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Differential Requirements for Soluble and Transmembrane Tumor Necrosis Factor in the Immunological Control of Primary and Secondary *Listeria monocytogenes* Infection

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The relative contributions of transmembrane tumor necrosis factor (memTNF) and soluble tumor necrosis factor (solTNF) in innate and adaptive immunity are poorly defined. We examined the capacities of wild-type (WT) mice, TNF$^{-/-}$ mice, and memTNF mice, which express only transmembrane TNF, to control primary and secondary *Listeria monocytogenes* infections. Soluble TNF was not required for induction or maintenance of protective immunity against a low-dose (200-CFU) *Listeria* infection. In contrast to TNF$^{-/-}$ mice, both WT and memTNF mice cleared the bacilli within 10 days and were fully protected against rechallenge with a lethal infective dose. Furthermore, T cells transferred from immune mice, but not from naïve, WT, and memTNF mice, protected TNF$^{-/-}$ recipients against an otherwise lethal infection. By contrast, infection with a higher dose of *Listeria* (2,000 CFU) clearly demonstrated that solTNF is required to coordinate an optimal protective inflammatory response. memTNF mice were more susceptible to a high-dose infection, and they exhibited delayed bacterial clearance, increased inflammation, and necrosis in the liver that resulted in 55% mortality. The dysregulated inflammation was accompanied by prolonged elevated expression of mRNAs for several chemokines as well as the macrophage effector molecules inducible nitric oxide synthase and LRG-47 in the livers of memTNF mice but not in the livers of WT mice. These data demonstrated that memTNF is sufficient for establishing protective immunity against a primary low-dose *Listeria* infection but that solTNF is required for optimal control of cellular inflammation and resistance to a primary high-dose infection. By contrast, memTNF alone is sufficient for resolution of a secondary, high-dose infection and for the transfer of protective immunity with memory T cells.

Listeria monocytogenes is a ubiquitous, facultative intracellular coccobacillus that is associated with septic abortion in pregnant women and disease in immunocompromised patients (31). Immunity to listerial infection is due to rapid activation of neutrophils and macrophages (6, 18, 25), in addition to *Listeria*-specific CD4$^+$ and CD8$^+$ T cells, particularly those targeting the pore-forming exotoxin listeriolysin O (1, 11, 13, 23, 28). Tumor necrosis factor (TNF), a pleiotropic cytokine that mediates a broad range of proinflammatory activities, is expressed predominantly by activated macrophages and T cells and has an essential protective role in *Listeria* infection. TNF$^{-/-}$ mice, as well as animals deficient in TNFRI, are highly susceptible to listeriosis (24, 26). In contrast, administration of recombinant TNF rescued animals from an otherwise lethal dose of *Listeria* (12, 19, 24, 26).

There are two forms of TNF, transmembrane TNF (memTNF) and soluble TNF (solTNF), which function physiologically by interacting with the receptors TNFRI and TNFRII. TNF is initially synthesized as a nonglycosylated, transmembrane protein, which exists as a homotrimer. Membrane-bound TNF may be cleaved by the matrix metalloprotease, tumor necrosis factor alpha-converting enzyme, which leads to the release of soluble TNF (16). The two TNF receptors are expressed on a diverse range of cell types, but they have distinct downstream effects (22). Furthermore, identification of a casein kinase I motif in the intracellular domain of memTNF suggests that memTNF itself is also able to transduce signals as a receptor (14, 36).

The relative contributions of memTNF and solTNF to inflammation during infection or autoimmune disease have been difficult to elucidate. The availability of mice which express only memTNF on a TNF$^{-/-}$ or TNF$^{-/-}$/lymphotoxin$^{-/-}$ background permits analysis of this question. Mice which express memTNF in the absence of solTNF (27) or in the absence of both solTNF and lymphotoxin (20) were protected against acute *Mycobacterium tuberculosis* infection, but they succumbed to late progressive infection (21, 29). Furthermore, in a model of autoimmune encephalomyelitis, the disease progression in memTNF mice was indistinguishable from the disease progression in wild-type (WT) mice, whereas TNF$^{-/-}$ mice showed delayed disease onset (27).

In this study, we used memTNF mice to determine the relative contributions of soluble and memTNF to the control of primary and secondary *L. monocytogenes* infections. memTNF mice cleared a low-dose *Listeria* infection with kinetics similar to those of WT mice, and they were fully protected against an otherwise lethal secondary challenge. However, following primary infection with a higher dose of *Listeria*, memTNF mice exhibited delayed clearance of bacteria with slower T-cell ac-

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cumulation and increased inflammation and mortality, indicating that soluble TNF is required for optimal control of a primary infection. In contrast, expression of memTNF alone by immune T cells was sufficient to transfer immunity to *Listeria* infection.

MATERIALS AND METHODS

Animals. C57BL/6 mice that were 6 to 10 weeks old were purchased from the Animal Resource Centre (Perth, Australia). TNF-deficient mice ($TNF^{-/-}$) were generated by targeted disruption of TNF in C57BL/6 mice as previously described (15). memTNF mice, in which the gene expressing memTNF was placed into $TNF^{-/-}$ mice, were generated at DNAX Research Institute, Palo Alto, CA (27). All mice were kept in specific-pathogen-free conditions at the Centenary Institute Animal Facility. All experiments were undertaken with the approval of the University of Sydney Animal Ethics Committee.

Experimental infection. *L. monocytogenes* strain EGD was prepared as previously described (24). WT, memTNF, and $TNF^{-/-}$ mice were infected with *Listeria* intravenously via the lateral tail vain. At specified times, the numbers of viable bacteria in the spleen and liver were determined by plating serial dilutions of organ homogenates on tryptic soy agar (Difco, Detroit, MI) and incubating the preparations overnight. Heat-killed *L. monocytogenes* was prepared by incubating *Listeria* cells at 80°C for 2 h. For transfer experiments, $TNF^{-/-}$ mice were irradiated with 500 rads prior to infection. Groups of mice were injected intravenously with 200 bacilli and 7.5×10^6 purified T cells. Bone marrow-derived macrophages were cultured with 15% L929 supernatant for 6 days before overnight prestimulation with combinations of 200 U/ml gamma interferon (IFN-γ) and 10 ng/ml of lipopolysaccharide. Macrophages were infected for 1 h with *Listeria* at a multiplicity of infection of 1, they were washed, and bacterial loads were determined after 4 h of incubation.

Cytokine production and phenotypic analysis of cellular infiltration. Single-cell suspensions were prepared from mouse livers perfused with phosphate-buffered saline (PBS) containing heparin (10 U/ml; Sigma, St. Louis, MO) and from splenocytes. Erythrocytes were lysed, and cells were suspended in RPMI medium (Cytosystem, Sydney, Australia) containing 10% fetal calf serum (Trace, Sydney, Australia), 2 mM L-glutamine, 0.5 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Trace), and 100 μg/ml streptomycin (CSL, Melbourne, Australia). Leukocytes were incubated on ice with anti-CD16/CD32 monoclonal antibodies (MAbs) (BD Pharmingen, San Diego, CA) and then stained with fluorescently labeled MAbs. In addition, some leukocytes were cultured overnight on anti-CD3 MAb-coated plates (BD Pharmingen) to which brefeldin A (Sigma) was added for the final 4 h; the surface markers were stained, and the cells were permeabilized for intracellular staining. The fluorescent MAbs used for phenotypic analysis with a FACSCalibur (Becton Dickinson, San Jose, CA) were CD4-allophycocyanin, CD8a–peridinin-chlorophyll protein (PerCP), CD8b.2-phycoerythrin, Gr-1–fluorescein isothiocyanate, Mac-1–allophycocyanin, NK1.1-phycoerythrin, CD62L-phycoerythrin (BD Pharmingen), IFN-γ–fluorescein isothiocyanate, and isotype controls (Caltag, Burlingame, CA). The percentage of apoptosis was determined by annexin-V staining (BD Pharmingen), performed according to the manufacturer's instructions. Splenocytes were cultured with heat-killed *L. monocytogenes* or medium (control) for 72 h. IFN-γ production in the culture supernatant was determined by a capture enzyme-linked immunosorbent assay, as previously described (29).

T-cell purification. T cells were enriched from single-cell spleen suspensions by magnetic cell sorting with indirect microbeads (Miltenyi Biotec, Gladbach, Germany). Briefly, cells were incubated with a combination of phycoerythrin-conjugated anti-B220 and anti-major histocompatibility complex class II MAb, followed by anti-phycoerythrin microbeads, before negative selection using an autoMACs (Miltenyi Biotec). Purification was confirmed by staining for the leukocyte markers CD3, CD4, CD8, Mac-1, and B220, using fluorescently labeled antibodies (BD Pharmingen) and analysis with a FACSCalibur. This procedure resulted in acquisition of T-cell populations whose purity was greater than 93%.

RNA purification and RTQ-PCR. Liver tissue was homogenized in 1 ml RNAzol trireagent (Sigma) and stored at −70°C. Extraction of total RNA, RNA purification, reverse transcription, and real-time quantitative PCR (RTQ-PCR) were performed as previously described (29). Primers for all target genes (Table 1) were designed using the Primer Express 1.5 software (Applied Biosystems, Foster City, CA) and were made by Proligo (Sydney, Australia). RTQ-PCR was performed with a PE Applied Biosystems model 7700 sequence detector. The identity and purity of the PCR product were confirmed by melting curve analysis. All data were analyzed using the PE Applied Systems Sequence Detector 1.7 software and were plotted as the increase in fluorescence intensity of the SYBR green reporter dye versus the cycle number. The threshold cycle number was used to quantify the target gene expression for each sample, using the comparative threshold cycle method. The results represented the expression of the target gene relative to the expression in WT uninfected mice.

TABLE 1. RTQ-PCR primer sets

Molecule	Direction	Primer sequence (5′→3′)
Murine GAPDH[a]	Forward	CTC CAC TCA CGG CAA ATT CA
	Reverse	CGC TCC TGG AAG ATG GTG AT
Murine CCL3	Forward	CCA AGT CTT CTC AGC GCC AT
	Reverse	TCC GGC TGT AGG AGA AGC AG
Murine CCL4	Forward	AGG GTT CTC AGC ACC AAT GG
	Reverse	GCT GCC GGG AGG TGT AAG A
Murine CXCL1	Forward	CCC TCA ACG GAA GAA CCA AA
	Reverse	AGG CAC ATC AGG TAC GAT CCA
Murine iNOS	Forward	TGC CCC TTC AAT GGT TGG TA
	Reverse	ACT GGA GGG ACC AGC CAA AT
Murine LRG-47	Forward	AAA GGT CCA CAG ACA GCG TCA CTC G
	Reverse	CAG GGG AGC ATA ATG GGT CTC TGC

[a] GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Histology. Liver tissue samples were perfused and fixed in 10% neutral buffered formalin (Fronine, Sydney, Australia) and embedded in paraffin blocks, and 5-μm sections were cut. The sections were stained with hematoxylin and eosin for histopathological examination.

Immunofluorescence. Perfused liver tissue samples were snap frozen in optimal cutting temperature compound (Tissue-Tek, Sakura, Japan). Then 5-μm sections of the tissue were cut with a Cryostat (Microm, Walldorf, Germany), adhered to poly-L-lysine-coated (Sigma) slides, and fixed in acetone (BDH, Melbourne, Australia) for 10 min. The samples were rehydrated in PBS and then kept in a humidity chamber at 37°C, with two PBS washes between steps. Samples were blocked with 30% horse serum for 10 min before polyclonal rabbit anti-mouse inducible nitric oxide synthase (iNOS) (Upstate Biotech, Lake Placid, NY) was added for 30 min. Anti-rabbit immunoglobulin G-fluorescein isothiocyanate (Silenus, Melbourne, Australia) was added for 30 min, and then antifade mountant [0.3% 1,4-diazabicyclo(2,2,2)octan (Merck, Darmstadt, Germany), 90% glycerol (BDH)] was applied.

Statistical analysis. Statistical analyses of the results of immunological assays and log-transformed bacterial counts were conducted using analysis of variance (ANOVA) or Student's *t* test. Fisher's least protected significance difference post hoc test was used for pairwise comparison of multigroup data sets. Survival was calculated with a Kaplan-Meier nonparametric survival plot, and significance was assessed by the log rank Mantel-Cox test. A P value of <0.05 was considered significant.

RESULTS

Delayed bacterial clearance and increased mortality in memTNF mice following high-dose, but not low-dose, primary *Listeria* infection. In order to determine if transmembrane TNF alone is sufficient to induce protective immunity against a primary *Listeria* infection, WT, $TNF^{-/-}$, and memTNF mice were infected intravenously with a high dose of *L. monocytogenes* (2,000 CFU). The bacterial loads increased initially in the WT mice, peaking at days 3 to 5, before rapid clearance by day 10 (Fig. 1A and B). In contrast, $TNF^{-/-}$ mice were unable to control the growth of *Listeria*; these mice became moribund (mean survival time, 4.5 days; $P < 0.0001$) (Fig. 1C), and the bacterial loads were 2 and 4 $\log_{10}$ higher in the spleen and liver, respectively, by day 5 ($P < 0.0001$) (Fig. 1A and B). In memTNF mice, the bacterial growth initially resembled that in their WT counterparts. From day 4, however, the memTNF mice diverged into two phenotypes: those that were able to control and clear the *Listeria* infection, albeit with a significant delay ($P < 0.02$) compared to WT mice, and those that developed an overwhelming infection and became moribund be-

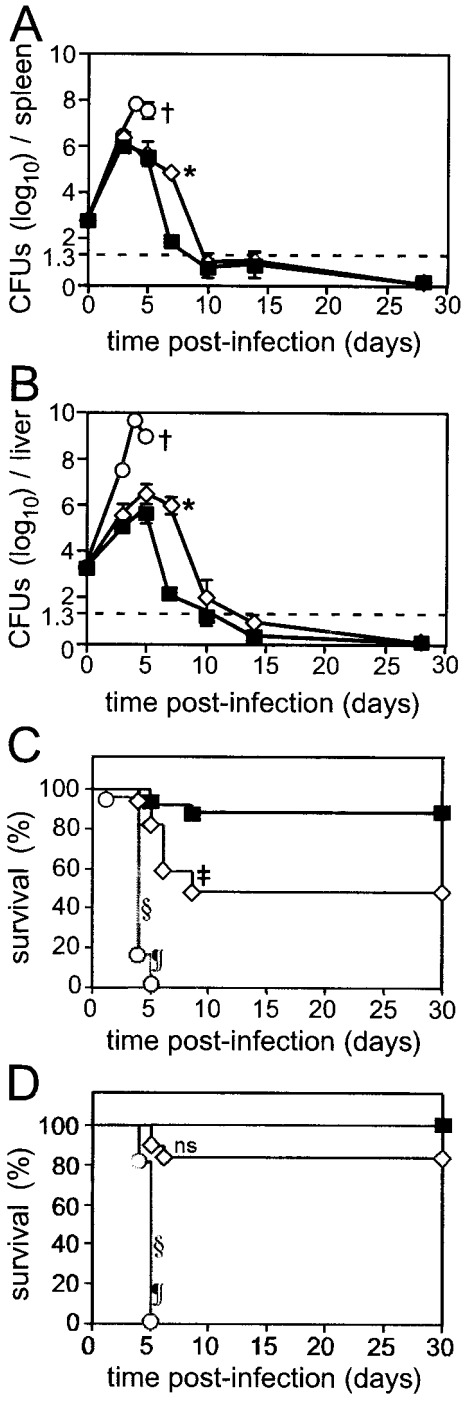

FIG. 1. Delayed bacterial clearance and increased susceptibility of memTNF mice to *Listeria* infection. WT (■), memTNF (◇), and $TNF^{-/-}$ (○) mice were infected with 2,000 (A to C) or 200 (D) CFU of *Listeria* intravenously. The bacterial loads in the spleen (A) and liver (B) were determined by serial dilution of organ homogenates at different times. The dotted line indicates the limit of detection. The data are the means and standard errors for five mice per group from one of three representative experiments. Significance was determined by ANOVA. An asterisk indicates that the P value was <0.02 for a comparison of memTNF and WT mice, and a dagger indicates that the P value was <0.0001 for a comparison of $TNF^{-/-}$ and WT mice. Additional groups of mice were monitored twice daily and euthanized if they displayed signs of declining health. (C) Time to euthanasia in three experiments following infection of WT (n = 19), memTNF (n = 19), and $TNF^{-/-}$ (n = 11) mice with 2,000 CFU of *Listeria*. (D) Time to euthanasia in two experiments following infection of WT (n = 21), memTNF (n = 29), and $TNF^{-/-}$ (n = 19) mice with 200 CFU of *Listeria*. Significance was determined by the log rank Mantel-Cox test. A double dagger indicates that the P value was <0.009 for a comparison of memTNF and WT mice; a section sign indicates that the P value was <0.0001 for a comparison of $TNF^{-/-}$ and WT mice; and a paragraph sign indicates that the P value was 0.0001 for a comparison of memTNF and $TNF^{-/-}$ mice. ns, not significant.

tween 4 and 8 days postinfection. This resulted in an overall mortality rate of 55% ($P < 0.009$) (Fig. 1C). The listerial burdens in moribund mice (7.5 log_{10} in the spleen and 8.5 log_{10} in the liver) were markedly increased and similar to those in $TNF^{-/-}$ mice (7.5 log_{10} and 9.0 log_{10}, respectively). When mice were infected with a 10-fold-lower dose of *Listeria* (200 CFU), WT and $TNF^{-/-}$ mice responded with patterns similar to those observed with the higher dose. WT mice cleared the infection, while $TNF^{-/-}$ mice became moribund (Fig. 1D). The majority of memTNF mice also survived a low-dose infection (Fig. 1D) and cleared the bacilli without the delay seen in memTNF mice given 2,000 CFU (data not shown).

Delayed accumulation of leukocytes in memTNF mice during primary *Listeria* infection. The primary site of *Listeria* infection is the liver. To determine if the increased susceptibility of the memTNF mice to a high-dose infection was due to alterations in the inflammatory response, the influx of leukocytes into the liver was measured throughout the course of infection. The numbers of macrophages and neutrophils increased in all groups by day 3 and peaked in WT and memTNF mice at day 7 (Fig. 2). In WT mice, despite the early development of small inflammatory foci (described below), the total numbers of leukocytes did not increase significantly until day 5, when they rapidly expanded, reaching a peak at day 7 before decreasing (Fig. 2). Leukocyte recruitment to the livers of memTNF mice, however, was delayed ($P < 0.04$ at day 7), and the population did not reach the maximal size until day 14, 1 week after the peak of infection. These differences were largely due to a delay in the recruitment of T cells into the liver. In WT mice, the number of $CD4^+$ T cells rose sharply after day 5 and remained elevated from day 7 to day 28, while the number of $CD8^+$ T cells increased greatly beginning on day 5, peaked at day 10, and fell to preinfection levels by day 14. In contrast, the maximal increases in the numbers of $CD4^+$ and $CD8^+$ T cells in memTNF mice did not occur until days 10 to 14 (Fig. 2 and 3). At day 7, significantly fewer T cells were isolated from the livers of memTNF mice than from the livers of WT mice (for $CD4^+$ T cells, $P < 0.002$; for $CD8^+$ T cells, $P < 0.03$). Despite the differences in the total numbers of T cells isolated from the liver, the percentages of T cells that were positive for the apoptotic marker annexin-V were not significantly different between the WT and memTNF mice (Table 2).

Delayed T-cell responses in memTNF mice during listerial infection. Both $CD4^+$ and $CD8^+$ T cells contribute to protective antilisterial immunity. The numbers of $CD4^+$ and $CD8^+$ T cells in the spleens and livers of infected WT mice increased rapidly beginning on day 5. These cells displayed the activated phenotypes $CD62L^{lo}$ (Fig. 3A) and $CD44^{hi}$ (data not shown). Elevated numbers of activated $CD4^+$ T cells were still detect-

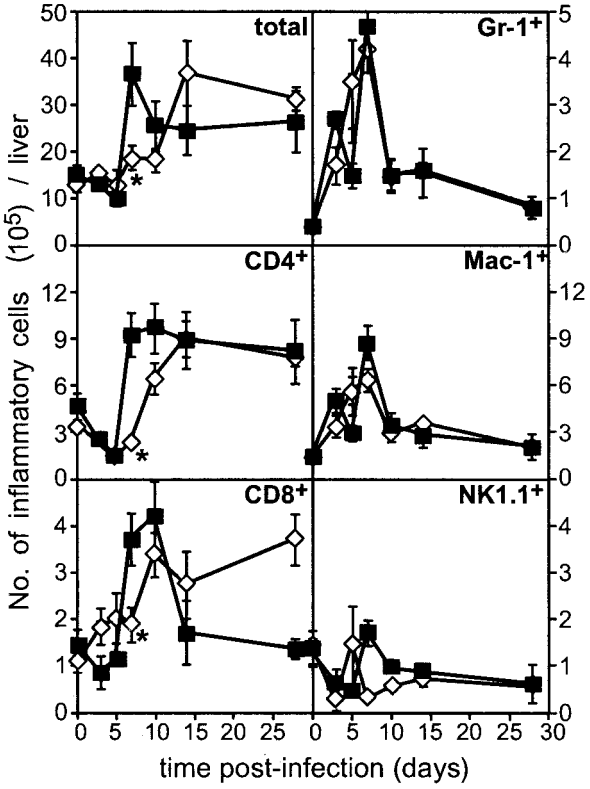

FIG. 2. Delayed influx of T cells in livers of infected memTNF mice. WT (■) and memTNF (◇) mice were infected with 2,000 CFU of *Listeria* intravenously. Livers were perfused, and single-cell suspensions were prepared from uninfected and infected mice at different times. Leukocytes were enumerated, stained for CD4, CD8, Gr-1, Mac-1, and NK1.1, and analyzed by flow cytometry. The data are the means and standard errors for five mice per group from one of two representative experiments. Significance was determined at day 7 by Student's *t* test. An asterisk indicates that the *P* value was <0.04 for a comparison of memTNF and WT mice.

able in the liver at day 28, but the numbers had declined to preinfection levels in the spleen by day 14. The recruitment of activated $CD4^+$ T cells to the livers of memTNF mice was delayed to day 10 ($P < 0.02$) and then followed the pattern of the WT response. $CD4^+$ T-cell activation was significantly delayed in the spleens of memTNF mice (at day 7, $P < 0.003$). Furthermore, the numbers of activated $CD8^+$ T cells, which are required for protection against *Listeria* infection, peaked at day 10 in the livers of both memTNF and WT mice and then declined to preinfection levels in WT mice but remained elevated in memTNF mice at 28 days postinfection. In the spleen, the numbers of activated $CD8^+$ T cells peaked between days 7 and 10 in both WT and memTNF mice.

IFN-γ production is the hallmark of an activated Th1 response. In the spleens of both WT and memTNF mice, the peak number of IFN-γ-producing T cells occurred by day 7 (Fig. 3B), although beginning on day 5, cultures of spleen cells, which contained similar numbers of T cells, from the two strains of mice produced similar levels of IFN-γ in response to heat-killed *L. monocytogenes* (Fig. 3C). Examination of the livers showed that while in WT mice the number of IFN-γ-secreting $CD4^+$ T cells had risen beginning on day 5 and remained elevated, in memTNF mice the expansion of this population was delayed to day 7 postinfection ($P < 0.0004$ at day 7). Infiltration of IFN-γ-secreting $CD8^+$ T cells was comparable in the livers and spleens of WT and memTNF mice.

Enhanced and prolonged macrophage activation in memTNF mice. Activation of macrophage antibacterial functions is a key requirement for successful elimination of the invading pathogen. It has previously been demonstrated that the enzymes iNOS and LRG-47 are crucial for the development of protective immunity against *Listeria* (4, 17). The mRNA expression in the livers of WT and memTNF *Listeria*-infected mice revealed that the levels of both iNOS and LRG-47 increased in WT and memTNF mice by 3 days postinfection and remained elevated in memTNF mice but not in WT mice at day 7 (Fig. 4). Staining for iNOS expression in the liver demonstrated that in WT mouse livers, iNOS expression was confined to a few discrete foci at day 3 and that there was slight enlargement by day 7 (Fig. 5A and B). However, iNOS was present throughout several larger lesions in memTNF mice at day 3 and remained prevalent in larger regions of tissue at day 7 (Fig. 5C and D). In vitro killing assays demonstrated that IFN-γ-stimulated bone marrow-derived macrophages from memTNF mice were as effective as WT macrophages in killing *Listeria* (for WT mouse macrophages, 36.3% killing compared to unstimulated macrophages; for memTNF mouse macrophages, 35.0% killing compared to unstimulated macrophages).

Increased cellular inflammation and necrosis in livers of memTNF mice. The livers of *Listeria*-infected WT mice contained small discrete inflammatory foci, in addition to the occasional (<one lesion/section) small necrotic lesion at day 5 (Fig. 5E). The foci were predominantly composed of tight aggregates of macrophages and lymphocytes, with the occasional necrotic lesion containing primarily neutrophils, circumscribing the area of necrosis (Fig. 5F). The number and size of inflammatory foci were greatest at day 5, and the lesions progressively cleared throughout the course of infection (data not shown). By day 14, only very occasional small cellular foci were still evident, and the rest of the liver appeared normal. In contrast, in the livers of infected $TNF^{-/-}$ mice there were large areas of cellular infiltration and extensive tissue destruction, and neutrophils were the dominant cell type (Fig. 5G and H). Analysis at a higher magnification revealed that the cells at the circumference of necrosis were heavily infected with coccobacilli (data not shown).

In contrast to the distinct pathological phenotypes of infected WT and $TNF^{-/-}$ mice, memTNF mice infected with 2,000 CFU of *Listeria* exhibited a spectrum of pathologies, particularly at day 5. Mice that appeared to be healthy exhibited an immunological response similar to that of the WT mice but showed increased pathology. The livers contained increased numbers of small compact foci per section, along with more frequent larger necrotic lesions (Fig. 5I and J). In addition, mice that appeared to be physically moribund (with ruffled fur, reduced mobility, and weight loss) showed liver pathology similar to that of the $TNF^{-/-}$ mice (Fig. 5K and L). The memTNF mice that survived the early infection proceeded to clear the bacilli, and there was a progressive reduction in inflammatory lesions. Overall, clearance of the inflammatory foci was delayed in the memTNF mice compared with the WT mice, and larger numbers of inflammatory foci were still evi-

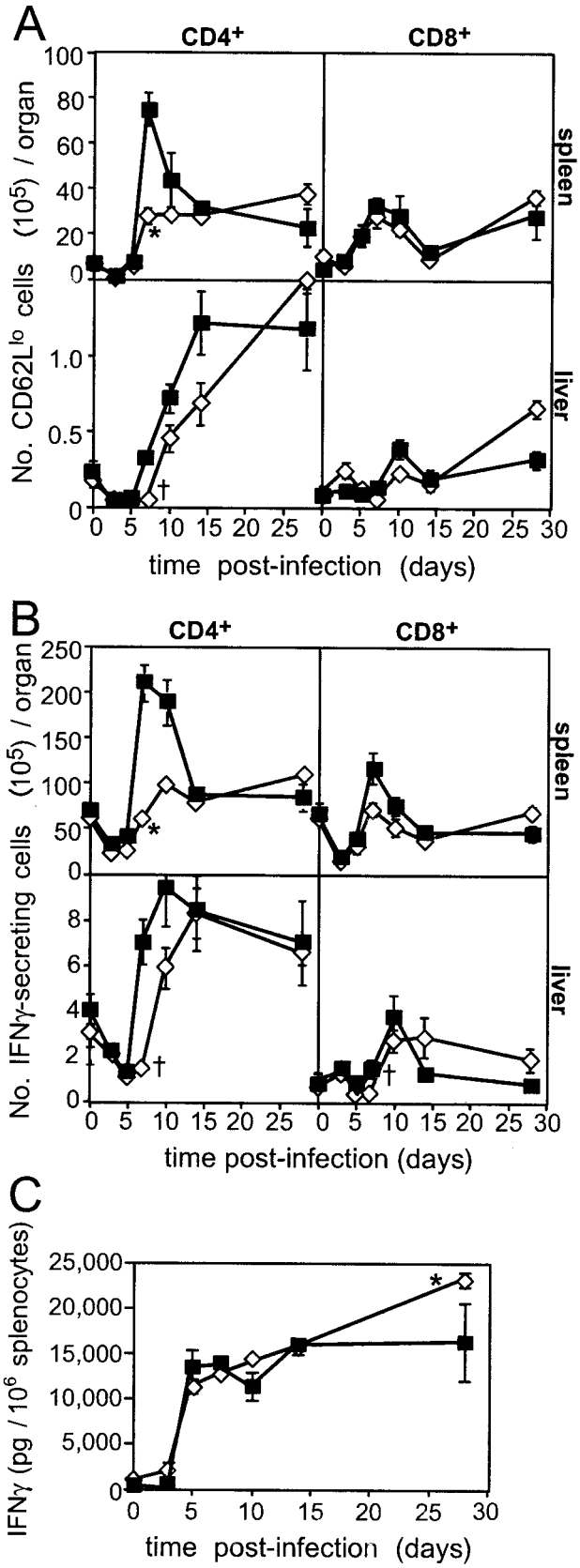

FIG. 3. T-lymphocyte activation and IFN-γ secretion in spleens and livers of infected memTNF mice. WT (■) and memTNF (◇) mice were infected with 2,000 CFU of *Listeria* intravenously. (A and B) Single-cell suspensions were prepared from splenocytes and leukocytes from perfused livers of uninfected and infected mice at different times. Leukocytes were enumerated, stained for coexpression of CD4 or CD8 and surface CD62L (A) or intracellular IFN-γ (B), and detected using flow cytometry. (C) Splenocytes were cultured for 72 h with heat-killed *L. monocytogenes* or medium alone, and IFN-γ production was measured in the culture supernatant by an enzyme-linked immunosorbent assay. The data are the means and standard errors for five mice per group from one of two representative experiments. Significance for the spleen values was determined by ANOVA, and an asterisk indicates that the P value was <0.05 for a comparison of memTNF and WT mice. Significance for the liver values at day 7 was determined by Student's t test, and a dagger indicates that the P value was <0.03 for a comparison of memTNF and WT mice.

dent in memTNF mouse livers at 14 days postinfection (data not shown).

memTNF mice that received a low dose of *Listeria* also showed increased inflammatory responses in the liver, even though there was no delay in the clearance of bacteria. The inflammatory response in WT mice peaked at 5 to 7 days postinfection and was resolved by day 10. In $TNF^{-/-}$ mice, extensive necrosis occurred in low-dose infections, as well as in high-dose infections (data not shown). The inflammatory response in the memTNF mice resembled that in the livers of memTNF mice that survived a high-dose infection. The inflammatory foci were discrete and localized but more extensive than those in WT mice. Moreover, the inflammatory response resolved more slowly in the memTNF mice than in the WT mice, and there were increased numbers of inflammatory foci in the liver at 10 days postinfection (data not shown).

Enhanced and prolonged expression of chemokine mRNA in memTNF mice. In order to determine whether the differences in accumulation of leukocytes observed in the *Listeria*-infected livers were due to altered chemokine expression, mRNAs for the monocyte/lymphocyte-attracting chemokines CCL3 and CCL4 and the neutrophil attractant CXCL1 were examined. Early neutrophil recruitment is essential for optimal protective immunity against *Listeria* infection (5), so initially we examined the response in the liver after only 24 h of infection. At this time, WT mice expressed 3-fold more CXCL1 mRNA than memTNF mice expressed (for WT mice, 15.56-fold increase compared with uninfected mice [range, 7.9- to 19.3-fold]; for memTNF mice, 5.48-fold increase [range, 4.3- to 6.3-fold]), although there was no early increase in CCL3 expression (for WT mice, 0.51-fold increase; for memTNF mice, 1.3-fold increase). We also examined expression of the chemokines CXCL1, CCL3, and CCL4 over the course of the listerial infection (Fig. 6). Expression of these three chemokines was elevated in all three strains of mice at day 3 and returned to the basal levels by day 7 in only the WT mice. In the memTNF mice, mRNA expression remained elevated at day 7 and did not return to the basal levels until day 14.

T cells from immune memTNF mice protect against secondary *Listeria* infection. To determine whether memTNF is sufficient for the development and expression of memory responses, WT and memTNF mice were infected with 200 CFU of *Listeria* and 8 weeks later challenged with 10^5 *Listeria* CFU/mouse. This dose was lethal for both WT and memTNF naïve mice, which had highly elevated bacterial loads at day 3 (Fig. 7)

TABLE 2. Percentages of apoptotic cells in the livers of WT and memTNF mice[a]

Day	% of apoptotic cells			
	$CD4^+$		$CD8^+$	
	WT mice	memTNF mice	WT mice	memTNF mice
0	28.5 ± 3.0	39.5 ± 19	9.0 ± 5.1	6.5 ± 3.7
3	30.7 ± 12.9	21.1 ± 10.7	2.9 ± 2.1	5.4 ± 3.8
7	43.3 ± 5.1	41.9 ± 5.8	19.0 ± 2.3	15.9 ± 1.9

[a] WT and memTNF mice were infected with 2,000 CFU of *Listeria*. Leukocytes from the liver were purified at different times and stained for annexin-V. The data are the means ± standard deviations for four mice per group.

and succumbed to infection between days 2 and 4 (Fig. 8A). Both WT and memTNF mice which had previously cleared a low-dose infection controlled this otherwise lethal infection; there were 4-$\log_{10}$-fewer bacteria the liver and spleen at day 3 than in naïve mice (Fig. 7), and the level of survival was 100% (Fig. 8A). Furthermore, we examined the rate of proliferation of immune T cells from both WT and memTNF mice following restimulation. T cells from immune mice were purified, labeled with carboxyfluorescein diacetate succinimidyl ester, and transferred into WT and memTNF naïve recipients, which were then challenged with a lethal dose of *Listeria*. There was no difference in the patterns of migration or rates of proliferation of immune T cells from WT and memTNF mice (data not shown). At 72 h postinfection, we found that immune T cells from both strains of mice had preferentially migrated to the liver, and $CD8^+$ T cells in the liver were the only cells that underwent more than one round of division (data not shown).

T cells from immune memTNF mice transfer protection to TNF-deficient mice. Finally, we examined the role of memTNF expressed on T cells in conferring protection to $TNF^{-/-}$ mice. T cells from naïve or immune mice that had been infected with 200 CFU of *Listeria* 14 days previously were transferred into $TNF^{-/-}$ recipients at the time of challenge with 200 CFU of *Listeria*. $TNF^{-/-}$ mice that received naïve T cells from either WT or memTNF mice succumbed to infection after 4 to 5 days (Fig. 8B), whereas over 70% of $TNF^{-/-}$ mice that received immune T cells from either WT or memTNF mice survived infection. When these mice were culled 28 days after infection, they had completely eradicated the bacterial infection, their livers were normal, with no discernible inflammation remaining, and they exhibited strong antigen-specific IFN-γ responses (data not shown).

DISCUSSION

This study established the differential requirements for soluble and transmembrane TNF for the control of primary and secondary *Listeria* infections. TNF was required for optimal control of a primary *Listeria* infection, acting principally through coordination of the inflammatory response. Transmembrane TNF was sufficient to resolve a low-dose infection, although there was increased hepatic inflammation, but at a higher infective dose of *L. monocytogenes*, a bimodal response to infection developed between days 3 and 6. While the initial bacterial loads in WT and memTNF mice were comparable, differences in cell recruitment, with more diffuse cellular influx and increased necrosis in the livers of the memTNF mice, were visible by day 3. About one-half of the memTNF mice developed a rapidly progressive course of infection with florid hepatic destruction (Fig. 5I and K) and subsequent mortality. The surviving memTNF mice exhibited delayed bacterial clearance (Fig. 1A and B) and a dysregulated inflammatory response with delayed recruitment of T cells and increased pathology. Indeed, the inflammation in the memTNF mice was more severe than that in the WT mice at all stages of infection. This inflammation resolved more slowly after clearance of the bacteria in mice that survived a primary infection, indicating that soluble TNF may contribute to restoration of the normal homeostatic mechanisms after bacterial clearance.

This study is the first study to demonstrate that protective immunity to *Listeria* infection can be conferred by memTNF-expressing T lymphocytes. Thus, T-cell expression of transmembrane TNF is sufficient to permit effective T-cell migration and activation of infected macrophages with resolution of infection.

TNF plays an important role in establishing coordinated immune responses, in part through regulated recruitment of inflammatory cells, stimulation of adhesion molecule expression on endothelial cells, and induction of chemotaxis (32, 33). Our data confirm that neither soluble nor transmembrane TNF is essential for the upregulation of chemokine expression, but the regulation of chemokine mRNA is altered in the absence of soluble TNF. The initial expression of the neutrophil-recruiting chemokine CXCL1 was higher in WT mice at 24 h postinfection than in memTNF mice. As neutrophils play a major protective role in resistance to *Listeria* infection, the capacity of WT mice to recruit neutrophils quickly to the site of

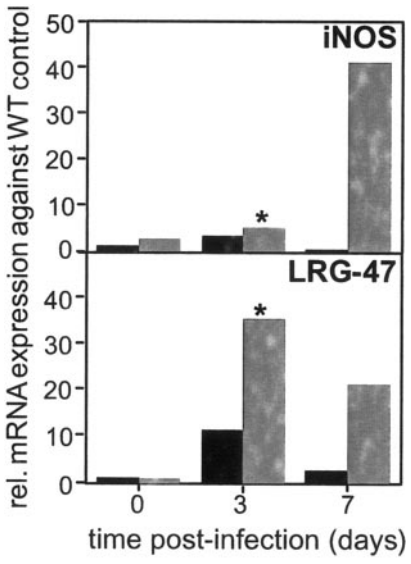

FIG. 4. Enhanced macrophage activation in livers of infected memTNF mice. WT (black bars) and memTNF (gray bars) mice were infected with 2,000 CFU of *Listeria* intravenously. mRNA expression in the livers from uninfected mice and infected mice was measured by RTQ-PCR to determine the relative (rel.) expression of LRG-47 and iNOS. The data are the means and standard errors for five mice per group from one of two representative experiments. Significance was determined by ANOVA. An asterisk indicates that the *P* value was <0.05 for a comparison of memTNF and WT mice.

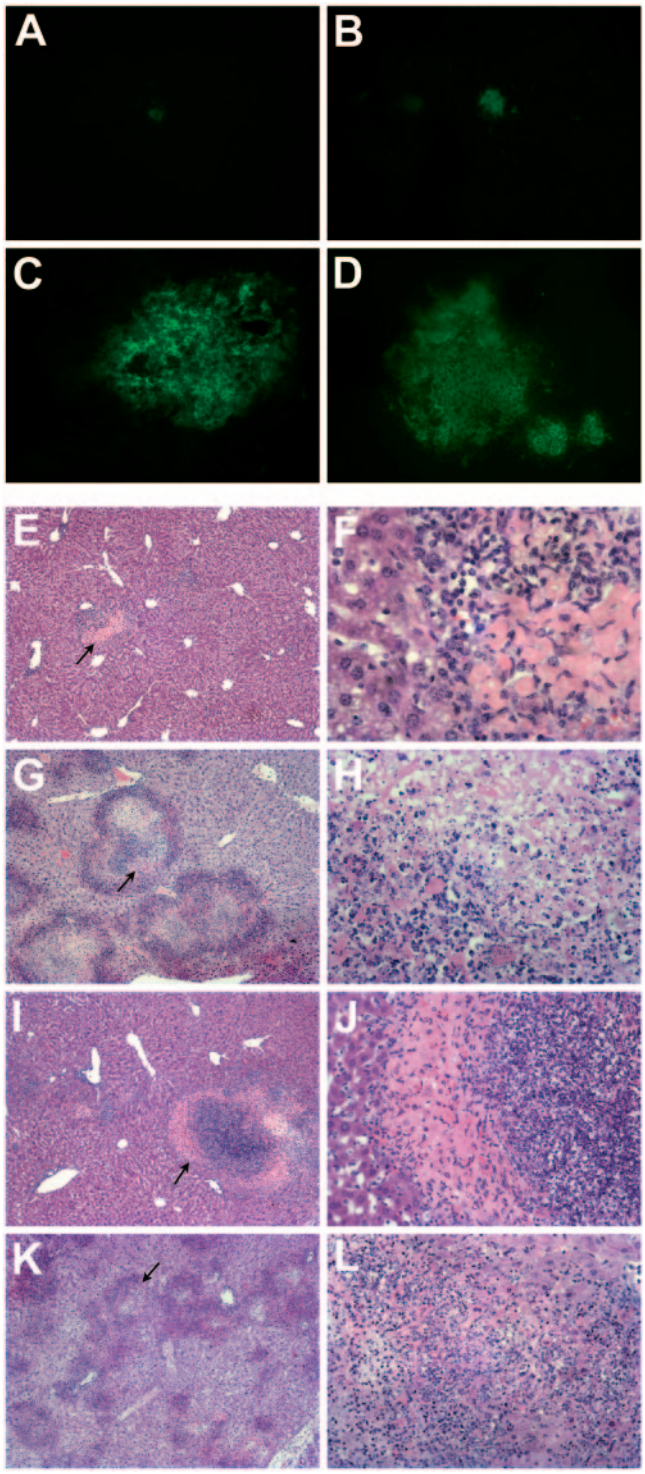

FIG. 5. Increased inflammation, necrosis, and iNOS expression in memTNF mice. WT, memTNF, and $TNF^{-/-}$ mice were infected with 2,000 CFU of *L. monocytogenes* intravenously. The photographs show typical sections from one of five mice in two representative experiments. (A to D) Perfused liver sections were frozen in optimal cutting temperature compound, and 5-μm sections were stained for iNOS expression and examined at a magnification of ×200. (A and B) WT livers on day 3 (A) and day 7 (B). (C and D) memTNF livers on day 3 (C) and day 7 (D). (E to L) Perfused liver sections were fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (E, G, I, and K) Magnification, ×25. (F and H) Magnification, ×400. (J and L) Magnification, ×200. (E and F) WT mice 5 days postinfection. (E) Inflammatory foci, scattered throughout the tissue, were mostly discrete and localized and consisted of tightly apposed mononuclear cells. Several necrotic lesions also were evident. (F) The necrotic lesions mostly consisted of a central core of degraded tissue surrounded by neutrophils and mononuclear cells. (G and H) $TNF^{-/-}$ mice 5 days postinfection. (G) Overwhelming hepatic destruction with intense inflammatory involvement was observed throughout the preparation. Darkly staining neutrophils appeared to radiate out from a central region of tissue degradation through the remaining viable liver. (H) High-power view of the interface (arrow in panel G) revealed disintegrated tissue, which was surrounded by cellular debris and pyknotic matter. (I and J) Nonmoribund memTNF mice 5 days postinfection. (I) Inflammatory foci mostly consisted of mononuclear leukocytes and neutrophils, but they were more frequent, larger, less organized, and less compact in memTNF mice than in their WT counterparts. Most necrotic lesions comprised a small area of purulent matter circumscribed by neutrophils and mononuclear cells. Other necrotic lesions consisted of a central core of degraded cells, predominantly neutrophils, surrounded by a region of disintegrated tissue, which was in turn encircled by inflammatory cells. (J) Enlargement (arrow in panel I) showing a central core of dying inflammatory cells and hepatocytes surrounded by completely necrotized tissue and a thin outer layer of leukocytes. (K and L) Moribund memTNF mice 5 days postinfection. (K) Rampant cellular infiltration and hepatic destruction. Darkly staining collections of cells, scattered throughout the tissue, were composed primarily of apoptotic bodies, neutrophils, and necrotic matter. (L) Enlargement of a section (arrow in panel K) showing areas of complete tissue destruction apparent throughout the preparation.

infection may be an important survival advantage. Furthermore, the relative expression of both CXC and CC chemokines was elevated in the liver 3 days postinfection, at the time of maximum listerial burden, but returned to basal levels in WT mice by day 7, when maximal recruitment of monocytes and granulocytes occurred. However, memTNF mice had increased levels of CCL3, CCL4, and CXCL1 mRNAs in the liver, which remained elevated at day 7 and did not return to basal levels until day 14 (Fig. 6). This increased chemokine expression in memTNF mice may have been a manifestation of a perturbed feedback loop, in which chemokine secretion was sustained until inflammatory stimuli were removed from infected cells by recruited leukocytes. Indeed, there was delayed listerial clearance in memTNF mice during a high-dose primary infection, and at day 7 postinfection memTNF mice showed a 40- to 50-fold increase in mRNA expression for the lymphocyte- and macrophage-recruiting chemokines CCL3 and CCL4. Overall, memTNF alone was less efficient than the combination of soluble and transmembrane TNF for initiating cell recruitment. Thus, while neither soluble nor transmembrane TNF is essential for chemokine induction, these molecules may instead be involved in establishing the multiple overlapping chemokine gradients in the extracellular matrix that tightly regulate the orientation of leukocytes (33). While soluble TNF can rapidly diffuse to reach target cells and stimulate production of chemotactic gradients, transmembrane TNF relies on cell-cell contact, which is unlikely to be as efficient on its own.

In addition to recruiting leukocytes, TNF is required for organization of the inflammatory infiltrate into discrete organized lesions (32). Consistent with previous reports (12), most inflammatory foci in $TNF^{-/-}$ mice consisted of groups of

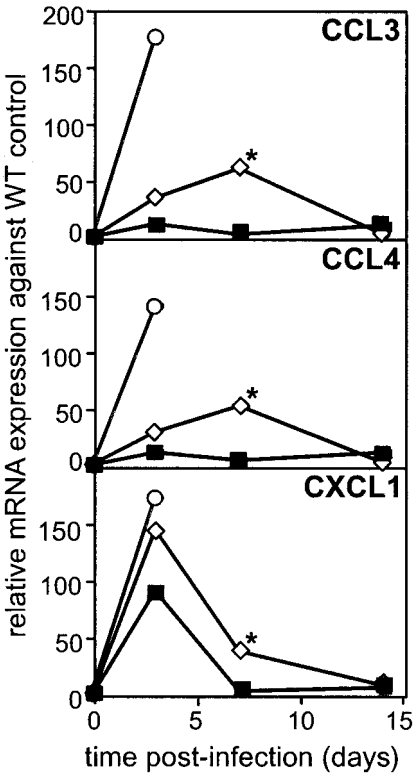

FIG. 6. Enhanced and prolonged chemokine production in livers of infected memTNF mice. WT (■), memTNF (◇), and $TNF^{-/-}$ (○) mice were infected with 2,000 CFU of *Listeria* intravenously. mRNA expression in livers was measured by RTQ-PCR to determine the relative expression of the chemokines CCL3, CCL4, and CXCL1. The data are the means for five mice per group from one of two representative experiments. The significance of differences at day 7 was determined by Student's *t* test. An asterisk indicates that the *P* value was <0.04 for a comparison of memTNF and WT mice.

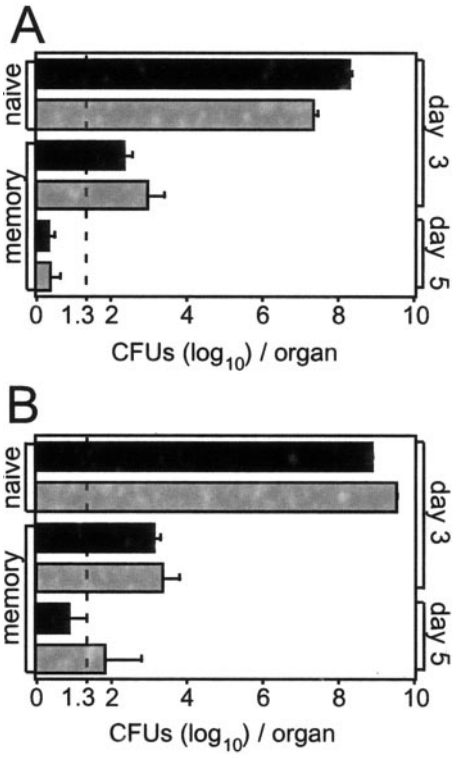

FIG. 7. Transmembrane TNF is sufficient to control bacterial growth following a secondary challenge with a lethal dose of *Listeria*. WT (black bars) and memTNF (gray bars) mice were infected with 200 CFU of *Listeria* intravenously. Eight weeks after the initial infection, naïve and immune mice were infected with 10^5 CFU of *Listeria*. The bacterial loads in the spleen (A) and liver (B) were determined at 3 and 5 days postinfection. The dotted line indicates the limit of detection. The data are the means and standard errors for five mice per group from one of two representative experiments.

heavily infected hepatocytes with predominantly neutrophilic involvement and rampant necrosis by day 4. memTNF mice also exhibited greater necrosis and had larger, more frequent and diffuse inflammatory lesions than WT mice, even though the total leukocyte numbers from digested livers were similar for the two strains. This may have been a reflection of the diffuse structure of inflammatory lesions in memTNF mice. The increased number of neutrophils, which was evident histologically in the lesions of memTNF and $TNF^{-/-}$ mice, was not confirmed by the cytometric analysis, but this may have been due to the death of activated neutrophils sensitive to the mechanical stress of purification. Indeed, in our flow cytometric analysis, forward/side scatter gating on cellular infiltrate indicated that there was a higher percentage of dead cells in memTNF and $TNF^{-/-}$ mice than in their WT counterparts.

In WT mice, a reduction in the bacterial load led to contraction of the response with a decline in the numbers of splenocytes and leukocytes in the liver. This homeostatic mechanism is crucial for minimizing immunopathology. By contrast, in memTNF mice, there was a delay in the clearance of $CD4^+$ and $CD8^+$ T cells and in resolution of inflammatory lesions in the liver. Depletion of $CD4^+$ or $CD8^+$ T cells from $TNF^{-/-}$ mice leads to reduced immunopathology and necrosis and to prolonged survival following *Mycobacterium avium* or *Mycobacterium bovis* BCG infection (7, 37). This suggests that soluble TNF has an immunoregulatory function that limits excessive Th1-type inflammatory responses.

The differential requirements for soluble TNF and transmembrane TNF may also be partially due to a signaling bias for either of the two TNF receptors. memTNF is thought to signal predominantly through the proinflammatory receptor TNFRII and not through the proapoptotic signaling receptor TNFRI (10). Furthermore, memTNF can also function as a receptor. Studies have demonstrated that macrophages become refractory to lipopolysaccharide stimulation and unresponsive to proapoptotic signals following reverse signaling through memTNF (14). However, the percentages of apoptotic T cells isolated from the liver during infection did not differ significantly for the WT and memTNF mice. This suggests that apoptotic signaling through TNFRI either was not reduced in the memTNF mice or was adequately compensated for by other available apoptotic pathways. Along with regulating inflammation, TNF also acts at other stages in the response to intracellular infection to provide optimal activation of macrophages (22, 37). TNF, in synergy with IFN-γ, induces macrophage activation with consequent increased expression of iNOS and reactive nitrogen intermediates (Fig. 4)

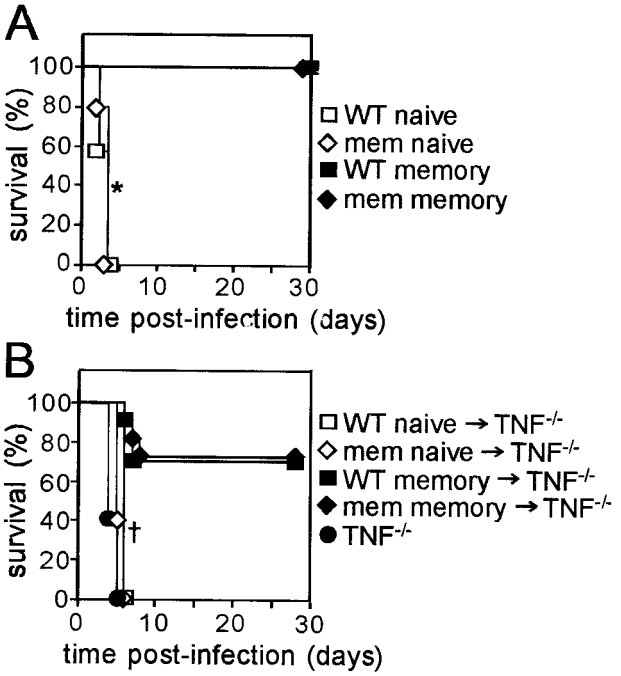

FIG. 8. Protection against secondary *Listeria* infection does not require soluble TNF. (A) WT and memTNF mice were infected with 200 CFU of *Listeria* intravenously, and 8 weeks after the initial infection, naïve and immune mice were infected with 10^5 CFU of *Listeria*. The data are the time to euthanasia for between 8 and 10 mice/group. Significance was determined by the log rank Mantel-Cox test. The asterisk indicates that the *P* value was <0.001 for a comparison of immune and naïve mice. (B) WT and memTNF mice were infected with 200 CFU of *Listeria* intravenously, and 14 days later, single-spleen-cell suspensions were prepared from immune and naïve mice, and T cells were isolated by negative selection ($CD3^+$, >90%). Two hundred colony-forming units of *Listeria* and 5×10^6 purified T cells were injected intravenously into $TNF^{-/-}$ mice that had been irradiated 24 h previously with 500 rads. The data are the time to euthanasia for 11 to 15 mice/group. Significance was determined by the log rank Mantel-Cox test. The dagger indicates that the *P* value was <0.002 for comparisons of WT immune and WT naïve mice and memTNF immune and memTNF naïve mice.

(3, 8). These data demonstrate that T-cell expression of memTNF alone is sufficient for T-cell migration and subsequent macrophage activation. Furthermore, these data demonstrate that macrophages do not need to express TNF to control an otherwise lethal infection but instead can be adequately activated by surface TNF expression on immune T cells alone.

Finally, the differential requirements for soluble and transmembrane TNF provide a potential explanation for the increase in infectious diseases, especially tuberculosis, reported in individuals who receive anti-TNF therapy. The recently introduced anti-TNF therapies, which have proven to be highly successful in the treatment of chronic inflammatory processes, including Crohn's disease, rheumatoid arthritis, and psoriasis (30), have been hampered by their interference with host cell immunity. The two commonly used TNF antagonists are the monoclonal antibody infliximab and the soluble TNFRII fusion protein etanercept (30). Infliximab, which has high affinity for both solTNF and memTNF, has been associated with a higher frequency of infectious complications, such as tuberculosis reactivation and listerial meningitis, than etanercept, which binds predominantly to solTNF (2, 9, 35). Indeed, this is consistent with our findings that memTNF is sufficient to orchestrate protective immunity against a low-dose *Listeria* infection but cannot fully compensate for the lack of solTNF during a high-dose infection. The development of inhibitors of soluble TNF which leave functional memTNF may allow TNF inhibition of inflammatory disease while sufficient protective immunity is maintained by memTNF (34).

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