., >40 days post vaccination) could provide superior protection against subsequent tumor challenge bearing the immunizing Ag. Antitumor immune responses can be elicited by LM-expressing Ags from tumor-inducing viruses, such as papillomavirus, as well as tumor-associated antigens such as tyrosinase-related protein-2 (TRP2), which is expressed in its native form by the murine melanoma B16 cell line (29). It was shown that LM engineered to express TRP2 (rLM-TRP2) efficiently induces the activation of TRP2-specific T cells, as demonstrated by cytolytic activity against B16 tumors in vitro that correlated with s.c. tumor protection in vivo (29). Therefore, LM can be used as a vaccination vehicle for inducing effective antitumor T cell responses. Interestingly, we found that TNFR2−/− mice that were vaccinated with rLM-OVA were superior in controlling the growth of E.G7 tumor cells than similarly vaccinated WT mice. Although secondary expansion of OVA-specific TNFR2−/− CD8 effector memory T cells was similar to WT memory CD8 T cells, TNFR2-deficiency conferred resistance of activated TNFR2−/− CD8 memory cells to AICD upon restimulation. It is likely that this resistance to AICD contributes to the more effective control of growth of E.G7 tumor cells in rLM-OVA-vaccinated TNFR2−/− mice.

We propose the following speculative model to explain the resistance of Ag-activated TNFR2−/− CD8 T cells to AICD. Although TNFR1 contains a death domain, previous studies have shown that TNFR1 activation can lead to the induction of apoptosis and/or activation of NFκB (1). Furthermore, translocation of TNFR1 to lipid rafts is essential for TNF-mediated NFκB activation (30). We propose that TNF-induced signaling via TNFR2 results in either the degradation or sequestration of TRAF2, thereby changing the nature of the signaling complexes that are recruited by TNFR1. Under normal conditions, TNFR2 limits the amount of TRAF2 that is available to TNFR1, and the net result is that TNFR1 promotes the formation of death-inducing complexes comprising TRADD, FADD, and caspase 8 and 10 (1, 31). However, in the absence of TNFR2, we propose that higher recruitment of TRAF2 to TNFR1 facilitates their translocation to lipid rafts, thereby favoring activation of the prosurvival NFκB pathway, leading to greater resistance of activated TNFR2−/− CD8 T cells to AICD. Our preliminary data provide support for this hypothesis. We found that 2C11 plus IL-2 activated WT CD8 T cells preferentially degrade TRAF2 after TNF-α stimulation relative to similarly activated TNFR2−/− CD8 T cells (our unpublished data). Furthermore, the high susceptibility of 2C11 plus IL-2 activated WT CD8 T cells to AICD correlates with TNFR2-induced degradation of TRAF2 (our unpublished data). Thus, TNFR2 may play a critical role in regulating AICD in activated CD8 T cells by titrating the amount of TRAF2 that is available for binding TNFR1. Alternatively, because studies have demonstrated that T cell growth lymphokines cause the entry of T cells into the S phase of the cell cycle where receptor ligation causes apoptosis (32), the reduced levels of endogenously produced IL-2 by TNFR2−/− CD8 T cells during initial T cell activation may selectively keep the activated T cells in the G1 phase of the cell cycle where they are protected from AICD. Consistent with this explanation, IL-2 has been shown to predispose mature T lymphocytes to undergo apoptosis after Ag-receptor stimulation, a process which is blocked when T cells are prevented from entering into IL-2-induced S phase by rapamycin (33, 34). Regardless of which hypothesis is correct, our findings reveal a novel mechanism for enhancing secondary responses to tumor-associated Ags by influencing the susceptibility of activated CD8 T cells to AICD.

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Disclosures

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