Brief Definitive Report

Secreted Lymphotoxin-α Is Essential for the Control of an Intracellular Bacterial Infection

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Abstract

Although the essential role of tumor necrosis factor (TNF) in the control of intracellular bacterial infection is well established, it is uncertain whether the related cytokines lymphotoxin-α ($LT\alpha_3$) and lymphotoxin-β (LTβ) have independent roles in this process. Using C57Bl/6 mice in which the genes for these cytokines have been disrupted, we have examined the relative contribution of secreted $LT\alpha_3$ and membrane-bound LTβ in the host response to aerosol *Mycobacterium tuberculosis* infection. To overcome the lack of peripheral lymph nodes in $LT\alpha^{-/-}$ and $LT\beta^{-/-}$ mice, bone marrow chimeric mice were constructed. $LT\alpha^{-/-}$ chimeras, which lack both secreted $LT\alpha_3$ and membrane-bound LTβ ($LT\alpha1\beta2$ and $LT\alpha2\beta1$), were highly susceptible and succumbed 5 wk after infection. $LT\beta^{-/-}$ chimeras, which lack only the membrane-bound LTβ, controlled the infection in a comparable manner to wild-type (WT) chimeric mice. T cell responses to mycobacterial antigens and macrophage responses in $LT\alpha^{-/-}$ chimeras were equivalent to those of WT chimeras, but in $LT\alpha^{-/-}$ chimeras, granuloma formation was abnormal. $LT\alpha^{-/-}$ chimeras recruited normal numbers of T cells into their lungs, but the lymphocytes were restricted to perivascular and peribronchial areas and were not colocated with macrophages in granulomas. Therefore, $LT\alpha_3$ is essential for the control of pulmonary tuberculosis, and its critical role lies not in the activation of T cells and macrophages per se but in the local organization of the granulomatous response.

Key words: lymphotoxin • TNF • tuberculosis • granuloma • lung

Introduction

The control of chronic intracellular bacterial infection is dependent on the activation and expression of cellular immunity (1). In the case of *Mycobacterium tuberculosis* infection, infected APCs activate T cells in the context of IL-12, leading to a Th1 pattern of T cell responses. Mycobacteria-specific T cells are recruited back to the initial site of infection in the lung (2), where they initiate the cascade of cellular and molecular events that control the infection. The expression of immunity requires the continued recruitment of T cells and macrophages into the lung, the migration and aggregation of these cells to form granulomatous lesions, and the release of IFN-γ by T cells (3), which in concert with TNF activate mycobactericidal mechanisms in infected macrophages (4). Granulomatous lesions are the hallmark of the inflammatory response to mycobacteria and are essential to control infection (1). The cytokine and chemokine signals modulating this inflammatory response are poorly understood, although TNF plays a critical role in orchestrating the movement of these cells once they enter infected tissues (5).

Lymphotoxin (LT) comprises two members of the TNF superfamily, LTα and LTβ. LTα is active as a secreted homotrimeric molecule ($LT\alpha_3$, also known as TNF-β) (6), which binds not only to both of the TNF receptors (TNFRI and TNFRII) (7, 8) but also to the newly identified herpes virus entry mediator (HVEM) receptor (9). In comparison to TNF, which is produced by a wide range of cell types, $LT\alpha_3$ is produced primarily by $CD4^+$ T cells, B cells, and NK cells (10). As $LT\alpha_3$ and TNF bind to TNFRI with similar affinity (11) and have ~30% homology in amino acid sequence (12), the functional activity for $LT\alpha_3$ independent of TNF has been unclear. By contrast, LTβ is a heterotrimeric molecule that exists in two forms, the major

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being LTα1β2 and the minor LTα2β1 (13). Both forms of LTβ bind the unique LTβ receptor (LTβR) (14), and LTβ signaling through LTβR is essential for the formation of the peripheral lymphoid organs (15). Mice deficient in either LTβ or LTβR lack peripheral lymph nodes and Peyer's patches and display disorganized splenic architecture.

Recently, the activity of $LT\alpha_3$ during immune responses has been reexamined. Transgenic expression of the LTα gene under control of the rat insulin promoter led to the formation of chronic inflammatory lesions at the sites of transgene expression (16). These lesions resembled secondary lymphoid tissue, not only in their cellular composition and structure, but also in their ability to respond to Ag. Interestingly, TNFRI was the receptor mediating the formation of these lesions. LTα- and LTβ-deficient mice have also been employed to investigate the independent functions of $LT\alpha_3$ and LTβ in inflammatory pathologies. Targeted disruption of the gene for LTα results in loss of both secreted $LT\alpha_3$ and cell surface LTβ complexes, whereas targeted disruption of the LTβ gene results in loss of only cell surface LTβ. Disruption of either LTα or LTβ also results in the phenotypic loss of secondary lymphoid tissue. Using radiation bone marrow chimeras with gene-targeted and wild-type (WT) animals, normal secondary lymphoid organ function has been reconstituted, enabling the study of cytokine effector function at the target tissue level (17). In this manner, $LT\alpha_3$ was shown to have no independent role in the cellular inflammation or clinical course of experimental autoimmune encephalomyelitis (EAE), a model of tissue-specific autoimmune disease (17).

To determine whether $LT\alpha_3$ or LTβ independently contribute to the host response to *M. tuberculosis* infection, we have constructed radiation bone marrow chimeras, using as donors C57Bl/6 mice in which the genes for LTα or LTβ have been disrupted. Transfer of bone marrow into irradiated mice resulted in repopulation of peripheral lymphoid tissues with leukocytes deficient in both $LT\alpha_3$ and LTβ or deficient in LTβ alone. Analysis of low dose aerosol *M. tuberculosis* infection in these mice demonstrated the essential role of $LT\alpha_3$ in the host response to mycobacterial infection. Although there was evidence of induction of Ag-specific T cell responses and activation of macrophages, mycobacterial growth was unrestrained, leading to the death of $LT\alpha^{-/-}$ chimeric mice. By contrast, $LT\beta^{-/-}$ chimeric mice were able to control the infection normally. The inflammatory response in the lungs of $LT\alpha^{-/-}$ chimeric mice was markedly abnormal, with failure to form distinct granulomas, indicating that $LT\alpha_3$ has an essential role in the cellular recruitment and organization underlying this process.

Materials and Methods

Mice. C57Bl/6 (Ly5.2), C57Bl/6.Ly5.1, and C57Bl/6.RAG-$1^{-/-}$ mice were obtained from Animal Resources Centre, and LTα and LTβ gene knockout mice on a C57BL/6 background have been previously described (17, 18). Adult (>6 wk old) mice were used in all experiments. Mice were housed under specific pathogen–free conditions at the Centenary Institute animal facility, or after infection in a Level 3 physical containment facility.

Generation of Radiation Bone Marrow Chimeras. Radiation bone marrow chimeric mice were generated as in Riminton et al. (17). To monitor engraftment, C57Bl/6.Ly5.1 bone marrow was transferred into C57Bl/6.Ly5.2 recipient mice. Peripheral blood was analyzed by flow cytometry for the presence of the Ly5.1 congenic marker, and reconstitution was considered satisfactory when >95% of leukocytes were of donor type.

Aerosol Infection of Mice with M. tuberculosis. A Middlebrook airborne infection apparatus (Glas-Col Inc.) delivered ~100 bacilli of *M. tuberculosis* H37Rv (American Type Culture Collection no. 27294) to each mouse. The number of viable bacteria in target organs was determined by plating serial dilutions of organ homogenates on supplemented Middlebrook 7H11 nutrient agar (Difco Labs.). The data are expressed as the $\log_{10}$ of the mean number of bacteria recovered per organ.

Lung Preparations. Mice were killed at several time points after aerosol *M. tuberculosis* infection. One lung was homogenized and serial dilutions spread on duplicate quadrants of bacterial plates. The other lung was minced and incubated with collagenase (10 U/ml; Worthington) and DNase (13 μg/ml; Boehringer Mannheim) to produce a cell suspension, as previously described (5).

Phenotypic Analysis of Pulmonary Infiltrates. mAbs used for flow cytometry were: CD4 (CT-CD4; Caltag Laboratories), CD8 (CT-CD8; Caltag Laboratories), CD16/32 (2.4G2; BD PharMingen), CD44 (IM 7.8.1; Sigma-Aldrich), CD45RB (16A; Sigma-Aldrich), Ly-6G (RB6-8C5; BD PharMingen), biotinylated anti-Ly5.1, and streptavidin–PE (Sigma-Aldrich).

T Cell Responses to Mycobacterial Ags. Mediastinal lymph node or lung cell suspensions were suspended in culture medium (RPMI-1640; Sigma-Aldrich), 10% FCS (CSL Bioscience), 2 mM L-glutamine (Flow Laboratories), 50 mM 2-ME (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), and 10 mM sodium bicarbonate (BDH) and cultured alone or with purified protein derivative (PPD; 10 μg/ml) of *M. tuberculosis* (Statens Seruminstitut) for 72 h, and total IFN-γ production was measured by capture ELISA (5). The frequency of IFN-γ–producing cells was determined by ELISpot assay, as previously described (5). Delayed-type hypersensitivity (DTH) reactions were determined as the difference in swelling between PBS-injected footpads and PPD-injected footpads, as described in reference 5.

Generation of Bone Marrow–derived Macrophages. Macrophages were derived from bone marrow by culture in medium supplemented with 20% L929 fibroblast culture supernatant for 7 d at 37°C. Cells were stimulated with LPS (10 μg/ml; Sigma-Aldrich) and IFN-γ (100 U/ml; Boehringer Mannheim) for 72 h. In infection studies, cells were prestimulated with IFN-γ (100 U/ml) or culture medium for 16 h and infected with *M. tuberculosis* H37Rv (multiplicity of infection 1:1), and the supernatants were harvested after 54 h.

Nitrite Measurements. Serum nitrite was obtained by reducing serum nitrate to nitrite with nitrate reductase, and the nitrite concentrations in culture supernatants levels were determined using the Greiss reagent (reference 19; 3% phosphoric acid, 1% *p*-aminobenzene-sulphonamide, 1% *n*-1-napthylethylenediamide; Sigma-Aldrich).

Measurement of Bioactive TNF and Analysis of TNF mRNA by Reverse Transcriptase PCR. Levels of biologically active TNF were measured using the WEHI 164 cytotoxicity assay (31). For analysis of TNF mRNA, total lung RNA was prepared from 3

wk postinfection–perfused lungs using RNAzol B (Tel-Test). cDNA was synthesized using SUPERSCRIPT II RNase H reverse transcriptase (RT; GIBCO BRL) and analyzed by RT-PCR using primers and methods as previously described (17).

Histological and Immunohistochemical Analysis. Lung and liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at 4 μm, and stained with hematoxylin and eosin. Coded slides were assessed blind on 37 criteria for differences in cellular infiltrate. A granuloma in the peripheral lung was defined as a collection of 10 or more macrophages and T lymphocytes.

Statistical Analysis. Differences between the mean number of CFUs, number of granulocytes, and levels of nitrite and TNF were analyzed by Student's *t* tests.

Results

LTα- and LTβ-deficient Chimeric Mice Generate Normal T Cell Responses after M. tuberculosis Infection. To confirm the immune competence of the chimeric mice, their ability to mount comparable mycobacterial Ag-specific T cell responses after aerosol *M. tuberculosis* infection was examined. Mediastinal lymph node cells from all chimeric groups produced significant and comparable amounts of IFN-γ after both PPD stimulation (Fig. 1 A) and mitogenic stimulation (data not shown). The frequency of Ag-specific IFN-γ–producing cells in both the lymph nodes and lungs of chimeric mice was also analyzed. All chimeric mice had comparable numbers of IFN-γ–producing cells both in the lungs (Fig. 1 B) and in the draining lymph nodes (data not shown). The ability of the chimeric mice to mount Ag-specific DTH reactions was measured at 4 wk after infection. Chimeric mice were challenged with 10 μg of PPD in one footpad and the vehicle control in the other. Chimeric mice from all groups mounted similar DTH responses to mycobacterial proteins (Fig. 1 C). Therefore, the deficiencies in either soluble or membrane forms of LT do not affect the potential of these chimeric mice to mount Ag-specific T cell responses after infection with *M. tuberculosis*.

LTα-deficient Chimeras Rapidly Succumb to Aerosol Tuberculosis. Chimeric mice and normal unmanipulated WT mice were infected with virulent *M. tuberculosis*, and their clinical condition and bacterial growth was monitored over time. LTα$^{-/-}$ chimeras appeared healthy to day 28 but then rapidly deteriorated, and all succumbed by day 38 (Fig. 2 A). By contrast, both WT and LTβ$^{-/-}$ chimeras controlled the same infectious dose and survived for >150 d. The number of mycobacteria recovered from the lungs of LTα$^{-/-}$ chimeras was comparable to that in WT chimeras for the first 3 wk of infection but was significantly increased from this time. At the time of death, there were 3log$_{10}$ more bacteria in the lungs of LTα$^{-/-}$ chimeras compared with WT chimeras (Fig. 2 B; $P < 0.0001$). In contrast, LTβ$^{-/-}$ chimeras had comparable bacterial loads in the lungs to WT chimeras over the course of the infection. There was also increased dissemination of organisms into the spleens (Fig. 2 C) and livers (Fig. 2 D) of LTα$^{-/-}$, but not LTβ$^{-/-}$, chimeras compared with WT chimeras. *M. tuberculosis*–infected control WT chimeric mice and normal

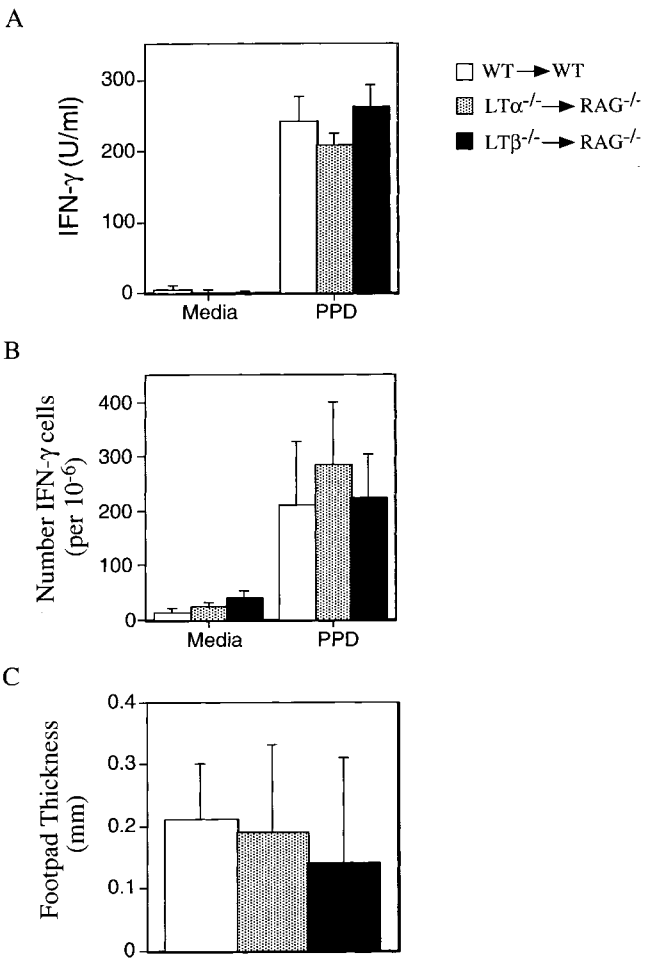

Figure 1. LTα$^{-/-}$ chimeric mice have the potential to mount normal T cell responses. (A) Mediastinal lymph node cells from chimeric mice, 3 wk after *M. tuberculosis* infection, were cultured in vitro in the presence of PPD or PBS for 72 h, and IFN-γ was measured in supernatants by ELISA. The data represent the means and SD of triplicate culture from four mice. (B) Lung cell homogenates from 3 wk infected chimeric mice were cultured overnight in the presence of PPD or PBS, and the frequencies of IFN-γ–producing cells were analyzed by ELISpot assay. The data represent means and SD of triplicate cultures from four mice. (C) The ability of the chimeric mice to mount DTH responses was examined. Chimeric mice were challenged in left hind footpad with 10 μg of PPD and the right hind footpad with PBS on day 28 after infection. The data represent the means and SD of the differences in footpad swelling between the PBS- and PPD-challenged footpad for each of four mice.

WT mice showed comparable rates of survival and bacterial loads (data not shown).

Recruitment of Leukocytes in Chimeric Mice. The recruitment of leukocytes to the sites of infection is a critical event in the host response to tuberculosis. Therefore, the numbers of different lymphocyte subpopulations in the lungs were assessed over the course of infection. Comparable numbers of CD4$^+$ (Table I) and CD8$^+$ (data not shown) T cells were recruited into the lungs of LTα$^{-/-}$, LTβ$^{-/-}$, and WT chimeric mice over the course of infection. Similar numbers of B lymphocytes were also observed (data not shown). To investigate the activation state of these T cells, the expression of CD44 and CD45RB was

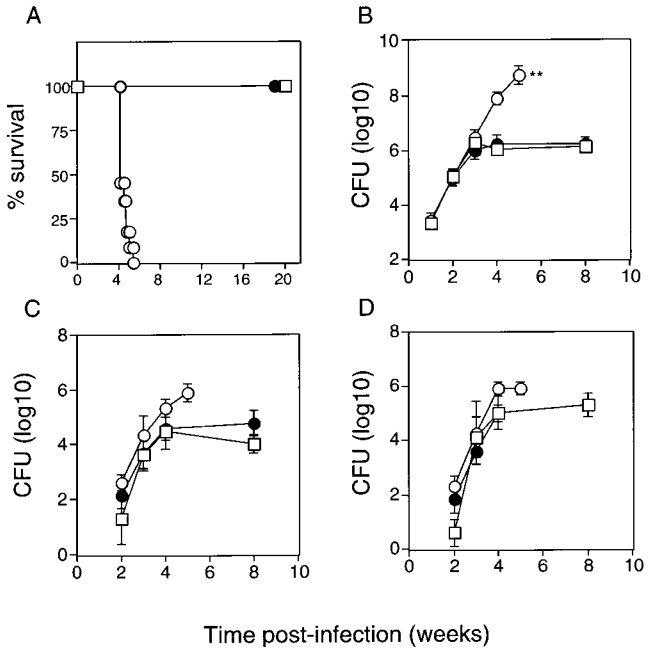

Figure 2. LTα$^{-/-}$ chimeric, unlike LTβ$^{-/-}$ chimeric mice, cannot control *M. tuberculosis* infection. (A) LTα$^{-/-}$ chimeric mice (n = 13; ○), LTβ$^{-/-}$ chimeric mice (n = 15; ●) and WT chimeric mice (n = 15; □) were exposed to a low dose aerosol of *M. tuberculosis* and survival monitored over time. Unmanipulated WT mice showed 100% survival (data not shown). The numbers of viable bacteria present in lungs (B), spleen (C), and liver (D) of WT chimeras (□), LTα$^{-/-}$ chimeras (○), and LTβ$^{-/-}$ chimeras (●) were determined over time. The data represent the means and SD of the mycobacterial CFUs from four or five mice per time point. These data are representative results from one of two similar experiments (**$P < 0.0001$).

examined on CD4$^+$ T cells. CD4$^+$ T cells from LTα$^{-/-}$, LTβ$^{-/-}$, and WT chimeric mice expressed similar levels of CD44^{high}CD45RB^{low} expression (data not shown). Interestingly, the numbers of granulocytes (Ly-6G$^+$ cells) recruited to the lungs of the chimeric mice differed. At 4 wk after infection, there were significantly more granulocytes ($P < 0.005$) in the lungs of LTα$^{-/-}$ chimeric mice compared with both WT and LTβ$^{-/-}$ chimeric mice (Table I).

LTα-deficient Chimeric Mice Produce Normal Amounts of Reactive Nitrogen Intermediates. The antimicrobial mechanisms of activated mouse macrophages include the production of reactive nitrogen intermediates (RNI). Therefore, nitrite levels in the sera of the chimeric mice were analyzed throughout the course of infection to determine if LTα$_3$ plays a critical role in regulating this antimicrobial mechanism. LTα$^{-/-}$, LTβ$^{-/-}$, and WT chimeric mice all had similar levels of serum nitrite (Fig. 3 A). To determine if macrophages deficient in LTα and LTβ have the potential to produce comparable amounts of RNI to WT macrophages, bone marrow–derived macrophages from the three types of mice were generated. These macrophages were stimulated with LPS and IFN-γ or were infected with *M. tuberculosis*, with and without pre-stimulation with IFN-γ (Fig. 3 B). Macrophages derived from LTα$^{-/-}$ and LTβ$^{-/-}$ bone marrow produced amounts of RNI comparable to those of WT macrophages.

TNF mRNA Expression and TNF Production by Macrophages Is Normal in LTα-deficient Mice. To exclude the possibility that the failure of LTα$^{-/-}$ chimeric mice to control infection was due to a reduced production of TNF, both the expression of mRNA for TNF during infection of chimeric mice and the production of TNF by LTα$^{-/-}$ and LTβ$^{-/-}$ macrophages were examined. Comparable levels of TNF mRNA was expressed in the lungs of LTα$^{-/-}$, LTβ$^{-/-}$, and WT chimeric mice during infection (Fig. 3 C). Macrophages derived from LTα$^{-/-}$, LTβ$^{-/-}$, and WT mice produced similar amounts of TNF after either stimulation with LPS and IFN-γ or infection with *M. tuberculosis* after prestimulation with IFN-γ (Fig. 3 D).

Granulomatous Response in LTα-deficient Chimeric Mice. The lungs of the different chimeric mice were examined histologically to determine whether the rate of development and pattern of cellular responses differed across the groups of chimeric mice. In WT chimeras and LTβ$^{-/-}$ chimeric mice, the granulomatous response resembled that seen in normal WT mice after aerosol tuberculosis, so that

Table I. *Recruitment of CD4$^+$ T Cells and Granulocytes (Ly6-G$^+$) to the Lungs of LTα$^{-/-}$, LTβ$^{-/-}$, and WT Chimeras after Infection with M. tuberculosis*

	No. CD4$^+$ cells (×10^3)*			No. granulocytes (×10^3)‡		
Time	WT→WT	LTα$^{-/-}$→RAG$^{-/-}$	LTβ$^{-/-}$→RAG$^{-/-}$	WT→WT	LTα$^{-/-}$→RAG$^{-/-}$	LTβ$^{-/-}$→RAG$^{-/-}$
wk						
1	24.4 (7.9)	46.2 (17)	36.9 (10)	118 (59)	182 (78)	83 (25)
2	252 (21)	364 (39)	402 (20)	262 (283)	410 (283)	450 (265)
3	444 (10)	487 (10)	678 (25)	361 (52)	484 (141)	488 (275)
4	414 (17)	538 (22)	474 (13)	371 (200)	1,814§ (377)	376 (172)

*Means ± SD of numbers of cells staining positive for antibody directed against CD4.
‡Means ± SD of numbers of cells staining positive for antibody directed against Ly6-G.
§Significant differences between WT and LTα$^{-/-}$ chimeras ($P < 0.005$).

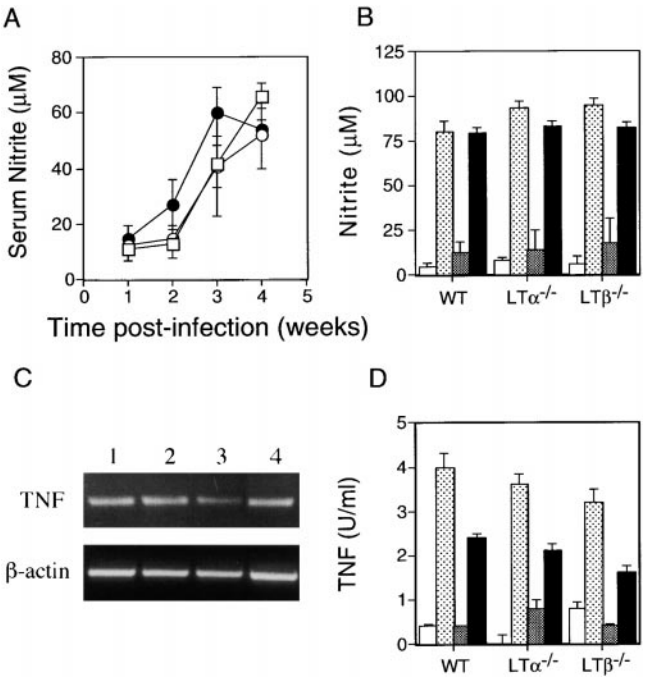

Figure 3. LTα and LTβ are not essential for the host production of RNI, and their deficiency does not affect the potential to produce TNF. (A) The nitrite concentrations in the sera of WT (□), LTα$^{-/-}$ (○), and LTβ$^{-/-}$ (●) chimeric mice were analyzed throughout the course of infection. Serum nitrate was reduced to nitrite and nitrite levels determined using the Greiss reagent. The data represents the means and SD of nitrite levels for four or five mice (*$P < 0.05$). (B) Bone marrow–derived macrophages were stimulated either with medium alone (open column) or LPS (10 μg/ml) and IFN-γ (100 U/ml) (stippled column), or infected with *M. tuberculosis* with (black column) and without (hatched column) prestimulation with IFN-γ (100 U/ml). Nitrite levels in culture supernatants were determined by the Greiss reagent. The data represents the means and SD for triplicate cultures. (C) TNF mRNA expression in the lungs of chimeric mice 3 wk after infection. RNA was extracted from lung homogenates, and cDNA was synthesized for RT-PCR as described in Materials and Methods. Lane 1, WT chimera; lane 2, LTα$^{-/-}$ chimera; lane 3, LTβ$^{-/-}$ chimera; lane 4, WT control. β-actin PCR product, 304 bases; TNF PCR product, 256 bases. (D) Bone marrow–derived macrophages were stimulated with either medium alone (open column) or LPS (10 μg/ml) and IFN-γ (100 U/ml) (stippled column) or infected with *M. tuberculosis* with (black column) and without (hatched column) prestimulation with IFN-γ (100 U/ml). TNF production was measured using the WEHI 164 cytotoxicity assay. The data represent the means and SD for triplicate cultures.

by 4 wk after infection the granulomas in these groups were 0.4–0.8 mm in diameter (Fig. 4, A and C). These granulomas consisted of intact macrophages, some lymphocytes, and only occasional neutrophils, with no necrosis (Fig. 4, D and F). The lesions that formed in the lungs of LTα$^{-/-}$ chimeric mice were markedly different. The peripheral lung lesions had twice the diameter (Fig. 4 B) and consisted of collections of neutrophils and fewer macrophages than WT chimeric mice (Fig. 4 E). Lymphocytes were absent from the lesions (Fig. 4 E), and a moderate amount of necrosis was evident. In contrast to WT and LTβ$^{-/-}$ chimeras, the lymphocytes in LTα$^{-/-}$ chimeras were restricted to perivascular and peribronchial regions (Fig. 4 E). This pattern was confirmed by immunohistochemical identification of the lymphocytes with anti-CD3 staining (data not shown).

Discussion

Although the genes for LTα_3 and TNF were identified at the same time (12), the functions of TNF have been more clearly defined than those for LTα_3. LTα_3 has been considered either as a mediator of inflammation or a functionally redundant form of TNF (20). Recently, with the advent of recombinant forms of LTα_3, in vitro studies have delineated proinflammatory properties for LTα_3 (21), although in vivo activity of LTα_3, independent of TNF, has not been defined. This study demonstrates, for the first time, an essential function for secreted LTα_3, but not membrane-bound LTβ, in the control of pulmonary tuberculosis. TNF production was not impaired in the LTα- and LTβ-deficient chimeric mice, with TNF mRNA being produced in the lungs of *M. tuberculosis*–infected LTα$^{-/-}$ and LTβ$^{-/-}$ chimeric mice (Fig. 3 C). Importantly, macrophages from LTα- and LTβ-deficient mice demonstrated normal TNF secretion in response to both nonspecific activation and infection with *M. tuberculosis* (Fig. 3 D). Moreover, LTβ-deficient chimeric mice, produced in a similar fashion to LTα$^{-/-}$ chimeras, controlled *M. tuberculosis* infection in the same manner as chimeras reconstituted with WT bone marrow (Fig. 2 A). Therefore, LTα_3 is essential to control this chronic bacterial infection and acts independently of TNF, possibly through an additional receptor or mechanism. LTα_3 has the potential to act at a number of steps in the host response to tuberculosis.

First, LTα_3 deficiency may cause T cell dysfunction. However, T cell activation and IFN-γ release was normal in all chimeric groups (Fig. 1). Second, LTα_3 may participate in the T cell–mediated activation of macrophage bactericidal mechanisms, such as the induction of inducible nitric oxide synthase (iNOS) and production of RNI. IFN-γ synergizes with TNF (22) or LTα_3 (23) to produce maximal activation of murine macrophages and the production of RNI, which have mycobactericidal activity both in vivo and in vitro (22). Both TNF and LTα_3 can induce in vitro the nuclear translocation of nuclear factor (NF)-κB, resulting in the induction of iNOS gene transcription (24, 25). During *M. tuberculosis* infection, WT and LTα$^{-/-}$ chimeric mice demonstrated comparable levels of serum nitrite (Fig. 3 A). Also, macrophages derived in vitro from LTα$^{-/-}$ and WT bone marrow produced similar levels of nitrite after nonspecific activation or infection with mycobacteria (Fig. 3 B). Therefore, reduced capacity for macrophage activation, as measured indirectly by increased nitric oxide production, does not explain the susceptibility of LTα$^{-/-}$ chimeric mice to tuberculosis.

Third, LTα_3 may contribute to the inflammatory component of the effector response to mycobacterial infection. A striking feature of the LTα$^{-/-}$ chimeric mice was the failure to develop a normal granulomatous response after mycobacterial infection. Containment of *M. tuberculosis* infection in the lungs requires the recruitment of CD4$^+$ and

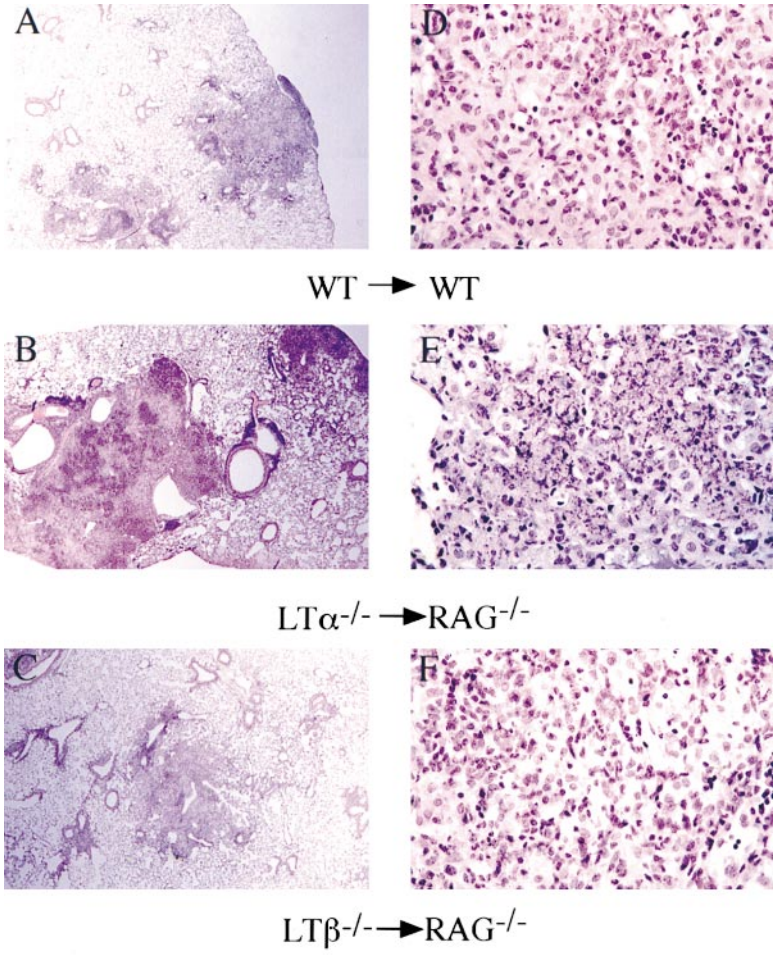

Figure 4. LTα$^{-/-}$ chimeric mice have defective granuloma formation. Lung tissue from chimeric mice 28 d after infection was fixed in buffered formalin, sectioned, and stained with hematoxylin and eosin. Panels A–C represent low power views of the lungs (magnification ×50), and panels D–F represent high power views of the lesions (×400). A and C show small, discrete granulomas in WT and LTβ$^{-/-}$ chimeric mice respectively, in contrast to the large necrotic lesions seen in the LTα$^{-/-}$ chimeric mice (B). D and F show the colocalization of macrophages and lymphocytes in the granulomas found in the WT and LTβ$^{-/-}$ chimeras, respectively. This is in contrast to the large numbers of neutrophils and necrotic debris seen in the lesions of the LTα$^{-/-}$ chimeric mice (E).

CD8^{+} T cells across the pulmonary endothelium, leukocyte migration through the infected tissues, and the colocalization of macrophages and lymphocytes to form granulomas. LTα$^{-/-}$ and WT chimeras displayed equivalent accumulation of T cells in the lungs (Table I), and these cells showed similar patterns of activation/memory marker expression (data not shown) and IFN-γ production (Fig. 1). In the LTα$^{-/-}$ chimeras, however, the lymphocytes accumulated in the perivascular and peribronchial regions of the lung and failed to migrate into the infected tissues. As a result, the well defined granulomas evident in WT chimeras were replaced by large accumulations of neutrophils and necrotic material. These changes in cell migration and granuloma formation in LTα$^{-/-}$ chimeric mice may relate to the recently described in vitro proinflammatory activities of LTα$_3$. Recombinant murine (rm)LTα$_3$ induced the expression of the cell adhesion molecules vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E-selectin, and mucosal addressin cell adhesion molecule on endothelial cells in vitro (26). Furthermore, rmLTα$_3$ induces the expression of T cell and monocyte attracting chemokines RANTES (regulated upon activation, normal T cell expressed and secreted), IFN-γ–inducible protein-10, and monocyte chemoattractant protein-1 (26). Therefore, LTα$_3$ has the potential to regulate the chemotactic signals that organize the cells into a mature granuloma, even though it is not a chemoattractant itself. As few studies into the chemokine-producing activity of LTα$_3$ have been undertaken, it is possible that LTα$_3$ may induce as yet unidentified chemokines and cytokines. Interestingly, LTα deficiency did not affect the acute cellular inflammation in the brain during EAE (18). This may represent differences in the intensity and chronicity of the inflammatory response, which are stronger and more prolonged after aerosol tuberculosis than that occurring during EAE, in addition to differences in the responding cell types and the organ involved in the inflammatory response.

Another facet of the dysregulated inflammatory response was the marked increase in granulocytes in the lungs of LTα$^{-/-}$ compared with LTβ$^{-/-}$ chimeric and WT chimeric control mice (Table I). This contrasts with the mild influx of neutrophils that may have a beneficial role early after *M. tuberculosis* infection in normal mice (27). In other genetically deficient mice with increased susceptibility to *M. tuberculosis*, such as TNF$^{-/-}$ and IFN-γ$^{-/-}$ mice, a similar large influx of neutrophils occurs (5, 28). The accumulation of activated neutrophils may contribute to the marked damage to the lungs during infection. Neutrophil migration and activation contributes to lung injury in acute respiratory distress syndrome patients (29). The 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 contributed to the death of these mice.

In contrast to the absolute requirement for $LT\alpha_3$ to control pulmonary tuberculosis, chimeric mice deficient in only membrane-bound LTβ were able to mount a normal granulomatous response and contained *M. tuberculosis* infection. A recent study in which the action of LTβ was blocked with a LTβR–Ig fusion protein during *M. bovis* (BCG) infection, found a modest, two- to threefold increase in bacterial numbers (30). These modest effects contrast with the profound susceptibility of $LT\alpha^{-/-}$ chimeras, which showed greater than 1,000-fold increases in bacterial loads (Fig. 3). Furthermore, any effects of LTβR–Ig therapy may be due to the neutralization of not only LTβ, but also the cytokine LIGHT, which also signals through LTβR (31).

$LT\alpha_3$ has been considered to mediate its activity through TNFRI and TNFRII. Nevertheless, TNF, which was expressed normally in the lungs of the $LT\alpha^{-/-}$ chimeric mice (Fig. 3 C), was unable to compensate for the lack of $LT\alpha_3$. Therefore, it is possible that $LT\alpha_3$ is signaling through an additional receptor. One possibility is the HVEM receptor, which has a wide tissue distribution being expressed predominantly on T cells, B cells, and monocytes (32). Signaling through HVEM not only induces activation of the transcription factors NF-κB and activator protein 1 (33) but also is thought to play a role in T cell activation (34). An alternate possibility is that $LT\alpha_3$ may be signaling through the same receptors as TNF, but the timing and site of production of $LT\alpha_3$ may be different to that for TNF. Although T cells, which are the major source of $LT\alpha_3$, are also able to produce TNF, the relative importance of T cell–derived TNF, as compared with macrophage-derived TNF, in the immune response to tuberculosis is unclear. In summary, this model of chimeric mice deficient in individual members of the TNF/LT family of cytokines has identified an independent function for $LT\alpha_3$ in the host control of aerosol tuberculosis and will permit the further dissection of the mode of action of $LT\alpha_3$.

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