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TNF Receptor Type 2 (p75) Functions as a Costimulator for Antigen-Driven T Cell Responses In Vivo

Edward Y. Kim, John J. Priatel, Soo-Jeet Teh, and Hung-Sia Teh

Naive T cells require costimulation for robust Ag-driven differentiation and survival. Members of the TNFR family have been shown to provide costimulatory signals conferring survival at distinct phases of the T cell response. In this study, we show that CD4 and CD8 T cells depend on TNFR type 2 (p75) for survival during clonal expansion, allowing larger accumulation of effector cells and conferring protection from apoptosis for a robust memory pool in vivo. We demonstrate using the MHC class I-restricted 2C TCR and MHC class II-restricted AND TCR transgenic systems that TNFR2 regulates the threshold for clonal expansion of CD4 and CD8 T cell subsets in response to cognate Ag. Using a novel recombinant Listeria monocytogenes (rLM) expressing a secreted form of the 2C agonist peptide (SIY) to investigate the role of TNFR2 for T cell immunity in vivo, we found that TNFR2 controls the survival and accumulation of effector cells during the primary response. TNFR2−/− CD8 T cells exhibit loss of protection from apoptosis that is correlated with diminished survivin and Bcl-2 expression. Null mutant mice were more susceptible to rLM-SIY challenge at high doses of primary infection, correlating with impaired LM-specific T cell response in the absence of TNFR2-mediated costimulation. Moreover, the resulting memory pools specific for SIY and listeriolysin O epitopes derived from rLM-SIY were diminished in TNFR2−/− mice. Thus, examination of Ag-driven T cell responses revealed a hitherto unknown costimulatory function for TNFR2 in regulating T cell survival during the differentiation program elicited by intracellular pathogen in vivo. The Journal of Immunology, 2006, 176: 1026–1035.

The adaptive immune response comprises a myriad of spatially and temporally regulated interactions between receptors and ligands. The numerous interactions are mediated by receptors on the T cell surface (TCR, adhesion molecules, cytokine receptors, costimulatory receptors), and their counterparts derived from activated APCs: MHC-peptide Ag complex, cytokines (IL-2, IL-7, IL-15), and costimulatory ligands (B7, TNF superfamily members). All components are orchestrated in sequence for effective adaptive immunity. Examination into minimal requirements for a productive T cell response revealed that brief duration of antigenic stimulation can commit CD8 T cells to several rounds of cell division and concomitant effector/memory differentiation (1, 2). These studies demonstrate that initial exposure of T cells to threshold-crossing activation signals triggered by antigenic stimulation culminates in a differentiation program used by T cells for their expansion, effector, and memory function. However, recent studies have shown that execution of this program for CD8 T cell differentiation also relies on external signals in addition to antigenic stimuli, indicating a dynamic process that yields flexibility for developing an appropriate adaptive response depending on all variables concerning host defense. For instance, greater duration of antigenic stimulation leads to increased survival of primed T cells for the Ag-independent proliferative phase of the differentiation program (3), demonstrating that TCR-mediated signals provide important input for imprinting cellular survival. External factors such as IL-2 have been shown to be important for enhancing Ag-independent proliferation after brief priming of CD8 T cells (2), strongly suggesting that external signals can integrate into the program to govern quantitative outcomes during clonal expansion. Given that the extent and magnitude of clonal expansion is directly related to survival after primary activation (4), a key regulatory modality for the progression of productive Ag-driven T cell response may be derived from both internal (i.e., TCR-mediated) and external (i.e., cytokine-mediated and costimulatory) interactions that control survival.

Costimulation has been extensively studied as an integral component of the differentiation program during the primary response (5, 6). Two families of costimulatory receptors have been identified for T cells. CD28 and TNFR superfamilies have been shown to modulate discrete aspects of T cell function, with each member playing a role in transition points during T cell differentiation. For instance, CD28 plays an established role in modulating the threshold of T cell activation, augmentation of IL-2 production, and conferring survival for sustaining the T cell response (6). TNFR superfamily members such as OX40 and 4-1BB have been implicated in effector to memory transition, acting at a distinct phase downstream of CD28 (5, 7). Mice deficient in either of these TNFR family members display a marked decrease in the memory pool due to defects in survival during and after primary expansion (8, 9). These effects have been attributed to the regulation of apoptotic molecules such as survivin and Bcl-2 mediated by costimulatory signals during clonal expansion (10). Several recent studies have investigated the role of costimulation during infection with Listeria monocytogenes (LM). CD28-deficient mice have reduced LM-specific CD8 T cell responses, compared with wild-type (WT)
mice, but differentiation into effector and memory T cells appears intact (11), suggesting that CD28 sets a critical threshold for the accumulation of LM-specific CD8 T cells. Mice that lack CD137 ligand (4-1BB ligand) also display diminished activation of CD8 T cells, compared with WT mice (12). Thus, costimulation mediated by members of two distinct superfamilies of receptors provides multiple checkpoints in the differentiation program that confers flexibility in the adaptive immune response.

LM is a well-characterized model system for studying T cell-mediated immunity against intracellular bacteria (13). LM rapidly triggers an innate response that is essential for host survival; early resistance is conferred by production of IFN-γ and TNF-α, as mice lacking these cytokines or their cognate receptors succumb to infection (14, 15). Adaptive immunity is crucial for clearance and long-term protective immunity after LM infection. SCID mice are able to control low-dose LM infection but fail to clear it (16), and mice lacking Ab T cells rapidly succumb to high-dose LM infection within 5 days (17), demonstrating the critical role that T cells play for bacterial clearance. As an intracellular bacterium, LM induces a potent CD8+ T cell response that is critical for anti-listerial defense (18, 19). Indeed, MHC class I- and class II-restricted epitopes derived from LM have been characterized facilitating the tracking of both CD4+ and CD8+ T cell responses to intracellular bacterial challenge, thereby providing a powerful system for studying cellular processes and mechanisms that regulate T cell-mediated immunity in vivo (13).

We have recently reported that TNFR type-2 (TNFR2), also referred to as p75, is an important costimulator for the differentiation program triggered by TCR-mediated stimulation. However, the role of TNFR2 in Ag-driven CD4 and CD8 T cell responses was poorly characterized. To investigate the role of TNFR2 in Ag-driven CD4 and CD8 T cell responses, we used two well-characterized TCR transgenic systems that are restricted to either MHC class I (2C) (24, 25) or MHC class II (AND) (26), as described in our Abstract. Our data establish a critical role for TNFR2 as a costimulator of Ag-driven T cell responses in vivo, and demonstrates a key modality by which progression of the differentiation program can be regulated.

Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2b) and B6-TNFR2-deficient mice were obtained from The Jackson Laboratory. B6 mice deficient in TNFR2 have been previously described (27). TNFR2-deficient mice were genotyped using a PCR strategy. Mice 4–7 wk of age were used for all experiments. Breeders for the H–2b 2C TCR transgenic mice were provided by Dr. D. Y. Loh (previously at Washington University, St. Louis, MO). The 2C TCR transgenic mice (24, 25) were bred onto the C57BL/6 (H-2b) background and crossed with TNFR2−/− mice to generate 2C TNFR2−/− mice. B10.AND TCR transgenic mice (26) were obtained from The Jackson Lab-

oratory, and subsequently crossed with TNFR2−/− mice to generate AND TNFR2−/− mice. Animal studies were approved by our institutional review board.

Cells

Lymph nodes were harvested and single cell suspensions prepared from each of the mouse lines. The CD4+ CD8− (CD8+) T cell and CD4+ CD8− (CD8+) T cell subsets were purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotech). CD4+ and CD8+ T cells from AND and 2C TCR transgenic mice, respectively, were positively selected using a MACS MS+ Separation column and MiniMACS magnet, as per manufacturer’s protocol (Miltenyi Biotech), achieving >95% purity. Splenocytes from B6 mice were irradiated for use as APCs to present the SIYRYYGL peptide (abbreviated as SIY) in complex with H-2K+ MHC class I molecules, and alone as feeder cells as indicated. The SIY/K+ complex is a cognate ligand for the 2C TCR (25). The DCEK cell line was used as APCs for displaying pigeon cytochrome c peptide (amino acid residues 119–128) in complex with H-2K+ in p75 TNFR2−/− mice. Cells were cultured at 37°C and 5% CO2 in IMDM (Invitrogen Life Technologies) supplemented with 10% (v/v) FBS (Invitrogen Life Technologies), 5 × 10−3 mM 2-ME, and antibiotics (1-media).

Abs and intracellular staining

Abs against CD4, CD8, 2C TCR (clone 1B2 mAb), Thyl.1, Thyl.2, and IFN-γ were from E Bioscience, anti-survivin (NB500-201) was from Novus Biologicals, and anti-β-catenin was from BD Pharmingen. Cell staining and flow cytometry were performed according to standard procedures. Briefly, cells were incubated with the relevant Abs for >15 min at 4°C and subsequently washed twice with FACS medium (PBS plus 2% FCS). Annexin V-FITC (BD Biosciences) staining was conducted at room temperature for 15 min in saline containing 10 mM HEPES (pH 7.2) and 2.5 mM CaCl2. For intracellular cytokine staining, cells were incubated in a fixation/permeabilization solution (2% paraformaldehyde, 0.2% Tween 20 in PBS). An- ticytokine-, CD8-, and Thy1.2-specific Abs were added to cells in PBS containing 0.2% Tween 20. The CellQuest program (BD Biosciences) and FACSscan was used for data acquisition and analysis.

Recombinant LM (rLM)-SIY and infection

A rLM-SIY was constructed to express a secreted form of an SIY-bearing peptide (J. J. Priatel, L. Zenevich, H. Shen, and H.-S. Teh, manuscript in preparation). Briefly, the Ag cassette containing the SIY-peptide, a sequence previously shown to induce strong anti-SIY responses (28), was introduced into the bacterial genome by homologous recombination as previously described (29). Mice were infected by i.v. injection of the tail vein with indicated doses in PBS. Bacterial doses were determined by plating the injected stock on brain-heart infusion agar.

In vitro restimulation for quantification of T cells specific for epitopes derived from LM

Splenocytes from infected and uninfected mice were harvested at various time points postinfection (p.i.) and restimulated with SIY, listeriolysin O (LLO)190–201, or anti-CD3 (plate-bound, 10 ng/ml) for 72 h in the presence of GolgiStop (BD Biosciences) and 10 ng/ml Golgi inhibitor (GolgiStop; BD Biosciences). Briefly, the Ag preparation (25) was added to the injected stock on brain-heart infusion agar.

Results

TNFR2 regulates threshold for clonal expansion of Ag-specific CD8 and CD4 T cells

2C is a well-characterized transgenic TCR that recognizes cognate Ag (SIY peptide) in the context of MHC class I (Kb) (24, 25). We used this transgenic system to investigate whether TNFR2 plays an important role for the CD8 T cell response to cognate Ag. Using an assay in which the number of peptide-loaded APCs (irradiated B6 splenocytes) was titrated with proportionate numbers of feeder cells (irradiated B6 splenocytes not coated with peptide) so as to keep a total cell number in culture constant, we found that 2C TNFR2−/− CD8 T cells are hyporesponsive relative to WT cells at
limiting Ag conditions. As shown in Fig. 1A, 2C CD8 T cells initiated a program of clonal expansion in response to $1 \times 10^5$ peptide-loaded APCs, undergoing multiple rounds of cell division. In contrast, 2C TNFR2 $^{-/-}$ CD8 T cells failed to undergo any cell division at this dose. This result is consistent with our previous findings that TNFR2 lowers the threshold of T cell activation (20): increasing antigenic stimulation (i.e., dose of peptide-loaded APCs) results in an increase in the proportion of cells that crossed this threshold to initiate a program of CD8 T cell differentiation and cell division, such that 2C TNFR2 $^{-/-}$ CD8 T cells required excess peptide-loaded APCs to undergo robust proliferation. Although 2C TNFR2 $^{-/-}$ CD8 T cells underwent multiple rounds of cell division when stimulated with $4 \times 10^5$ peptide-loaded APCs, we noted that the total number of cells in culture was diminished, compared with WT cells (Fig. 1B) suggesting that the survival of CD8 T cells undergoing cell division may be controlled by TNFR2.

AND is a well-characterized transgenic TCR that recognizes cognate Ag (pigeon cytochrome c peptide, amino acid residues 88–104) in the context of MHC class II (H-2 I-E$^k$) (26). To determine whether TNFR2 is important for costimulating CD4 T cell response to cognate Ag, the peptide dose was titrated in culture with APCs (DCEK cell line expressing MHC class II-E$^k$) in the presence or absence of exogenous IL-2. As shown in Fig. 1C, AND TNFR2 $^{-/-}$ CD4 T cells failed to undergo clonal expansion at the lowest Ag dose tested (0.01 $\mu$M), whereas 63% (100 $\pm$ 37) of AND CD4 T cells underwent multiple rounds of cell division. AND TNFR2 $^{-/-}$ CD4 T cells required greater doses of cognate

**FIGURE 1.** TNFR2 regulates clonal expansion of Ag-specific CD4 and CD8 T cell responses in vitro. A, 2C TNFR2 $^{-/-}$ CD8 T cells display defective clonal expansion under Ag-limiting conditions that is associated with increased threshold for cell division and survival. A total of $1 \times 10^6$ 2C or 2C TNFR2 $^{-/-}$ CFSE-labeled CD8 T cells was cultured with indicated ratios of peptide-loaded APCs (irradiated B6 splenocytes pulsed with 1 mM SIY peptide) to feeder cells (irradiated B6 splenocytes), harvested at day 3 and analyzed by FACS (see Materials and Methods). B, The total number of 2C (1B2$^+$) TNFR2 $^{-/-}$ CD8 T cells in culture was significantly reduced, compared with WT cells. The percentage of 1B2$^+$ CD8$^+$ cells was multiplied by total numbers of live cells in culture. Statistical significance (*, $p < 0.05$) of three independent experiments is shown. C, AND TNFR2 $^{-/-}$ CD4 T cells display defective clonal expansion that is associated with increased threshold for cell division, and only partially rescued by exogenous IL-2. A total of $1 \times 10^6$ AND or AND TNFR2 $^{-/-}$ CFSE-labeled CD4 T cells was cultured with peptide-loaded APCs (irradiated DCEK cells pulsed with indicated concentration of pigeon cytochrome c (PCC) peptide), harvested on day 3 and analyzed by FACS (see Materials and Methods). Data are representative of three independent experiments.
Ag to achieve an equivalent response as WT counterparts, demonstrating the role of TNFR2 in regulating threshold for clonal expansion. Interestingly, exogenous IL-2 provided only a modest increase in the clonal expansion of AND TNFR2−/− CD4 T cells at the lowest Ag dose, whereas AND CD4 T cells underwent robust cell division (Fig. 1C). These data demonstrate that TNFR2 is an important costimulatory molecule for CD4 and CD8 subsets in regulating the threshold for clonal expansion in response to Ag.

**CD8 T cells depend on TNFR2 for survival during the early phase of the T cell response to rLM-SIY**

LM is a well-characterized intracellular pathogen infection model for measuring T cell responses in vivo. We engineered a rLM that expresses the 2C agonist peptide (SIY) (Priatel et al., manuscript in preparation) to investigate whether TNFR2 functions as a costimulator for T cell responses to intracellular pathogens. Following adoptive transfer of CFSE-labeled 2C or 2C TNFR2−/− CD8 Thy1.2 T cells into B6-Thy1.1 mice, rLM-SIY was used to infect hosts (10⁴ CFU) and spleens were subsequently harvested on days 3 and 7 p.i. (Fig. 2, A and B respectively). Adoptively transferred cells were tracked using the congenic marker Thy1.2 and the clonotypic transgenic 2C TCR (with mAb 1B2), analyzed for cell division using CFSE, and differentiation into effectors using intracellular cytokine staining of IFN-γ. The first rounds of cell division by 2C Thy1.2 CD8 T cells in the spleens of response to rLM-SIY was visualized by CFSE on day 3 p.i.; the WT population comprised ~0.58% of the total spleen (Fig. 2A), or ~90 × 10⁴ 2C CD8 T cells (total numbers of transferred cells per spleen) (Fig. 2C). In contrast, 2C TNFR2−/− Thy1.2 CD8 T cells exhibited a marked reduction in clonal expansion, comprising only 0.13% of the total spleen of infected mice, or ~20 × 10⁴ 2C TNFR2−/− CD8 T cells (Fig. 2C), resulting in ~4.5-fold reduction in clone size, compared with WT population. However, CFSE dilution analysis showed that 2C TNFR2−/− CD8 T cells were able to undergo multiple rounds of cell division at this dose of rLM-SIY infection; the diminished clone size therefore suggests that TNFR2 controls T cell survival once the program of cell division is initiated. Interestingly, the differentiation program for transiting 2C TNFR2−/− CD8 T cells into effectors appeared intact because surviving cells expressed IFN-γ after brief restimulation with SIY peptide in vitro (Fig. 2A). These data indicate that TNFR2 regulates the accumulation of effector cells in response to intracellular pathogen and suggest that the survival of CD8 T cells during the first rounds of cell division is dependent on TNFR2.

We next quantified the frequency of adoptively transferred cells at the peak of the T cell response to LM challenge (1 wk p.i.) (13). Examination of the numbers of Thy1.2+ cells in infected spleens at 1 wk p.i. revealed a significant reduction in 2C TNFR2−/− CD8 T cells (1.02 vs 0.33%; Fig. 2C). Whereas 2C CD8 T cells continued to expand to reach peak numbers at 1 wk p.i. (~150 × 10⁴ 2C Thy1.2+ CD8 T cells), the frequency of 2C TNFR2−/− CD8 T cells in the spleens of infected mice was reduced ~5-fold (Fig. 2C). 2C CD8 T cells underwent multiple rounds of cell division as demonstrated by complete dilution of CFSE; strikingly, 2C TNFR2−/− CD8 T cells were virtually devoid of this clonally expanded population, suggesting a loss of protection from apoptosis during expansion (Fig. 2C). This dramatic defect is highlighted by the substantial reduction in the frequency of 2C TNFR2−/− IFN-γ+ CD8 effector cells in the spleens of infected mice. Furthermore, the small residual population of TNFR2−/− 2C CD8 T cells present at day 7 p.i. did not produce IFN-γ after Ag stimulation (Fig. 2B), suggesting that this population is either anergic or Ag inexperienced.

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**FIGURE 2.** TNFR2 is critical for clonal expansion of CD8 T cells in response to secreted peptide expressed by rLM. A total of 2 × 10⁶ CFSE-labeled 2C or 2C TNFR2−/− CD8 Thy1.2 T cells was adoptively transferred into B6-Thy1.1 mice (transferred cells were tracked using the congenic marker Thy1.2) and analyzed for cell division using CFSE and differentiation into effectors using intracellular cytokine staining of IFN-γ. One day after transfer, rLM-SIY was used to infect hosts (10⁴ CFU) and spleens were subsequently harvested on days 3 (A) and 7 (B) p.i. Uninfected mice (open histogram) were used to denote a negative control. C, Total numbers of B220+ (2C) IFN-γ+ CD8+ T cells were enumerated by multiplying the percentage of the population in the spleen and the total number of viable splenocytes. Statistical significance (*) is shown for three mice per group. D, Loss of protection from apoptosis during the first rounds of cell division in TNFR2−/− CD8 T cells. Spleens were harvested on day 3 p.i. using the adoptive transfer-infection model as described, and CFSE vs annexin V was analyzed using flow cytometry (gated on Thy1.2+ cells, all of which were CD8+B220+). Data are representative of three independent experiments.

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We next tested the susceptibility of TNFR2−/− T cells to apoptosis during clonal expansion. Annexin V staining in conjunction with CFSE dilution revealed that a large proportion of 2C TNFR2−/− CD8 T cells undergo cell death during the first few rounds of cell division; virtually all of the cells that were recruited to the dividing population were annexin V⁺, whereas WT counterparts contained an annexin Vneg population that persisted through multiple rounds of division (Fig. 2D). Thus TNFR2 confers protection from apoptosis during the first rounds of cell division. In this staining we noted a population of annexin V⁺ cells from spleens of infected WT mice, which we believe correspond to T cells that die by attrition early during the response to LM. Adoptively transferred cells in uninfected mice remained CFSEhigh and annexin Vneg (data not shown). This is consistent with previously reported observations of selective depletion of T cells en masse via apoptosis in the spleens of mice infected with proinflammatory pathogens such as LM (30). Our results suggest that costimulation mediated via TNFR2 is important in rescuing Ag-specific T cells from generalized depletion that occurs during the early stages of LM infection.

We previously showed that TNFR2 lowers the threshold of T cell activation and survival (21), and so we investigated whether increasing antigenic doses of primary challenge with rLM-SIY would increase the proportion of 2C TNFR2−/− CD8 T cells that survive to form the effector pool. As shown in Fig. 3A, increasing doses in log increments of LM infection led to increased numbers of 2C and 2C TNFR2−/− CD8 T cells in the spleens of infected mice on day 4 p.i. However, TNFR2 deficiency led to a >8-fold reduction in frequency of SIY-specific 2C CD8 T cells even at the highest dose of rLM-SIY tested. CFSE dilution revealed that 2C TNFR2−/− CD8 T cells were able to undergo multiple rounds of cell division (Fig. 3B), confirming that 2C TNFR2−/− CD8 T cells were activated by rLM-SIY infection but failed to survive during clonal expansion. These data suggest that the Ag-driven differentiation program can be aborted via apoptosis in the absence of TNFR2.

Song et al. (10) recently showed that survivin expression promotes proliferation and antagonizes apoptosis, leading to enhanced accumulation of effector T cells. T cell survival during Ag-driven response is also dependent on costimulation-mediated expression of antiapoptotic Bcl-2 family members (31, 32), particularly after the phase of cell division (10). We further investigated TNFR2-mediated survival by examining the expression kinetics of antiapoptotic molecules survivin and Bcl-2 during T cell activation in vitro (Fig. 4). TNFR2 deficiency led to a dramatic reduction in the percentage of survivin⁺ CD8 T cells in culture: 87% for WT vs 67% for TNFR2−/− on day 2 and 67% for WT vs 23% for TNFR2−/− on day 4. Bcl-2 expression was also regulated by TNFR2 during T cell activation, as the percentage of Bcl-2high CD8 T cells was dramatically lower in TNFR2−/− CD8 T cells on day 2 of culture: 82% for WT vs 51% for TNFR2−/−. Moreover, TNFR2 regulated the proportion of Bcl-2high T cells as well as Bcl-2 expression per cell on day 4: ~64% of 2C CD8 T cells were Bcl-2mean fluorescence intensity of 361), whereas only ~5% of 2C TNFR2−/− CD8 T cells were Bcl-2high (mean fluorescence intensity of 199). These data indicate that TNFR2 regulates the expression of antiapoptotic molecules that confer survival during and after cell division, and correlates with its role for conferring protection against apoptosis during the Ag-driven T cell response. TNFR2 is important for endogenous CD4 and CD8 T cell responses to rLM-SIY

The adoptive transfer-infection model we describe allowed us to track the response of a clonotypic population of CD8 T cells to rLM-SIY in vivo. We were next interested in investigating the endogenous CD4 and CD8 T cell response to epitopes derived from rLM-SIY simultaneously in the same animal. We noted that infection of mice with rLM-SIY elicited robust endogenous responses specific for SIY (MHC class I-restricted peptide) and LLO190–201 (MHC class II-restricted, endogenous peptide derived from LLO) peptides in host animals (33, 34). This way we could examine the importance of TNFR2 in the endogenous CD8 and CD4 T cell responses to MHC class I- and class II-restricted epitopes of rLM-SIY in the same host. TNFR2−/− or WT animals were infected with indicated doses of rLM-SIY, and the frequency of SIY-specific CD8 and LLO-specific CD4 T cells were determined by intracellular cytokine staining of IFN-γ in response to brief peptide restimulation in vitro. We investigated the course of T cell response in WT and TNFR2−/− mice to gain insight on the total numbers of CD4 and CD8 T cells specific for epitopes derived from rLM-SIY as a function of time after primary challenge. Examination of the kinetics of CD8 and CD4 T cell responses confirmed the defect in clonal expansion of TNFR2−/− T cells specific for epitopes derived from rLM-SIY, as indicated by dramatic reductions across the time points tested (SIY-specific CD8 T cell responses to rLM-SIY).
TNFR2 regulates clonal expansion of CD8 and CD4 T cells against MHC class I- and class II-restricted epitopes of rLM-SIY. WT and TNFR2−/− CD4 and CD8 T cells were adoptively transferred into B6 or TNFR2−/− hosts and subsequently infected with rLM-SIY. Transferred cells underwent robust clonal expansion in both WT and TNFR2−/− hosts (Fig. 6), indicating that Ag presentation was intact and that the reduction in the T cell response was not due to a T cell extrinsic defect. Taken together, these data strongly suggest that TNFR2 can regulate the response of CD8 and CD4 T cell subsets to intracellular bacterial pathogen by promoting the accumulation of effector cells and lowering the threshold of antigenic stimulation required for optimal clonal expansion.

**TNFR2 is important for immunity against high doses of rLM-SIY challenge**

Previous reports have suggested that T cell-mediated immunity is important for clearance of LM infection when challenged with high doses of bacteria (16, 17). We therefore examined bacterial load in the spleens of infected mice to determine whether the substantial reduction in the T cell response observed for TNFR2−/− mice correlates with increased susceptibility to rLM-SIY infection. When challenged with lower doses of rLM-SIY, bacterial load in the spleen peaked around day 3 p.i. in WT and TNFR2−/− mice (Fig. 7). However, TNFR2−/− mice were more susceptible when challenged with high doses of rLM-SIY; bacterial load persisted in the spleens of TNFR2−/− mice at day 5, whereas WT mice were able to clear bacteria. Thus, the diminished clonal expansion of CD8 and CD4 T cells specific for epitopes derived from rLM-SIY between days 3 and 5 p.i. correlates with increased susceptibility to infection when challenged with a high dose. It also suggests that the bacterial burden on day 5 postinfection provided increased antigenic stimulation in driving expansion of rLM-SIY-specific T cells in TNFR2−/− mice observed at day 7 p.i., which in turn coincided with clearance of rLM-SIY in the spleens of TNFR2−/− mice.

We were also interested in whether TNFR2 deficiency correlates with increased susceptibility to virulent LM challenge. To address this question we used the WT strain of LM, which possesses >10-fold greater virulence than rLM-SIY (data not shown). Consistent with rLM-SIY challenge, TNFR2−/− mice displayed a dramatic decrease in the frequency of LLO-specific CD4 T cell effectors (Fig. 8A) in the spleens of infected mice, compared with WT, corresponding to a 6-fold reduction in the number of LLO-specific CD4 T cells at the peak of the primary response against WT LM (Fig. 8B). WT mice were able to clear bacteria by 1 wk p.i. In...
contrast, TNFR2−/− mice displayed delayed clearance of bacteria, demonstrated by titers that persisted at day 7 p.i. (data not shown). These data indicate that TNFR2 is important for mounting a strong T cell response to high-dose challenge with virulent LM, which correlates with efficient clearance of bacteria from the spleens of infected mice.

**FIGURE 7.** TNFR2-deficient mice display delayed bacterial clearance upon challenge with high dose of rLM-SIY. Bacterial load in spleens of infected mice at indicated doses (Lo = 1 × 10⁵, Med = 1 × 10⁶, Hi = 1 × 10⁷ CFU) of primary challenge with rLM-SIY (see Materials and Methods). Statistical significance (*) is denoted. Data is representative of three independent experiments.

**Generation of memory T cells against rLM-SIY is compromised in TNFR2−/− mice**

After expansion of Ag-specific T cells and concomitant effector function against intracellular pathogen, massive contraction by apoptosis ensues leaving a relatively small population of memory T cells (reviewed in Ref. 35). Pope et al. (36) showed that the magnitude of the primary response to LM is correlated with the size of the resulting Ag-specific memory population. We therefore examined whether the diminished T cell response in TNFR2−/− mice against rLM-SIY would correlate with a decrease in the resulting memory pool 3 mo after primary challenge. As shown in Fig. 9, A and B, the frequency of SIY-specific CD8 and LLO-specific CD4 T cells, respectively, was decreased 3-fold in the spleens of TNFR2−/− mice, compared with WT mice. Consistent with results reported by Pope et al. (36), we found that increasing the dose of primary challenge led to an increase in the frequency of Ag-specific memory T cells (Fig. 9, A and B). The percentage of SIY-specific CD8 and LLO-specific CD4 T cells that survived from the peak of the primary response to form the resulting memory pools were ~13 and 10% for WT mice, respectively, whereas TNFR2−/− mice were 6 and 4%, respectively for SIY-specific (Fig. 9C) and LLO-specific (Fig. 9D) memory T cells. Although higher doses of primary challenge with rLM-SIY led to an increase in frequency of the memory populations, TNFR2−/− mice were still compromised, compared with WT mice suggesting that TNFR2 promotes survival during the generation of the memory population. These data indicate that TNFR2 can regulate the size of the resulting memory pool against intracellular pathogen by promoting optimal primary expansion and subsequent survival following bacterial clearance.

**Discussion**

The Ag-driven T cell differentiation program consists of clonal expansion and concomitant differentiation into effector and memory cells. The threshold of T cell activation is dependent on interactions besides TCR and MHC-peptide complexes, namely costimulation by molecules such as CD28 (37). Indeed, CD28 has been reported to reduce the time necessary for antigenic stimuli to activate naive T cells, as well as to augment the magnitude of T cell responses for both naive and primed T cells (38). Previous reports have suggested a molecular link between cell division and effector cytokine expression (39, 40), indicating that T cell immunity is functionally productive once a threshold has been reached for initiating a differentiation program of multiple rounds of cell division and subsequent acquisition of effector function. More recently however, members of the TNFR superfamily (such as OX40 and 4-1BB) have been shown to function at distinct phases during the T cell response (7); notably, costimulation appears to confer T cell survival for driving clonal expansion of cells that progress through the differentiation program (10, 32). These studies broaden
the roles for costimulation to include temporal and spatial regulation of distinct receptor-ligand interactions that profoundly influence T cell fate during differentiation by controlling survival.

The T cell response is a dynamic and flexible process that depends on signals that maximize the pool of effectors and formation of memory by conferring survival through “check points.” Members of two different families of receptors (CD28-related Ig-like superfamily and the TNFR superfamily) appear to govern specific outcomes of T cell fate: CD28 and CD27 appear to function in promoting T cell expansion during the early phase, whereas OX40 and 4-1BB control survival late in the primary response (5, 7). Our data show that TNFR2 plays a critical role during the early phase of the T cell response by conferring survival during the first rounds of cell division, and thereby regulate the extent of clonal expansion at the peak of the response. Interestingly, the expression of anti-apoptotic molecules survivin and Bcl-2 was dependent on TNFR2 and correlated with protection from apoptosis during and after cell division. Song et al. (10) showed that OX40-mediated expression of survivin antagonizes apoptosis during proliferation, whereas Bcl-2 was required for the phase after cell division. Antiapoptotic molecules are therefore common targets of costimulatory signals derived from distinct cell surface receptors for controlling survival at distinct phases of the T cell response.

The dramatic reduction in the clone size of Ag-specific TNFR2−/− T cells was associated with increased susceptibility to apoptosis as well as an increased requirement for greater antigenic stimulation (i.e., increased threshold of activation). In the absence of TNFR2, CD4 and CD8 T cells required greater antigenic stimulation and the clone size of effectors was thereby greatly diminished in vitro. However, the differentiation program as measured by activation markers such as CD44, CD69, and CD62 ligand appeared intact in TNFR2−/− T cells (data not shown), indicating that TNFR2 regulates threshold of activation, with surviving T cells retaining the ability to differentiate into effectors. Taken together, our data reveal a hitherto undefined costimulatory role for TNFR2 in regulating threshold of activation and early cell survival during Ag-specific T cell responses, regulating quantity rather than quality of effector cells generated.

We tested whether TNFR2-mediated costimulation was important for T cell-mediated immunity against LM challenge. Our data show that TNFR2 conferred a survival advantage for CD4 and CD8 T cells during challenge with LM, whereas null mutants exhibited a dramatic reduction in the T cell response that correlated with increased susceptibility to primary challenge. These findings appear in contrast with previous studies that showed that TNFR2 did not play a significant role in the susceptibility of mice to bacterial challenge (41, 42), as bacterial loads were cleared by day 5 p.i., and survival of TNFR2-deficient mice was comparable to WT control (42). Although these studies identified a critical role for TNFR1 (p55) in the inflammatory innate response to LM, they did not analyze the T cell response to LM; the endpoints measured in these studies using TNFR2−/− mice are largely attributable to innate immunity. By engineering rLM-SIY system to specifically monitor CD8 T cells specific for secreted epitope derived from intracellular bacteria as well as CD4 T cells specific for the endogenous epitope LLO, our data revealed a critical role for LM-specific CD4 and CD8 T cell expansion stemming from defective survival during the early phase of the response. The smaller pool

**FIGURE 8.** TNFR2−/− mice display reduced CD4 T cell response specific for WT strain of LM. A, WT or TNFR2−/− mice were infected with WT LM and spleens harvested 1 wk after. To quantify the frequency of CD4 T cells specific for LLO190–201, splenocytes were restimulated with this peptide in vitro for 5 h and then analyzed for IFN-γ expression via intracellular FACS staining (see Materials and Methods). As a positive control, splenocytes were also restimulated with 10 μM plate-bound anti-CD3. Values atop each gate represent the percentage in each dot plot of IFN-γ+ CD4+ cells in the total cell count from spleen. B, Total numbers of LLO190–201-specific CD4 T cells are diminished at the peak of the response to WT LM. Statistical analyses performed with three mice per group.
of effector T cells that resulted from TNFR2 deficiency correlated with increased susceptibility to higher doses of primary LM challenge, which has been previously shown to be dependent on T cell-mediated immunity (16, 17). Our results are consistent with a recent report that showed an important role for T cell-derived TNF-α, which found that TNF-α knocked out in the T cell lineage resulted in increased susceptibility to LM challenge, as well as inability to control bacterial load by day 4 p.i. (43). Moreover, our results are consistent with the implication that T cell-derived TNF-α possesses a distinct function in host defense by providing protection against high bacterial load (43), constituting a second line of defense. We extend these implications to suggest that TNF-TNFR2 interactions provide costimulation of the primary T cell response against LM, regulating threshold of activation and survival during the generation of effector T cells, which is important for mounting effective T cell-mediated clearance at a high dose of LM infection.

The role of costimulation during infection has been studied using gene-targeted mice (13). The costimulatory role of TNFR2 during LM infection appears similar to CD28; CD28−/− mice possess reduced LM-specific CD8 T cell responses, compared with WT mice, but differentiation into effector T cells is intact (11, 12). Moreover, CD28−/− mice exhibited increased susceptibility to LM as evidenced by persistent bacterial load even up to day 7 p.i. (11, 12). This result is in contrast to 4-1BB-deficient mice that were able to clear bacteria, and the activation of the CD4 T cell compartment appeared intact, whereas the number of Ag-specific CD8 T cells were slightly reduced compared with WT (12). The phenotypic similarity between CD28 and TNFR2 suggests an interesting functional link during the early phase of the T cell response to infection, as we had proposed previously based on commonality of signaling intermediates used for IL-2 induction and T cell survival (21). CD28 and TNFR2 likely perform nonredundant and complementary functions because signaling defects in TNFR2−/− T cells are not rescued by CD28 signaling (21). Two recently characterized costimulatory members of the TNFR superfamily, namely 4-1BB and OX40, were found to have spatially and temporally segregated roles during the T cell response: OX40 ligand-deficient mice exhibited decreased CD4 T cells late in the primary response and no detectable secondary expansion of adoptively transferred CD4 T cells, whereas 4-1BB ligand deficiency had a minor effect on the primary response of CD4 T cells, but affected the secondary response (44). OX40/4-1BB ligand double knockout mice were impaired in both the CD4 and CD8 T cell response to both protein Ag and influenza virus (44). Our data strongly suggest that TNFR2 is positioned early during the T cell response, regulating the threshold for clonal expansion and early survival during the first rounds of cell division. This role translates into optimal clonal expansion in response to intracellular bacterial pathogen and robust generation of the Ag-specific memory T cell population. These data support a dynamic model for the T cell

![FIGURE 9.](http://www.jimmunol.org/)

TNFR2−/− mice are compromised in the generation of Ag-specific memory populations against rLM-SIY. WT and TNFR2−/− mice were infected with indicated doses of rLM-SIY, and spleens were harvested 81 days p.i. Splenocytes were restimulated for 5 h with SIY (A) or LLO190–201 (B) peptides and IFN-γ expression analyzed by intracellular FACS. C and D, The frequency of SIY-specific CD8 and LLO-specific CD4 memory T cells that survived from the peak of the primary response is compromised in TNFR2−/− mice. Percentages (average of four mice per group) were determined by dividing the total numbers of SIY-specific CD8 or LLO-specific CD4 T cells at day 81 p.i. by the total number at day 7 p.i. The total numbers of SIY- and LLO-specific memory T cells at day 81 p.i. was determined by multiplying the percentage of IFN-γ+ cells in response to the respective peptide re-stimulation by the total number of splenocytes. Statistical significance (∗) of four mice per group is shown.
responses, with multiple interactions temporally and spatially segregated for determining T cell fate and conferring flexibility for adaptive immunity.

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Disclosures

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