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Tumor Necrosis Factor Receptor 1 Mediates Dendritic Cell Maturation and CD8 T Cell Response through Two Distinct Mechanisms

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Abstract

Tumor necrosis factor alpha (TNF α) and its two receptors (TNFR1 and 2) are known to stimulate dendritic cell (DC) maturation and T cell response. However, the specific receptor and mechanisms involved *in vivo* are still controversial. Here we show that in response to an attenuated mouse hepatitis virus (MHV) infection, DCs fail to mobilize and up-regulate CD40, CD80, CD86, and MHC class I in TNFR1^{-/-} mice as compared to the wild-type and TNFR2^{-/-} mice. Correspondingly, virus-specific CD8 T cell response was dramatically diminished in TNFR1^{-/-} mice. Adoptive transfer of TNFR1-expressing DCs into TNFR1^{-/-} mice rescues CD8 T cell response. Interestingly, adoptive transfer of TNFR1-expressing naïve T cells also restores DC mobilization and maturation and endogenous CD8 T cell response. These results show that TNFR1, not TNFR2, mediates TNF α stimulation of DC maturation and T cell response to MHV *in vivo*. They also suggest two mechanisms by which TNFR1 mediates TNF α -driven DC maturation: a direct effect through TNFR1 expressed on immature DCs and an indirect effect through TNFR1 expressed on naïve T cells.

Introduction

Initiation of a T cell response requires T cell-dendritic cell (DC) interactions through two sets of receptors: T cell receptor (TCR) recognition of antigen in the form of peptides presented by the major histocompatibility complex (MHC) molecules and binding of the co-stimulatory molecule CD28 on naïve T cells to its ligands CD80 (B7.1) and CD86 (B7.2) expressed on mature DCs (1, 2, 3). DCs are sentinels and normally reside in the tissues in an immature state without expressing CD80 and CD86 (5). Following microbial infection, immature DCs take up and process antigens to present them as peptides with MHC (5, 11).

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Immature DCs are also activated to express the co-stimulatory molecules through pathogen recognition receptors, such as toll-like receptors (TLR) (6), that recognize evolutionarily conserved microbial components or pathogen-associated molecular patterns (PAMPs) (7). By the time DCs migrate from the site of infection to the draining lymph nodes, they have matured, present antigenic peptides and express co-stimulatory molecules (4, 8). In the draining lymph node, the mature DCs activate cognate naïve T cells by providing two stimulating signals (8).

In addition to PAMPs, cytokines can also stimulate DC maturation and expression of CD80 and CD86 (9). Tumor necrosis factor α (TNF α) and its family member lymphotoxin α (LT α) are potent cytokines secreted by activated macrophages and T cells (10, 11, 12) and exerts pleiotropic effects on inflammation and immunity through two receptors: TNFR1 (p55) and TNFR2 (p75), which are ubiquitously expressed by most nucleated cells (13, 14). Treatment of DCs with TNF α , but not LT α , *in vitro* leads to up-regulation of surface expression of MHC class I, class II, CD80 and CD86 and an enhanced T cell stimulatory activity (12, 38). Transgenic expression of TNF α in DCs results in elevated levels of CD40, CD80 and ICAM-13 expression and induction of a stronger mixed lymphocyte reaction (15). In TNF α -deficient (TNF $\alpha^{-/-}$) mice, DC maturation is impaired and T cell response is diminished following infection with a replication deficient recombinant adenovirus (rAd) (16). Adoptive transfer of antigen-primed wild-type DCs into TNF $\alpha^{-/-}$ mice rescues T cell response. Although in LT $\alpha^{-/-}$ mice the number of DCs in the spleen was reduced, the observed deficiency is likely due to a lack of the membrane-bound LT α/β but not soluble LT α , that signals through LT β R (39, 40, 41, 42). Furthermore, the defective DC maturation and CD8 T cell responses in TNF $\alpha^{-/-}$ mice suggest that LT α does not replace TNF α function in these processes. Together, these results suggest a critical role of TNF α , but not LT α , in DC maturation *in vivo*.

The role of TNF α /TNFR in DC maturation and T cell response, however, is more complex. In contrast to the diminished T cell response to rAd in TNF $\alpha^{-/-}$ mice (16), following Mycobacterium infection, antigen-specific CD8 T cells, as identified by peptide-MHC tetramer staining, are significantly elevated at 14 and 27 days post infection (dpi) (17). Similarly, following acute or chronic infections with lymphocytic choriomeningitis virus (LCMV), while the magnitude of virus-specific CD8 T cell response is similar between wild-type and TNFR1 $^{-/-}$ or TNFR2 $^{-/-}$ mice 8 days post infection (dpi), the response is much greater in TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ double knockout mice (18, 19). Seven days post influenza virus infection, virus-specific CD8 T cell response is enhanced in TNFR2 $^{-/-}$ mice (20). These apparently contradictory results of CD8 T cell responses to different infections in TNF $\alpha^{-/-}$, TNFR1 $^{-/-}$, TNFR2 $^{-/-}$, and TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ double knockout mice raise at least two questions: What is the likely cause contributing to the different effects of TNF α and TNFR on CD8 T cell responses to different infections in knockout mice? Through which receptor does TNF α mediate DC maturation *in vivo*? Consistent with a much lesser important role of LT α in DC maturation, LT $\alpha^{-/-}$ mice make delayed but effective CD8 T cell response to influenza virus infection (43) and the defective CD8 T cell responses to LCMV likely result from abnormal lymphoid architecture (44).

Further complicating the delineation of the role of TNF α /TNFR in DC maturation is the observation that T cells are required for efficient DC maturation, including CD80 and CD86 expression. In *RAG1* $^{-/-}$ mice, which are deficient in T cells, the number of DCs is significantly reduced, and the residual DCs are defective in expressing CD80 and CD86 and activating naïve T cells (21). Both the deficiency in DC number and function is restored by adoptive transfer of naïve T cells into the *RAG1* $^{-/-}$ mice (22). Recently, B7-H1 (PD-L1) expressed by naïve T cells is shown to condition immature DCs to undergo efficient maturation following stimulation by influenza virus (23). Given the complex effects of

TNF α /TNFR on CD8 T cell responses, it is possible that TNF α or TNFRs may affect DC maturation and T cell response through multiple pathways.

In this study, we have constructed an attenuated mouse hepatitis virus A59 (MHV-A59) that infects mice but does not cause apparent liver damage. By comparing DC maturation and CD8 T cell response in wild-type, TNFR1^{-/-} and TNFR2^{-/-} mice following infection with the attenuated MHV-A59, we show that CD8 T cell response is impaired in the absence of TNFR1, but not TNFR2. The impaired CD8 T cell response is associated with an impaired DC maturation and mobilization and can be corrected by adoptive transfer of wild-type DCs into TNFR1^{-/-} mice. Furthermore, DC mobilization and maturation and CD8 T cell response in TNFR1^{-/-} mice are also restored by adoptive transfer of TNFR1-expressing naïve T cells. These findings suggest that TNFR1 mediates DC maturation through two mechanisms: a direct effect through TNFR1 expressed on immature DCs and an indirect effect through TNFR1 expressed on naïve T cells. Our results also suggest a possible explanation for the divergent effects of TNF α and TNFR deficiencies on CD8 T cell responses to different infections.

Materials and Methods

Viruses, mice and infection

Wild-type MHV-A59, referred to as A59/WT, was engineered to express eGFP, ISQAVHAAHAEINEAGR (OVA₃₂₃₋₃₃₉) and SIYRYYYGL (SIY), referred to as RA59/GOS. The recombinant RA59/GOS was constructed by replacing ORF4 in A59/WT with a sequence coding for eGFP-OVA-SIY fusion protein through targeted RNA recombination (24, 33). Briefly, a 90 bp DNA fragment containing OVA-SIY sequences was cloned in frame into the 3' end of eGFP. The DNA fragment encoding eGFP-OVA-SIY fusion protein was then cut out and cloned into pMH54 plasmid (25, 33) via Sal I and Not I sites, referred to as pMH54-eGFP-OVA-SIY. *In vitro* transcription using pMH54-eGFP-OVA-SIY plasmid as a template led to production of corresponding RNA that was flanked by A59/WT sequences. To construct RA59/GOS virus, AK-D cells were infected for 4 hrs with feline MHV (fMHV) (25, 33), which is a recombinant MHV-A59 containing the ectodomain of the feline infectious peritonitis virus spike glycoprotein in place of the A59/WT spike protein. The infected AK-D cells were electroporated with *in vitro* transcribed RNA for eGFP-OVA-SIY and plated on a layer of murine 17Cl-1 cells. Targeted recombination between fMHV and *in vitro* transcribed RNA led to generation of recombinant MHV where ORF4 of A59/WT was replaced by eGFP-OVA-SIY. The correct recombinant RA59/GOS was identified by eGFP expression and sequencing, and plaque purified twice on 17Cl-1 cells. Both A59/WT and RA59/GOS viruses were amplified and titrated by plaque assay in 17Cl-1 cells (26). MHV-A59, fMHV, pMH54 plasmid, 17Cl-1 and AK-D cell lines are all kindly provided by Dr. Ralph Baric (Chapel Hill, USA).

C57BL/6 (B6) mice were purchased from Vitalriver (Beijing, China). TNFR1^{-/-} mice on a mixed genetic background of 129/Sv/Ev and B6 were kindly provided by Dr. Horst Bluethmann (27). TNFR2^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, USA). Both strains of mice were backcrossed for more than 18 generations onto the B6 background. Thy1.1⁺ B6 mice were a gift from Dr. Shengdian Wang. Mice were housed under specific-pathogen-free conditions in the animal facilities at the Institute of Biophysics, Chinese Academy of Sciences. All mouse work was performed according to the guidelines of the Animal Ethics and Experimentation Committee of the Institute of Biophysics. Every batch of mice used in this study was sampled to ensure no prior MHV infection (i.e. negative for anti-MHV antibody in the serum). Mice at 6–8 weeks of age were infected with A59/WT or RA59/GOS virus intraperitoneally (i.p.) in 500 μ l phosphate-buffered saline (PBS).

Antibodies, tissue preparation and flow cytometry

Mice were sacrificed and perfused with 5 ml of PBS. Spleens were removed and gently homogenized through a nylon filter with a syringe plunger in RPMI 1640 medium supplemented with 2% fetal calf serum (FCS). Red blood cells in splenocyte suspension were lysed with 144 mM ammonium chloride and 17 mM Tris-HCl, pH7.4 solution. Single-cell suspension from liver was obtained by cutting liver tissue into pieces followed by 1mg/ml collagenase IV digestion at 37°C for 30 minutes. The lysate was centrifuged at 50g for 1 minute to remove debris, re-suspended in 5 ml of 40% Percoll solution and then layered over 5 ml of 70% Percoll solution and centrifuged at 1500 g for 20 minutes without break at room temperature. Cells were removed from the interface, washed, and counted by trypan blue exclusion.

All antibodies were purchased as direct conjugates. Anti-CD3-FITC, CD8-APC, CD40-PE, CD80-PE, CD86-PE, and H-2K^b:Ig fusion protein-PE were from BD Biosciences. B220-FITC, Gr-1-FITC, CD11c-FITC and Thy1.1 (CD90.1)-FITC were from eBiosciences. To load SIY peptide, SIY and H-2K^b:Ig fusion protein at a molar ratio of 40:1 were incubated in 37°C for 6 hours. The SIY-K^b was used within one week. For staining, 0.5–2 × 10⁶ cells from spleen and liver were incubated with anti-CD16/32 (Fc blocker) at 4°C for 5 minutes and then incubated with a cocktail of appropriate antibodies at 4°C for 30 minutes in PBS supplemented with 2% FCS and 0.1% azide. Dead cells were excluded by 7AAD staining. Stained cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences). Data analysis was carried out using FlowJo™ software (Tree Star, Inc., Ashland, OR).

For intracellular IFN γ and TNF α staining, splenocytes and intrahepatic lymphocytes (1 × 10⁶) were stimulated with or without SIY peptide (1 μ g/ml) for 5h at 37°C in the presence of 10U of human recombinant IL-2 and brefeldin A (Golgiplug; PharMingen) in a total volume of 200 μ l of RPMI 1640 medium supplemented with 10% FCS. As positive controls, cells were stimulated by PMA and ionomycin in the presence of brefeldin A. As negative control, cells were cultured in medium only with brefeldin A. Cells were stained for surface expression of CD8 and SIY-K^b dimer, then were fixed and permeabilized with the Cytotfix/Cytoperm kit (PharMingen) and stained with APC-conjugated monoclonal rat anti-mouse IFN γ antibody or FITC-conjugated monoclonal rat anti-mouse TNF α antibody. Cells were analyzed with a FACSCalibur cytometer.

Differentiation and transfer of dendritic cells

Bone marrow cells from B6 mice were cultured at 4 × 10⁵ cells per ml in RPMI 1640 supplemented with 10% FCS, β -mercaptoethanol, L-glutamine, streptomycin, penicillin and 20ng/ml GM-CSF in 6-well plates (28). At day 2 and 4, half of the culture medium was replaced with fresh medium. At day 7, non-adherent and loosely adherent cells were collected by gentle pipetting, and centrifuged at 400g for 5 min. Cells were counted by trypan blue exclusion and analyzed for CD11c expression (~90% CD11c⁺) by flow cytometry. For adoptive transfer, 8 × 10⁵ cells in 200 μ l of PBS were administrated per mouse by tail vein injection.

Purification and transfer of T cells

Total T cells were purified from lymph nodes of B6 mice (Thy1.1⁺) by negative depletion using FITC-conjugated anti-CD11c, B220, or Gr-1 antibodies followed with anti-FITC magnetic beads (Miltenyi Biotech). CD4⁺ and CD8⁺ T cells were purified from lymph nodes of B6 mice by positive selection using anti-CD4 or anti-CD8 conjugated magnetic beads. Purified cells were counted and analyzed for CD3 by flow cytometry. Purified T cells (~95% CD3⁺) were injected intravenously into Thy1.2⁺ TNFR1^{-/-} mice (5–8 × 10⁶ per recipient in 200 μ l of PBS).

Statistical analyses

Statistical significance between groups was assessed by Student t test using Prism 5 software (GraphPad Software). A P-value of <0.05 was considered significant.

Results

Construction and characterization of recombinant MHV

Although a CD8 epitope, RCQIFANI (29), has been identified in wild-type MHV-A59 (A59/WT), its binding to MHC class I K^b is weak. K^b tetramer loaded with RCQIFANI failed to stain CD8 T cells in the spleen of MHV infected C57BL/6 (B6) mice (data not shown). To facilitate studying T cell responses to A59/WT, we constructed a recombinant MHV-A59 expressing eGFP fused to a CD4 epitope, ISQAVHAAHAEINEAGR (OVA₃₂₃₋₃₃₉), and a CD8 epitope, SIYRYYGL (SIY). The recombinant MHV, referred to as RA59/GOS, was constructed by targeted RNA recombination replacing a non-essential gene, open reading frame 4 (ORF4), with sequence encoding eGFP-OVA-SIY fusion protein (Fig. 1A, see Materials and Methods). Recombinant virus was selected via eGFP-positive cells (Fig. 1B) and further plaque purified.

The recombinant RA59/GOS virus was further characterized for its replication *in vitro* and infection in mice. Murine fibroblasts 17Cl-1 were inoculated with 5×10^5 pfu of either A59/WT or RA59/GOS (MOI=1) and virus titer in the culture supernatants was measured every 4 hrs for the next 24 hrs. The titer of A59/WT increased steadily, reaching a peak level of 10^8 pfu/ml around 20 hrs post infection (Fig. 1C). In contrast, the titer of RA59/GOS only reached 10^6 pfu/ml 24 hrs post infection, indicating a reduction of replication capacity by approximately 100-fold. Furthermore, B6 mice were inoculated intraperitoneally (i.p.) with A59/WT or RA59/GOS and virus titers were measured in the liver 1, 3, and 5 days post infection (dpi). Following inoculation with 5×10^5 pfu of A59/WT, virus was readily detected in the liver at all three time points, peaking at 3 dpi (Fig. 1D). In contrast, RA59/GOS virus was detected in the liver of inoculated mice only at 1 dpi and only when 1×10^6 pfu was used for the inoculation. No virus was detected at 3 or 5 dpi or when lower doses of virus were inoculated. Correlating with virus replication, elevated levels of serum alanine aminotransferase (ALT) were detected in B6 mice infected with A59/WT virus at 1, 3, and 5 dpi (Fig. 1E), whereas no elevated ALT level was detected in RA59/GOS inoculated mice at any day or virus doses. Taken together, these results suggest that RA59/GOS is an attenuated virus with dramatically reduced capacity to replicate in cultured cells and mice.

RA59/GOS induces CD8 T cell responses in a dose dependent manner

To study CD8 T cell response to MHV infection, we inoculated B6 mice with 10^2 , 10^4 or 10^6 pfu of RA59/GOS. Seven dpi, SIY-specific CD8 T cells were identified in the spleen and liver by staining with anti-CD8 antibody and a H-2K^b:Ig fusion protein, the K^b component of which was loaded with SIY peptide (SIY-K^b), followed by flow cytometry. When mice were infected with A59/WT virus, a background level (0.1–0.2%) of SIY-positive CD8 T cells was detected in the spleen and liver (Fig. 2A). Similar background level of SIY-positive CD8 T cells was detected in the spleen and liver of RA59/GOS infected mice when H-2K^b:Ig fusion protein used for staining was not loaded with SIY peptide (Fig. 2A far right panel). In contrast, the frequency of SIY-positive CD8 T cells was increased at least 10-fold over the background level in the spleen and liver of RA59/GOS infected mice that were stained with both anti-CD8 and SIY-K^b. Notably, the frequency of SIY-K^b-positive CD8 T cells increased with the increasing inoculation virus doses. Similarly, the total numbers of SIY-positive CD8 T cells in the spleen and liver increased with the increasing inoculation virus doses (Fig. 2B). In addition, a significant fraction of SIY-K^b-positive CD8 T cells from both the spleen and liver was induced to express IFN γ or

TNF α by SIY peptide in vitro (Fig. 2C). These results suggest that attenuated RA59/GOS virus can cause infection in mice but probably does not replicate significantly, resulting in functional CD8 T cell responses proportional to the doses of inoculating virus.

Defective CD8 T cell response to RA59/GOS in TNFR1-deficient mice

To investigate the effect of TNFR in CD8 T cell response to MHV, we compared SIY-specific CD8 T cell responses to RA59/GOS among B6 mice, B6 mice deficient in TNFR1 (TNFR1^{-/-}), and B6 mice deficient in TNFR2 (TNFR2^{-/-}). Seven dpi similar frequency and number of SIY-specific CD8 T cells were detected in the spleen and liver of B6 and TNFR2^{-/-} mice (Fig. 3A and B). However, only background level of SIY-specific CD8 T cells was detected in the spleen and liver of TNFR1^{-/-} mice.

To exclude the possibility that CD8 T cell response in TNFR1^{-/-} mice was delayed, the frequency and total number of SIY-specific CD8 T cells were measured in the spleen and liver 11 dpi. Again, while similarly robust SIY-specific CD8 T cell responses were detected in the spleen and liver of B6 and TNFR2^{-/-} mice, no significant SIY-specific CD8 T cell response was detected in TNFR1^{-/-} mice (Fig. 3C and D). These results show that TNFR1, but not TNFR2, is required for efficient CD8 T cell response to MHV infection.

Impaired DC mobilization and maturation in TNFR1-deficient mice following RA59/GOS infection

Because of the critical role of DCs in mediating CD8 T cell responses, we determined DC numbers and maturation status in TNFR1^{-/-} mice following RA59/GOS infection. Three dpi, cells from spleen and liver of B6 and TNFR1^{-/-} mice were enumerated and analyzed for CD11c plus CD40, CD80, CD86 or MHC class I. Compared to CD11c⁺ cells from spleen or liver of B6 mice, a significantly lower percentage of CD11c⁺ cells from spleen and liver of TNFR1^{-/-} mice expressed CD40, CD80, CD86 or class I (Fig. 4A and B, p<0.05). More dramatically, while the number of CD11c⁺ DCs was similar in the spleen or liver between PBS-injected B6 and TNFR1^{-/-} mice (Fig. 4C), the number of CD11c⁺ DCs was increased approximately 2 fold in both spleen and liver of B6 mice following RA59/GOS infection. However, the number of CD11c⁺ DCs was not increased at all in the spleen and liver of TNFR1^{-/-} mice following RA59/GOS infection. Thus, in the absence of TNFR1, DC mobilization and maturation are impaired following RA59/GOS infection.

Next, we determined whether SIY-specific CD8 T cell response to RA59/GOS infection can be rescued by transfer of TNFR1-expressing DCs into TNFR1^{-/-} mice. GM-CSF induced bone marrow DCs from B6 mice were adoptively transferred into TNFR1^{-/-} mice, followed by infection with RA59/GOS and analysis at 7 dpi. Without DC transfer, virtually no SIY-specific CD8 T cells were detected in the spleen or liver of TNFR1^{-/-} mice. With DC transfer, SIY-specific CD8 T cells were detected in the spleen and liver of TNFR1^{-/-} mice (Fig. 5). Statistically, there was no difference in both the frequency and the number of SIY-specific CD8 T cells in the spleen or liver between B6 mice and TNFR1^{-/-} mice that were transferred with DCs. These results show that the impaired DC maturation and mobilization is a major factor contributing to the defective CD8 T cell response to RA59/GOS infection in TNFR1^{-/-} mice.

Transfer of TNFR1-expressing T cells also rescues endogenous CD8 T cell response to RA59/GOS in TNFR1-deficient mice

We also tested whether TNFR1-expressing T cells can rescue CD8 T cell response to RA59/GOS infection in TNFR1^{-/-} mice. Total T cells were purified from lymph nodes of Thy1.1⁺ B6 mice. Purified Thy1.1⁺ T cells (>95% CD3⁺) were transferred into TNFR1^{-/-} mice on Thy1.2⁺ background (5×10^6 cells per recipient), followed by RA59/GOS infection and

analysis for Thy1.1, CD8 and SIY-K^b at 7 dpi. In both spleen and liver, less than 0.5% of live cells were transferred Thy1.1⁺ T cells (Fig. 6A). As expected, without T cell transfer, the frequency and number of SIY-specific endogenous (Thy1.1⁻) CD8 T cells were minimal in the spleen or liver of TNFR1^{-/-} mice. With T cell transfer, significant levels of SIY-specific Thy1.1⁻CD8 T cells were detected in the spleen and liver of TNFR1^{-/-} mice (Fig. 6B, C). Statistically, there was no difference in both the frequency and the number of SIY-specific Thy1.1⁻ CD8 T cells in the spleen or liver between B6 mice and TNFR1^{-/-} mice transferred with T cells. Furthermore, when purified CD4 and CD8 T cells were transferred separately into TNFR1^{-/-} mice, endogenous CD8 T cell responses to MHV were also elevated to similar levels as in B6 mice (Fig. 6D). These results show that transfer of TNFR-expressing T cells into TNFR1^{-/-} mice also restores the endogenous CD8 T cell response to RA59/GOS infection.

TNFR1-expressing T cells restore DC mobilization and maturation in TNFR1-deficient mice

We further determined whether DC mobilization and maturation in TNFR1^{-/-} mice were restored by adoptive transfer of TNFR1-expressing T cells. Purified Thy1.1⁺ T cells were transferred into TNFR1^{-/-} mice (5×10^6 cells per recipient), followed by RA59/GOS infection and analysis for CD11c, CD40, CD80, CD86 and MHC I at 3 dpi. As shown in Fig. 4A and B, DCs from T cell-transferred TNFR1^{-/-} mice up-regulated CD40, CD80, CD86 and MHC I to the similar levels as those in B6 mice. In addition, the numbers of DCs in the spleen and liver were restored to the similar levels as those in B6 mice (Fig. 4C). These results show that transferred T cells rescue the virus-specific CD8 T cell response in TNFR1^{-/-} mice through DC activation and mobilization.

Discussion

Studies have shown a critical role of TNF α in DC maturation both *in vitro* and *in vivo* (12, 15, 16, 38). Although LT α also binds to TNFR1 and TNFR2, its effect on DC accumulation and/or homeostasis in the spleen is primarily through signaling via LT β R by membrane LT α/β but not soluble LT α (39, 40, 41, 42). Consistently, LT α ^{-/-} mice produce delayed but effective CD8 T cell responses to influenza virus infection (43) and the defective CD8 T cells responses to LCMV likely result from abnormal lymphoid architecture (44). Furthermore, the defective DC maturation and CD8 T cell responses in TNF α ^{-/-} mice suggest that LT α does not replace TNF α function in these processes. These findings suggest that signaling through TNFR by soluble LT α is unlikely critical for DC maturation or CD8 T cell responses. Nevertheless, because LT α ^{-/-} mice lack both soluble LT α and membrane LT α/β and because there is no reagent for selectively blocking soluble LT α , current studies have not conclusively ruled out an essential involvement of soluble LT α in DC maturation and CD8 T cell responses.

Results presented here reveal two distinct mechanisms by which TNFR stimulates DC maturation and initiates T cell response following MHV infection. First, our findings suggest that TNF α can stimulate DCs maturation *in vivo* directly through TNFR1 expressed on immature DCs. Although maturation of DCs is impaired in TNF α ^{-/-} mice following infection with a replication defective rAd (16), which receptor mediates TNF α 's effect on DC maturation *in vivo* was not identified. Our observation that DC maturation and CD8 T cell response are impaired in TNFR1^{-/-}, but not TNFR2^{-/-}, mice suggests that it is TNFR1 that mediates DC maturation *in vivo*. Our findings are consistent with and further extend the studies showing that TNF α stimulates DC maturation *in vitro* via TNFR1 (30). Furthermore, because of complex cell-cell interactions *in vivo*, previous studies did not address whether TNF α can directly stimulate DC maturation through TNFRs expressed on immature DCs. For example, T cell response to rAd in TNF α ^{-/-} mice is restored by adoptive transfer of antigen primed mature wild-type DCs. Because TNF α is secreted primarily by activated

macrophages and T cells (10, 11, 12), the transferred DCs is unlikely to secrete TNF α to activate maturation of endogenous DCs to restore T cell response. It is more likely that the transferred DCs bypass endogenous DCs and directly activate T cell response. Like TNF $\alpha^{-/-}$ mice, TNFR1 $^{-/-}$ mice have normal numbers of DCs in the spleen and liver and normal T cell and macrophage development. Unlike TNF $\alpha^{-/-}$ mice, TNF α is still present in TNFR1 $^{-/-}$ mice. Because only the transferred DCs can respond to TNF α in TNFR1 $^{-/-}$ mice, restoration of CD8 T cell response in TNFR1 $^{-/-}$ mice by adoptive transfer of bone marrow derived wild-type DCs (not antigen-primed) suggests that the transferred DCs can respond directly to TNF α in TNFR1 $^{-/-}$ mice. Together, these results suggest that *in vivo* TNF α also directly stimulates DC maturation through TNFR1 expressed on immature DCs.

Second, our results reveal an alternative T cell-dependent pathway by which TNF α may stimulate DC maturation and CD8 T cell response *in vivo*. We found that in TNFR1 $^{-/-}$ mice DC mobilization and maturation and CD8 T cell response to the attenuated MHV are also restored by adoptive transfer of TNFR1-expressing naïve T cells. Because in these mice, only the transferred T cells can respond to TNF α , restoration of DC maturation and mobilization and response by endogenous CD8 T cells must go through the transferred T cells. Macrophages play a critical role in the clearance of MHV (31). It can be envisioned that TNF α secreted by activated macrophages could stimulate the transferred naïve T cells, resulting in expression of molecules that can in turn stimulate DC mobilization and maturation. For example, CD40L is expressed by activated T cells and can engage CD40 expressed on immature DCs to stimulate DC maturation (32). Nevertheless, the molecular mechanism mediating the T cell-dependent pathway of DC maturation and mobilization has yet to be elucidated.

Our results also provide a possible explanation for the apparently contradictory results of CD8 T cell responses to different infections in TNF $\alpha^{-/-}$, TNFR1 $^{-/-}$, TNFR2 $^{-/-}$, and TNFR1 $^{-/-}$ and TNFR2 $^{-/-}$ double knockout mice (16, 18, 22). The recombinant RA59/GOS was constructed by replacing a 283 bp fragment of ORF4 with eGFP-OVA-SIY. The resulting virus is attenuated based on the observation that the virus titer was reduced over 100 fold in both 17C1-1 cells and mice when compared to the wild-type MHV-A59 virus (Figure 1B, C). In addition, the duration of viremia was reduced to the first day post infection with RA59/GOS whereas viremia was detected for three days post infection with RA59/WT (Figure 1C). Consistent with the minimal viral replication, no serum ALT was detected following inoculation of RA59/GOS. However, virus infection and limited translation occur because CD8 T cell response to SIY can be detected in a dose-dependent manner (Figure 2). Das Sarma *et al* have reported a recombinant MHV-A59 in which ORF4 was replaced with GFP. The resulting virus replicated similarly as wild-type A59 *in vitro* (33). However, Sperry *et al* reported that single-amino-acid substitutions in ORF1b-nsp14 and ORF2a of the mouse hepatitis virus are attenuating *in vivo* (34). The difference between the two recombinant viruses is the addition of OVA-SIY sequences to the GFP sequences in our virus, resulting in an insert of 860 bp fragment in our virus versus an insert of 720 bp in Das Sarma's virus. As the replaced fragment of ORF is only 283 bp, the increased insert size could have interfered with virus replication, resulting in an attenuated RA59/GOS.

It is notable that T cell response is impaired in TNF $\alpha^{-/-}$ mice following infection with a replication defective rAd (16) and in TNFR1 $^{-/-}$ mice following infection with an attenuated MHV. In contrast, T cell responses were all enhanced in TNF $\alpha^{-/-}$ or TNFR $^{-/-}$ mice following acute or chronic infection with replication competent pathogens, such as LCMV, influenza virus and Mycobacterium (16, 17, 18, 19, 20). Studies have shown that DCs can be stimulated to mature independent of TNF α (35), probably through direct interaction between microbial components and TLRs (6) or other pathogen recognition receptors (36, 37). The TNF α -independent pathway of DC maturation is more likely to occur when

pathogen can replicate, whereas the replication defective or highly attenuated pathogens may not produce sufficient amount of microbial components to directly engage TLRs and other pathogen recognition receptors to activate DCs directly. Thus, T cell response is selectively impaired in TNF α ^{-/-} or TNFR1^{-/-} mice only in response to infection with replication defective rAd or highly attenuated MHV. It is also possible that different pathogens, such as MHV, rAd, LCMV, influenza virus and Mycobacterium express different microbial components, infect different cell types in mice and have different replication cycles; these differences may also contribute to the observed different outcomes of CD8 T cell responses in TNF α - or TNFR-deficient mice.

In summary, our results show that *in vivo* TNFR1 mediates TNF α -dependent DC maturation either through a direct binding of TNF α to TNFR1 on immature DCs or through an indirect T cell-dependent pathway. The divergent CD8 T cell responses to different infections in TNF α - and TNFR-deficient mice may also relate to the pathways by which DCs are activated.

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Abbreviations used in this paper

DCs	dendritic cells
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
TLRs	toll-like receptors
PAMPs	pathogen-associated molecular patterns
A59/WT	wild-type mouse hepatitis virus A59
RA59/GOS	recombinant mouse hepatitis virus A59 expressing eGFP-OVA-SIY fusion protein
fMHV	feline mouse hepatitis virus
rAd	recombinant adenovirus
LCMV	lymphocytic choriomeningitis virus
dpi	days post infection
MOI	multiplicity of infection
ALT	alanine aminotransferase

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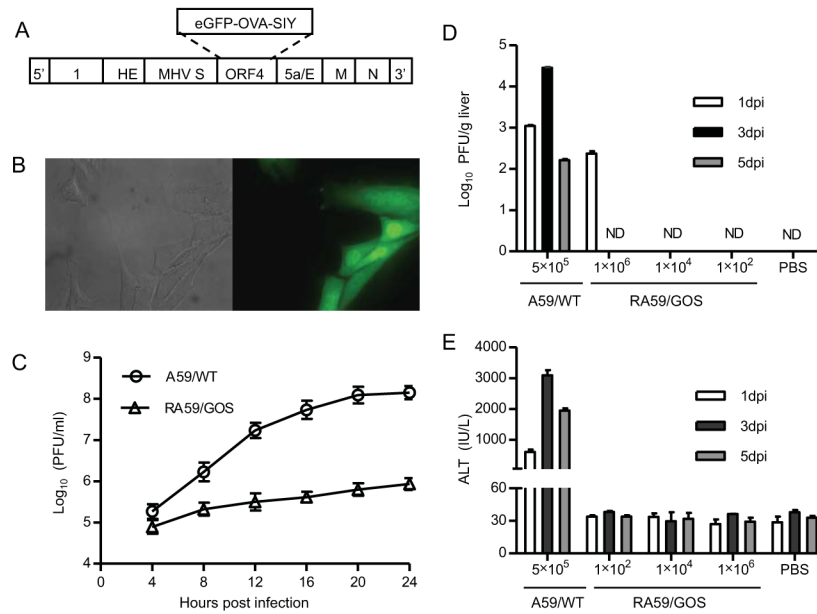


FIGURE 1. Construction and characterization of recombinant MHV-A59 expressing an eGFP-OVA-SIY fusion protein

(A) Schematic diagram of recombinant MHV virus. Targeted RNA recombination was used to replace ORF4 of MHV-A59 with a sequence encoding eGFP-OVA-SIY fusion protein. The eGFP-OVA-SIY fusion gene was first cloned into pMH54 plasmid via Sal I and Not I sites, and then transcribed into RNA for recombination with feline MHV in AK-D cells. See Materials and Methods for details.

(B) eGFP fluorescence of RA59/GOS infected cells. 17Cl-1 cells were seeded on cover glass in 6-well plate followed by RA59/GOS infection at MOI of 1. Eight hours post infection, cells were fixed and visualized by fluorescence microscopy. Bright field and fluorescent images of the same area are shown.

(C) Comparison of replication of wild-type and recombinant MHV-A59 in cultured cells. 17Cl-1 cells were infected in triplicates with A59/WT or RA59/GOS at MOI of 1. Culture supernatants were harvested every 4 hrs and assayed for virus titer by plaque assay. The mean virus titer \pm standard deviation (SD) of triplicate samples is shown. Representative results from one of three experiments are shown.

(D, E) Comparison of virus titers and ALT levels in B6 mice infected with A59/WT or RA59/GOS. B6 mice were inoculated i.p. with A59/WT (5×10^5 pfu/mouse) or the indicated doses of RA59/GOS. PBS injected mice were used as control. At 1, 3 and 5 dpi, sera and livers were harvested for assaying ALT levels and virus titers, respectively. Virus titers (D) and ALT levels (E) are shown as mean \pm SD of 5–6 mice per group. ND, not detectable. Representative results from one of three experiments are shown.

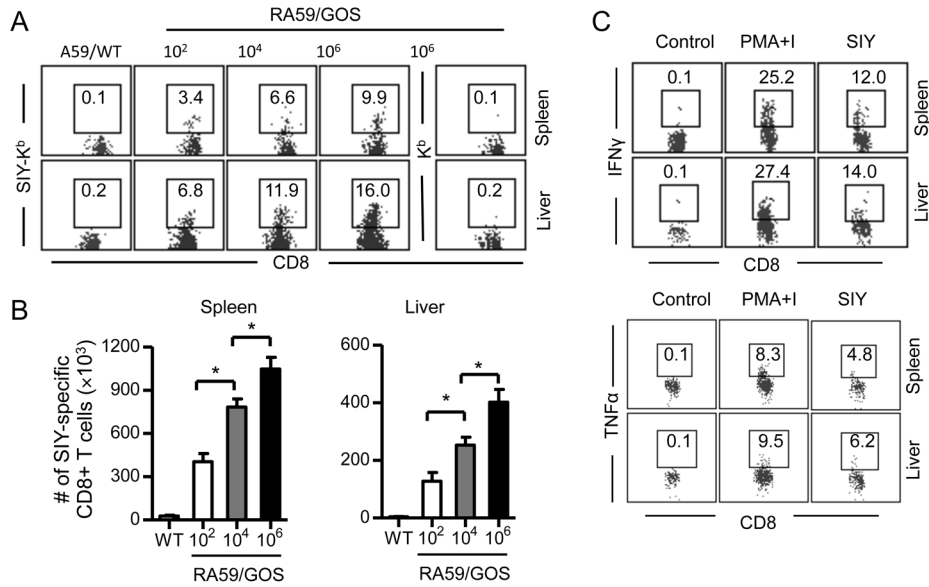


FIGURE 2. RA59/GOS induces CD8 T cell responses in a dose dependent manner
 Groups of B6 mice were infected i.p. with different doses of RA59/GOS virus or with 5×10^5 pfu of A59/WT virus. Seven dpi, cells from spleen and liver were enumerated and analyzed for CD3, CD8, SIY-K^b and 7AAD (A and B). Alternatively, cells were stimulated with SIY peptide or PMA plus ionomycin (PMA+I) or without any stimulation (Control) for 5 hrs and stained for CD8, SIY-K^b and intracellular IFN γ or TNF α (C). K^b indicates the same stains except that H-2K^b:Ig fusion protein was not loaded with SIY peptide. (A) Representative SIY-K^b (or K^b) versus CD8 staining profiles of CD3⁺ CD8⁺ live cells (7AAD⁻) from spleen and liver are shown. The number indicates percentage of SIY-K^b-positive cells among CD8⁺ cells. (B) Comparison of mean \pm SD of SIY-K^b⁺ CD8⁺ cells in the spleen (left panel) and liver (right panel) of 4 mice per group. Data from one of two similar experiments are shown. * indicates p value of <0.05. (C) Intracellular staining of IFN γ and TNF α gating on SIY-K^b⁺ and CD8⁺ T cells in the spleen and liver. Representative data from two independent experiments are shown.

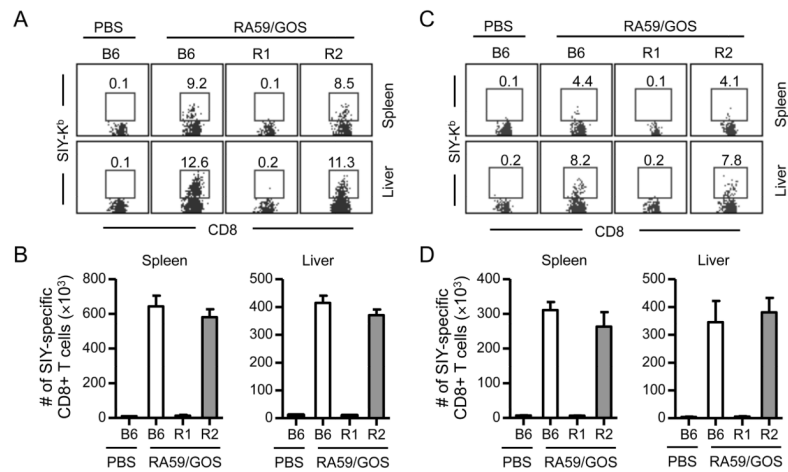


FIGURE 3. Defective CD8 T cell response to RA59/GOS in TNFR1-deficient mice

(A, B) B6, TNFR1^{-/-} (R1) and TNFR2^{-/-} (R2) mice were inoculated i.p. with 1×10^6 pfu of RA59/GOS or the same volume of PBS. Seven dpi, cells from spleen and liver were enumerated and analyzed for CD3, CD8, SIY-K^b and 7AAD as in Fig. 2. (A) Representative SIY-K^b versus CD8 staining profiles of CD3⁺ CD8⁺ live cells from spleen and liver. (B) Comparison of mean \pm SD of SIY-K^b+ CD8⁺ cells in the spleen and liver of 4 mice per group. Combined data from two experiments are shown. (C, D) B6, TNFR1^{-/-} and TNFR2^{-/-} mice were infected and analyzed as in A and B, except analysis was done 11 dpi. Data shown are from 3–4 mice per group.

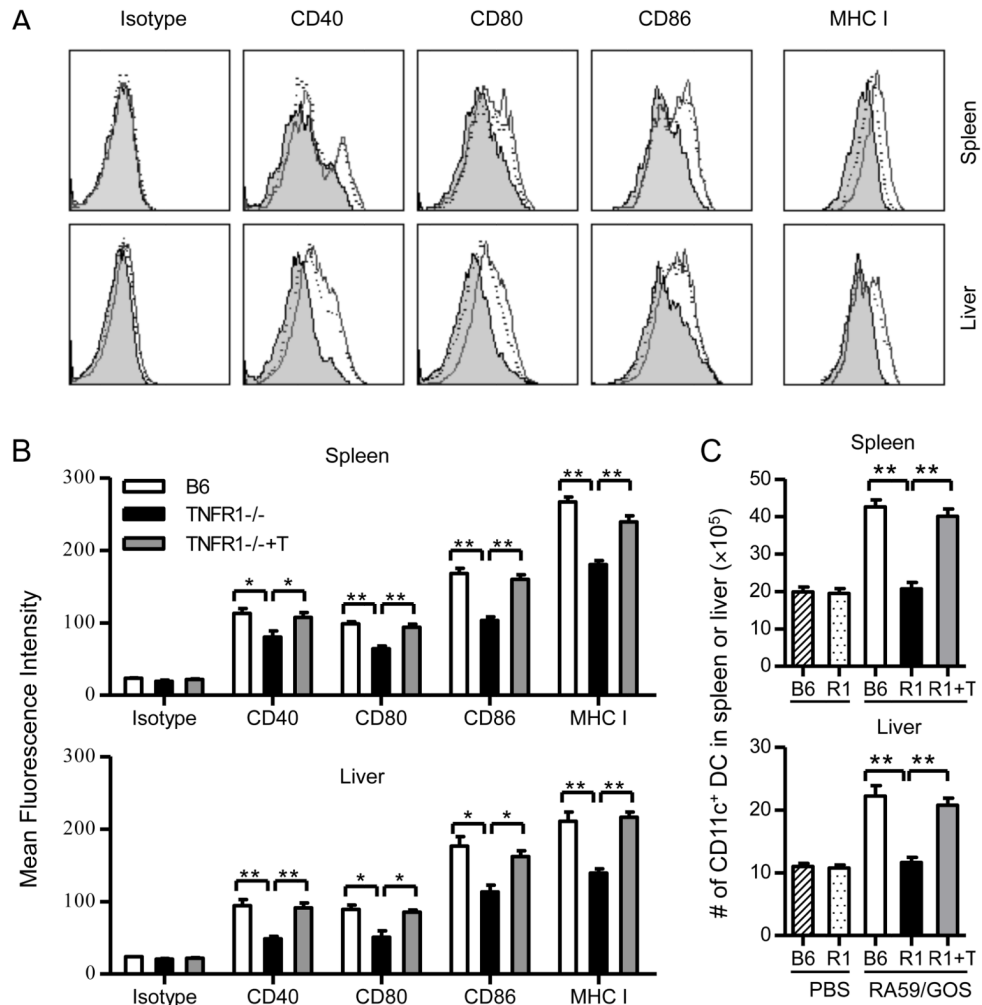


FIGURE 4. DC maturation and mobilization is impaired in TNFR1-deficient mice and the impaired DC response is restored by adoptive transfer of TNFR1-positive T cells
 B6 mice, TNFR1^{-/-} mice and TNFR1^{-/-} mice that were transferred with purified CD3⁺ T cells (TNFR1^{-/-}+T or R1+T) one day earlier were inoculated with 1 × 10⁶ pfu of RA59/GOS virus or the same volume of PBS. Three days later, cells from spleen and liver were enumerated and analyzed for CD11c plus CD40, CD80, CD86 or MHC class I. (A) Comparison of CD40, CD80, CD86 and MHC I expression by CD11c⁺ cells from B6 mice (histograms with solid lines), TNFR1^{-/-} mice (shaded histograms), and TNFR1^{-/-} mice injected with T cells (histograms with dotted lines). (B) Comparison of mean fluorescence intensity (MFI) of CD40, CD80, CD86, and MHC I by CD11c⁺ cells from the spleen (upper panel) or liver (lower panel) from B6 mice, TNFR1^{-/-} mice, and TNFR1^{-/-} mice injected with T cells. (C) Comparison of the total numbers of CD11c⁺ cells in the spleen (upper panel) and liver (lower panel) of B6 mice, TNFR1^{-/-} mice (R1), and TNFR1^{-/-} mice injected with T cells (R1+T). * p < 0.05; ** p < 0.01.

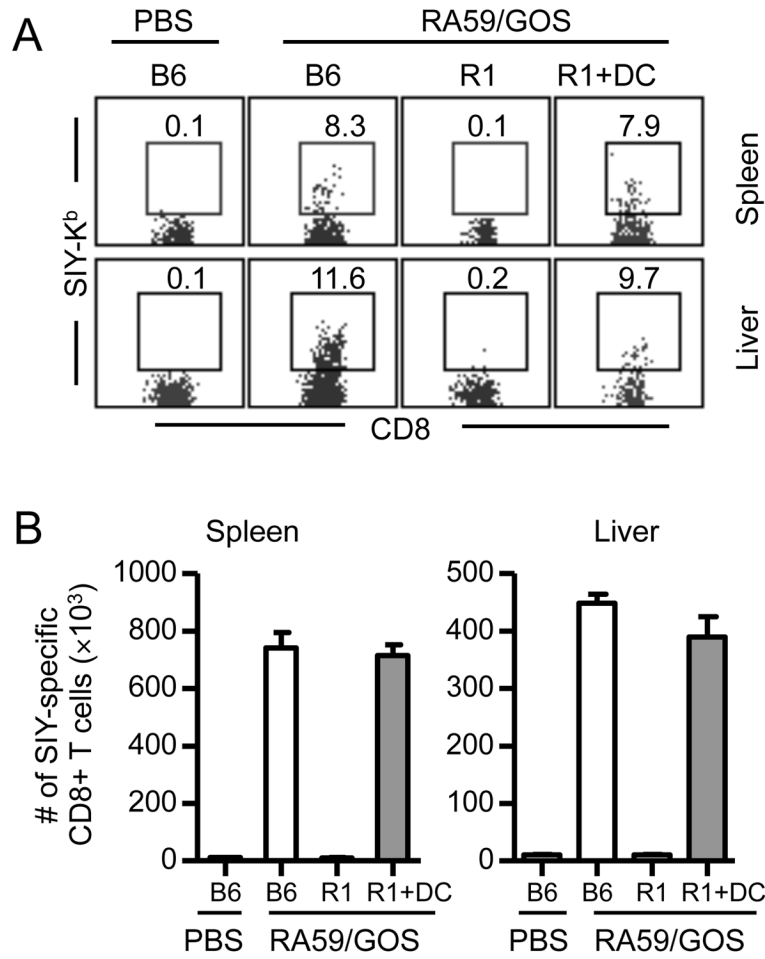


FIGURE 5. Rescue of CD8 T cell response in TNFR1-deficient mice by adoptive transfer of TNFR1-expressing DCs
 Bone marrow cells from B6 mice were cultured in the presence of GM-CSF for 7 days. DCs (75% CD11c⁺) were injected intravenously into TNFR1^{-/-} mice (R1+DC, 8 × 10⁵ per recipient). One day later, mice were infected with 1 × 10⁶ pfu of RA59/GOS virus and the frequency and the number of SIY-specific CD8 T cells were analyzed in the spleen and liver at 7 dpi as in Fig. 2. (A) Representative SIY-K^b versus CD8 staining profiles of CD3⁺ CD8⁺ cells from spleen and liver. (B) Comparison of mean ± SD of SIY-K^b⁺ CD8⁺ cells in the spleen and liver of 4 mice per group. Data shown are from one of two independent experiments.

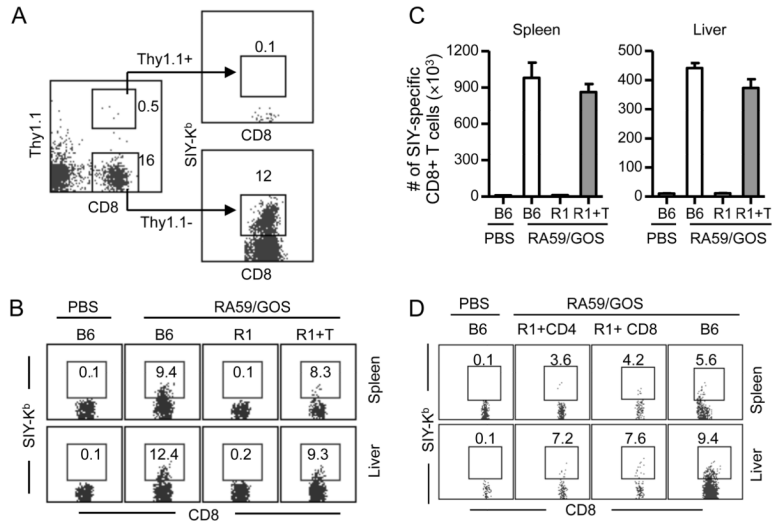


FIGURE 6. Rescue of CD8 T cell response in TNFR1-deficient mice by adoptive transfer of TNFR1-expressing T cells

Total, CD4⁺ and CD8⁺ T cells (>95% CD3⁺) were purified from lymph nodes of B6 mice (Thy1.1⁺) and injected intravenously into TNFR1^{-/-} mice (R1+T, 5–8 × 10⁶ per recipient). One day later, mice were infected with 1 × 10⁶ pfu of RA59/GOS virus and 7 dpi cells from spleen and liver were enumerated and analyzed for Thy1.1, CD8, and SIY-K^b. (A) Representative Thy1.1 versus CD8 staining profiles of live cells from liver. Note, very few transferred T cells (Thy1.1⁺) were positive for SIY-K^b. (B) Representative SIY-K^b versus CD8 staining profiles of endogenous (Thy1.1⁻) CD8⁺ cells from spleen and liver as gated in A. The number indicates percentage of cells in the gated regions. R1, TNFR1^{-/-} mice; R1+T, TNFR1^{-/-} mice transferred with purified total T cells. (C) Comparison of mean ± SD of SIY-K^b⁺ CD8⁺ Thy1.1⁻ endogenous T cells in the spleen and liver of B6 mice, TNFR1^{-/-} mice and TNFR1^{-/-} mice transferred with total T cells. (D) Representative SIY-K^b versus CD8 staining profiles of endogenous (Thy1.1⁻) CD8⁺ cells from spleen and liver as gated in A. The number indicates percentage of cells in the gated regions. R1+CD4, TNFR1^{-/-} mice transferred with purified CD4 T cells; R1+CD8, TNFR1^{-/-} mice transferred with purified CD8 T cells. Data shown are from one of two independent experiments with 4 mice per group per experiment.