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Transmembrane TNF Is Sufficient to Initiate Cell Migration and Granuloma Formation and Provide Acute, but Not Long-Term, Control of *Mycobacterium tuberculosis* Infection

Bernadette M. Saunders,* Stephen Tran,* Sigrid Ruuls, ‡ Jonathon D. Sedgwick, ‡ Helen Briscoe, ‡ and Warwick J. Britton*‡

TNF is critical for immunity against *Mycobacterium tuberculosis* infection; however, the relative contributions of the soluble and transmembrane forms of TNF in this immunity are unknown. Using memTNF mice, which express only the transmembrane form of TNF, we have addressed this question. Wild-type (WT), TNF−/−, and transmembrane TNF (memTNF) mice were infected with *M. tuberculosis* by aerosol. TNF−/− mice developed overwhelming infection with extensive pulmonary necrosis and died after only 33 days. memTNF mice, like WT mice, contained bacterial growth for over 16 wk, developed an Ag-specific T cell response, and initially displayed compact granulomas, comprised of both lymphocytes and macrophages. Expression of mRNA for the chemokines CXCL10, CCL3, CCL5, and CCL7 was comparable in both WT and memTNF mice. As the infection progressed, however, the pulmonary lesions in memTNF mice became larger and more diffuse, with increased neutrophil accumulation and necrosis. This was accompanied by increased influx of activated memory T cells into the lungs of memTNF mice. Eventually, these mice succumbed to infection with a mean time to death of 170 days. The expression of memTNF on T cells is functionally important because the transfer of T cells from memTNF, but not TNF−/− mice, into either RAG−/− or TNF−/− mice conferred the same survival advantage on the *M. tuberculosis*-infected recipient mice, as the transfer of WT T cells. Therefore, memTNF, in the absence of soluble TNF, is sufficient to control acute, but not chronic, *M. tuberculosis* infection, in part through its expression on T cells. *The Journal of Immunology*, 2005, 174: 4852–4859.

**T**umor necrosis factor is a pleiotropic cytokine critical to controlling a vast array of immunological responses. The importance of TNF in the generation and maintenance of a protective immune response against *Mycobacterium tuberculosis* and a host of other bacterial and viral pathogens has been clearly demonstrated (1–3). Studies in gene-deleted mice, infected with mycobacteria species of varying virulence, have demonstrated that, although TNF is not required for the generation of an Ag-specific T cell response, it is essential for controlling the recruitment of inflammatory cells to sites of infection and the development of a protective granulomatous response, with containment of bacilli growth and the survival of infected animals (2, 4–7). TNF is involved at almost every stage of the inflammatory response, from the initial macrophage response to the presence of *M. tuberculosis*, to the attachment, migration, and trafficking of leukocytes through blood vessels, and to their retention at the site of infection (8–10). However, excess TNF is associated with debilitating autoimmune diseases, such as rheumatoid arthritis and Crohn’s disease. Neutralizing TNF is effective therapy for both conditions (1, 11, 12); however, this treatment leaves *M. tuberculosis*-infected individuals at an enhanced risk for reactivation of latent tuberculosis (TB) infection (13–15). This confirms the ongoing requirement for TNF to maintain protective immunity against TB.

TNF is synthesized as a nonglycosylated, transmembrane protein, that forms an active homotrimer with two other TNF chains (16–18). TNF is initially membrane-bound, and then it undergoes cleavage by the specific metalloproteinase TNF-converting enzyme (TACE) to form a soluble trimer (10). Both the transmembrane and soluble forms of TNF function through binding to one of two receptors (TNFRI and TNFRII), which are found on almost all cell types (17, 19). In addition, transmembrane TNF (memTNF) also contains a casein kinase I motif and itself can act as a receptor (20, 21).

The memTNF mice used in this study were generated by replacing the endogenous C57BL/6 TNF allele with the mutant Δ1–9,K11E TNF allele (22). This mutant retains normal cell surface expression and functional capacity of wild-type (WT) memTNF, but is resistant to TACE-mediated cleavage (22). memTNF mice showed reduced disease severity in the murine model of autoimmune encephalomyelitis with less inflammation in the spinal cord than WT mice (22). The availability of these mice has allowed us to examine contributions of memTNF and soluble TNF in mediating immunity to mycobacterial infection. We report here that the expression of memTNF alone was sufficient to control TB infection for >16 wk. Moreover, transmembrane expression of TNF on T cells promoted T cell migration and enhanced the survival of
RAG$^{-/-}$ and TNF$^{-/-}$ recipients of memTNF T cells, as compared with recipients of TNF-deficient T cells. Nevertheless, although memTNF alone was adequate to establish a strong protective immune response to TB infection, it was insufficient for the maintenance of long-term protective immunity following M. tuberculosis infection.

Materials and Methods

Animals

Specific pathogen-free, female C57BL/6 and RAG-deficient (RAG$^{-/-}$) mice at 6–8 wk of age were purchased from the Animal Research Centre (Perth, Australia). TNF gene-deficient mice (TNF$^{-/-}$) were generated and maintained under specific pathogen-free conditions in the Centenary Institute Animal Facility, as previously described (23). memTNF mice were generated at DNAX Research Institute (22). These mice express a TNF gene lacking the TACE cleavage site; they display normal levels of membrane-bound TNF, but without a functional cleavage site, no soluble TNF is produced. All mice were maintained in the Centenary Institute Animal facility under ethical approval from the Sydney University Animal Ethics committee.

T cell purification

Single-cell spleen suspensions were prepared by sieving through 200-μm mesh and resuspending the cells in culture medium. To purify CD4$^+$/H11001 nT mice of TNF-deficient T cells. Nevertheless, although memTNF mice were generated at DNAX Research Institute (22). These mice express a TNF gene lacking the TACE cleavage site; they display normal levels of membrane-bound TNF, but without a functional cleavage site, no soluble TNF is produced. All mice were maintained in the Centenary Institute Animal facility under ethical approval from the Sydney University Animal Ethics committee.

Enrichment and infection of peritoneal exudate-derived macrophages

Ten milliliters of cold PBS with 20 U/ml heparin was injected into the peritoneal cavity of mice. Peritoneal exudate was collected, and 10$^6$ cells/ml were cultured in 24-well plates with 100 U of IFN-γ per well. Following overnight incubation and then removal of nonadherent cells, adherent macrophages were infected with Mycobacterium bovis bacillus Calmette-Guérin (BCG) (multiplicity of infection, 1) or stimulated with LPS (30 μg/ml). Supernatants were collected after 24 h, and biologically active TNF was assayed by bioassay as previously described (24).

Animal irradiation and infection

WT, TNF$^{-/-}$, and memTNF mice were infected via aerosol with 70–100 M. tuberculosis H37Rv bacilli using a Middlebrook airborne infection apparatus (Glas-Col). In transfer experiments, TNF$^{-/-}$ recipient mice were irradiated with 500 rad before infection. Groups of WT, TNF$^{-/-}$, or RAG$^{-/-}$ mice were infected i.v. with 1 × 10$^7$ M. tuberculosis H37Rv at the time of transfer of 1 × 10$^5$ purified T cells. At determined time points, bacterial load was enumerated in the spleen, liver, and lung following homogenization and serial dilution of organ homogenates onto 7H11 agar and incubation for 2–3 wk at 37°C.

Histology

Liver and lung sections were inflated and fixed with 10% neutral buffered formalin for at least 2 wk before embedding in paraffin and sectioned at 5 μm. Sections were stained with H&E. To determine the percentage of the lung area with inflammatory cell infiltration, whole lung sections were scanned using Image-Pro Plus software (Media Cybernetics), and Adobe Photoshop was used to calculate the percentage of the whole lung with inflammatory cell involvement after subtracting the background cellularity in normal lung.

Lymphocyte responses to mycobacterial Ags

Single-cell suspensions of lung and mediastinal lymph nodes were prepared as previously described (25). In brief, lungs were inflated through the aorta with 10 ml of PBS with 20 U/ml heparin. Lungs were sliced and incubated at 37°C with 50 U/ml collagenase I type 4197 (Worthington) and 13 μg/ml DNAse I (Boehringer Mannheim) for 60 min before passing the suspensions through 200-μm mesh and resuspending the cells in culture medium. Single-cell spleen and lymph node suspensions were prepared by passing cells through a mesh sieve, centrifuging, and resuspending the cells in culture medium. RBC were lysed and cell suspensions were distributed at 2 × 10$^5$ cells per well of 96-well plates and incubated with Con A (Sigma-Aldrich) or H37Rv culture filtrate proteins (CFP) (5 μg/ml) for 72 h before measuring IFN-γ production. CFP was kindly provided by Dr. J. Belisle (Colorado State University, Fort Collins, CO) under the National Institutes of Health TB research material and vaccine testing Contract N01-A1-75320. The concentrations of IFN-γ in culture supernatants were determined by capture ELISA using a capture assay with Abs R4-6A2 and XMG2 biotin and using a rIFN-γ standard (Roche).

Phenotypic analysis

Leukocyte subpopulations in the spleen were determined by staining for cell markers using fluorescently labeled Ab enumerated on a FACSCalibur: CD4, CD8 (Caltag), CD44, and CD45R (BD Pharmingen).

RNA purification and synthesis

Lung tissue was lysed in 1 ml of RNazol Bee (Tel-Test) and immediately stored at −70°C. Total RNA extractions were performed using the acid phenol-guanidine method (26). All samples were DNase treated with DNA-free kit (Ambion) following manufacturer’s instructions. For reverse transcription, 5 μg of RNA were mixed with oligo(dT) primers (0.1 μg) (Invitrogen Life Technologies) at 70°C for 10 min, and cooled at 4°C afterward. Reverse transcription was performed at 37°C for 60 min in a total volume of 20 μl containing 100 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies), first-strand buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2) (Invitrogen Life Technologies), 20 mM DTT (Invitrogen Life Technologies), 2 mM dNTPs (Promega), and 20 U of RNase OUT RNase inhibitor (Invitrogen Life Technologies). All cDNA samples were diluted 1/25 in diethylpyrocarbonate-treated water.

Real-time PCR quantification using SYBR Green 1

PCR were performed with Platinum qPCR Supermix-UDG (Invitrogen Life Technologies), containing 0.5× concentration of SYBR Green 1 (Molecular Probes). The total volume of each PCR is 20 μl, comprising the following: 8 μl of cDNA sample, 10 μl of Platinum qPCR Supermix-UDG with SYBR Green 1, 100 nM each primer, and 1 μl of 6-carboxyfluorescein (ROX; 50× concentration) (Invitrogen Life Technologies). Primers for all target genes were designed using Primer Express 1.5 software (Applied Biosystems) and are listed in Table I. PCR were performed with Model 7700 Sequence Detector (Applied Biosystems) using the following thermal conditions: stage 1, 50°C for 2 min, and 95°C for 10 min; stage 2, 95°C for 25 s, and 60°C for 1 min. Stage 2 was repeated for 40 cycles. The identity and purity of the PCR product were confirmed by melting curve analysis. All data were analyzed using the Sequence Detector 1.7 software (Applied Biosystems) to calculate the threshold cycle number (C$_T$) used to quantify target gene expression of each sample using the comparative C$_T$ method (27). Results represent the expression of target gene relative to WT uninfected.

Statistical analysis

Statistical analysis of the results from immunological assays and log-transformed bacterial counts were conducted using ANOVA. Fisher’s protected least significant difference ANOVA post hoc test was used for pairwise comparison of multigrouped data sets. Differences with $p < 0.05$ were considered significant. Survival was calculated on a Kaplan Meier non-parametric survival plot, and significance was assessed by the log rank (Mantel-Cox) test.

Results

Macrophages from memTNF mice do not release soluble TNF following BCG infection

Activated macrophages are known to produce large amounts of TNF following mycobacterial infection. To confirm the pattern of TNF response to mycobacterial infection, adherent macrophages derived from peritoneal cells of WT, memTNF, and TNF$^{-/-}$ mice were activated with IFN-γ before infection with M. bovis BCG (multiplicity of infection, 1) or stimulation with LPS (50 ng/ml). WT macrophages released over 333 pg/ml TNF following BCG infection or LPS stimulation, whereas unstimulated macrophages produced <10.4 pg/ml. Macrophages derived from memTNF or TNF$^{-/-}$ mice released no detectable TNF.
memTNF contains acute M. tuberculosis replication in the absence of soluble TNF.

TNF is an essential component for sustained protective immunity against M. tuberculosis infection. To explore the relative contributions of memTNF and soluble TNF in this immunity, WT, TNF⁻/⁻, and memTNF mice were infected via aerosol with a low dose of M. tuberculosis H37Rv, and the course of infection was examined. TNF⁻/⁻ mice were unable to control mycobacterial growth, showing a 2 log₁₀ increase in bacterial numbers in the lung by day 28 (Fig. 1). However, the transmembrane expression of TNF was sufficient to control the growth of M. tuberculosis during the first 16 wk of infection. WT and memTNF mice displayed equivalent bacterial loads in the lungs, spleen, and liver during this time (Fig. 1).

M. tuberculosis infection stimulates T cell activation in the absence of both soluble TNF and memTNF.

At 4 wk postinfection, TNF⁻/⁻ mice showed a marked increase in CD4 and CD8 T cell numbers as part of the gross cellular infiltrate that occurs late in infections (Fig. 2A; Ref. 2). There was a small rise in CD4 and CD8 T cells in memTNF mice at this time point. Then, later in infection after 12 wk, the numbers of activated CD4 and CD8 T cells within the lungs of the memTNF-infected mice increased significantly when compared with WT mice, although bacterial load remained equivalent. The majority of the recruited T cells are CD4⁺ and CD8⁺, and CD44hiCD45lo cells are a clear marker of activated cells (Fig. 2B; Ref. 2).

Table I. PCR primer sets for real-time PCR assays

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<th>Target Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Length (bp)</th>
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<td>90</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CGCTCTCTGGAAGATGGTGAT</td>
<td></td>
</tr>
<tr>
<td>CXCL10 (IP-10)</td>
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<tr>
<td></td>
<td>Reverse</td>
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</tr>
<tr>
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<td></td>
<td>Reverse</td>
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</tr>
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<tr>
<td></td>
<td>Reverse</td>
<td>CCACCTCTCTGCTTTTGTACGA</td>
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FIGURE 1. MemTNF is sufficient to control the growth of M. tuberculosis. WT (□), TNF⁻/⁻ (■), and memTNF (●) mice were infected via aerosol with 70–100 M. tuberculosis bacilli. The course of infection was examined in the lung (A), spleen (B), and liver (C) at the times indicated. The means and SDs of bacterial loads were determined by ANOVA (*, p < 0.001).

FIGURE 2. MemTNF is sufficient to stimulate normal initial cellular influx and the generation of Ag-specific T cells. WT, TNF⁻/⁻, and memTNF mice were infected via aerosol with 70–100 M. tuberculosis bacilli. The times indicated, single-cell lung suspensions were prepared from infected mice. A, Cells were stained for T cell markers, CD4 and CD8, and CD44 hiCD45 low phenotype was determined by flow cytometry. WT (□), TNF⁻/⁻ (■), and memTNF (●) mice. B, At 28 (a) and 56 (b) days postinfection, lung cells from WT (□), TNF⁻/⁻ (■), and memTNF (●) mice were cultured at 2 × 10⁵ cells/well with CFP for 72 h. IFN-γ levels in culture supernatants were determined via ELISA. Cultures of uninfected lung cells produced <100 pg/2 × 10⁵ cells of IFN-γ.
FIGURE 3. memTNF is sufficient to control early inflammation and granuloma formation in M. tuberculosis infection, but is insufficient to control inflammation in chronically infected mice. WT, TNFα−/−, and memTNF mice were infected via aerosol with 70–100 M. tuberculosis bacilli. At the times indicated, the lung lobes were perfused with 10% neutral buffered formalin, fixed in paraffin, and processed. A and B, WT mice at 4 wk postinfection. Lesions are discrete and localized with minimal interstitial pneumonia evident in the rest of the lobe. Lesions are composed of macrophages and lymphocytes in close juxtaposition throughout the granuloma and only very occasional neutrophils are seen. C and D, Lungs of TNFα−/− mice 4 wk postinfection. Massive necrosis of the infected lung with large sections of dead tissue, pyknotic material, and pneumonia, evidenced by blocked airways (arrow). Darkly stained collections of cells are predominantly neutrophils. E and F, memTNF mice at 4 wk postinfection. As in WT mice, the pulmonary lesions are discrete and compact surrounded by largely normal lung with minimal interstitial pneumonia. Macrophages and lymphocytes, closely interspersed throughout the lesions, are the two predominant cell types, and very few neutrophils are evident. G and H, WT mice 16 wk postinfection. Lesions are still discrete, although somewhat larger than at 4 wk postinfection, and surrounded by apparently normal lung alveoli. Dense accumulations of lymphocytes surrounded by large epithelioid macrophages with occasional neutrophils are evident, as are occasional multinucleated giant cells. I and J, memTNF mice at 16 wk postinfection. Lesions are larger and more diffuse than at 4 wk and are larger than those in WT mice at this time point. They are still composed predominantly of macrophages and lymphocytes, although increased accumulations of neutrophils (arrow) are visible compared with WT mice. Dense lymphocytic accumulations are found dispersed throughout the lesions and are also accumulated around blood vessels. H&E-stained sections. A, C, E, G, and I, ×25 magnification. B, D, F, H, and J, ×400 magnification. Sections are representative of lung sections from four to five mice per group and from one of two experiments.

Histological analysis of the lungs of the infected mice revealed that, by 4 wk postinfection, WT mice had developed compact lesions composed predominantly of lymphocytes and macrophages with very few neutrophils present (Fig. 3, A and B). In striking contrast, the lungs of the TNFα−/− mice infected with M. tuberculosis displayed massive inflammation with extensive necrosis involving the majority of the lung (Figs. 3, C and D, and 4). Neutrophils were the predominant infiltrating cell type, and extensive cellular destruction was evident within the lesions, which also contained areas of pyknotic material, neutrophils, and some macrophages. Lymphocytes were found around the perivascular cuff of blood vessels, but not within the central inflammatory lesions. The CD4 and CD8 T cells in both the WT and memTNF mice displayed an activated phenotype being CD44+CD45low. Mediastinal lymph node cultures from memTNF mice also displayed a significant increase in cell numbers evident from 8 wk postinfection (data not shown). Cultures of lung cells from WT and memTNF mice produced similar levels of IFN-γ in response to M. tuberculosis CFP at days 28 and 56 postinfection (Fig. 2B). Lung cells from TNFα−/− mice produced IFN-γ with and without additional CFP stimulation at day 28, probably because the lungs of these mice had such a high bacterial load within pulmonary macrophages. At 16 wk postinfection, lung cells from infected mice continued to produce significant amounts of IFN-γ following CFP stimulation, although levels were lower than those seen at 4 and 8 wk postinfection (WT, 1353.6 ± 54 pg/2 × 105 cells; memTNF, 2160 ± 337 pg/2 × 105 cells). Furthermore, a similar pattern of IFN-γ production was observed in mediastinal lymph nodes cultures (data not shown).

memTNF contributes to T cell migration and initial granuloma formation

FIGURE 4. MemTNF is not sufficient to control inflammation in chronically infected mice. WT (■), TNFα−/− (●), and memTNF (○) mice were infected as described in Fig. 1. At the times indicated, a lobe of the left lung was perfused in formalin, mounted, sectioned, and stained with H&E. The percentage of the lung area involved with inflammatory cell infiltration was calculated using Image-Pro Plus software (Media Cybernetics) and Adobe Photoshop, and is represented as the percentage of the whole lung involved after subtracting the background cellularity in normal lung. The statistical significance of differences between memTNF and WT mice was determined by ANOVA (*, p < 0.05, WT vs TNFα−/−, WT vs memTNF).
mice developed pneumonia, evidenced by the filling of airways with cellular debris. However, the expression of memTNF was sufficient to prevent this initial inflammatory damage (Figs. 3, E and F, and 4). The lesions in the memTNF mice resembled those seen in the WT mice, with compact granulomas, composed predominantly of lymphocytes and macrophages. Lesions were discrete, interstitial pneumonia was minimal, and large sections of the lung appeared normal.

As the infection progressed, the inflammatory response in the WT mice remained largely confined to discrete lesions containing lymphocytes and macrophages. Tight wedges of lymphocytes were visible, interspersed between large epithelioid macrophages (Fig. 3, G and H). During the first 8 wk of infection, memTNF mice developed lesions of similar size and structure to those of WT mice (Figs. 3 and 4). However, memTNF mice were unable to maintain these discrete lesions long term, and by 12 wk postinfection, they were markedly larger than those seen in WT mice, and small pockets of neutrophils were evident (data not shown). By 16 wk, lesions were noticeably more diffuse in memTNF mice (Fig. 3, I and J), compared with their WT counterparts (Fig. 3, G and H). The cellular composition of the granulomas was still predominantly macrophages and lymphocytes, but an increased neutrophil infiltrate was evident. Neutrophil numbers were smaller than those observed in TNF−/− mice before their demise. Some lymphocyte wedges remained visible within lesions, but increasingly, lymphocytes remained localized around the perivascular cuff. Quantitative assessment revealed significantly increased inflammatory cell involvement by 12 wk postinfection in the lungs in memTNF mice compared with WT mice (Fig. 4) (p < 0.04), even though there was no difference in bacterial load at this time.

Reduced long-term survival of memTNF-infected mice

As previously described, (2) TNF−/− mice, deficient in both the soluble and transmembrane forms of TNF, succumb rapidly to M. tuberculosis infection with a mean survival time of only 33 days (Fig. 5). The expression of memTNF increased resistance to TB, but mice were unable to control infection long term and became moribund with a mean survival time of 170 days. By contrast, WT mice all survived to 300 days postinfection at which time the experiment was terminated.

Enhanced chemokine mRNA expression in WT and memTNF-infected mice

The formation of protective granulomas requires the production of an array of chemokines that permit the recruitment, migration, and retention of inflammatory cells at the site of infection. We have previously demonstrated, using RNase protection assays, that TNF−/− mice infected i.v. with M. tuberculosis show a delay in the induction of a number of chemokines within the lung, with a corresponding delay in the recruitment of inflammatory cells to the site of infection (28). However, two weeks following aerosol infection, there was no significant difference in the early expression of mRNA for the chemokines CXCL10, CCL3, and CCL7 measured in the lungs of WT, TNF−/−, and memTNF mice (Fig. 6A). By 4 wk postinfection, both WT and memTNF lungs displayed increased mRNA for the four chemokines measured, with a similar decline in levels by 12 wk postinfection in three independent experiments. In one of these experiments, the levels of mRNA were actually higher in the memTNF lungs than those seen in the WT lung, although they declined to similar levels by 12 wk postinfection. Therefore, the induction of chemokines was not impaired in the memTNF mice. Furthermore, we showed that the expression of mRNA for TNF follows a similar pattern in the lungs of both WT and memTNF mice (Fig. 6B).

**FIGURE 5.** memTNF alone is insufficient to control chronic M. tuberculosis infection. WT (□), TNF−/− (○), and memTNF (○) mice were infected via aerosol with 70–100 M. tuberculosis bacilli. Infected mice were monitored daily and euthanized when they showed signs of ill health. Data represent the times to euthanization of 8–10 mice from two similar experiments. *p < 0.001, memTNF vs WT, TNF−/− vs WT, and TNF−/− vs memTNF; log rank (Mantel-Cox).

**FIGURE 6.** Presence of memTNF is associated with induction of chemokines in the lung during M. tuberculosis infection. WT (□), TNF−/− (○), and memTNF (●) mice were infected as described in Fig. 1. At the times indicated, a lobe of lung was removed and digested in RNAzol. RNA was extracted, and cDNA was transcribed. A, mRNA levels, for the chemokines CXCL10, CCL3, CCL5, and CCL7, were measured by quantitative real-time PCR as described in Materials and Methods. Data are the means of chemokine mRNA expression compared with the levels in WT uninfected mice for five mice/group. Data are representative of one of three experiments. B, mRNA expression for TNF was measured by quantitative real-time PCR as described. Data are the means of infected vs WT uninfected lungs from five mice/group and are representative of one of three experiments.
RAG-- recipients of TNF-deficient T cells (mean survival times: RAG-- 26 days; TNF--→RAG-- 46 days; WT→RAG-- 72 days; memTNF→RAG-- 86 days) (Fig. 7A). Increased survival was not associated with any significant difference in bacterial loads between any of the groups of RAG-- infected mice (Fig. 7B). Nor was it associated with a failure of the transferred CD4 and CD8 T cells to proliferate within their RAG-- hosts following M. tuberculosis infection (Fig. 7C). Interestingly, we found that T cells from TNF-- mice proliferated more extensively than T cells from WT mice.

T cells expressing memTNF increase the survival of TNF--/ mice

Finally, we investigated whether T cell expression of memTNF alone was effective at controlling mycobacterial infection in TNF-deficient animals. The transfer of 10^6 purified memTNF or WT T cells into irradiated (irr) TNF-- mice at the time of i.v. M. tuberculosis infection conferred an equivalent and significant survival advantage to these mice, (Fig. 8A). Mean survival times in mice were as follows: TNF-- mice, 32 days; irr TNF-- mice, 30 days; TNF--→irr TNF-- mice, 32 days; WT→irr TNF-- mice, 46 days; and memTNF→irr TNF-- mice, 46 days. This survival advantage was not associated with a reduction in bacterial growth, because there were no significant differences in the bacterial loads in the spleens and livers of any of groups of the TNF--/ M. tuberculosis-infected mice (Fig. 8B). Histological examination of liver tissue revealed that RAG-- and TNF--/ recipients of WT and memTNF T cells formed a small number of compact lesions after infection, whereas in recipients of TNF--/ T cells, the lesions were diffuse, larger, and more numerous (data not shown).

Discussion

The study identifies distinct functional roles for soluble TNF and memTNF in the control of M. tuberculosis infection. We demonstrate that protective immunity is established in the absence of soluble TNF; however, memTNF alone is insufficient to provide lasting protection during chronic M. tuberculosis infection.

Control of TB infection requires activation of the bactericidal activity of infected macrophages to kill or control bacterial replication and the concurrent containment of these macrophages to prevent further dissemination of the bacilli. There is strong evidence that TNF is required for both arms of this response. Macrophage killing of mycobacteria is more efficient when macrophages are pretreated with both TNF and IFN-γ (30–32), and inhibition of TNF with neutralizing Ab reduces mycobacterial killing. Furthermore, the inflammatory response generated following M. tuberculosis infection is highly dysregulated in TNF--/ mice. Although T cells and macrophages are recruited to the lungs of M. tuberculosis-infected mice, the T cells collect around the perivascular cuff and fail to migrate into central lesions, thereby limiting their contact with infected macrophages (2). Moreover, infection of TNF-- or TNFR--/ mice with mycobacteria of varying virulence leads to progressive fatal necrosis in the lung and liver (2, 4, 6, 7, 33–35). T cells contribute to the development of this necrosis, because depletion of either CD4 or CD8 T cells during Mycobacterium avium or BCG infection increased the survival of
TNFRp55\(^{-/-}\) mice (34, 36) and reduced the overwhelming hepatic necrosis. Therefore, T cells are essential for development of protective immunity to mycobacterial infection (24, 37, 38), but they also contribute to the increased pathology in the absence of TNF. The reasons for this duality are uncertain; however, a reduction in TNFRI signaling is associated with reduced T cell apoptosis and increased inflammation, not only in mycobacterial infections, but in infection with Leishmania major or Rhodococcus equi and in autoimmune encephalomyelitis (34, 39, 40).

This current study demonstrates that memTNF alone permits the migration of T cells and their localization within infected lesions in mice infected with aerosolized \textit{M. tuberculosis}. Furthermore, memTNF is sufficient to prevent the development of the early necrosis seen in TNF\(^{-/-}\) mice. Ag-specific, IFN-\(\gamma\)-producing T cells were found within the lungs of WT and memTNF-infected mice (Fig. 2), and the lymphocytes were in close juxtaposition with macrophages in the granulomatous lesions. Lymphocyte accumulations are clearly visible within the lesions seen in WT and memTNF mice by 4 wk postinfection (Figs. 3, A and E). Furthermore, the association of lymphocytes and macrophages was maintained in memTNF even as the lesions become larger and more diffuse by 12 wk (Figs. 3, G and I, and 4). This suggests that failure of T cell recruitment was not responsible for the eventual demise of the memTNF animals. Therefore, memTNF, in the absence of soluble TNF, is sufficient both for T cell migration and the stimulation of the bactericidal activity in infected macrophages. Indeed, it is clear that macrophages were efficiently and sufficiently activated during the first 16 wk of infection because there was no increase in mycobacterial numbers in memTNF compared with WT mice (Fig. 1).

The importance of memTNF expression on T cells was further emphasized in the adoptive transfer studies. Transfer of TNF-deficient (TNF\(^{-/-}\)) T cells conferred only a small degree of protection to RAG\(^{-/-}\) recipients infected with 10\(^5\) \textit{M. tuberculosis} organisms and no protection after high-dose (10\(^6\) organisms) infection (29). However, transfer of memTNF T cells to RAG\(^{-/-}\) (Fig. 7A) or TNF\(^{-/-}\) (Fig. 8B) mice provided a significantly greater survival advantage than TNF\(^{-/-}\) T cells, commensurate with that conferred by WT T cells. These data, particularly from the experiments performed in the TNF\(^{-/-}\) recipients where the sole source of TNF was that membrane-bound cytokine on transferred T cells, support our conclusion that memTNF is sufficient to orchestrate T cell migration and granuloma formation and provide initial protection against \textit{M. tuberculosis}.

Another critical parameter in the inflammatory response to mycobacterial infection is the production of chemokines. These are required for the recruitment and migration of leukocytes into the site of infection, acting in concert with the up-regulation of adhesion molecules, such as ICAM-1 and VCAM (8, 10). We have previously shown (28) that induction of several chemokines in the liver is delayed in the absence of TNF during mycobacterial infection. Reduced chemokine expression has also been demonstrated in purified lung macrophages from TNF\(^{-/-}\) compared with WT mice at 2 wk post-\textit{M. tuberculosis} infection (41). In addition, we show here that, in the presence of memTNF, the expression of mRNA for selected chemokines (CCL3, -5, -7, and CXCL10) was detected at 2 wk postinfection and rose in a similar pattern to WT mice. In the chronic phase of infection, at 12 and 16 wk, there were equivalent levels of mRNA for the four chemokines measured in both the memTNF and WT mice. These data provide unambiguous evidence that soluble TNF is not required for the induction and maintenance of chemokines following \textit{M. tuberculosis} infection.

However, despite the adequate chemokine and cellular response during the initial phase of \textit{M. tuberculosis} infection, memTNF alone failed to provide long-term protection. Two principal differences were noted between the WT and memTNF mice as the infection progressed. First, there were increased numbers of activated T cells present in the lung during the chronic phase of infection (Fig. 2). Second, an increasing proportion of the lung was involved by the inflammatory cell influx (Fig. 4); even though the bacterial load was not raised in the lungs at 16 wk, the mice eventually succumbed, presumably due to the detrimental pathology in the lung. These observations suggest that soluble TNF has distinct functions late in \textit{M. tuberculosis} infection, perhaps by exercising negative feedback to limit the inflammatory cells present in the lung or through facilitating macrophage activation during chronic infection (36). The increased size and density of the granulomas in memTNF mice late in infection may not permit sufficient direct interaction between memTNF on T cells and TNFRs on macrophages. Therefore, soluble TNF may be necessary for macrophage activation and sustained protective immunity.

The complex regulation of protective responses to mycobacteria by members of the TNF superfamily was exemplified by studies in memTNF knockin mice that were deficient in both soluble TNF and lymphotoxin-\(\alpha\) (LT-\(\alpha\)). These mice developed a 5-fold increase in bacterial growth when compared with WT animals after 4 wk infection with \textit{M. tuberculosis} (42). This may be the result of deficits in both LT-\(\alpha\) and soluble TNF, because LT-\(\alpha\) plays an essential role in protective immunity to TB, which is not compensated for by TNF (43). A similar effect of LT-\(\alpha\), independent of memTNF, was observed in the development of cerebral malaria in susceptible mice (44).

The majority of clinical TB in humans is due to reactivation of latent TB infection. The mechanisms involved in the containment of latent bacilli and the contribution of TNF superfamily members to this process is incompletely understood. However, studies in the murine model of chronic TB infection have proven that TNF is essential for the containment of the infection, because neutralization of TNF resulted in the fatal reactivation of \textit{M. tuberculosis} disease (33, 35). Furthermore, transgenic mice expressing soluble TNFRII-Fc in the lungs (45) demonstrated a more rapid progression of disease with a reduced mean survival time. This increased susceptibility was associated with a more marked inflammatory response and necrosis in the lungs. Recent use of anti-TNF therapy in humans has also highlighted the importance of TNF in control of latent TB infection. A significant number of patients with rheumatoid arthritis and Crohn’s disease receiving anti-TNF therapy developed reactivation of latent TB within 3 mo of commencing therapy (13, 46). Interestingly, patients treated with the anti-TNF mAb, infliximab, which blocks both soluble TNF and memTNF, may be at greater risk of reactivation of TB than patients treated with the soluble TNFRII-Fc, etanercept, which blocks soluble TNF but binds weakly to memTNF (15, 47). These findings parallel our observations in mice and suggest that, in humans, signaling through memTNF may be sufficient for sustained resistance to TB disease in some individuals. Understanding the mechanisms of action of both soluble and membrane-bound forms of TNF may contribute to the refinement of therapies for human diseases as well as shedding light on the activity of soluble TNF and memTNF throughout the acute, latent, and reactivation phases of TB disease.

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References


