Differential Requirements by CD4+ and CD8+ T Cells for Soluble and Membrane TNF in Control of *Francisella tularensis* Live Vaccine Strain Intramacrophage Growth

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Differential Requirements by CD4+ and CD8+ T Cells for Soluble and Membrane TNF in Control of Francisella tularensis Live Vaccine Strain Intramacrophage Growth

Siobhán C. Cowley,† Jonathon D. Sedgwick, and Karen L. Elkins

During primary infection with intracellular bacteria, the membrane-associated form of TNF provides some TNF functions, but the relative contributions during memory responses are not well-characterized. In this study, we determined the role of T cell-derived secreted and membrane-bound TNF (memTNF) during adaptive immunity to Francisella tularensis live vaccine strain (LVS). Although transgenic mice expressing only the memTNF were more susceptible to primary LVS infection than wild-type (WT) mice, LVS-immune WT and memTNF mice both survived maximal lethal secondary Francisella challenge. Generation of CD44high memory T cells and clearance of bacteria were similar, although more IFN-γ and IL-12(p40) were produced by memTNF mice.

To examine T cell function, we used an in vitro tissue coculture system that measures control of LVS intramacrophage growth by LVS-immune WT and memTNF-T cells. LVS-immune CD4+ and CD8+ T cells isolated from WT and memTNF mice exhibited comparable control of LVS growth in either normal or TNF-α knockout macrophages. Although the magnitude of CD4+ T cell-induced macrophage NO production clearly depended on TNF, control of LVS growth by both CD4+ and CD8+ T cells did not correlate with levels of nitrite. Importantly, intramacrophage LVS growth control by CD8+ T cells, but not CD4+ T cells, was almost entirely dependent on T cell-expressed TNF, and required stimulation through macrophage TNFRs. Collectively, these data demonstrate that T cell-expressed memTNF is necessary and sufficient for memory T cell responses to this intracellular pathogen, and is particularly important for intramacrophage control of bacterial growth by CD8+ T cells. The Journal of Immunology, 2007, 179: 7709–7719.

Francisella tularensis is a small Gram-negative facultative intracellular bacterium, and the causative agent of an acute febrile illness known as tularemia (1). The most virulent strains of this organism are highly infectious when acquired via the aerosol route, with an estimated mortality rate of 30–60% in untreated patients (2). For these reasons, this organism has been classified as a category A bioterrorism agent. The attenuated live vaccine strain (LVS)2 of F. tularensis was derived by repeated passage of a virulent F. tularensis strain on agar (3); LVS has been studied as an investigational product but it is not currently licensed for use in humans in the United States. Therefore, the development of a new F. tularensis vaccine, as well as better understanding of the unlicensed LVS strain, remain high priorities. Furthermore, immunity to Francisella has much in common with other more clinically important intracellular pathogens, such as Mycobacterium tuberculosis (4). Thus, studies that identify the correlates of protection for tularemia can be used to further our understanding of immunity to intracellular pathogens in general.

Although LVS is avirulent for humans when administered via most routes, it is highly virulent for laboratory mice and causes a fulminant infection in the organs of the reticuloendothelial system (5). The outcome of a primary LVS infection in mice is dependent on the route of inoculation: the LD50 for infections initiated via the intradermal (i.d.) route is ∼104 bacteria in BALB/cByJ mice, whereas the LD50 for infections administered via the i.v. or i.p. routes approaches a single bacterium (5, 6). Mice given a sublethal i.d. LVS dose can typically survive and clear the infection, and survive secondary i.p. challenge doses as high as 100,000 LD50 (1, 5, 7). Consequently, LVS murine infection is a convenient model in which to analyze immunity to tularemia. As for many intracellular pathogens, TNF is essential for survival of Francisella infection: mice treated with neutralizing anti-TNF Abs quickly succumb to sublethal doses of LVS during both primary and secondary infections (8). TNF is initially produced as soluble and membrane-bound TNF (memTNF) during adaptive immunity to this intracellular pathogen, with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 Abbreviations used in this paper: LVS, live vaccine strain; i.d., intradermal; memTNF, membrane-bound TNF; WT, wildtype; KO, knockout; BMMφ, bone marrow-derived macrophage; cDMEM, complete DMEM.

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differentially neutralize soluble and membrane TNF (17), it is important to define the relative contributions of soluble and membrane TNF in intracellular infections, including *Francisella*.

We have recently shown that mice expressing only memTNF are less susceptible to sublethal i.d. LVS infection than totally TNF-deficient mice (TNF knockout (KO) mice), but significantly more susceptible to LVS infection than WT mice (S. Cowley, M. Goldberg, A. Ho, and K. Elkins, submitted for publication). The memTNF mice succumbed during the first several days of a primary i.d. LVS infection in a dose-dependent manner; mice that survived lower doses of LVS through the early innate immune response ultimately cleared the infection. Thus, although memTNF cannot contribute to resistance to LVS infection, it is not sufficient to compensate for all of the functions of soluble TNF during the innate immune response. Because TNF is also used by T cells during secondary adaptive immune responses to control LVS intracellular growth, in this study, we investigate the role of memTNF and soluble TNF in the function of LVS-immune T cells, by focusing on the ability of LVS-immune T cells to inhibit LVS growth in macrophages.

Materials and Methods

**Bacteria**

*F. tularensis* LVS (ATCC 29684; American Type Culture Collection) was grown and frozen as previously described (7, 18). Viable bacteria were quantified by plating serial dilutions on Mueller Hinton agar plates.

**Animals and infections**

Male specific-pathogen-free C57BL/6J mice and TNFR 1/2 KO mice were purchased from The Jackson Laboratory. TNF KO and memTNF mice were obtained via material transfer agreement with Schering Plough BioPharma (formerly DNAX Research), and bred at the Center for Biologics Research and Evaluation, U.S. Food and Drug Administration (CBER/FDA). All animals were housed in a barrier environment at CBER/FDA, and procedures were performed according to approved protocols under animal care and use committee guidelines. Throughout, ‘LVS-immune’ refers to splenocytes obtained from mice given a sublethal priming dose of 10^2 LVS i.d. 1–3 mo before sacrifice. For in vivo secondary LVS challenges, all mice were given a sublethal i.d. dose of 10^5 LVS i.d. 1–3 mo before sacrifice. For in vivo secondary LVS chal-

**In vitro assessment of control of intracellular bacterial growth in bone marrow-derived macrophages (BMMφ)**

The in vitro culture systems used, and validation of the culture system’s abilities to reflect known parameters of T cell activities during in vivo control of bacterial growth, have been described in detail elsewhere (19, 20). BMMφ were used as the target cells. Bone marrow was flushed from femurs of healthy adult mice with DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone). 10% L-929 conditioned medium, 0.2 mM L-glutamine (Invitrogen Life Technologies), 1 mM HEPES buffer (Invitrogen Life Technologies), and 0.1 mM nonessential amino acids (Invitrogen Life Technologies) at a concentration of 2 × 10^5 spleenocytes/ml. One milliliter of spleenocytes was cultured overnight, and supernatants were collected for quantification of cytokine and nitrite levels as described.

**Quantitation of cytokines and NO in BMMφ supernatants**

Culture supernatants were assayed for IFN-γ, IL-12(p40), and TNF by standard sandwich ELISAs, using reagents obtained from BD Pharmingen and quantified by comparison to recombinant standards, as per the manufacturer’s instructions. NO was detected in culture supernatants by the Griess reaction as previously described, using commercial Griess reagent (Sigma-Aldrich). Limits of detection for IFN-γ, IL-12(p40), and TNF ELISAs were 100, 78, and 100 pg/ml, respectively. The limit of detection for the Griess reaction was 1.5 μM nitrite/ml.

**Flow cytometry analyses**

Single-cell suspensions were prepared and stained for a panel of murine cell surface markers and analyzed using a LSR II flow cytometer (BD Biosciences) and FACS Diva or FlowJo software essentially as previously described (18–21). Clones used included RM4-5 (anti-CD4), 53-6.7 (anti-CD8a), GL3 (anti-γδ TCR), PK136 (anti-NK1.1), 53-2.1 (anti-Th1), H57-597 (anti-TCR-β chain), 17A2 (anti-CD3), M1/70 (anti-CD11b), RA3-6B (anti-CD45/B220), DX5 (anti-CD49b), and IM7 (anti-CD44 Ab). All Abs

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overnight ex vivo, and the levels of IFN-γ to infection within 1 wk (Table I). The same 5/H11003 memTNF mice given a primary 5 and cleared the infection within 2 wk, whereas LVS-naive WT and

after primary infection, mice were administered the indicated dose of LVS i.p. and monitored for survival; the dose of infection was

challenge. As shown in Fig. 1,

isotype control Abs. However, the memTNF cultures consisted of significantly higher levels of IFN-γ and IL-12(p40) as compared with WT mice.

presented are representative of two to three experiments of similar design.

above and Fc block were obtained from BD Pharmingen, and optimal concentrations were determined in separate experiments for use in three- to six-color staining protocols as required using appropriate fluorochrome-labeled isotype control Abs.

Results

memTNF-transgenic mice survive a secondary lethal LVS challenge, but produce increased levels of IL-12(p40) and IFN-γ as compared with WT mice

To determine the capacity of immunized memTNF mice to withstand maximal secondary LVS challenge, LVS-immune WT and memTNF mice were given a secondary LVS infection. Previous results have shown that WT mice that survive a primary sublethal LVS infection are solidly immune to secondary lethal challenges of >10^6 LVS i.p. (this dose is >10^5 LD_{50} for a naive WT mouse) (22). Furthermore, the majority of naive memTNF mice can survive and clear a primary 10^5 LVS i.d. infection (Table I). Thus, memTNF and WT mice were given a sublethal 10^5 LVS i.d. primary infection. Four weeks after the initial primary infection, mice were challenged with 10^5 LVS i.p., and the LVS burdens in the spleens and livers were enumerated during the first 4 days after challenge. As shown in Fig. 1, A and B, the numbers of LVS in the spleens and livers of the WT mice peaked on days 1–2 postchallenge, and diminished to around the limit of detection by day 4 (<30 CFU/spleen and <100 CFU/liver). Similarly, the LVS CFUs in the spleens and livers of the memTNF mice were highest on days 1 and 2 after challenge, and the memTNF mice cleared the infection by day 4 after challenge. The slightly higher LVS organ burden in the memTNF mice on day 1 after challenge was observed in repeated experiments, but it was never significantly different from WT mice. All LVS-immune WT and memTNF mice given a maximal secondary 5 × 10^5 LVS i.p. challenge survived and cleared the infection within 2 wk, whereas LVS-naive WT and memTNF mice given a primary 5 × 10^5 LVS i.p. dose succumbed to infection within 1 wk (Table I). The same 5 × 10^5 LVS dose given to mice via the i.d. route was sublethal for WT mice, but 100% lethal for memTNF mice (Table I).

To characterize the cytokine environment within the spleen during a 10^5 LVS i.p. secondary challenge, spleen cells from the LVS-challenged mice were harvested on selected days and cultured overnight ex vivo, and the levels of IFN-γ, IL-12(p40), and NO were measured in the culture supernatants. As shown in Fig. 1, C and D, spleen cell production of IFN-γ and p40 was significantly higher in the memTNF mice than the WT mice on days 1 (p40) and 3 (IFN-γ and p40) after infection (p < 0.01). In contrast, no significant differences in NO production were observed between the memTNF and WT mice (Fig. 1E). To further examine the nature of the responding T cells during in vivo secondary challenge, the numbers of activated CD44_{high}CD4^{+} and CD8^{+} T cells in the spleens of infected mice were enumerated on day 3 following a 10^5 i.p. secondary infection. As shown in Fig. 1F, in both the memTNF and WT mice, the numbers of CD44_{high}CD4^{+} and CD8^{+} T cells present in the spleens increased ~3-fold by day 3 after secondary challenge, as compared with naive WT mice. There were no significant differences between the memTNF and WT mice in the numbers of CD44_{high}CD4^{+} and CD8^{+} T cells present in the spleens on day 3 after secondary challenge (p > 0.05). Thus, during secondary lethal challenge, memTNF mice recruit WT levels of activated CD4^{+} and CD8^{+} T cells, and control and clear a secondary LVS challenge similar to WT mice; however, splenocytes from the memTNF produce significantly higher levels of IFN-γ and IL-12(p40) as compared with WT mice.

memTNF mediates control of LVS intramacrophage growth in vitro, but maximal NO production requires soluble TNF

To directly assess the role of memTNF in control of LVS intramacrophage growth, we compared the ability of LVS-immune spleen cells harvested from memTNF and WT mice to control LVS growth in BMMφ in an in vitro system. Splenocytes were obtained from LVS-immune WT or memTNF mice that were primed 4 wk earlier with a sublethal i.d. LVS infection. Immune splenocytes were cocultured with LVS-infected WT or memTNF BMMφ monolayers, and control of LVS growth was assessed after 3 days in the two different BMMφ monolayers. As seen in Fig. 2A, addition of LVS-immune, but not naive, WT or memTNF splenocytes to their cognate infected BMMφ monolayers resulted in readily measurable and significant (p < 0.01) inhibition of LVS growth compared with cultures containing either naive splenocytes or no splenocytes. LVS replicated to a similar extent in both the WT and memTNF BMMφ, and growth was not significantly affected by the addition of naive splenocytes to the cultures. Coculture of LVS-immune WT splenocytes with WT BMMφ resulted in a 2.65 log_{10} reduction in growth of the bacteria, while coculture of memTNF splenocytes with memTNF BMMφ resulted in a similar 2.79 log_{10} reduction of LVS growth. To assess the contribution of memTNF to the observed inhibition of LVS growth in the memTNF cultures, we tested the impact of abrogation of TNF activity on the control of LVS growth in the coculture system. As shown in Fig. 2A, addition of neutralizing anti-TNF Abs to both WT and memTNF cocultures resulted in a significant reversal of control of LVS growth as compared with cultures containing isotype control Abs to both WT and memTNF cocultures resulted in a significant reversal of control of LVS growth as compared with cultures containing isotype control Ab, demonstrating that TNF functions to inhibit LVS intracellular growth when it is available only in the membrane-bound form.

Several in vitro studies have previously shown that TNF-dependent anti-LVS activity is mediated primarily through induction of macrophage NO (5, 23). To determine whether memTNF also acts primarily through induction of NO, we compared the levels of nitrite (as a surrogate marker of NO) in the supernatants of the memTNF and WT cultures. As seen in Fig. 2B, both the WT and memTNF cocultures with immune spleen cells contained measurable levels of NO, and this NO production was greatly reduced by addition of anti-TNF Abs, but not isotype control Abs. However, the memTNF cultures consistently produced significantly less NO than the WT cultures (p < 0.01). Thus, although memTNF can facilitate macrophage

Table I. Survival of primary and secondary LVS infection by memTNF mice

| Mouse Strain | Primary i.d. LVS Dose | Number of Deaths/Total | Secondary i.p. LVS Challenge | Number of Deaths/Total |
|--------------|-----------------------|------------------------|-----------------------------|------------------------|
| C57BL/6J     | 5 × 10^4               | 0/5                    | N/A                         | N/A                    |
|              | 1 × 10^5               | 0/5                    | 5 × 10^4                    | 0/5                    |
| memTNF       | 5 × 10^4               | 5/5                    | N/A                         | N/A                    |
|              | 1 × 10^5               | 3/10                   | 5 × 10^4                    | 0/7                    |
| TNF KO       | 5 × 10^4               | 10/10                  | N/A                         | N/A                    |
|              | PBS                    | 5 × 10^4               | 5/5                         |                        |

a C57BL/6J wild-type, TNF KO, or memTNF male mice were administered the indicated doses of LVS i.d. and monitored for survival; the dose of infection was confirmed by simultaneous plate counts and is expressed as total CFU. One month after primary infection, mice were administered the indicated dose of LVS i.p. and monitored for survival for 1 mo. Surviving mice were sampled to confirm clearance of infections. N/A, not applicable. These data are representative of two to three experiments of similar design.

b Mean time to death for C57BL/6 mice was 5.5 ± 1.0 days.

c Mean time to death for memTNF mice was 5.2 ± 1.3 days.

d Mean time to death for TNF KO mice was 5.0 ± 2.1 days.

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production of NO, the levels produced in the absence of soluble TNF were significantly lower.

CD8$^+$ T cells require macrophage TNFRs to control LVS intracellular growth

We next sought to identify the T cell subpopulations that use memTNF to control LVS intracellular growth. First, we assessed the ability of CD4$^+$ and CD8$^+$ T cells isolated from LVS-immune memTNF mice to inhibit LVS growth in memTNF BMM$^+$, as compared with similar cultures containing WT T cells and WT BMM$^+$. As shown in Fig. 3A, control of LVS growth was not significantly different between the WT CD4$^+$ and CD8$^+$ T cells, and the memTNF CD4$^+$ and CD8$^+$ T cells ($p > 0.05$). Addition of neutralizing anti-TNF Abs to these cultures resulted in a significant reversal in the control of growth by CD4$^+$ and CD8$^+$ T cells from both WT and memTNF mice. In repeated experiments, the magnitude of this reversal of control of growth by addition of anti-TNF Abs was consistently less for the memTNF cells as compared with the WT cells. This suggests that either the anti-TNF Abs are less efficient at neutralizing the activity of memTNF, or conversely, that the memTNF cells use additional compensatory mechanisms to control LVS growth in the absence of soluble TNF. Nevertheless, these results clearly demonstrate that memTNF contributes to the control of LVS intracellular growth mediated by both CD4$^+$ and CD8$^+$ LVS-immune T cells, and that CD8$^+$ T cells are particularly dependent on TNF activity.

The IL-12-IFN-$\gamma$ axis is a tightly regulated feedback loop that includes macrophage production of IL-12 and TNF, followed by T cell production of IFN-$\gamma$, culminating in macrophage activation and control of bacterial intracellular growth (24). Because we
observed higher levels of both IFN-γ and IL-12(p40) production in ex vivo-cultured splenocytes harvested from memTNF mice during secondary challenge, we also assessed the coculture supernatants for cytokine production. Interestingly, both IFN-γ and IL-12(p40) production in the memTNF cocultures were significantly greater than that of the WT cocultures (Fig. 3, B and C); this was true for cocultures containing whole memTNF spleen cells, as well as purified memTNF CD4⁺ and CD8⁺ T cells as compared with their WT counterparts. The lower NO levels (Fig. 2B) coupled with higher IFN-γ and IL-12 production found in the memTNF cultures suggest that, although control of LVS growth was equivalent to that of WT cells, there is a defect in the regulation of the IL-12-IFN-γ axis in the memTNF cultures as compared with the WT cultures. Thus, soluble TNF is required to regulate IL-12 and IFN-γ production and/or use appropriately.

Because both macrophages and T cells express TNFRs and can respond to TNF, we next determined the role of macrophage TNF stimulation in the control of LVS growth. To this end, we cocultured LVS-immune CD4⁺ and CD8⁺ T cells purified from both WT and memTNF mice with BMMϕ derived from mice genetically deficient for TNFRs 1 and 2 (TNFR1/2 KO). Here, CD4⁺ and CD8⁺ T cells can respond to TNF while the LVS-infected BMMϕ cannot, permitting measurement of the relative importance of macrophage TNF stimulation, as compared with TNF T cell stimulation, in control of LVS intracellular growth. As seen in Fig. 4, a trend toward a small reversal of control of LVS growth was evident for all cell types tested in both the WT and memTNF cocultures containing TNFR1/2 KO BMMϕ, as compared with their control BMMϕ cocultures. However, in repeated experiments, reversal of control of growth was consistently significant only for the CD8⁺ T cell cocultures. Elimination of macrophage TNFRs resulted in an almost complete loss of the ability of WT and memTNF CD8⁺ T cells to control LVS intracellular growth. Together with the results of TNF blockade (Fig. 3A), these results indicate that CD8⁺ T cells control LVS intracellular growth primarily via stimulation of macrophage TNFRs, and that CD8⁺ T cell memTNF mediates this activity.

Given the importance of TNF stimulation in the induction of macrophage NO, we further measured the nitrite levels in the WT and memTNF spleen cell cocultures containing TNF R1/2 KO macrophages. As shown in Fig. 5, A and B, macrophage NO production was severely diminished in all cultures containing TNFR1/2 KO macrophages, regardless of whether a significant effect on control of LVS growth was observed or not. This reduction was evident in both the memTNF and WT spleen cell cultures, confirming that memTNF is sufficient to induce macrophage NO production; however, as observed earlier (Fig. 2B), the levels of NO produced in the memTNF cocultures were significantly less than that of the WT cultures. Furthermore, cocultures containing immune CD8⁺ cells from both WT and memTNF mice exhibited only low levels of nitrite, and these levels were not significantly diminished further by the absence of macrophage TNFRs. Conversely, NO production by the TNFR1/2 KO BMMϕ in the CD4⁺

FIGURE 2. memTNF spleen cells control LVS intracellular growth similarly to WT spleen cells, but exhibit defective NO production. A. Control of LVS intramacrophage growth by LVS-immune memTNF splenocytes. BMMϕ from WT and memTNF mice were infected with LVS and cocultured with splenocytes from either uninfected mice (naïve spleen), mice infected i.d. with LVS 4 wk previously (primed spleen), or no spleen cells (LVS + macrophages). Immediately following LVS infection of the BMMϕ, splenocytes were added to the indicated wells at a ratio of 1:2 (splenocyte:BMMϕ). In the indicated wells, either anti-TNF Abs or control IgG Ab (25 μg/ml) were added to the cultures at the time of addition of the splenocytes. Seventy-two hours after infection, the BMMϕ were washed, lysed, and plated to determine the levels of intracellular bacteria. B. Secretion of NO into culture supernatants following 72 h coculture of LVS-infected BMMϕ and immune splenocytes. Values shown are the mean numbers of CFU per milliliter ± the SEM of viable bacteria (A) or mean micromoles per milliliter of nitrite (B) (triplicate samples). Asterisks (*) indicate p < 0.01 as compared with cocultures containing control Ab. Symbols (+) indicate p < 0.05 between WT and memTNF cocultures. These data are representative of five experiments of similar design.
T cell cocultures was diminished almost to background levels; however, this had only a small and insignificant effect on LVS growth control by CD4\(^+\) T cells (Fig. 4). Thus, the high levels of NO detected in the CD4\(^+\) T cell cocultures are mediated via stimulation of macrophage TNFRs, although it does not significantly affect the ability of CD4\(^+\) T cells to inhibit LVS growth. However, because the ability of CD4\(^+\) T cells to control LVS growth was significantly reduced by the addition of anti-TNF Abs (Fig. 3A), CD4\(^+\) T cells clearly possess a TNF-dependent anti-LVS activity.

To evaluate the contributions of IFN-\(\gamma\) and IL-12 to NO production, the coculture supernatants were assessed for cytokine production. As shown in Fig. 5, C and D, significantly higher levels of IFN-\(\gamma\) (Fig. 5, C and D), but not IL-12 (Fig. 5, E and F), were detected in all the TNFR1/2 KO BMM\(\phi\) cocultures containing either WT or memTNF CD4\(^+\) and CD8\(^+\) T cells, as compared with cocultures with WT BMM\(\phi\). Thus, in the absence of macrophage TNFRs, IFN-\(\gamma\) levels in the culture supernatants were in excess. This indicates that the ability of macrophages to express TNFRs is essential to maintain NO production and proper regulation of IFN-\(\gamma\) production and/or use.

**T cell-derived, but not macrophage-derived, TNF is required for control of LVS intramacrophage growth in vitro**

Because macrophage TNFRs are a critical component in the effective control of LVS intracellular growth by CD8\(^+\) T cells, and macrophage-derived TNF is known to operate in an autocrine fashion, we investigated whether macrophage TNF expression is a critical component of control of LVS intracellular growth by either CD4\(^+\) or CD8\(^+\) T cells. To this end, LVS-immune CD4\(^+\) and CD8\(^+\) T cells were purified from WT and memTNF mice, and cocultured in vitro with LVS-infected BMM\(\phi\) derived from total TNF KO mice. Thus, the only available TNF is derived from the T cells in these cocultures. As shown in Fig. 6A, control of LVS growth by WT CD4\(^+\) and CD8\(^+\) T cells was not significantly different in cocultures containing either WT or TNF KO BMM\(\phi\) (\(p > 0.05\)). Similarly, memTNF CD4\(^+\) and CD8\(^+\) T cells controlled LVS growth equally well in memTNF and TNF KO BMM\(\phi\) (Fig. 6B). Loss of macrophage TNF had a small effect on the ability of the WT whole primed spleen cocultures to produce NO (Fig. 6C). However, this reduction varied in magnitude and significance in repeated experiments, and was not observed in the WT cultures using CD4\(^+\) and CD8\(^+\) enriched T cells. Furthermore, the memTNF cocultures did not exhibit a significant reduction in NO production during coculture with TNF KO BMM\(\phi\) as compared with memTNF BMM\(\phi\) (Fig. 6D). Thus, CD4\(^+\) and memTNF-transgenic cocultures. Infected BMM\(\phi\) were cocultured with splenocytes obtained from either uninfected mice (naive spleen), whole primed splenocytes from immune WT or memTNF mice (primed spleen), or the indicated T cell subsets enriched from immune WT or memTNF mice (CD4\(^+\) cells, CD8\(^+\) cells). All splenocyte populations were added at a 1:2 ratio (splenocyte to BMM\(\phi\)). Anti-TNF Abs (25 \(\mu\)g/ml) were added to the wells at the time of addition of the splenocytes to the infected BMM\(\phi\). Seventy-two hours after infection, the BMM\(\phi\) were washed, lysed, and plated to determine the levels of intracellular bacteria. Culture supernatants harvested at 72 h were tested for levels of IFN-\(\gamma\) (B) and IL-12(p40) (C). Values shown are the mean numbers of CFU per milliliter ± the SEM of viable bacteria (A) or mean nanograms per milliliter of IL-12(p40) (\(B\) and \(C\)) (triplicate samples). Asterisks (+) indicate \(p\) values <0.01 as compared with cognate cocultures containing no Ab. Hatches (#) indicate CFU \(p\) values <0.01 as compared with cognate cocultures containing naive spleen cells. Symbols (+) indicate \(p\) values <0.05 between WT and memTNF cocultures. These data are representative of three experiments of similar design.
CD8^+ T cell-derived TNF, even when present only in the membrane-bound form, is sufficient to mediate control of LVS intracellular growth in the absence of macrophage production of TNF.

**FIGURE 4.** LVS-immune CD8^+, but not CD4^+, T cells are dependant on macrophage TNFRs for control of LVS intramacrophage growth. Ability of different T cell subsets isolated from LVS-immune WT (A) and memTNF (B) mice to control LVS growth in WT (A), memTNF (B), or TNFR1/2 KO (A and B) BMMφ. Infected BMMφ were cocultured with splenocytes obtained from either uninfected mice (naive spleen), whole primed splenocytes from immune WT or memTNF mice (primed spleen), or various T cell subsets enriched from immune WT or memTNF mice (CD4^+ cells, CD8^+ cells). All splenocyte populations were added at a 1:2 ratio (splencyte to BMMφ). Seventy-two hours after infection, the BMMφ were assessed for levels of intracellular LVS growth. Values shown are the mean numbers of CFU per milliliter ± the SEM of viable bacteria (triplicate samples). Asterisks (*) indicate p values <0.01 of cocultures containing TNFR1/2 KO BMMφ as compared with cocultures containing either WT (A) or memTNF (B) BMMφ. These results are representative of three experiments of similar design.

**FIGURE 5.** Macrophage TNFRs are required for NO production and IFN-γ regulation in WT and memTNF cocultures. Secretion of cytokines and NO into culture supernatants following coculture of LVS-infected TNFR1/2 KO BMMφ with WT and memTNF LVS-immune splenocytes. Culture supernatants were collected from the WT, memTNF, and TNFR1/2 KO BMMφ cocultures described in Fig. 4, after 72 h, and tested for NO (A and B), IFN-γ (C and D), and IL-12(p40) (E and F). Values shown are the mean micromoles per milliliter of nitrite (A and B) or the mean nanograms per milliliter ± the SEM of the indicated cytokine (C–F) (triplicate samples). Asterisks (*) indicate p values <0.01 of cocultures containing TNFR1/2 KO BMMφ as compared with their cognate cocultures containing either WT (A) or memTNF (B) BMMφ. These results are representative of three experiments of similar design.

Examination of IFN-γ and IL-12(p40) production (Fig. 6, E–H) in the TNF KO BMMφ cultures revealed a mild CD4^+ T cell-specific defect in the IL-12-IFN-γ axis in the absence of macrophage-derived TNF. Significantly higher levels of IFN-γ, but not IL-12, were present in both the memTNF and WT cocultures containing CD4^+ T cells. This effect was not observed consistently, or to a significant extent, in the CD8^+ T cell cocultures. The increase...
in IFN-γ production was not as pronounced as that seen in the TNFR1/2 KO BMMφ cultures (Fig. 5, C and D), and varied in magnitude from 2- to 3-fold between different experiments. However, this consistent increase in IFN-γ suggests a small but strict role for macrophage-derived TNF in the regulation of IFN-γ and IL-12 production and/or uptake in the CD4+ T cell cultures.

**Discussion**

Development of effective vaccines against intracellular pathogens is limited by an incomplete understanding of the mechanisms that contribute to protective immunity. For an intracellular pathogen such as *F. tularensis*, a critical aspect of protective immunity occurs during the interaction of Ag-specific T cells with infected host cells. To study this interaction in greater detail, we have previously developed an in vitro coculture model system to assess the mechanisms used by LVS-immune T cells to limit *Francisella* intracellular growth inside macrophages (19). This in vitro system is designed to reflect T cell activities that operate during secondary exposure to Ag. Here, we have applied this in vitro system to study the relative contributions of macrophage and T cell-derived soluble TNF, as well as memTNF, to the control of *F. tularensis* LVS intramacrophage growth. Using this system, we demonstrate that both CD4+ and CD8+ T cells can efficiently use memTNF to control LVS intracellular growth. Furthermore, we show that, unlike LVS-immune CD4+ T cells, LVS-immune CD8+ T cells rely heavily on the presence of TNF to mediate LVS growth control.

From the data presented here, it is possible to draw several conclusions regarding the role of TNF in the interaction of LVS-infected macrophages with immune T cells. First, TNF is essential for maximal control of LVS growth mediated by both LVS-immune CD4+ and CD8+ T cells (Fig. 3A); furthermore, both cell types can inhibit LVS intracellular growth when TNF is available only in the membrane-bound form (Figs. 3A, 4, and 6, A and B). However, the role of TNF in this process differs significantly between CD4+ and CD8+ T cells. CD8+, but not CD4+, T cells are almost entirely dependent on the activity of TNF to control LVS intracellular growth (Figs. 3A and 4). This is evidenced by the finding that only CD8+ T cells strictly require interaction with macrophage TNFRs to mediate LVS growth control (Fig. 4). In contrast, the ability of CD4+ T cells to control LVS intramacrophage growth was only partially dependent on TNF (Fig. 3A), and CD4+ T cell function was not significantly diminished in the absence of macrophage TNFRs (Fig. 4). Although the magnitude of CD4+ T cell-induced macrophage NO production was clearly dependent on TNF, control of LVS growth by both CD4+ and CD8+ T cells did not correlate with the levels of NO measured in the cocultures (Figs. 4 and 5, A and B). In particular, CD8+ T cell cocultures with TNFR1/2-deficient macrophages resulted in loss of LVS growth control but no change in NO production, strongly suggesting that TNF-mediated control of LVS growth by CD8+ T cells.

**FIGURE 6.** Macrophage TNF is not required for control of LVS intramacrophage growth or NO production. Ability of various T cell subsets isolated from LVS-immune WT (A) and memTNF (B) mice to control LVS growth in WT (A), memTNF (B), or TNF KO (A and B) BMMφ. Infected BMMφ were cocultured with splenocytes obtained from either uninfected mice (naive spleen), whole primed splenocytes from immune WT or memTNF mice (primed spleen), or various T cell subsets enriched from immune WT or memTNF mice (CD4+ cells, CD8+ cells). All splenocyte populations were added at a 1:2 ratio (spleocyte to BMMφ). Seventy-two hours after infection, the BMMφ were assessed for levels of intracellular LVS growth. Values shown are the mean numbers of CFU per milliliter ± the SEM of viable bacteria (triplicate samples). C–H, Secretion of NO and cytokines into culture supernatants following coculture of LVS-infected TNFR1/2 KO BMMφ with WT and memTNF LVS-immune splenocytes. Culture supernatants were collected from the WT, memTNF, and TNF KO BMMφ cocultures after 72 h, and assessed for NO (C and D), IFN-γ (E and F), and IL-12(p40) (G and H). Values shown are the mean micrograms per milliliter of nitrite (C and D) or the mean nanograms per milliliter ± the SEM of the indicated cytokine (E–H) (triplicate samples). Asterisks (*) indicate p values <0.05 of cocultures containing TNF KO BMMφ as compared with their cognate cocultures containing either WT (C and E) or memTNF (F) BMMφ. These results are representative of three experiments of similar design.
cells may act via mechanisms other than NO. Cell surface TNF has been shown to mediate cytotoxic activities by CD4\(^+\) T cells, as measured via l929 fibroblast lysis assays (25) and thus can act via mechanisms other than up-regulation of NO. Furthermore, TNF in combination with IFN-\(\gamma\) can synergistically activate a diverse number of other macrophage antimicrobial activities, such as expression of the tryptophan-limiting enzyme indolamine 2, 3-oxogenicase (26, 27), or induction of apoptosis (28). The combined action of these two cytokines may also redirect the intracellular trafficking of LVS to a compartment that does not support replication of the organism. The identity of other possible macrophage mechanisms that limit LVS intracellular growth will be the subject of ongoing studies.

The in vivo role of memTNF during primary infection has recently been explored in the \(M.\) \(tuberculosis\), \(L.\) \(monocytogenes\), and \(F.\) \(tularensis\) LVS murine infection models. In all cases, although memTNF contributed to resistance to infection, mice expressing only memTNF were more susceptible to infection than WT mice. The increased susceptibility of the memTNF mice to \(L.\) \(monocytogenes\) infections was attributed to a requirement for soluble TNF to regulate chemokine expression and coordinate an effective inflammatory response (29–31). In the presence of only memTNF, there was significant overproduction of chemokines during primary infection. Transient overproduction of IL-12(p40) was also observed in \(M.\) \(tuberculosis\)-infected memTNF mice (32, 33). This dysregulated inflammatory response was the likely cause of increased inflammatory lesions containing neutrophils and necrotic tissue destruction in the lung (\(Mycobacterium\) or liver (\(Listeria\)) of infected animals, resulting in mortality. Collectively, these studies indicate that soluble TNF has a regulatory function that limits excessive Th1 proinflammatory responses during primary intracellular infections.

The role of memTNF during \(Mycobacterium\) and \(Listeria\) secondary challenges has also been explored (29, 30). Similar to the results observed here (Fig. 1), \(Listeria\)-primed memTNF mice were fully protected against a lethal secondary \(Listeria\) challenge (29). Furthermore, in both the \(Mycobacterium\) and \(Listeria\) murine models, adoptive transfer of immune WT or memTNF-expressing T cells to TNF KO mice was sufficient to confer protection against challenge (29, 30), indicating that, similar to our in vitro observations, macrophage-derived TNF is not essential for T cell-mediated control of bacterial intracellular growth (Fig. 6, A and B). Thus, in all three of the \(Mycobacterium\), \(Listeria\), and \(Francisella\) murine infection models, memTNF is capable of mediating full resistance to secondary challenges. Here, we further demonstrate that there is a defect in the regulation of the IFN-\(\gamma\)–IL-12 axis, as evidenced by overproduction of both cytokines in the memTNF mice as compared with the WT mice. This defect was observed in vivo (Fig. 1, C and D), as well as in vitro (Fig. 3, B and C), and was not specifically explored in either the \(Mycobacterium\) or \(Listeria\) secondary challenge memTNF murine studies. The results presented here demonstrate that soluble TNF is an important component in the regulation of the IL-12-IFN-\(\gamma\) axis during secondary LVS infection.

Dysregulation of the IL-12-IFN-\(\gamma\) circuit can have deleterious in vivo effects, artificially inflating the proinflammatory response. Indeed, overproduction of these cytokines, in the presence and absence of TNF, can affect survival during infection: depletion of T cells from TNF KO mice in the \(Mycobacterium\) \(bovis\) bacillus Calmette-Guérin infection model prolonged survival by limiting excessive Th1-type inflammatory responses (34, 35). Acute toxoplasmosis is accompanied by overproduction of Th1 cytokines that is ultimately lethal (35). Similarly, it is well-known that patient therapies using excessive IL-12 treatment can be detrimental to the host (36). Additionally, TNFR1 KO mice were acutely susceptible to \(Mycobacterium\) \(avium\) infection in a manner that was dependent on T cells and excessive production of IL-12 (37). Most of these examples describe primary exposures to infectious agents, where the levels of IFN-\(\gamma\) and IL-12 are significantly higher than those observed here during secondary LVS challenge. Thus, because the secondary LVS infection was quickly resolved in the memTNF mice, and the levels of IFN-\(\gamma\) measured were low as compared with primary LVS infections, the observed disruption of the IL-12-IFN-\(\gamma\) circuit was not detrimental to the host. Although we did not examine the effect of IL-12-IFN-\(\gamma\) dysregulation on pathology in this study, it clearly did not have an impact on optimal protection against LVS secondary challenge. The results presented here underscore the differential requirements for key cytokines such as IFN-\(\gamma\), IL-12, and TNF during primary and secondary intracellular infections, and the need to explore both types of infections for a complete understanding of immunity to intracellular pathogens.

TNF has a critical role in activating macrophages to control LVS intramacrophage growth. For example, in vitro, the addition of neutralizing anti-TNF Abs to LVS-infected macrophages abolished the ability of IFN-\(\gamma\) treatment to induce NO production and control intracellular growth (5, 23). Similar in vitro observations have been made for other intracellular pathogens, including \(Leishmania\), \(Listeria\), and \(Mycobacterium\) species (38–40). Thus, one important role of TNF in intracellular infections is the direct control of bacterial intramacrophage growth via NO production. Here, we demonstrate that although memTNF can induce macrophage production of NO through stimulation of macrophage TNFRs, it is not as efficient as soluble TNF (Figs. 2B and 5, A and B). Previous studies have shown both reduced and normal levels of inducible NO synthase activity in the spleens of memTNF mice given a primary i.v. bacillus Calmette-Guérin infection (32, 33). Other studies have demonstrated overproduction of inducible NO synthase mRNA in memTNF mice during \(Listeria\) primary infection (29). This is the first study to show directly that T cell-derived memTNF is less efficient at activating macrophages to produce NO in the absence of soluble TNF during intracellular infection.

The differential abilities of memTNF and soluble TNF to control intracellular infections has important implications for anti-TNF treatment therapies that are currently on the market. Treatment regimens that target TNF function have been highly successful in improving chronic inflammatory diseases such as rheumatoid arthritis and Crohn’s disease. However, patients receiving these treatments have an increased occurrence of granulomatous infections such as \(M.\) \(tuberculosis\), \(L.\) \(monocytogenes\), and \(Histoplasma\) \(capsulatum\) (41). Tularemia is not included in this list, likely because it is not a common public health problem. However, \(F.\) \(tularensis\) is an intracellular pathogen that causes granulomatous lesions in humans, and the results obtained here are likely representative of other similar intracellular pathogens. Infliximab is a chimeric anti-TNF mAb that binds both soluble and membrane TNF (42). In contrast, etanercept is a dimeric fusion protein consisting of the extracellular portion of TNFRp75 linked to the Fc domains of human IgG1, binding mostly soluble trimeric TNF, and exhibiting low avidity for memTNF (42). As shown here, complete neutralization of TNF with an anti-TNF mAb significantly diminishes the ability of LVS-immune CD4\(^+\) and CD8\(^+\) T cells to control \(Francisella\) infection. However, cultures containing only memTNF still maintain control of LVS intracellular growth in vitro. In keeping with these observations, the highest incidences of infectious complications are associated with infliximab, which binds both soluble and membrane TNF (43). Although LVS-immune T cells expressing memTNF can control LVS growth in vitro
and in vivo, the observed dysregulation of IFN-γ and IL-12 pro-
duction could lead to unexpected complications in vivo, even when
memTNF is available.

Identification of correlates of protection during T cell-mediated
protective immune responses to intracellular pathogens is a major,
and thus far elusive, goal. Although IFN-γ is often proposed as a
correlate, the bulk of data accumulated to date suggest that vac-
cine-induced IFN-γ production may be a necessary, but not suf-
ficient, indicator of secondary protection (44). Frequently, the
presence of IFN-γ either in sera or in stimulated culture superna-
tants does not reliably predict immune status. The studies pre-
sented here clearly demonstrate that T cell, but not macrophage,
expression of memTNF is critical to the ability of immune T cells
to control and eliminate intracellular bacterial growth, particularly
for CD8+ T cells. Whether memTNF is typically used by T cells
to control LVS intracellular growth, or whether it is only func-
tionally important in the absence of soluble TNF, remains to be
determined. Production of TNF has also been proposed as a po-
tential alternate or complementary T cell correlate (45). The find-
ings presented here have important implications for the application
of TNF as a correlate; measurement of memTNF is much more
difficult technologically than measurement of secreted TNF (46). Se-
creted TNF quantities may not reflect the most relevant T cell
activities, but thus far attempts to measure cell surface TNF on
activated LVS-immune T cells using flow cytometry have been
inconclusive (data not shown). Therefore, future studies will be
directed at efforts to quantitate each form of this important medi-
ator, with a view toward facilitating better understanding of the
relative importance of membrane and soluble TNF, and determin-
ing its use as a practical correlate of protection for intracellular
infections.

Disclosures

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