Structural Deficiencies in Granuloma Formation in TNF Gene-Targeted Mice Underlie the Heightened Susceptibility to Aerosol Mycobacterium tuberculosis Infection, Which Is Not Compensated for by Lymphotoxin


TNF and lymphotoxin-α (LTα) may act at various stages of the host response to Mycobacterium tuberculosis. To dissect the effects of TNF independent of LTα, we have used C57BL/6 mice with a disruption of the TNF gene alone (TNF−/−). Twenty-one days following aerosol M. tuberculosis infection there was a marked increase in the number of organisms in the lungs of TNF−/− mice, and by 28–35 days all animals had succumbed, with widespread dissemination of M. tuberculosis. In comparison with the localized granulomas containing activated macrophages and T cells in lungs and livers of C57BL/6 wild-type (wt) mice, cellular infiltrates in TNF−/− mice were poorly formed, with extensive regions of necrosis and neutrophilic infiltration of the alveoli. Phenotypic analysis of lung homogenates demonstrated similar numbers of CD4+ and CD8+ T cells in TNF−/− and wt mice, but in TNF-deficient mice the lymphocytes were restricted to perivascular and peribronchial areas rather than colocated with macrophages in granulomas. T cells from TNF−/− mice retained proliferative and cytokine responses to purified protein derivative, and delayed-type hypersensitivity to purified protein derivative was demonstrable. Macrophages within the lungs of TNF−/− and wt mice showed similar levels of MHC class II and inducible nitric oxide synthase expression, and levels of serum nitrite were comparable. Thus, the enhanced susceptibility of TNF−/− is not compensated for by the presence of LTα, and the critical role of TNF is not in the activation of T cells and macrophages but in the local organization of granulomas.


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5 Abbreviations used in this paper: RNI, reactive nitrogen intermediates; LT, lymphotoxin; wt, wild-type; PPD, purified protein derivative; DTH, delayed-type hypersensitivity; ZN, Ziehl-Neelsen; iNOS, inducible nitric oxide synthase.

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ellular immunity to Mycobacterium tuberculosis infection is characterized by the emergence of Ag-specific T cells that mediate the recruitment and activation of macrophages to kill the intracellular bacteria and form granulomas (1, 2). Control of the infection relies on this granulomatous response, and disturbances to the immune response that prevent granuloma formation result in a failure to resolve the infection (3, 4). Granuloma formation is dependent on the T cell response, as illustrated by the absence of granulomas in T cell-deficient mice (5, 6). T lymphocytes are recruited by chemotactic cytokines to the site of infection (5, 7) where they release cytokines that activate macrophages and drive epithelioid cell differentiation, resulting eventually in granulomas. A variety of cytokines and chemotactic factors are required for this recruitment of lymphoid cells and efficient activation of macrophages. In vitro models of mycobacterial infection demonstrate synergy between IFN-γ and TNF for the stimulation of macrophage intracellular killing through the induction of high levels of reactive nitrogen intermediates (RNI)5 (8). The major source of TNF is mononuclear phagocytes, although T cells, particularly after appropriate stimulation, are capable of producing substantial amounts of TNF (9). As a highly potent proinflammatory cytokine, TNF has broad-ranging activities from the up-regulation of adhesion molecules on endothelium to the induction of apoptosis. TNF release correlates with antimycobacterial mechanisms in the murine model (10), as the peak of production coincides with a decrease in the bacillary load. Pretreatment of murine macrophages cultured in vitro with TNF and IFN-γ induces significant bactericidal activity, with a 100-fold reduction in the survival of intracellular mycobacteria (11). TNF is required for this mycobacteriostatic effect, as treatment of mycobacterium-infected macrophage cultures with anti-TNF antiserum or soluble anti-TNFRI abrogated the inhibition of mycobacterial growth and the release of RNI (8, 12, 13).

The in vivo role of TNF in protective responses to mycobacterial infection has been assessed using neutralization with anti-TNF antiserum and mice deficient in the TNFRI (4, 14). Both approaches resulted in the decreased development of granulomas, failure to restrict mycobacterial growth, and decreased survival. These experiments implicated a role for TNF in the control of mycobacterial infection, but the relative contributions of TNF and the related cytokine lymphotoxin-α (LTα) to protection are uncertain. There is ~30% amino acid identity between TNF and LTα,
which is secreted in a trimeric form as LTα3 (15). Both TNF and LTα, however, bind TNFRI with similar affinities (16), so experiments in TNFRI-deficient mice fail to distinguish between the roles of TNF and LTα. Moreover, the effects observed with the polyclonal anti-TNF antiserum may have been due to neutralization of either TNF or LTα. To define the contribution of TNF to the course of M. tuberculosis infection we have used mice in which the TNF gene has been disrupted directly in the C57BL/6 mouse strain (17) and that retain normal expression of LTα (18). This avoided the problems associated with using F1 hybrids of the 129 strain mice or the requirement for extensive backcrossing. Using a model of low dose M. tuberculosis aerosol infection, which mimics the major route of infection in humans, we demonstrate that TNF itself is crucial for the development of protective immunity against mycobacteria. Although there was evidence of the Ag-specific T cell activation and activation of macrophages, the growth of mycobacteria was unrestrained, leading to the death of TNF−/− mice. Activated T cells were present in the lungs of TNF−/− mice in similar proportions as in wild-type (wt) mice, but the failure of these T cells to form well-defined granulomas in TNF-deficient animals indicates that TNF is essential for the cellular recruitment and organization underlying this process.

Materials and Methods

Mice

Control wt mice were 6- to 8-wk-old C57BL/6 strain mice obtained from Animal Resources Center (Perth, Australia). TNF gene knockout mice prepared on the C57BL/6 background have been previously described (17). All mice were housed under specific pathogen-free conditions at the Centenary Institute animal facility until infection with M. tuberculosis, when they were transferred and maintained in a level 3 physical containment facility.

Aerosol infection of mice with tuberculosis

A Middlebrook airborne infection apparatus (Glas-Col, Terre Haute IN) was used to infect mice with M. tuberculosis H37Rv (ATCC no. 27294, American Type Culture Collection, Manassas, VA) strain that had been grown from a low passage seed lot in Proskauer-Beck liquid medium to midlog phase, aliquoted, and frozen at −70°C. The mice were placed in the exposure chamber of the apparatus, and a 10^5/ml suspension of the bacteria was placed into the nebulizer. This concentration delivered 100 bacilli into the lungs of exposed mice. The numbers of viable bacteria in target organs were followed over time by plating serial dilutions of whole organ homogenates on supplemented Middlebrook 7H11 nutrient agar (Difco, Detroit, MI) and counting bacterial colony formation after incubation for 20 days at 37°C. The data are expressed as the log_{10} value of the mean number of bacteria recovered per organ (n = 4 animals).

Lang preparations

Animals were sacrificed by carbon dioxide narcosis at appropriate time points. The lung vascular bed was cannulated and perfused with a warm solution containing PBS, 0.02% BSA (Sigma, St. Louis, MO), and 20 U/ml heparin (Fisons Pharmaceuticals, Sydney, Australia). One lung was homogenized, and a 100-μl aliquot was used for serial dilutions from 10^−1 to 10^−8 and spread in duplicate quadrants of bacterial plates. The other lung was chopped into small pieces using scissors and incubated in RPMI 1640 (Sigma) containing optimal concentrations of collagenase (Sigma) and DNAse (Sigma) for 60 min at 37°C. At the end of this incubation period, any large particulate debris were removed from the suspension by passage through sterile 100-μm pore size mesh, and cells were collected by centrifugation (480 × g at 4°C for 5 min).

Phenotypic analysis of pulmonary infiltrates

The following mAb were used: CD4 (CT-CD4; Caltag, South San Francisco, CA), CD8 (CT-CD8; Caltag), CD16/32 (2.4G2; PharMingen, La Jolla, CA), CD44 (IM 7.8.1; Sigma), CD45RB (16A; Sigma), Ly-6G (RB6-8C5; PharMingen) for flow cytometry. Staining of cells was performed in a 96-well round-bottom plate (Becton Dickinson, Lincoln Park, NJ) with 2 × 10^4 cells/well. Cells were pelleted by centrifugation (480 × g at 4°C for 1 min), the supernatant was aspirated, and Fc receptors were blocked by labeling with CD16/32 (for 15 min at 4°C). The cells were washed by centrifugation (2% BSA and 0.1% NaCl in PBS, 480 × g at 4°C for 1 min), and diluted Ab combinations were added (for 15 min at 4°C). Following washing, the samples were fixed overnight in 10% neutral buffered formalin (Fronine, Riverstone, Australia) and analyzed on the FACScan (Becton Dickinson, San Jose, CA). The expression of MHC class II and iNOS within tissues was investigated with indirect immunofluorescence staining with TRITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). Ab incubations were conducted for 20 min in a moist chamber at room temperature, and after each incubation the sections were washed three times for 5 min each time with PBS. The distribution of lymphocytes was demonstrated by immunoperoxidase staining of deparaffinized sections with biotinylated rat anti-Thy-1.2 (30H12) and streptavidin-conjugated horseradish peroxidase (Sigma) followed by color development with Sigma Fast diaminobenzidine. Sections were examined on a Leica DMRBE (Deerfield, IL).

T cell responses to mycobacterial Ags

 Mediastinal lymph node cells were harvested from wt and TNF−/− mice. A single cell suspension was prepared by sieving through 200-μm pore size mesh and resuspending the cells in culture medium (RPMI 1640; Sigma), 10% FCS (CSL Bioscience, Melbourne, Australia), 2 mM l-glutamine (Flow Laboratories, Sydney, Australia), 50 mM 2-ME (Sigma) buffered with 10 mM HEPES (Sigma), and 10 mM sodium bicarbonate (BDH, Melbourne, Australia). Cell suspensions were plated at 2 × 10^5 cells/well in 96-well plates and incubated for 72 h at 37°C in 5% CO2. The cells were stimulated with medium alone, purified protein derivative (PPD) of M. tuberculosis (Statens Seruminstitut, Copenhagen, Denmark), or PMA (Sigma). The proliferation of lymphocytes was determined using the Alamar Blue assay (Astral Scientific, Gymea, Australia). Briefly, for the last 16 h of a 72-h culture, Alamar Blue dye was added to a concentration of 10% (v/v), and Alamar Blue fluorescence (oxidation-reduction) was measured by absorbance at 540 nm (reduced form) and 620 nm (oxidized form) using a Titertek Multiscan Mcc/340 plate reader (ICN Pharmaceuticals, Costa Mesa, CA). The concentrations of IFN-γ in culture supernatants were determined by ELISA using an mAb capture assay with Abs RA-4,6A2 and XMG1.2-biotin (Endogen, Woburn, MA) following the manufacturer’s instructions. Avidin-alkaline phosphatase (Sigma) and n-nitro-phenylphosphate (1 mg/ml in 10 mM NaHCO3 and 0.1 mM MgCO3, pH 6.3) were used as the colorimetric reagents, and absorbance was measured at 405 nm.

Serum nitrite measurements

Serum nitrite was assayed by a modification of the nitrate kit for food analysis (Boehringer Mannheim, Mannheim, Germany). Briefly, serum nitrate was reduced to nitrite using nitrate reductase. Nitrite levels were determined using the Greiss reagent (19) (3% phosphoric acid, 1% p-aminobenzene-sulfonamide, and 1% n-naphthylethenediamide (Sigma)); 100 μl of this was added to 30 μl of reduced sera. Samples were incubated for 5 min at room temperature, and absorbance was read at 540 nm.

Determination of delayed-type hypersensitivity (DTH) response to PPD

Infected mice were challenged in one footpad with 10 μg of PPD in 50 μl of PBS and in the other with PBS alone. The swelling in each footpad was measured at 24 h using callipers (Mitutoyo 97227-10; Extech, Boronia, Australia), and the Ag-specific DTH response was determined as the difference in swelling between PBS-injected footpad against PPD-injected footpads. The PPD preparation did not induce swelling in the footpads of uninfected animals.

Histological and immunohistochemical analyses

Lung and liver tissue samples were fixed in 10% neutral buffered formalin, set in paraffin blocks, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin or the Ziehl-Neelsen (ZN) stain for acid fast bacilli. Slides were examined blind and analyzed using 37 criteria for differences in cellular infiltrate. Granulomas were defined as collections of 10 or more macrophages and T lymphocytes within the peripheral lung or liver. The average number of granulomas in liver sections was determined for five random high power fields (×400). The expression of MHC class II and iNOS within tissues was investigated with indirect immunofluorescence labeling. Air-dried frozen sections (4–6 μm) were double stained with rabbit anti-mouse iNOS polyclonal Ab (Upstate Biotechnology, Lake Placid, NY) and rat anti-mouse Ia mAb (P7/7), followed by F(ab')2 of affinity-purified goat anti-rat Ig conjugated to FITC (Caltag) in combination with TRITC-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL). Ab incubations were conducted for 20 min in a moist chamber at room temperature, and after each incubation the sections were washed three times for 5 min each time with PBS. The distribution of lymphocytes was demonstrated by immunoperoxidase staining of deparaffinized sections with biotinylated rat anti-Thy-1.2 (30H12) and streptavidin-conjugated horseradish peroxidase (Sigma) followed by color development with Sigma Fast diaminobenzidine. Sections were examined on a Leica DMRBE (Deerfield, IL).
Results

TNF and not LTα is crucial for the control of aerosol M. tuberculosis infection

TNF−/− and wt mice were infected with virulent M. tuberculosis, and their clinical condition and the growth of bacteria were followed over time. TNF−/− mice remained well until day 25, but then rapidly deteriorated, and all succumbed between days 28 and 35 of infection (Fig. 1). By contrast, wt mice controlled the same infectious dose and survived for >161 days. The number of mycobacteria recovered from the lungs of TNF−/− mice increased from day 17, and by day 28 this was 105 times greater than that in wt mice (Fig. 2). There was increased dissemination of organisms to the liver and spleen of TNF−/− mice (Fig. 2), and M. tuberculosis was recovered from the kidneys, bone marrow, and other organs not usually infected in wt mice (data not shown).

Lung T cell number and phenotype are unaltered by the deficiency in TNF

Both CD4+ and CD8+ T cells are vital to the resolution of M. tuberculosis infection in the lung. Therefore, the cell number and phenotype of the responding cells in TNF−/− mice were assessed throughout the course of the infection. Similar numbers of CD4+ and CD8+ T cells were present in the lungs of both wt control and TNF−/− mice. At 28 days postinfection, ~30% of lung lymphocytes were CD4+, while nearly 20% were CD8+ (Fig. 3, a and b). To investigate the activation state of these cells, CD4+ T cells were stained for the activation/memory markers CD45RB and CD44 (Fig. 3, c and d). The CD4+ T cells from both wt and TNF−/− showed similar levels of CD44high/CD45RBlow expression.

While the T cell compositions of the lung and draining lymph nodes were unaffected, a difference was observed in the numbers of Ly-6G+ cells (Gr-1, a marker of neutrophils). From as early as day 7 postinfection the number of Ly-6G+ cells was increased in the TNF−/− mice compared with that in wt controls (Fig. 3, e and f), and they were a significant component of the total leukocyte population by day 28.

Differential cellular infiltrate and granulomatous response in the lungs of infected TNF−/− mice

As normal numbers of T cells were detected in lung cellular homogenates from the TNF−/− mice throughout the course of the disease, the lungs and livers of TNF−/− and normal mice were examined histologically to determine whether the rate of development and the pattern of cellular responses were different between the TNF−/− and wt mice. In wt mice a few small granulomas were present throughout the lungs on day 14, and by day 28 these had grown into several granulomas, 1–1.2 mm in diameter (Fig. 4a), containing intact macrophages, some lymphocytes, and only occasional neutrophils with no necrosis (Fig. 4c). In TNF−/− mice the earliest differences in the lungs were mild to moderate perivascular and peribronchial accumulations of lymphocytes and plasma cells evident on days 7 and 14. Consistent with the cytometric analysis, by day 21 the cellular accumulations in TNF−/− mice were clearly different, consisting of groups of alveoli filled with neutrophils and few macrophages and no lymphocytes. By day 28 these collections were larger in TNF−/− mice, being 2.2–2.8 mm in diameter (Fig. 4b). The central regions contained necrotic debris and fibrin overlying outlines of alveoli with peripheral margins of intact neutrophils and macrophages. On days 21 and 28 there were prominent perivascular lymphocytic accumulations and milder peribronchial infiltrates of mixed mononuclear cells in both control and TNF−/− mice (Fig. 4, c and d). In areas free of cellular inflammation there was extensive exudation of proteinaceous fluid into the alveolar lumens of TNF−/− mice. On ZN staining, the granulomas in wt mice contain no more than several hundred bacilli per high power field at 28 days postinfection (Fig. 4e), whereas in TNF−/− mice the number of bacilli was greatly increased to about several thousand per high power field (Fig. 4f).
Although normal granuloma formation was retarded in TNF−/− mice, a degree of activation of the macrophages within the lungs was observed. Fig. 4, g and h, indicates the colocalization of MHC class II and iNOS labeling in wt and TNF−/− mice. The iNOS staining was generally within or around the granulomas in wt mice and the margins of the necrotic regions in TNF−/− mice. Increased MHC class II staining was also evident on alveolar macrophages and the endothelium of blood vessels in other parts of the lung parenchyma in both groups.

The location of lymphocytes was confirmed with immunoperoxidase staining. Thy1.2-positive lymphocytes were mixed with macrophages in the granulomas of normal mice (Fig. 5a). By contrast, in TNF−/− mice the Thy1.2-positive lymphocytes were restricted to perivascular and peribronchial regions and were absent from infiltrates in peripheral lung (Fig. 5, b and d). The failure of TNF−/− mice to develop normal granulomas was also evident in the liver, with a profound reduction in the number of granulomas during the course of M. tuberculosis infection (Fig. 6). In addition, at 28 days numerous foci of extramedullary hemopoiesis were present in the livers of TNF−/−, but not in wt mice.

Production of RNIs in TNF−/− mice

The antimicrobial mechanisms of activated mouse macrophages include the production of RNIs. Therefore, the nitrite levels in the serum of wt and TNF−/− mice were analyzed throughout the course of M. tuberculosis infection (Fig. 7). TNF−/− mice had levels of serum nitrite similar to those in wt mice. This was consistent with the observed expression of iNOS within pulmonary macrophages in infected TNF−/− mice (Fig. 4, g and h). Thus, at the time when wt mice begin to control infection and TNF−/− mice fail to do so, nitrite and iNOS levels were similar in these mice (Fig. 7).

T cell proliferation and IFN-γ release in infected mice are unaffected by TNF deficiency

To determine the reason for the inability to control mycobacterial growth, T cell proliferative responses in infected mice were examined. Fig. 8a demonstrates that in an in vitro restimulation assay at 3 wk, T cells from the mediastinal draining lymph nodes of both wt and TNF−/− infected mice proliferated to the same degree in response to stimulation with PPD. IFN-γ is crucial to the protective response to M. tuberculosis infection. Draining lymph node cells from infected animals were stimulated with PPD, and IFN-γ was measured. Although TNF−/− mice were unable to control M. tuberculosis infection they were still able to produce IFN-γ in an Ag-specific recall response (Fig. 8b).

TNF−/− mice develop DTH to mycobacterial proteins

It is uncertain which molecules are required for generating a DTH response to PPD. This was highlighted by the ability of IFN-γ-deficient mice to mount Ag-specific DTH (20). To determine whether TNF−/− mice could also mount this response, infected mice were challenged in one footpad with 10 μg of PPD, and the response was compared with that in wt mice. Although TNF−/− mice could not mount a protective response in the lungs, they were still able to mount a DTH response to mycobacterial proteins (Fig. 9).

Discussion

This study demonstrates that TNF is essential to prevent unrestrained growth of M. tuberculosis bacteria in all target organs following aerosol infection. These TNF−/− mice have normal expression of LTα mRNA (18), but LTα is unable to compensate for the deficiency of TNF. The susceptibility to M. tuberculosis correlates with a paucity in granuloma formation even though there is evidence of both T cell and macrophage activation. Ag-specific proliferation and IFN-γ production by T cells were at levels similar to those in wt animals, while macrophage activation, as reflected by expression of iNOS and serum nitrite production, was also comparable in TNF−/− and wt mice. Earlier studies using TNFRI−/− mice or neutralizing anti-TNF Ab suggested a role for TNF in the control of infection with M. tuberculosis, but these used either very high dose i.v. M. tuberculosis infection or M. bovis Calmette-Guérin bacillus infection (4, 14). In this study low dose inocula of M. tuberculosis were delivered by the aerosol route and resulted in disseminated fatal infection, confirming that TNF is essential for the protective response against tuberculosis. Moreover, as TNFRI binds both TNF and LTα, and polyclonal anti-TNF Abs may neutralize both cytokines, earlier studies (4, 14) were unable to distinguish between the protective effects of TNF and LTα. Infection of mice with disruption of the TNF molecule alone has defined the essential contribution of TNF to protective immunity.

TNF may operate at a number of steps in the host response to M. tuberculosis infection. First, TNF synergizes with IFN-γ in vitro for the maximal activation of bactericidal mechanisms in infected macrophages, including the production of RNI (8, 12). TNF together with IFN-γ was considered necessary for the expression of
iNOS by macrophages (21), although IFN-γ can induce low levels of iNOS activity on its own (22). Inhibition of TNF with soluble TNFRI in vitro abrogates NO release and the inhibition of the growth of Calmette-Guérin bacillus (12). Intriguingly, the TNF−/− mice are still able to produce NO (Fig. 7), and iNOS is detectable within their lungs (Fig. 4h). During M. tuberculosis infection other activating signals may be sufficient to induce iNOS. This is consistent with the presence of iNOS mRNA in lungs of M. tuberculosis-infected TNFRI-deficient mice (14) and immunoreactive iNOS in the brains of TNF−/− mice with experimental allergic encephalitis (18, 23). The inhibition of iNOS function in vivo (24, 25) and the deletion of its gene (26) confirm that RNI are essential for host resistance to mycobacteria, but RNI synthesis alone is not sufficient to control mycobacterial infection in TNF−/− mice.

**FIGURE 4.** TNF−/− mice have defective lung granuloma formation, although MHC class II expression and iNOS production are detectable. The left panels show lung tissue from 28-day postinfection controls (a, c, e, and g), while the right panels represent TNF−/− mice (b, d, f, and h). Tissue was fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin (a and b, low power, ×120; c and d, high power, ×240), the ZN method for acid fast bacilli (e and f, bacilli stain red; magnification, ×240; arrow indicates few remaining bacilli in wt), or Abs directed against MHC class II (green staining) and iNOS (red staining; g and h, ×240 magnification; arrows indicate granuloma structure).
Thus, failure of the activation of macrophage bactericidal mechanisms alone does not explain the profound susceptibility of these mice to tuberculosis.

Second, TNF appears crucial for the structural organization of the cellular response. Despite the presence of activated T cells in the lungs of TNF−/− in similar numbers and proportions to those in wt mice, the normal pattern of granuloma formation is disrupted (Fig. 4b). Previous studies have demonstrated neutralizing anti-TNF Ab inhibits granuloma formation in the liver (4, 14). This is confirmed by the markedly depressed granulomatous response in the liver of the TNF−/− mice (Fig. 6). Granuloma formation requires the activation of Ag-specific T cells, the accumulation of T cells and monocytes at the site of infection, and their local organization into a mature granuloma that limits the infection. In healthy wt mice, this process reduces the bacterial burden, but does not eliminate infection, so that at 23 wk postinfection mycobacteria are still demonstrable in the lungs, contained within granulomas

**FIGURE 5.** In wt mice T cells are present within granulomas in infected lungs, while in TNF−/− mice T cells are restricted to perivascular and peribronchial regions and are absent from peripheral lung. The left panels show lung tissue from 28-day postinfection controls (a and c), while the right panels represent TNF−/− mice (b and d). Tissue was fixed in buffered formalin, sectioned, and stained with an Ab directed against Thy1.2 (magnification, ×200). a and b represent peripheral lung; c and d represent perivascular and peribronchial areas, respectively.

**FIGURE 6.** TNF−/− mice have decreased numbers of granuloma throughout the course of *M. tuberculosis* infection. The values indicate the mean number of granulomas in five low power fields in the liver. Control (solid bars) and TNF−/− (open bars) mice are shown. *, p < 0.05, by Student’s *t* test.

**FIGURE 7.** TNF−/− mice produce RNI in response to *M. tuberculosis* infection. The nitrite concentrations in the sera of control (closed squares) and TNF−/− mice (open squares) were analyzed throughout the course of infection. Nitrite levels were determined by comparison with a standard sodium nitrite curve using the Greiss reagent.
numbers of CD4+ and CD8+ T cells (Fig. 3), the lymphocytes accumulated in the perivascular and peribronchial regions of TNF−/− mice. This is the earliest detectable histological difference between TNF−/− and wt mice. Activated CD44highCD45RBlow T cells appear able to cross the endothelium, but the T cells fail to migrate from the perivascular region into the inflamed lung, and this contributes to the deficiency in granuloma formation.

TNF may participate in both the recruitment and migration of T cells into inflamed tissues. This occurs through the TNF-stimulated expression of chemotactic molecules on endothelium and the up-regulation of surface homing molecules on T cells (27, 28), both of which contribute to the recruitment of T cells into inflamed tissue (29). However, in the TNF−/− mice there was normal recruitment of T cells into the lung, but failure of movement of the T cells into the infected alveoli to form granulomas. During the evolution of experimental autoimmune encephalitis in the central nervous system of TNF−/− mice, a similar failure of leukocyte migration was observed, with cells accumulating in the perivascular spaces of the brain but not moving normally into the parenchyma (18, 23).

Recent studies with IL-12−/− mice have indicated that these mice are unable to generate a DTH response to PPD during M. tuberculosis infection (30). By contrast, IFN-γ-deficient mice can mount a DTH response despite their increased susceptibility to M. tuberculosis (20). This suggested that other soluble factors, such as TNF, may be more important in generating a DTH response. The preservation of DTH despite the absence of TNF indicates that this cytokine is not essential for this response. DTH requires the presence of Ag-reactive T cells and their capacity to cross endothelium in response to signals and cause dermal edema. Ag-specific T cells are present in TNF−/− mice, as evidenced by specific IFN-γ responses in draining lymph nodes and the expression of T cell activation markers on lung T cells, but they are still unable to control the disease. Although the activation and production of IFN-γ by T cells are essential for the resolution of M. tuberculosis infection, clearly this response must occur within a localized, structurally normal granuloma at the site of infection for it to be successful.

The movement of mononuclear phagocytes as well as lymphocytes may be defective in the absence of TNF. Macrophages are normally activated by TNF to produce a range of locally active chemokines (28). These chemokines attract monocytes to the sites of infection and activate their development into mature macrophages, leading to granuloma formation, epithelioid cell differentiation and multinucleated giant cell formation (28). Other chemokines are induced by TNF. For example, the release of macrophage inflammatory protein-1α from LPS-stimulated macrophages is dependent on TNF and IL-1, since coinubcation of LPS-stimulated PBMC with TNF binding protein or IL-1R antagonist abrogates macrophage inflammatory protein-1α secretion (31). These mechanisms contribute to the rapid, transient accumulation of leukocytes observed following the local injection of TNF (32). Reduction in monocyte recruitment also contributes to the failure of the development of an effective granulomatous response.

Another effect of TNF deficiency is the dysregulation of the inflammatory response. The increased influx of activated neutrophils in TNF−/− mice may account for the marked damage to the lungs during infection. Neutrophil migration and activation contributes to lung injury in acute respiratory distress syndrome patients (33). Neutrophils and their oxidative products cause endothelial and epithelial cell injury, leading to increased protein permeability into alveoli and impaired lung function. The combination of tissue necrosis and the increased protein exudation in areas without tissue destruction contributes to the death of these mice. This phenomenon also occurs during M. tuberculosis infection in other genetically deficient mice, such as IFN-γ and γδ
TCR−/− mice (20, 34). This neutrophil influx may be an ineffective compensatory response, activated when the correct T cell/macrophage interaction is disrupted. TNF may play an important role by influencing local cellular traffic, affecting the positioning of lymphocytes and monocytes, and limiting the actions of neutrophils that cause tissue damage rather than protection.

In conclusion, TNF is essential for the control of *M. tuberculosis* infection. Its central role appears to be in the generation of a structurally effective granulomatous response, and this cannot be compensated for by LTα alone. Even though Ag-specific lymphocytes are activated and cross the vasculature into the lung, they fail to migrate into the infected tissues and interact with monocytes to form granulomas and prevent the unrestrained growth of the bacilli. This effect of TNF deficiency may be mediated by dysregulation of the chemokine network, which is crucial to the movement of lymphocytes and monocytes within the lung.

References


