Transmembrane TNF Induces an Efficient Cell-Mediated Immunity and Resistance to *Mycobacterium bovis* Bacillus Calmette-Guérin Infection in the Absence of Secreted TNF and Lymphotoxin-α[1]

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The contribution of a transmembrane (Tm) form of TNF to protective immunity against *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) was studied in transgenic (tg) mice expressing a noncleavable Tm TNF but lacking the *TNF/lymphotoxin-α (LT-α)* locus (Tm TNF tg mice). These mice were as resistant to BCG infection as wild-type mice, whereas TNF/LT-$\alpha^{-/-}$, TNF$^{-/-}$, and LT-$\alpha^{-/-}$ mice succumbed. Tm TNF tg mice developed granulomas of smaller size but at 2- to 4-fold increased frequencies compared with wild-type mice. Granulomas were mainly formed by monocytes and activated macrophages expressing Tm TNF mRNA and accumulating acid phosphatase. NO synthase 2 activation as a key macrophage bactericidal mechanism was low during the acute phase of infection in Tm TNF tg mice but was still sufficient to limit bacterial growth and increased in late infection. While infection with virulent *Mycobacterium tuberculosis* resulted in very rapid death of TNF/LT-$\alpha^{-/-}$ mice, it also resulted in survival of Tm TNF tg mice which presented an increase in the number of CFU in spleen (5-fold) and lungs (10-fold) as compared with bacterial load of wild-type mice. In conclusion, the Tm form of TNF induces an efficient cell-mediated immunity and total resistance against BCG even in the absence of LT-α and secreted TNF. However, Tm TNF-mediated protection against virulent *M. tuberculosis* infection can also be efficient but not as strong as in BCG infection, in which cognate cellular interactions may play a more predominant role in providing long-term surveillance and containment of BCG-infected macrophages. *The Journal of Immunology,* 2002, 168: 3394–3401.

Tuberculosis is reemerging as one of the most important health problems. Development of improved vaccines against *Mycobacterium tuberculosis* is still hampered by the limited understanding of the mechanisms controlling innate and acquired immunity. The only vaccine currently available against *M. tuberculosis*, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG),[3] has shown variable protective efficacy ranging from 0 to 85% in different studies (1). BCG vaccine is normally well tolerated but can cause diseases in immunodeficient patients. In children with mutations of the *IFN-γR1*, *IL-12*, and *IL-12R* genes, vaccination or infection with BCG resulted in lethal dissemination or severe infection (2–5). The requirement of IFN-γ and IL-12 in mediating resistance to mycobacterial infections has been also demonstrated by using genetically modified animals such as IFN-$\gamma^{-/-}$ or IFN-γR$^{-/-}$ and IL-12p40$^{-/-}$ mice, which were unable to control *M. tuberculosis* and *M. bovis* infections (6–12).

Mycobacterial infections induce a cell-mediated immune response, in which the contribution of macrophages and CD4 and CD8 T lymphocytes is essential (13, 14). Ag-specific T cells mediate the recruitment and activation of macrophages to form bactericidal granulomas. TNF and Th1 type cytokines play a critical role in the development of protective granulomas, which contain activated macrophages producing specific enzymes, such as inducible NO synthase (iNOS) or NOS2, and which are responsible for the elimination of bacteria. These mechanisms form the basis for protection against mycobacterial spreading (15, 16). Indeed, NOS2$^{-/-}$ mice are highly sensitive to *M. tuberculosis* and *M. bovis* infections (17, 18) but, in contrast, they show an enhanced resistance to *Mycobacterium avium* infection (19).

The importance of TNF in mycobacterial infections has been extensively reported. Neutralization of TNF with anti-TNF Abs or with soluble TNFR1-Ig fusion protein during *M. bovis* and *M. tuberculosis* infections or the absence of a functional TNF gene increased sensitivity by inhibiting macrophage differentiation and the development of well-differentiated granulomas, resulting in mycobacterial overgrowth and rapid animal death (20–24). Recently, it has been shown that lymphotoxin-α (LT-α) is also required for host defense against BCG and *M. tuberculosis* infections (25, 26). A contribution of cell surface-bound LT-$\alpha_1\beta_2$ in protective immunity against BCG infection has been reported (27).

Most TNF activities in defense mechanisms have been attributed to the secreted form of TNF, and little information is available on the

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Tm, transmembrane; tg, transgenic; NOS, NO synthase; LT-α, lymphotoxin-α; TACE, TNF-α-converting enzyme.

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contribution of the transmembrane (Tm) form. TNF is synthesized as a 26-kDa Tm precursor and is cleaved by membrane-bound metalloprotease(s), including the TNF-α-converting enzyme (TACE) or a disintegrin and metalloproteinase into 17-kDa monomers forming active homotrimers (28–32). Indications for distinct roles of Tm and secreted TNF have been obtained (33, 34). Transgenic (tg) mice expressing (instead of wild-type TNF) a Tm form of TNF, generated by deleting the first 12 amino acids of processed TNF, were partially resistant to *Listeria monocytogenes* infection (35). These mice were also prone to develop arthritis (36) and Con A-induced inflammatory liver disease (37). In addition, the inhibition of proteolytic TNF cleavage by TACE inhibitors protected mice completely from endotoxic shock (38). These results indicate that the TNF activities can be mediated by both the secreted and the Tm TNF molecules.

To dissect the functions exerted by secreted and Tm TNF in *M. bovis* BCG infection and also to assess the possibility of using TACE inhibitors in controlling deleterious effects of secreted TNF observed in active human tuberculosis, we have infected tg mice expressing a Tm murine TNF (Tm TNF tg) generated in TNF/LT-$\alpha^{-/-}$ mice (39). Mutations of this noncleavable TNF were introduced into a genomic clone of mouse TNF under the control of the mouse TNF promoter. The 3′ AU-rich elements of the genomic TNF locus were preserved to assure adequate gene regulation in vivo (40). Tm TNF tg mice were protected from LPS plus D-galactosamine-induced mortality, and no TNF bioactivity was found in the serum of these challenged mice (39).

Using this Tm TNF tg mouse line in systemic BCG infection, we demonstrate that, in the absence of LT-α and secreted TNF, tg mice are able to develop a protective immunity against *M. bovis* BCG and that, in the control of BCG infection, Tm TNF can compensate the absence of LT-α and exerts distinct activities from secreted TNF.

Materials and Methods

Animals

The generation of TNF/LT-$\alpha^{-/-}$ mice (C57BL/6 × 129/SVEV) tg for a noncleavable TNF gene has been described before (39). The transgene is under the control of the TNF promoter. The AU-rich regulatory elements at the 3′ end of the TNF gene have been maintained to assure appropriate gene regulation. To generate a noncleavable mutant of murine TNF, two deletions (Leu^{-12}–Leu^{-10} and Leu^{-2}–Leu^{1}) and an amino acid substitution ($Lys^{11} > Glu^{11}$) were introduced into a genomic TNF clone. Wild-type, Tm TNF tg mice and TNF/LT-$\alpha^{-/-}$ (41) were of a C57BL/6 × 129/SVEV genetic background. TNF$^{-/-}$ mice (42) were of C57BL/6 genetic background and LT-$\alpha^{-/-}$ were of 129/SVEV genetic background (43). Wild-type mice of C57BL/6 and 129/SVEV genetic background were included in survival and bacterial content experiments. Mice were maintained under conventional conditions in the animal facility of the Medical Faculty of the University of Geneva (Geneva, Switzerland), or under P3 conditions in Pasteur Institute (Paris, France).

Experimental infection and determination of BCG and M. tuberculosis *CFU*

Mice were inoculated into retro-orbital sinuses with 10^7 living BCG strain 1173 P2 or with 10^5 CFU of virulent *M. tuberculosis* strain H37Rv. The numbers of viable bacteria inoculated or recovered from organs after homogenization in saline containing 0.04% Tween 80 were determined by plating serial dilution of living bacilli onto Middlebrook 7H10 agar plates containing 10% oleic-albumin Dubos complex (Difco, Detroit, MI). Plates were incubated at 37°C for 21 days. No difference in the amount of CFU recovered from organs of BCG-infected C57BL/6 × 129/SVEV, C57BL/6, and 129/SVEV mice was found.

LPS challenge

Mice infected with 10^7 CFU of BCG were challenged i.p. with 1 μg/g body weight of LPS from *Escherichia coli* (serotype 0111: B4; Sigma-Aldrich, Schnelldorf, Germany) at day 14 after BCG inoculation. Blood samples for cytokine detection were obtained 2 h after LPS injection and mortality was monitored every 2 h.

Histologic analyses

A histopathologic analysis of infected organs was routinely performed in each experiment. Liver (at three different sites), lung (two lobes), and spleen (one-third) were fixed in 4% buffered formaldehyde and embedded in paraffin for subsequent H&E staining and Ziehl-Neelsen acid-fast staining.

Acid phosphatase activity

Acid phosphatase activity was determined on frozen tissue sections (cryostat sections of 5 μm) of liver as previously reported (20, 23, 27). The method used on tissue sections was modified for quantitative detection of acid phosphatase activity on the entire organ, as previously described (27).

In situ hybridization

A 1108-bp cDNA fragment of the murine TNF (positions 1–1108; obtained from Genentech, San Francisco, CA) was subcloned into pGEM-2. After the linearization of the plasmid, sense and antisense RNA probes were prepared using the appropriate RNA polymerases as described previously (39). Cryostat sections of organs were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, rinsed in PBS, and subsequently treated with proteinase K (Boehringer Mannheim, Mannheim, Germany) at 1 mg/ml at 37°C for 30 min. After post-fixation and acetylation, hybridization was performed with 2×10^5 cpm of ^{35}S-labeled RNA probe per microliter of hybridization solution for 18 h at 48°C (37).

Evaluation of serum levels of cytokines

Blood samples were obtained from retro-orbital sinuses at different time after infection. IFN-γ, IL-12p40, IL-10, TNF, and IL-18 levels were evaluated by ELISA with a sensitivity of 2–1000 pg/ml.

Bioactivity of TNF in mouse serum samples was measured on WEHI cells (clone 13) compared with standard murine TNF. WEHI cells (3×10^4/well) were incubated in the presence of actinomycin D (1 μg/ml) with mouse serum (dilution from 1/20 to 1/16,000) for 20 h in a 96-well plate. One picogram of standard TNF is able to kill 50% of WEHI cells. Cell viability was assessed as previously described (18).

Determination of NOS activity in spleen extracts

Evaluation of the induction of NOS activity was done on crude frozen spleen extracts of infected and uninfected mice. Spleens were homogenized in 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA (125 mg of tissue per milliliter of buffer). Crude supernatant was obtained by centrifugation of the homogenate at 10,000 × g for 5 min. NOS activity was measured by the ability of supernatant to convert radioactive L-[^{14}C]arginine (Amersham Pharmacia Biotech, Piscataway, NJ) to L-[^{14}C]citrulline as previously described (27, 44).

Ag-specific release of cytokines from spleen cells

Mice were infected with 10^7 BCG and sacrificed 22 wk later. The spleen cells were suspended in DMEM containing 10% FCS, treated for 5 min with a 0.155 M ammonium chloride/0.010 M potassium bicarbonate solution to lyse the erythrocytes, washed, and resuspended in DMEM plus 10% FCS. Cell suspensions were cultured in 96-well plates at 5×10^5 cells per well. The cells were stimulated with medium alone, living BCG (10^3 CFU/well), or BCG culture protein extracts (1.7 mg/ml) for 1, 3, or 6 days (11).

Nitrite determination

NO synthesis was assessed by measuring the accumulation of nitrite in cell supernatant as detected by the Griess reagent (1% sulfanilamide and 0.1% naphtylethylenediamide in 2.5% phosphoric acid). Absorption was measured at 550 nm and nitrite concentrations were determined by comparison with OD of the $NaNO_2$ standards.

Statistical analyses

The unpaired Student's t test was used for all analyses. Values of $p < 0.05$ were considered statistically significant.

Results

Tm TNF tg mice are protected from BCG infection and are able to kill intracellular bacteria

To explore the contribution of Tm TNF in host defense against *M. bovis* BCG we have used tg mice expressing a Tm TNF but lacking

the TNF/LT-α locus (Tm TNF tg mice; Ref. 39). In the previous study, Tm TNF tg mice were shown to be protected from LPS plus D-galactosamine-induced mortality due to acute hepatitis as a result of rapid release of TNF (39). To characterize Tm TNF tg mice in the context of the BCG infection, LPS was administrated to BCG-sensitized mice. Endotoxin administration induces a massive release of TNF, which results in fulminant hepatitis and death. TNF/LT-$\alpha^{-/-}$ mice as well as Tm TNF tg mice were protected from lethal effect of BCG/LPS treatment in contrast to wild-type mice, which all died 5–8 h after LPS challenge (Fig. 1*A*). Fig. 1*B* represents serum levels of TNF in BCG/LPS-treated mice 2 h after LPS. High amounts of serum TNF were found in wild-type but not in Tm TNF tg and TNF/LT-$\alpha^{-/-}$ mice as determined by ELISA (Fig. 1*B*) and bioactivity on WEHI cells (data not shown).

To study susceptibility of Tm TNF tg mice to BCG infection, wild-type, Tm TNF tg, TNF$^{-/-}$, LT-$\alpha^{-/-}$, and TNF/LT-$\alpha^{-/-}$ mice were infected i.v. with 10^7 living bacilli. Inoculation of sensitive wild-type mice with this dose of BCG leads to nonlethal and self-limiting infection. Mice deficient for TNF/LT-α, TNF, or LT-α alone died between 6 and 16 wk postinfection. In contrast, Tm TNF tg mice survived for at least 6 mo, similar to wild-type mice (Fig. 1*D*). No differences in the kinetics of weight alterations were observed between wild-type and Tm TNF tg mice during BCG infection. To further confirm resistance of Tm TNF tg mice to BCG infection, we quantified CFU by colony assay on tissues from wild-type, Tm TNF tg, and TNF/LT-$\alpha^{-/-}$ mice 2, 4, and 10 wk after infection. Wild-type and Tm TNF tg mice showed the same levels of living bacilli in infected organs, whereas high BCG loads were found in TNF/LT-$\alpha^{-/-}$ mice (Fig. 2). These data demonstrate that Tm TNF in mice lacking TNF/LT-α is sufficient for controlling BCG proliferation and ensures animal survival.

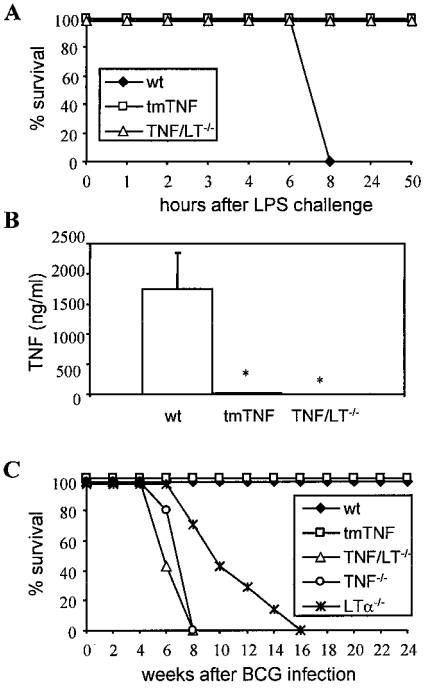

FIGURE 1. Tm TNF tg mice are protected from endotoxin-mediated lethality after BGC sensitization. *A*, Survival of mice after LPS challenge in BCG-sensitized mice. Mice were infected with 10^7 living BCG, and at day 14 LPS was administrated i.p. (1 μg/g body weight; $n = 6$ per group). These results are representative of two experiments. *B*, Serum levels of immunoreactive TNF at 2 h of BCG/LPS treatment ($n = 6$ per group). *C*, Tm TNF tg mice survive to BCG infection but not TNF$^{-/-}$, LT-$\alpha^{-/-}$, or TNF/LT-$\alpha^{-/-}$ mice. Shown is the long-term survival of mice infected with living BCG (10^7). Each group represents seven mice, except for TNF$^{-/-}$ mice ($n = 5$). Data from one of two representative experiments are shown.

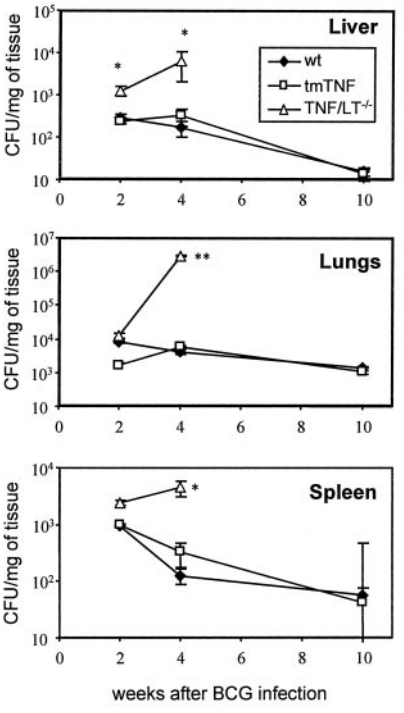

FIGURE 2. Tm TNF tg mice are able to control and eliminate BCG in infected organs. Mice were infected with living BCG (10^7) and killed after 2, 4, and 10 wk. Bacterial loads were determined in liver, lungs, and spleen. Data represent means ± SEM from five to seven mice per time point. Experiments were repeated twice or three times with comparable results. *, Statistically significant differences between wild-type (wt) and mutant mice: *, $p < 0.01$ or **, $p < 0.001$.

Upon BCG infection Tm TNF tg mice develop smaller but more granulomas

We have explored the role of Tm TNF in BCG granuloma formation. At 4 wk, wild-type mice showed well-formed granulomas mainly containing large and differentiated macrophages or epithelioid cells, T lymphocytes, and, occasionally, polymorphonuclear cells (Fig. 3*A*). Granulomas from Tm TNF tg mice were much smaller, containing few (one or two) epithelioid cells surrounded by activated monocytes, macrophages, and lymphocytes (Fig. 3*B*). These cells expressed TNF mRNA as evaluated by in situ hybridization of liver, spleen, and lung tissues. Fig. 3 shows the presence of TNF mRNA on liver tissue sections from wild-type (Fig. 3*D*) and Tm TNF tg mice (Fig. 3*E*), but not from TNF/LT-$\alpha^{-/-}$ mice (Fig. 3*F*). Macrophage activation within the granulomas was assessed by staining of tissue sections for acid phosphatase activity, an enzyme produced in large amounts by differentiated macrophages. We observed acid phosphatase activity on liver granulomas from wild-type mice (Fig. 3*G*) and also from Tm TNF tg mice (Fig. 3*H*), but rarely on TNF/LT-$\alpha^{-/-}$ mouse tissues (Fig. 3*I*). Quantification of the acid phosphatase activity of spleen enzyme extracts from BCG-infected mice showed the same activity in Tm TNF tg and wild-type mice through the infection (Fig. 4*A*). Assessment of the number of granuloma in the liver of Tm TNF tg mice revealed a 2-fold increase compared with wild-type mice after 4 wk of infection (Fig. 4*B*).

At 10 wk of BCG inoculation, the number of granulomas in the liver of Tm TNF tg mice was increased 4-fold when compared with wild-type mice (Fig. 4*B*). At this point of the infection, liver

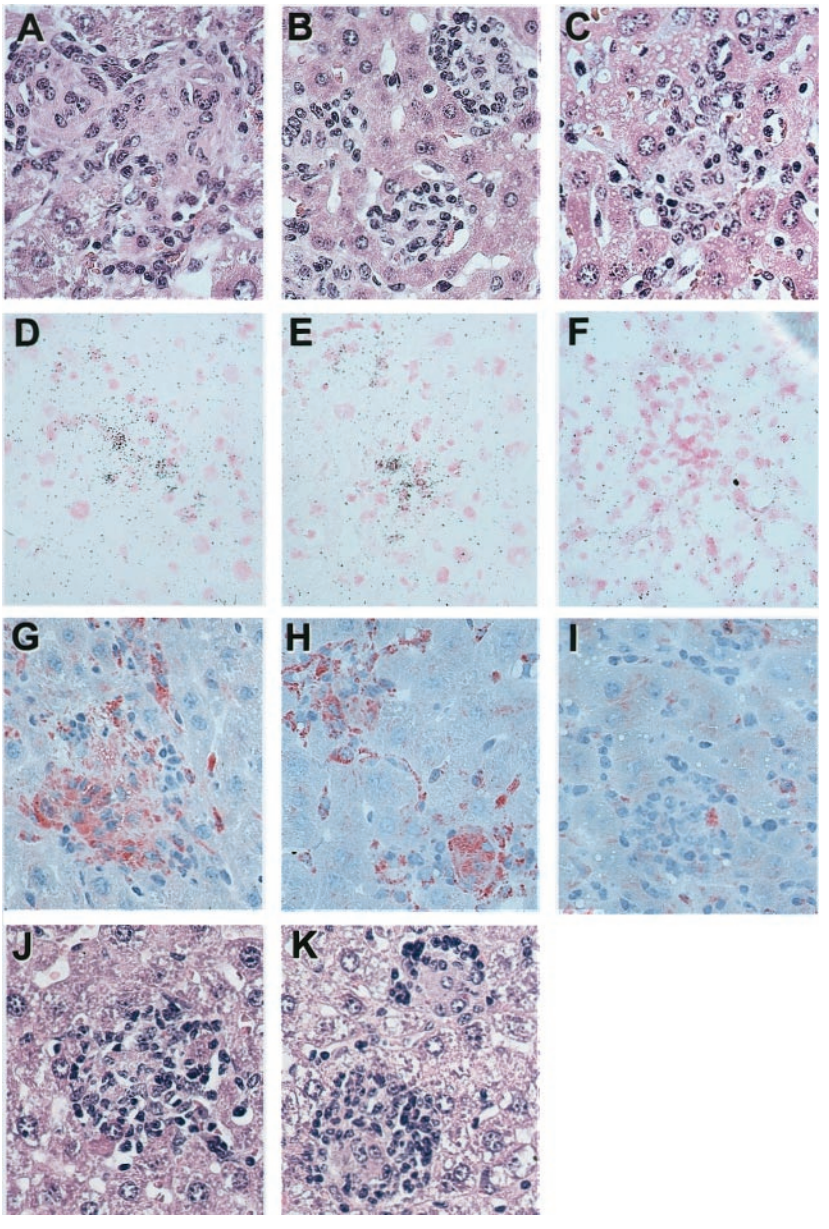

FIGURE 3. Tm TNF tg mice develop smaller granulomas containing activated macrophages producing TNF mRNA and acid phosphatase. *A–C*, Liver sections (stained with H&E) at 4 wk after BCG inoculation, show well-differentiated granulomas in wild-type mice (*A*) compared with small size and numerous granulomas in Tm TNF tg mice (*B*), whereas only very few, small granulomas are found in TNF/LT-$\alpha^{-/-}$ mice (*C*). These data are representative of two or three independent experiments with 8–10 mice per group and per experiment. *D–F*, In situ hybridization with ^{35}S-labeled TNF RNA antisense probes of liver sections from wild-type (*D*), Tm TNF tg (*E*), and TNF/LT-$\alpha^{-/-}$ (*F*) mice. TNF mRNA-positive cells can be identified by the dark silver grains on the tissue sections. These results are representative of one experiment with three mice per group. Magnification is ×400. *G–I*, Staining for acid phosphatase activity of liver sections from wild-type (*G*), Tm TNF tg (*H*), and TNF/LT-$\alpha^{-/-}$ (*I*) mice. *J* and *K*, H&E-stained liver sections after 10 wk of BCG infection from wt mice (*J*) and Tm TNF tg mice (*K*).

granulomas from wild-type mice started to disappear, signs of epithelioid cell death were apparent, and their number was reduced (Fig. 3*J*). In contrast, Tm TNF tg mice showed small but still well-differentiated granulomas, containing a central epithelioid cell or probably a multinucleated giant cell surrounded by monocytes and lymphocytes (Fig. 3*K*). These results demonstrate that the presence of Tm TNF results in the generation of numerous granulomas containing differentiated macrophages, but these remain of smaller size probably due to limited cell recruitment.

Bactericidal mechanisms are transiently reduced in Tm TNF tg mice upon BCG infection

Activation of NOS2 in macrophages represents one of the bactericidal mechanisms to eliminate intracellular bacteria. NOS2 is required for protection against BCG (18). We have evaluated the NOS activity from crude extracts of spleen at different time points after BCG inoculation by monitoring the conversion of radioactive L-arginine to L-citrulline and NO (18, 44). We observed a rapid NOS activation with maximal production 2 wk after infection in wild-type mice. In Tm TNF tg mice NOS was also activated, but lower levels were found at 2 wk of infection in comparison with wild-type mice (Fig. 4*C*). At 4 wk of infection, NOS activity in Tm TNF tg and wild-type was similar, and at 10 wk of infection Tm TNF tg mice showed a modest but statistically significant enhancement of NOS activity compared with wild-type mice, suggesting that the presence of Tm TNF sustains NOS activity (Fig. 4*C*). TNF/LT-$\alpha^{-/-}$ mice were unable to activate the NOS2 during the acute phase infection, and later activation appeared to be inefficient for BCG elimination.

Transient alteration of serum Th1 cytokines in Tm TNF tg mice

BCG induces a Th1 type of immune response, characterized by the release of IFN-γ and IL-12, which are required for host protection (8, 11). To investigate whether Tm TNF tg mice are able to produce these cytokines, we evaluated the serum concentrations of IFN-γ, IL-12, and also IL-18 during infection. At 2 wk after BCG inoculation, IFN-γ serum levels were lower in Tm TNF tg mice than in wild-type mice. However, at later points during infection, IFN-γ levels were similar in both groups of animals (Fig. 5). Serum concentrations of IL-12p40 were increased in Tm TNF tg

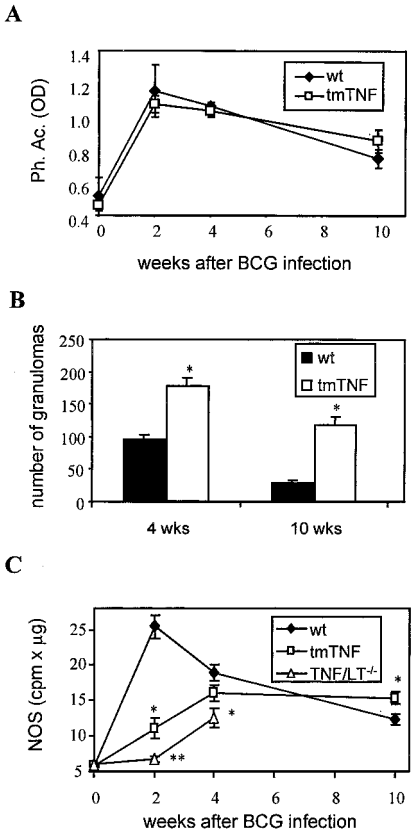

FIGURE 4. *A*, Quantification of acid phosphatase activity in spleen protein extracts. Acid phosphatase activity of spleen enzyme extracts from wild-type or Tm TNF tg mice, collected at different time points of BCG infection. Results represent means ± SEM of the OD_{570} values from five to six different samples per group and are representative of two independent experiments. *B*, Enhanced numbers of granulomas are observed in Tm TNF tg mice. Shown is quantification of liver granuloma in Tm TNF tg and wild-type mice ($n = 6$). Data are represented as means ± SEM of the number of granulomas per 1-mm^2 tissue section. These results are representative for two experiments. At 4 wk, values of p are <0.0002; at 10 wk, values of p are <0.000021. *C*, NOS activity in the spleen of BCG-infected mice. NOS activity was determined in crude spleen extracts from uninfected and infected mice ($n = 5$–6). Data are represented as means ± SEM of cpm per microgram of tissue. *, Value of $p < 0.010$; **, $p < 0.000002$. Experiment has been repeated twice with similar results.

mice up to 4 wk following BCG inoculation, and this increase in IL-12p40 was statistically significant (Fig. 5). IL-18 and IL-10 serum profiles were comparable in Tm TNF tg mice and wild-type mice. Cytokine profiles were totally different in TNF/LT-$\alpha^{-/-}$ mice, which were not able to early secrete IFN-γ, IL-12, IL-18, or IL-10, but at 4 wk of infection a complete disregulation of cytokine production was observed at more advanced stages of BCG infection, correlating with disease progression and death.

Ag-induced production of IFN-γ and NO by splenocytes in BCG-infected mice

To determined whether the presence of Tm TNF may affect Ag-specific production of IFN-γ, the amount of IFN-γ was assessed by culturing splenocytes from infected animals with BCG culture filtrate proteins and also with living BCG. There was only a marginal difference (1.5-fold reduction) between Ag-specific IFN-γ secretion from Tm TNF tg and wild-type splenocytes when culture filtrate proteins were used for priming (Fig. 6*A*). Addition of viable BCG to the splenocyte cultures, however, induced a 2.7-fold lower

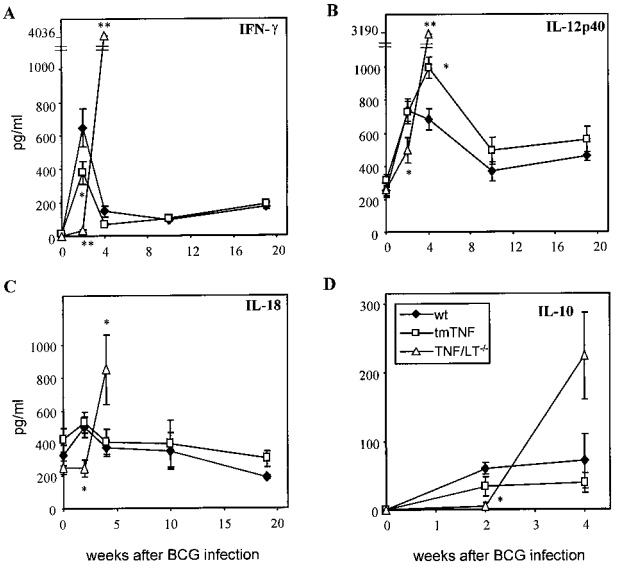

FIGURE 5. Alteration of cytokine serum levels in Tm TNF tg mice. Amounts of IFN-γ, IL-12p40, IL-18, and IL-10 were evaluated by ELISA in the serum of uninfected and infected mice. Data are represented as means + SEM (picogram of protein per milliliter of serum). These results are representative of two or three independent experiments. Asterisks indicate statistically significant differences between wild-type and indicated group: IFN-γ ($n = 5$–6 per group; *, $p < 0.03$), TNF (**, $p < 0.0003$), IL-12p40 ($n = 10$–11 per group; *, $p < 0.001$ and **, $p < 0.00013$), IL-18 ($n = 8$–9 per group; *, $p < 0.005$), IL-10 ($n = 5$–6 per group; *, $p < 0.001$).

IFN-γ release in Tm TNF tg cells than in wild-type splenocytes (Fig. 6*B*). These results correlate with the lower NO production of Tm TNF splenocytes compared with wild-type cells (Fig. 6*B*). Ag-induced NO production was similar in Tm TNF and wild-type

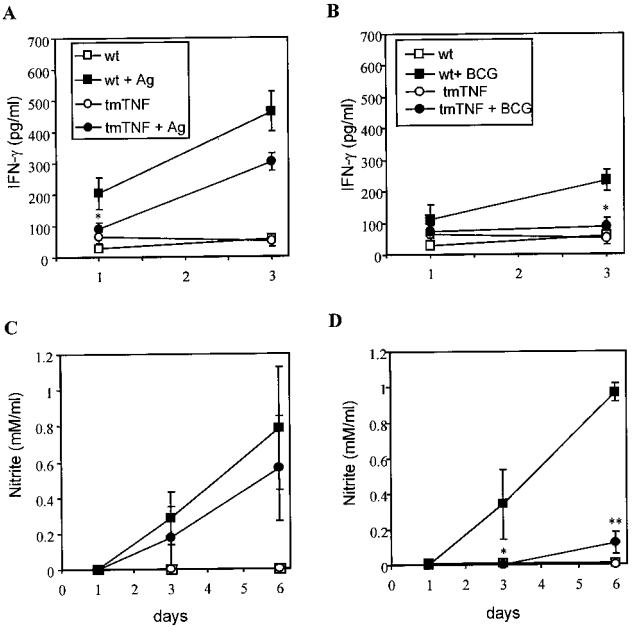

FIGURE 6. Ag-specific IFN-γ and NO release from splenocytes of BCG-infected mice. IFN-γ and NO were evaluated in the culture supernatant from cells incubated in the presence of Ags derived from BCG (*A* and *C*) or in the presence of 10^3 viable BCG (*B* and *D*). Control cells were incubated with medium only. Values are the mean ± SEM from five animals (assay in triplicates). *, Values of $p < 0.05$; **, $p < 0.0003$.

splenocytes. In contrast, BCG-induced NO production was dramatically reduced in Tm TNF tg cells (Fig. 6, *C* and *D*). Furthermore, the proportion of CD4-, CD8-, and F4/80-positive cells after 22 wk of BCG infection was similar in spleens from Tm TNF tg and wild-type mice as analyzed by flow cytometry (data not shown).

Infection with virulent M. tuberculosis *results in rapid death of TNF/LT-$\alpha^{-/-}$ mice and resistance of Tm TNF-tg mice, which show an increased bacterial load in infected organs*

Infection of wild-type ($n = 9$), Tm TNF tg ($n = 9$), and TNF/LT-$\alpha^{-/-}$ ($n = 5$) mice with virulent *M. tuberculosis* (H37Rv) resulted in rapid death of TNF/LT-$\alpha^{-/-}$ mice, which succumbed at day 20–23 postinfection. Transgenic mice for Tm TNF survived the infection and were healthy, with the exception of one mouse (one of nine) that presented wasting (10% of weight lost) at day 28 of infection. Bacterial load of wild-type and tg TNF mice were determined at day 28 as presented in Fig. 7. We observe an increase (5-fold) in the number of CFU in the spleen of Tm TNF tg mice. The number of CFU in the lung of wild-type mice was from 52×10^3 to 479×10^3 CFU per lung with an average of 150×10^3 CFU per lung. In Tm TNF tg mice the number of CFU observed was more heterogeneous, going from 540×10^3 to 3571×10^3 CFU per lung, and the average number was 1621×10^3 CFU per lung, or 10-fold higher that the number found in wild-type mice. These data suggest that Tm TNF tg mice are able to survive in conditions where TNF/LT-$\alpha^{-/-}$ mice rapidly die. However, the control of bacterial proliferation observed in BCG infection appears to be less efficient for *M. tuberculosis* infection.

Discussion

With the present study the specific role of Tm TNF in protective immunity against BCG infection has been directly addressed, using tg mice expressing noncleavable Tm TNF in the absence of wild-type TNF and LT-α. Tm TNF tg mice survived to BCG infection, whereas either TNF$^{-/-}$, LT-$\alpha^{-/-}$, or TNF/LT-$\alpha^{-/-}$ mice succumbed. These results thus suggest that Tm TNF confers protection even in conditions in which secreted wild-type TNF fails, as in LT-$\alpha^{-/-}$ mice, indicating that Tm TNF can also compensate for the absence of both wild-type TNF and LT-α. Indeed, in the absence of either the *TNF or LT-α locus*, mice are not protected from mycobacterial infection, and TNF$^{-/-}$ and LT-$\alpha^{-/-}$ mice eventually die from excessive intracellular bacterial proliferation. Surprisingly, in contrast to Tm TNF, secretable TNF, which plays a predominant role during the acute phase of infection, cannot substitute for the absence of LT-α as do surface-expressed Tm TNF. This may reflect the predominant role of cognate cellular interactions over soluble factors in providing long-term surveillance and control of BCG proliferation. This is probably less predominant in *M. tuberculosis* infection, in which the production of soluble factors results in more efficient response, although the unique presence of Tm TNF without soluble TNF or LT-α confers an important protection. Previously, it has been shown that inability to use TNF and LT-α in tg mice, TNF$^{-/-}$ mice, or LT-$\alpha^{-/-}$ mice renders animals highly susceptible to *M. tuberculosis* and BCG infections (23–26, 45).

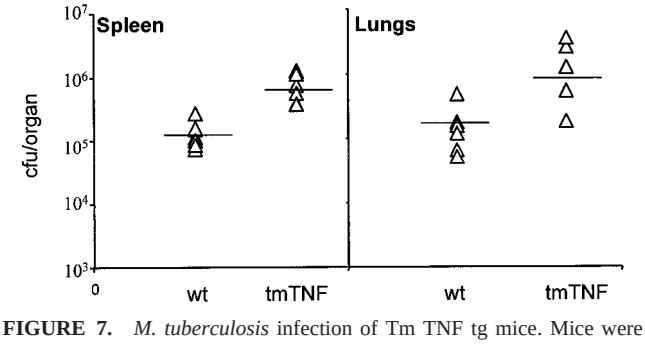

FIGURE 7. *M. tuberculosis* infection of Tm TNF tg mice. Mice were infected with virulent *M. tuberculosis* (10^5 CFU), and at day 28 bacterial loads were determined in spleen and lungs. Data represent individual values of infected mice (five to seven mice per group) and horizontal bars indicate means.

Reduction of immune surveillance in mycobacterial infections leads to the excessive proliferation of intracellular bacteria in macrophages. We show that tg mice expressing Tm TNF develop bactericidal mechanisms in macrophages which are able to control BCG proliferation like wild-type mice. Determination of bacterial loads in liver, lungs, and spleen demonstrates that Tm TNF tg mice can efficiently control and eliminate BCG as wild-type mice. Control of bacterial proliferation by Tm TNF tg mice was found to be less efficient in virulent *M. tuberculosis* infection and in particular in lung, in which the presence of soluble TNF may be more important than in BCG infection. Soluble TNF may probably mediate cell recruitment to lung tissue and more efficiently activate mycobactericidal mechanisms. Induction of bactericidal mechanisms, including NOS2 activation, which leads to the generation of NO and reactive nitrogen intermediates, is crucial for cellular immunity and protection against *M. tuberculosis* infection (17, 46). BCG infection in NOS2$^{-/-}$ mice resulted in an enhanced but inefficient cellular immune response with increased recruitment of inflammatory cells eventually causing tissue necrosis, and overproduction of TNF (18). NOS2 was activated in Tm TNF tg mouse spleen during the acute phase of infection, although this activation was lower than that found in wild-type mice. This can be explained by a limited or even delayed Th1 cell recruitment and differentiation and lower IFN-γ production, as observed in Tm TNF tg mice during acute phase of infection. Nevertheless, our data show that expression of Tm TNF during the presence of BCG is sufficient to sustain and enhance NOS2 activation in chronic infection.

TNF is synthesized as a 26-kDa Tm protein which is cleaved by the TACE to yield biologically active homotrimers. Strong evidence for a biologically active Tm precursor form of TNF in host defense has been reported (28–32). The 26-kDa Tm form of TNF on CD4 T cell clones was shown to provide a costimulatory signal for human B cell activation (47). Tm TNF was shown to play a role in antileishmanial and antimycobacterial defense in murine macrophages (48, 49). Tm TNF was also shown to be implicated in the polyclonal B cell activation induced by HIV-infected human T cells (50). A noncleavable Δ1–12 mutant of mouse TNF expressed as a transgene in TNF$^{-/-}$ mice was shown to confer a partial protection against *L. monocytogenes* infection (35). These mice were also found to be prone to develop arthritis (36). This latter effect, however, might be attributed to the enhanced expression of the transgene due to the absence of the AU-rich elements at the 3′ untranslated region of this Tm transgene. Absence of these regulatory sequences has been found to cause pathological alterations due to impaired TNF regulation (40).

Distinct functions of secreted and Tm TNF have been previously attributed to a differential binding to the two receptors TNFR1 and TNFR2 based on interactions with their receptors. Signaling through TNFR2 of Tm TNF has been reported for the mutant Tm TNF Δ1–12. However, the preference binding to TNFR2 of the Δ1–12 Tm TNF may be due to the deletion of a proline residue in position 12 (31). Because TNF requires signaling through TNFR1 for host defense against mycobacteria (51), the present results thus strongly indicate that Tm TNF expressed in our

Tm TNF tg mice, where the proline residue in position 12 is preserved, can also signal by binding to TNFR1.

Indications for bidirectional signaling processes via TNFR-Tm TNF, whereby Tm TNF may act like a receptor transducing cellular signals upon binding of soluble or membrane-bound receptors, have been recently obtained (52, 53). Reverse signaling through Tm TNF seems to play an active role in cellular interactions between endothelium and monocytes/macrophages (54). Reverse signaling processes through Tm TNF may probably play a role in macrophage activation of Tm TNF tg mice; this could also explain why Tm TNF mediates additional activities to those observed for soluble TNF.

Besides the protective role of TNF in infections, TNF production can be also associated with harmful effects such as fever and progressive weight loss. TNF was shown to be instrumental in fatal acute meningitis in rabbits when BCG-induced TNF or genetically engineered BCG strains produced TNF in the CNS (55). The release of excessive amounts of TNF can be prevented by using TACE inhibitors (38). Administration of a metalloproteinase inhibitor in healthy humans injected with a single dose of LPS strongly reduced LPS-induced TNF release but did not influence the increase in monocyte-bound TNF (56). These recent data from Dekkers et al. (56) suggest a beneficial effect of TACE inhibitors on the course of disorders where excessive production of TNF is considered to play an important role. Hence, our observation that Tm TNF can substitute for secreted TNF in host defense against BCG may indicate that TACE inhibitors may be used in human tuberculosis as therapeutic modality to attenuate harmful effects of TNF but preserving beneficial properties to maintain protective immunity.

In conclusion, our data show that in a strictly TNF-dependent model Tm TNF mediates long-term protection to *M. bovis* BCG intracellular pathogen, even in the absence of secreted TNF homotrimers. Furthermore, the biological activities exerted by Tm TNF in vivo may differ quantitatively from those mediated by secreted TNF and LT-α.

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