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Lymphotoxin- α and TNF Have Essential but Independent Roles in the Evolution of the Granulomatous Response in Experimental Leprosy

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Recent studies identified an association between genetic variants in the lymphotoxin- α (LT α) gene and leprosy. To study the influence of LT α on the control of experimental leprosy, both low- and high-dose *Mycobacterium leprae* foot pad (FP) infections were evaluated in LT α -deficient chimeric (cLT $\alpha^{-/-}$) and control chimeric (cB6) mice. Cellular responses to low-dose infection in cLT $\alpha^{-/-}$ mice were dramatically different, with reduced accumulation of CD4⁺ and CD8⁺ lymphocytes and macrophages and failure to form granulomas. Growth of *M. leprae* was contained for 6 months, but augmented late in infection. In contrast, tumor necrosis factor knockout and tumor necrosis factor receptor 1 knockout FPs exhibited extensive inflammatory infiltration with an increase in *M. leprae* growth throughout infection. Following high-dose infection, cB6 FP induration peaked at 4 weeks and was maintained for 12 weeks. Induration was not sustained in cLT $\alpha^{-/-}$ FPs that contained few lymphocytes and no granulomas. There was a reduction in the expression levels of inflammatory cytokines, chemokines, and chemokine receptors, including nitric oxide synthase 2, vascular cell adhesion molecule, and intercellular cell adhesion molecule. Furthermore, cLT $\alpha^{-/-}$ popliteal lymph nodes contained a higher proportion of naïve CD44^{lo}CD62L^{hi} T cells than cB6 mice, suggestive of reduced T cell activation. Therefore, both LT α and tumor necrosis factor are essential for the regulation of the granuloma, but they have distinctive roles

in the recruitment of lymphocytes and maintenance of the granulomatous response during chronic *M. leprae* infection. (*Am J Pathol* 2009, 174:1379–1389; DOI: 10.2353/ajpath.2009.080550)

On infection with many intracellular organisms, especially mycobacterial pathogens, a granulomatous response ensues. This complex process involves the participation of cell-mediated immunity (CMI), activation of endothelial cells, enhancement of adhesion molecule expression, management of macrophage and lymphocyte infiltration, and induction of the microbiostatic and microbicidal effects of macrophages.¹ In addition, extensive intercellular communication, chiefly through cytokine and chemokine signals, is required to orchestrate this cellular accumulation and results in a three-dimensional structure that limits or prevents dissemination of the pathogen and is largely protective.

The contributions of both soluble and membrane-bound tumor necrosis factor (TNF) to granuloma development have been established.^{2–5} Lymphotoxin (LT)- α is also a member of the TNF superfamily, but as compared with TNF, much less is known about its function. LT α is required for the development of secondary lymphoid tissue⁶ through its association with LT β , to form heterotrimeric LT $\alpha_1\beta_2$ and LT $\alpha_2\beta_1$, which enable interaction between lymphocytes and surrounding fibroblasts, and epithelial and myeloid cells that express the LT β receptor. Current evidence also suggests that LT α plays a regulatory role in CMI⁷ and serves as an initiating stimu-

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lus in chronic inflammation⁸ through the activation of signaling cascades involving adhesion molecules, cytokines, and chemokines, for the recruitment and retention of lymphocytes. LT α_3 , the secreted form of lymphotoxin, is produced by activated lymphocytes, primarily CD4⁺ T cells, natural killer cells, and B cells, and binds with equal affinity as TNF to TNF receptor (TNFR) 1 and TNFR2. LT α_3 also binds to the herpes virus entry mediator.⁹ This receptor has a wide tissue distribution, being expressed predominantly on T cells, B cells, and monocytes. Signaling through herpes virus entry mediator induces activation of nuclear factor- κ B and activator protein 1 and may play a role in T cell activation.

LT α signaling has proven important in controlling infections with diverse intracellular pathogens, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Candida albicans*, *Toxoplasma gondii*, *Leishmania donovani*, *Plasmodium chabaudi*, and influenza virus.^{10–16} The role of LT α in host defense against leprosy is unknown; however, LT α has been detected in leprosy lesions, primarily at the tuberculoid end of the immunopathological spectrum and in Type 1 reactions,^{17,18} which supports its purported role as a proinflammatory cytokine. Importantly, a recent study found that a polymorphism, LTA + 80 within the promoter region of the gene encoding LT α , is an important risk factor for developing leprosy per se.¹⁹

In an effort to understand the mechanisms underlying this association between LT α and leprosy, we used low and high dose models to examine *M. leprae* infection in mice deficient in this cytokine. Peripheral lymph nodes (LNs) do not develop in mice with a targeted disruption in the LT α gene.^{6,20,21} Therefore, to ensure that such defects did not contribute to our results, chimeric knockout (KO) mice were generated by reconstitution of irradiated RAG mice with LT $\alpha^{-/-}$ bone marrow cells (cLT $\alpha^{-/-}$). cLT $\alpha^{-/-}$ mice have intact peripheral lymphoid organs and are fully reconstituted with immune cells, but are deficient in hematopoietically derived soluble and membrane-bound forms of LT α .^{10,11,13,14,22} Irradiated RAG mice reconstituted with C57Bl/6 (B6) bone marrow cells served as chimeric controls (cB6). Our studies examined the long term effects of LT α deficiency into the chronic phase of *M. leprae* infection and its effect on the granulomatous response. In contrast to TNF KO (TNF $^{-/-}$) and TNFR1 KO (TNFR1 $^{-/-}$) mice, which showed extensive lymphocytic infiltration and augmented growth of *M. leprae* throughout the infection period, LT α -deficient mice were unable to recruit lymphocytes into the infected foot pad (FP), develop and maintain granulomas, or optimally control the growth of *M. leprae* in the chronic stage; thus, we show an aberrant phenotype in an animal model that has a defect in a gene that was found by whole genome screening to be significantly associated with the development of leprosy in humans.

Materials and Methods

Mice

Chimeric LT $\alpha^{-/-}$ (cLT $\alpha^{-/-}$) mice were generated by reconstitution of irradiated (5.5 Gy) C57Bl/6.RAG-1 $^{-/-}$

Table 1. Cellular Composition of the *M. leprae*-Infected FP

Marker	cB6	B6
CD3 ⁺	46.18 $\pm$ 3.43	51.85 $\pm$ 1.97
CD3 ⁺ CD4 ⁺	70.45 $\pm$ 0.60	58.55 $\pm$ 1.41
CD4 ⁺ CD44 ^{lo} CD62L ^{hi}	3.36 $\pm$ 1.09	5.77 $\pm$ 2.18
CD4 ⁺ CD44 ^{hi} CD62L ^{lo}	87.77 $\pm$ 1.62	77.17 $\pm$ 2.47
CD3 ⁺ CD8 ⁺	18.24 $\pm$ 1.91	20.33 $\pm$ 1.95
CD8 ⁺ CD44 ^{lo} CD62L ^{hi}	6.27 $\pm$ 2.73	9.18 $\pm$ 1.20
CD8 ⁺ CD44 ^{hi} CD62L ^{lo}	82.76 $\pm$ 1.93	71.84 $\pm$ 3.18
B220 ⁺	0.87 $\pm$ 0.26	1.80 $\pm$ 0.19
NK1.1 ⁺	2.21 $\pm$ 0.36	4.15 $\pm$ 0.71
CD11b ⁺ Ly6G-	76.63 $\pm$ 2.74	75.42 $\pm$ 2.45
CD11b ⁺ IA ^{b+}	76.73 $\pm$ 4.37	80.70 $\pm$ 1.71
Ly6G ⁺	5.42 $\pm$ 1.69	4.99 $\pm$ 0.60
CD11c ⁺	1.45 $\pm$ 0.27	1.73 $\pm$ 0.13

FP from individual B6 and cB6 mice were harvested at 12 weeks post infection and digested to yield single cell suspensions. Values are percentages of cells in the CD4, CD8, lymphocyte, or myeloid gate. Gates were determined based on forward- and side-scatter parameters.

mice with 1×10^7 LT $\alpha^{-/-}$ bone marrow cells. Irradiated RAG mice reconstituted with C57Bl/6 (B6) bone marrow (cB6) served as controls. Cellular reconstitution was verified by analysis of peripheral blood by flow cytometry (data not shown) as described previously¹³ and by comparison of immune responses of cB6 mice with C57Bl/6J (B6) mice (Table 1). TNF knockout (TNF $^{-/-}$) mice were generated as previously described.² TNFR1 $^{-/-}$ and B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Experiments were approved by the University of Sydney Animal Care and Ethics Committee and performed in compliance with all applicable state and federal laws and regulations in accordance with USPHS Policy on Humane Care and Use of Laboratory Animals. The National Hansen's Disease Program's Assurance of Compliance (#A3032-01) is registered with the Office of Laboratory Animal Welfare.

Maintenance of a Viable *M. leprae* Inoculum

M. leprae strain Thai-53 is maintained in continuous programmed passage in athymic *nu/nu* mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN). *M. leprae* viability was determined by radiorespirometry²³ and vital staining.²⁴ The bacilli were stored at 4°C and used within 24 hours.

Infection of Mice with *M. leprae*

Low dose (Shepard) growth model: Mice were infected with 6×10^3 *M. leprae* in 0.03 ml PBS in each hind FP. At 3, 6, and 9 months, and 12 months (TNFR1 $^{-/-}$ only), post-infection, FPs were harvested for enumeration of acid fast bacilli (AFB), and histopathological and immunohistochemical analyses; popliteal LN cells were analyzed by flow cytometry.

High dose infection model: Each hind FP was inoculated with 3×10^7 *M. leprae* in 0.03 ml PBS,²⁵ and induration was measured weekly using a Vernier caliper. At 3 months post-infection, FP tissues were harvested for histopathological and immunohistochemical analyses,

flow cytometry, and RNA expression; popliteal LN cells were evaluated by flow cytometry.

Enumeration of AFB

AFB were enumerated in individual FPs. Briefly, each hind FP was homogenized, three smears were prepared, and 20 microscopic fields per smear were counted to determine the yield for that individual mouse. The threshold of counting ability in this system is 4.8×10^3 AFB per FP, equivalent to 1 AFB in 60 microscopic fields.

Histopathology

The feet from *M. leprae*-infected mice were fixed in 10% buffered formalin, decalcified in a solution of 10% (w/v) sodium citrate and 22.5% (v/v) formic acid, and embedded in paraffin. Four- μ m sections were prepared of cross-sections at the distal, mid, and proximal area of the metatarsals of the infected foot and stained with H&E or Fite-Faraco acid fast stain. The histological feature of a granuloma was defined as an aggregation of macrophages with *M. leprae* and lymphocytes. In the sections the lymphocytic infiltrates, bacterial index of granuloma, and granuloma fraction were assessed as previously described.^{26,27} Sections were viewed and captured on a Zeiss Axioplan microscope (Zeiss, Germany) equipped with a MicroFire camera and PictureFrame software.²⁵

Immunohistochemistry

FP tissue was embedded in optimal cutting temperature medium (Tissue Tek, Inc., Torrance, CA), snap frozen in liquid nitrogen, and stored at -70°C . Frozen tissue was cut (Frigocut 2800E, Leica Microsystem, Inc., Bannockburn, IL) into 4- μ m sections and fixed in cold acetone. After blocking with avidin and biotin (Vector Laboratories, Burlingame, CA), rat anti-mouse CD4 (PharMingen, San Diego, CA) or rat anti-mouse CD8 (PharMingen) was applied to serial sections. Biotin-labeled mouse anti-rat F(ab)₂ fragments (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as the secondary antibody. Immunohistochemical staining was performed with the Vectastain Elite ABC kit (Vector), the AEC Substrate kit (Vector), and a hematoxylin counterstain.

Flow Cytometry

LN cells were prepared by passing LNs from individual mice through a tissue screen. FP tissues were digested with collagenase/DNase to produce single cell suspensions.²⁵ Cells were treated with Fc Block (CD16/CD32 [Fc γ III/II receptor]) (BD Biosciences, San Jose, CA) and stained with appropriate isotype control antibodies or anti-CD3 (clone 17A2), anti-CD4 (clone RM4-5), anti-CD8a (clone 53-6.7), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-CD244.2 (clone 2B4), anti-CD11b (clone M1/70), anti-I-A^b (clone 25-9-17), and anti-Ly-6G (clone 1A8), all from BD Biosciences. Analyses were

performed using a FACS Aria interfaced with a Hewlett Packard 4100 running FACS Diva software (BD Biosciences).

Isolation of total RNA and Real-Time Reverse Transcription PCR

RNA was extracted from FP tissue of individual mice using an RNagents kit (Promega Corp., Madison, WI). RNA was reconstituted in RNase-free distilled water, treated with DNase (DNA-free kit, Ambion, Austin, TX), and RNA concentrations were determined by reading the optical density at 260 nm in a SynergyHT Multidetector Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). cDNA was generated from 0.5 μ g RNA using random hexamers and an reverse transcription (RT) for PCR kit (Clontech, Palo Alto, CA) at 42°C for 1 hour in a thermocycler (9600, Perkin-Elmer Corp., Norwalk, CT). Controls for DNA contamination were prepared from RNA samples using the reverse transcription reagents minus the reverse transcriptase. All sample and control preparations were aliquoted and stored at -70°C . Real-time RT-PCR for cytokine and chemokine transcripts were performed on these cDNA via Taqman technology using specific primer sets and probes (Applied BioSystems, Foster City, CA) and Universal Master Mix (Applied BioSystems) in an ABI PRISM 7700 Sequence Detection System (Applied BioSystems). Semiquantitative analyses of the data were accomplished using the standard curve comparative method. Standard curves were generated using tenfold dilutions of cDNA from Concanavalin A-stimulated mouse spleen cells or interferon (IFN) γ and lipopolysaccharide-stimulated mouse macrophage. Data were normalized for template variation by dividing each gene transcript value by that of the glyceraldehyde-3-phosphate dehydrogenase RNA RT-PCR value for the same template.²⁵

Statistical Analyses

Growth of *M. leprae* was analyzed using the non-parametric Mann-Whitney test. Differences in induration were determined using pairwise *t*-tests of least square means. All other data were analyzed using unpaired *t*-tests with or without Welch's correction. Data were considered significant at $P < 0.05$.²⁵

Results

LT α -Deficient Chimeras Show Impaired Restriction of M. leprae Growth during Chronic Infection, Whereas TNF $^{-/-}$ and TNFR1 $^{-/-}$ FPs Exhibit Enhanced Bacillary Growth throughout Infection

Mice were infected with 6×10^3 viable *M. leprae* per FP and harvested at 3, 6, and 9 months, and 12 months (TNFR1 $^{-/-}$ only), post-infection. As shown in Figure 1A,

Figure 1. LT α -deficient chimeras show impaired restriction of *M. leprae* growth during chronic infection, whereas TNF $^{-/-}$ and TNFR1 $^{-/-}$ FPs exhibit enhanced bacillary growth throughout infection. cB6 (circles) and cLT $\alpha^{-/-}$ (inverted triangles) mice (A), B6 (squares) and TNF $^{-/-}$ (diamonds) mice (B), and B6 (squares) and TNFR1 $^{-/-}$ (triangles) mice (C), were infected in each hind FP with 6×10^3 viable *M. leprae* (low dose growth model). At 3, 6, and 9 months, and 12 months (C only), the number of AFB per FP was determined. Each symbol represents one mouse. The threshold of counting ability is 4.8×10^3 AFB per FP. Experiments shown are representative of two independent experiments each. ** $P < 0.01$; *** $P < 0.001$.

multiplication of *M. leprae* in cB6 mice reached approximately 10^5 bacilli by 3 months and remained at this level through 9 months. There was no difference between cB6 and cLT $\alpha^{-/-}$ mice in the number of AFB recovered at 3 and 6 months; however, *M. leprae* counts were significantly higher in the cLT $\alpha^{-/-}$ FPs at 9 months ($P = 0.0006$). Hence, LT α is not necessary for the restriction of acute *M. leprae* multiplication; however, the influence of LT α deficiency on bacterial growth is apparent as infection progresses into the chronic stage.

In contrast, growth of *M. leprae* was enhanced throughout infection in TNF $^{-/-}$ and TNFR1 $^{-/-}$ FPs. As shown in Figure 1, B and C, multiplication of *M. leprae* in the FPs of B6 mice reached on the order of 10^4 to 10^5 bacilli per FP at 3 months, peaked at 10^5 AFB by 6 months and remained at this level at 9 to 12 months. Numbers of *M. leprae* recovered from the FP of TNF $^{-/-}$ mice (Figure 1B) were $\sim$ tenfold higher at 3 months ($P = 0.0022$), 6 months ($P = 0.0022$), and 9 months ($P = 0.0013$). Likewise, AFB were markedly elevated in the TNFR1 $^{-/-}$ mice (Figure 1C) at all time points as compared with B6 mice (3 months, $P = 0.0087$; 6 months, $P = 0.0022$; 9 months, $P = 0.0043$; 12 months, $P = 0.0022$). Thus, TNF signaling is required for optimal restriction of *M. leprae* growth during both the acute and chronic stages of infection.

M. leprae-Infected cLT $\alpha^{-/-}$ FPs Exhibit a Reduced Granulomatous Response throughout Infection

The cellular infiltrates that developed in the cB6 mice on infection with *M. leprae* displayed characteristics similar to those seen in B6 FPs previously reported.^{25,28,29} cB6 FPs exhibited scattered macrophage infiltration with a nil to mild lymphocyte response at 3 months (Figure 2A), which by 9 months (Figure 2, A and B) developed into a moderate to dense lymphocyte infiltration among plump histiocytes and macrophages (Figure 2C) containing bacilli. In contrast, cLT $\alpha^{-/-}$ mice exhibited very poor cellular infiltration throughout infection (Figure 2A). At 3 months, the FPs showed scattered histiocytes and macrophages in the superficial dermis; many of the macro-

phages were spindle shaped. Lymphocytic infiltration was absent. By 6 months, FPs showed small perivascular and perifascicular clusters of macrophages with histiocytes in the superficial dermis, and in some areas the cells were disposed in streaks. Strikingly, in cLT $\alpha^{-/-}$ mice the lymphocyte response remained poor. This overall picture continued at 9 months (Figure 2, A and D), where the FPs exhibited spindled macrophages (Figure 2E) containing clumps of AFB (Figure 2E inset) and few lymphocytes. The poor lymphocytic response in cLT $\alpha^{-/-}$ FPs was supported by immunohistochemical staining. As shown in Figure 2F, cB6 FP infiltrates contained primarily CD4 $^{+}$ T cells with a lower influx of CD8 $^{+}$ T cells (Figure 2G). In stark contrast, there were very few T cells, either CD4 $^{+}$ (Figure 2H) or CD8 $^{+}$ (Figure 2I), present in the cLT $\alpha^{-/-}$ FPs. These data suggest that hematopoietically derived LT $\alpha^{-/-}$ is crucial for cellular recruitment into the *M. leprae*-induced granuloma.

M. leprae-Infected TNF $^{-/-}$ and TNFR1 $^{-/-}$ FPs Present an Intense and Diffuse Granulomatous Response

In B6 mice, scattered histiocytes and macrophages were present (Figure 3A), the lymphocytic response was nil to mild, and the inflammatory cells were predominantly in the superficial dermis at 3 months. By 6 months, focal accumulation of foamy macrophages, histiocytes, and few lymphocytes were observed, and this continued at 9 months (Figure 3, A–C). The cellular infiltrates were perifascicular and the nerves were not inflamed. Immunohistochemical staining showed the lymphocytes to be primarily CD4 $^{+}$ T cells (Figure 3H), with a lower level of infiltration of CD8 $^{+}$ T cells (Figure 3I).

TNF $^{-/-}$ and TNFR1 $^{-/-}$ mice, in contrast, exhibited a more rapid granulomatous response (Figure 3A). Early in infection, FPs contained large, unorganized cellular accumulations composed of histiocytes and macrophages scattered with lymphocytes around blood vessels and nerves and infiltrating the muscle bundles. The cellular infiltrates occupied up to 70% of the dermis. As the infection progressed, the granulomatous response con-

Figure 2. *M. leprae*-infected cLT $\alpha^{-/-}$ FP exhibit a reduced granulomatous response throughout infection. cB6 and cLT $\alpha^{-/-}$ mice were infected in each hind FP with 6×10^3 viable *M. leprae*. **A:** FP granulomas were scored for macrophage morphology (MΦ), lymphocyte infiltration (Lymph), granuloma fraction (GF), and bacilli in the granulomas (BIG). **B–E:** Histopathological (H&E) and **(F–I)** immunohistochemical staining in FP at 9 months post-infection. In cB6 mice, a mild chronic inflammatory infiltrate (arrows, **B** and **C**) was observed in the subcutis; in cLT $\alpha^{-/-}$ mice, the cellular infiltrate was greatly reduced (**D**), and spindle shaped MΦ (arrows, **E**) contained clumps of AFB (**E inset**, Fite-Faraco acid fast stain). In cB6 mice, the T cell infiltrate consisted primarily of CD4 $^{+}$ cells (arrow, **F**) with a lower influx of CD8 $^{+}$ cells (arrow, **G**). cLT $\alpha^{-/-}$ FP contained few CD4 $^{+}$ (hatched arrow, **H**) or CD8 $^{+}$ (hatched arrow, **I**) cells. For **B, D, F, H, I**, scale bars = 50 μ m; **C** and **E**, scale bars = 20 μ m; and **E inset**, scale bar = 10 μ m. Experiment shown is representative of two independent experiments.

sisted of diffuse and dense infiltration by lymphocytes (Figure 3A, D, and F) extending to the dorsal side of the foot. In areas there was well-defined granuloma formation with focal aggregation of histiocytic cells, small multinucleate giant cells and lymphocytes (Figure 3, E and G). The cellular infiltrate cuffed the nerves but there was no intraneural lymphocytic inflammation (Figure 3F). The lymphocytic response was composed of both CD4 $^{+}$ (Figure 3, J and L) and CD8 $^{+}$ (Figure 3, K and M) T cells, and occupied up to 80% of the dermis. These data indicate

Figure 3. *M. leprae*-infected TNF $^{-/-}$ and TNFR1 $^{-/-}$ FP present a diffuse granulomatous response. B6, TNF $^{-/-}$ and TNFR1 $^{-/-}$ mice were infected in each hind FP with 6×10^3 viable *M. leprae*. **A:** Differential granulomatous response was determined by scoring as in Figure 2. **B–G:** Histopathological (H&E) and **(H–M)** immunohistochemical staining in FP. B6 FP were comprised of histiocytes, MΦ, and a mild lymphocytic response (**B** and **C**, arrows in **C**); in TNF $^{-/-}$ and TNFR1 $^{-/-}$ mice, there was a diffuse and dense infiltration (**D** and **F**) with giant cell formation (arrows, **E** and **G**). In all three mouse strains, the T cell infiltrate consisted primarily of CD4 $^{+}$ (**H, J**, and **L**) cells with a lower influx of CD8 $^{+}$ (arrows, **I** and **K**) cells. For **(B, D, F, H–M)** scale bars = 50 μ m; **(C)** scale bar = 20 μ m; and **(E and G)** scale bars = 10 μ m. Experiment shown is representative of two independent experiments.

that TNF is essential for organizing and restraining the granulomatous response to *M. leprae* infection, and that TNF, rather than LT α , is the primary signal for the TNFR1 receptor in this model.

LT α -Deficient Chimeras Exhibit Impaired FP Induration and a Reduced Granulomatous Response Following High Dose Infection

To further analyze the mechanisms involved in the aberrant granulomatous response in cLT $\alpha^{-/-}$ FP, the effects of LT α deficiency were evaluated using a high dose infection model.²⁵ On inoculation of *M. leprae* into cB6 mice, the FP induration increased gradually over the first 5 weeks, peaked and remained relatively constant up to 12 weeks post-infection (Figure 4). This pattern of induration is similar to that seen in B6 mice²⁵ and LT α transcripts could be detected in these FPs (see supplemental Figure S1 at <http://ajp.amjpathol.org>). In contrast, an initial delay in FP induration was observed in cLT $\alpha^{-/-}$ mice, which increased to match that of cB6 at 3 to 4 weeks.

Figure 4. LT α -deficient chimeras exhibit impaired FP induration. cB6 (circles) and cLT α ^{-/-} (inverted triangles) FP were inoculated with 3×10^7 *M. leprae* and FP induration was measured using a Vernier caliper. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

Thereafter FP induration steadily waned in cLT α ^{-/-} mice and was significantly less than that in cB6 throughout the remainder of the infection period.

Histopathologically, high dose cB6 FPs, as in the low dose model, contained macrophages and a moderate lymphocyte infiltration around blood vessels, nerves, and muscle bundles (Figure 5, A and B). The lymphocytes

A

	cB6	cLT α ^{-/-}
MΦ	MΦ	MΦ
Lymph	2+	0-1+
GF	40-50%	10-20%
BIG	3-4+	5+

Figure 5. cLT α ^{-/-} mice exhibit a reduced granulomatous response following high dose infection. **A:** Differential granulomatous response was determined by scoring as in Figure 2. **B–C:** Histopathological (H&E) and **(D–G)** immunohistochemical staining in FP at 12 weeks post-inoculation. In cB6 mice, a moderate lymphocyte infiltration was observed (**B**); in cLT α ^{-/-} mice, there were few scattered lymphocytes and small collections of MΦ (**C**). In cB6 mice, the T cell infiltrate consisted primarily of CD4⁺ cells (**D**) with a lower influx of CD8⁺ cells (**E**). cLT α ^{-/-} FP contained few CD4⁺ (arrow, **F**) or CD8⁺ (arrow, **G**) cells. For (**B** and **C**) scale bars = 50 μ m; and (**D–G**) scale bars = 30 μ m. Experiment shown is representative of two independent experiments.

were primarily CD4⁺ T cells (Figure 5D) with a lower influx of CD8⁺ cells (Figure 5E). In contrast, LT α ^{-/-} FPs exhibited small collections of macrophages (Figure 5, A and C) with only a few scattered lymphocytes (Figure 5, F and G). There was no intraneural inflammation in either strain. Thus, the lack of cellular infiltration was not dependent on dose of *M. leprae* as the granulomatous response failed to develop in cLT α ^{-/-} mice even with a high level of infection.

Immune Cell Composition of the *M. leprae*-Infected FPs

We next examined the cellular composition of the *M. leprae*-infected FPs. Flow cytometric analyses of cB6 FP cells harvested at 12 weeks revealed a cellular makeup similar to that of B6 mice, further demonstrating that these chimeras were fully reconstituted and generated a comparable granulomatous response to *M. leprae* infection (Table 1, ²⁵). cB6 FP cellular infiltrates were composed primarily of a myeloid population, with a high forward/side scatter (9894.7 ± 384.0 gated events per 15,000 total events), and a lymphocyte population (1363.3 ± 169.1 gated event per 15,000 total events). In the myeloid gate, CD11b⁺ macrophages accounted for $76.63 \pm 2.74\%$ of the cells, and 76.63 ± 2.74 of these macrophages expressed I-A^b. Few neutrophils were present ($5.42 \pm 1.69\%$). The lymphocyte gate was comprised primarily of CD3⁺ T cells ($46.18 \pm 3.43\%$) with few natural killer cells ($2.21 \pm 0.36\%$) and fewer B cells ($0.87 \pm 0.26\%$). Of the CD3⁺ cells present, the majority were CD4⁺ ($70.45 \pm 1.05\%$) compared with CD8⁺ ($18.24 \pm 3.31\%$), confirming the results obtained by the immunohistochemical staining (Figures 2, 3, and 5). In addition, CD4⁺ and CD8⁺ cells were examined for T cell activation markers. Although the repertoire of T cell activation markers is rapidly expanding,³⁰ the relative levels of CD44 and CD62L are frequently used to distinguish naïve (CD44^{lo}CD62L^{hi}) from effector (CD44^{hi}CD62L^{lo}) T cells.^{31–37} In the *M. leprae*-infected FPs, the majority of CD4⁺ and CD8⁺ cells were of the effector phenotype, expressing CD44^{hi}CD62L^{lo} ($87.77 \pm 1.62\%$ and 82.76 ± 1.93 , respectively). We also attempted to evaluate the cellular composition of the cLT α ^{-/-} FPs by flow cytometry; however, we were unsuccessful as these preparations lacked sufficient numbers of leukocytes for a reliable assessment.

cLT α ^{-/-} Mice Exhibit Altered Lymphocyte Profiles in the Draining Popliteal LNs Following *M. leprae* Infection in the FPs

Since we were unable to evaluate the cLT α ^{-/-} FP populations, we examined the T cell profiles in the popliteal LN, which drains the FP, using our two infection models. The number and composition of lymphocytes in the LNs of cB6 mice was comparable to that seen in B6 LNs (unpublished data), again indicating that the chimeric mice were fully restored. In the low dose model (Figure

Figure 6. cLT $\alpha^{-/-}$ mice exhibit altered lymphocyte profiles in the draining popliteal LN following *M. leprae* infection in the FP. cB6 (circles) and cLT $\alpha^{-/-}$ (inverted triangles) FP were inoculated with 6×10^3 viable *M. leprae*. At 3, 6, and 9 months post-infection, popliteal LN from individual mice were harvested and single cell suspensions were prepared. For analysis by flow cytometry, the lymphocyte gate was determined based on forward- and side-scatter parameters. Cell numbers obtained from cB6 ($7.18 \times 10^6 \pm 4.82 \times 10^6$, $1.38 \times 10^7 \pm 1.61 \times 10^7$, and $4.66 \times 10^6 \pm 2.34 \times 10^6$, at 3, 6, and 9 months, respectively) and cLT $\alpha^{-/-}$ ($2.36 \times 10^6 \pm 1.66 \times 10^6$, $7.17 \times 10^5 \pm 3.76 \times 10^5$, and $3.77 \times 10^6 \pm 4.15 \times 10^6$, at 3, 6, and 9 months, respectively) mice were similar, except at 6 months post-infection when fewer cells were recovered from the deficient mice ($P = 0.0152$). The percentage of (A) CD3+ and B220+ cells within the lymphocyte gate, (B) CD3+ cells that were CD4+ or CD8+, and (C) CD4+ and CD8+ cells expressing CD44^{lo}CD62L^{hi} (Naïve [Nv]; closed symbols) and CD44^{hi}CD62L^{lo} (Effector; open symbols) were determined. Each graph depicts group means $\pm$ SE of 4 to 5 mice per group and is representative of two independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. Below each graph, scatter plots demonstrate flow cytometry data and percentages of an individual representative mouse from each group.

6), similar numbers of cells were also obtained from the LNs in both cB6 ($7.18 \times 10^6 \pm 4.82 \times 10^6$, $1.38 \times 10^7 \pm 1.61 \times 10^7$, and $4.66 \times 10^6 \pm 2.34 \times 10^6$, at 3, 6, and 9 months, respectively) and cLT $\alpha^{-/-}$ ($2.36 \times 10^6 \pm 1.66 \times 10^6$, $7.17 \times 10^5 \pm 3.76 \times 10^5$, and $3.77 \times 10^6 \pm 4.15 \times 10^6$, at 3, 6, and 9 months, respectively) mice, except at 6 months post-infection when fewer cells were recovered from the deficient mice ($P = 0.0152$). LNs from infected cB6 mice exhibited a roughly 40% T cell to 60% B cell ratio at 3 months, which decreased as infection progressed (Figure 6A). On infection of cLT $\alpha^{-/-}$ FPs, however, the T cell to B cell ratio in the LNs was essentially reversed with cLT $\alpha^{-/-}$ LNs containing significantly higher percentages ($\sim 60\%$) of CD3+ cells ($P = 0.0001$, $P = 0.0006$, $P = 0.0006$ at 3, 6, and 9 months, respectively) and lower percentages ($\sim 40\%$) of B220+ cells ($P = 0.0003$, $P = 0.0004$, $P = 0.0024$ at 3, 6, and 9 months, respectively). In addition, the CD4:CD8 ratio was altered in cLT $\alpha^{-/-}$ LNs. At 3 and 6 months in cLT $\alpha^{-/-}$ LNs, there was a higher CD4:CD8 T cell ratio as compared with cB6 LNs (Figure 6B). CD4+ and CD8+ cells were also examined for T cell activation markers. The ratio of naïve to effector CD4+ cells was high in both strains of mice early in infection at 3 months. In the cB6 LNs, this ratio inverted over the course of infection, and

by 9 months, the majority of CD4+ cells were of the effector phenotype. In contrast, in the absence of LT α , few effector CD4+ cells developed and the majority retained the naïve phenotype. Within the CD8+ population, although the reduction in the naïve phenotype was slower, the expression of the activation markers progressed in cLT $\alpha^{-/-}$ LNs as in control mice (Figure 6C). These data indicate an aberrant evolution of CD4+ lymphocytes in the cLT $\alpha^{-/-}$ mice in response to *M. leprae* infection.

We likewise compared the popliteal LN populations in the two strains of mice in the high dose infection model. The LNs from the *M. leprae*-infected cLT $\alpha^{-/-}$ mice were smaller than those from the cB6 mice and contained fewer total cells (cB6: $4.25 \times 10^7 \pm 2.32 \times 10^7$ cells; cLT $\alpha^{-/-}$: $1.50 \times 10^6 \pm 1.27 \times 10^6$ cells, $P = 0.004$). As shown in Figure 7A, the cB6 LNs were comprised primarily of B220+ cells ($68.79 \pm 9.39\%$) with a smaller population of CD3+ cells ($23.62 \pm 8.36\%$) present. In contrast, the popliteal LNs of cLT $\alpha^{-/-}$ mice contained a markedly higher percentage of CD3+ cells (62.25 ± 13.60 , $P = 0.0022$) with a corresponding reduction in the percentage of B220+ cells (25.05 ± 10.16 , $P < 0.0001$). While there was little difference in the ratio of CD4+ cells (43.49 ± 6.57 in cB6 and 52.10 ± 4.47 in cLT $\alpha^{-/-}$) to

Figure 7. Lymphocyte composition of the popliteal LN of cB6 and cLTα^{-/-} mice in the high dose infection model. cB6 (closed bars) and cLTα^{-/-} (hatched bars) FP were inoculated with 3×10^7 *M. leprae*. Popliteal LNs from individual mice were harvested and single cell suspensions were analyzed by flow cytometry. The lymphocyte gate was determined based on forward- and side-scatter parameters. The LNs from the *M. leprae*-infected cLTα^{-/-} mice were smaller than those from cB6 mice and contained fewer total cells (cB6: $4.25 \times 10^7 \pm 2.32 \times 10^7$ cells; cLTα^{-/-}: $1.50 \times 10^6 \pm 1.27 \times 10^6$ cells, $P = 0.004$). The percentages of (A) CD3⁺ and B220⁺ cells within the lymphocyte gate, (B) CD3⁺ cells that were CD4⁺ or CD8⁺, and (C) CD4⁺ and CD8⁺ cells expressing CD44 and CD62L were determined at 12 weeks post-infection. Experiment shown is representative of two independent experiments. Bars depict group means $\pm$ SE of four mice per group. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. Below each graph, corresponding scatter plots demonstrate flow cytometry data and percentages of an individual representative mouse from each group.

CD8⁺ cells (50.69 ± 6.86 in cB6 and 43.18 ± 3.84 in cLTα^{-/-}) between the two strains (Figure 7B), the percentages of both naïve CD4⁺CD44^{lo}CD62L^{hi} cells and naïve CD8⁺CD44^{lo}CD62L^{hi} cells were higher in the cLTα^{-/-} LNs (Figure 7C). Collectively, these data support a crucial role for LTα in T cell evolution and lymphocyte trafficking, not only within the local microenvironment of *M. leprae* infection in the FP but also in the regional lymphoid tissue, and demonstrate that its influence is sustained throughout long term infection.

Inability to Maintain the Granulomatous Response to *M. leprae* in cLTα^{-/-} Mouse FP Is Associated with Reduced Cytokine, Chemokine, and CAM Expression

Expression of various cytokine and chemokine transcripts in the FP was examined by real time RT-PCR. Like B6 mice,^{25,29} cB6 mice expressed Th1 cytokines, including interleukin (IL)-12/23p40, IL-12p35, IFNγ, IL-2, and TNF, as well as the activated macrophage product, NOS2 (Figure 8A). In contrast, there was a 6- to 33-fold lower level of expression of all tested cytokine transcripts in the cLTα^{-/-} FPs, with the exception of IL-12p35, which was expressed at a level similar to cB6 mice. As shown in Figure 8B, cB6 FPs expressed CCL2, CCL3, CCL4, and CXCL10 and the chemokine receptors, CXCR3 and CCR2. For each of these chemokines, there was a 9 to

50-fold lower level of expression in the cLTα^{-/-} FPs, together with reduced expression for CXCR3 and CCR2. In addition, there was a reduced expression of the cell adhesion molecules, VCAM and ICAM, in the LTα^{-/-} FP (Figure 8C) as compared with cB6 FPs.

Discussion

Clinical and histopathological criteria are used to classify a patient's disease status in the broad spectrum of leprosy.³⁸ In addition, associations have been made between tuberculoid leprosy and Th1 cytokine generation, and lepromatous leprosy and Th2 cytokine generation.¹⁷ However, whether the cytokine responses expressed across the leprosy spectrum are the cause or an effect of the disease is poorly understood.

In our ongoing studies, we use two models to analyze the effects of *M. leprae* infection in mice. In the low dose growth model, published by Shepard in 1960,³⁹ an inoculum of a few thousand *M. leprae* grows locally and plateaus with the onset of adaptive immunity by ≤ 6 months post-infection in immunocompetent mice. For many years, this infection model was the only method available for determining *M. leprae* viability and growth, and it was used to study basic immunological mechanisms and evaluate new anti-leprosy drugs.⁴⁰ We use this low dose model to compare growth of *M. leprae* among the KO

Figure 8. Inability to maintain the granulomatous response to *M. leprae* in cLT α ^{-/-} mouse FPs is associated with reduced cytokine, chemokine, and CAM expression. cB6 (closed bars) and cLT α ^{-/-} (hatched bars) FPs were inoculated with 3×10^7 *M. leprae* and FP tissue was harvested at 12 weeks post-infection. RNA was purified and subjected to real-time RT-PCR for (A) Th1 cytokines and NOS2, (B) chemokines and chemokine receptors, and (C) CAMs. Gene expression is reported as a ratio to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. Data shown are means $\pm$ SE of four mice per group and are representative of two independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P < 0.0001$.

strains, as well as to evaluate histopathological and immunohistochemical responses in the FP.

Recently, we described a second model loosely based on the Lepromin Test.²⁵ Unlike the 48-hours purified protein derivative test, which is diagnostic for *M. tuberculosis* exposure, the Lepromin Test in humans measures the ability to mount a granulomatous response to a large inoculum of dead *M. leprae*, and induration is generally measured at 28 days. The Lepromin Test has been used both clinically and in research settings to assess the host's capacity to mount a CMI response to the bacilli.³⁸ In our model, we inoculate a high dose of viable *M. leprae* into the FP. In immunocompetent mice (²⁵ this study, unpublished data), FP induration peaks and plateaus at approximately 28 days; however, we have found that the response in many strains of immunocompromised mice evolves dramatically over the course of several weeks, so we routinely monitor the infections for a longer duration. Furthermore, the FP and the draining popliteal LNs develop distinct and characteristic cellular profiles.²⁵ The FP, which contains immune cells recruited to this site only after *M. leprae* infection, consists of macrophages and

effector T cells, primarily CD4⁺; whereas, the LN is comprised of B cells and naive CD4⁺ and CD8⁺ T lymphocytes.

Since cytokines, as well as chemokines and CAMs, are instrumental for the induction, function, regulation, and maintenance of granulomas,¹ we have used our two models to examine *M. leprae* growth and especially granuloma formation in mouse strains with knockouts in various cytokines important in CMI, including IFN γ ^{-/-}, IL-12p40^{-/-}, and TNF^{-/-}, as well as NOS2^{-/-} mice. While the contribution of each of the cytokines to CMI is well established, none of these knockouts allowed an unrestricted growth of the bacilli as occurs when CMI is totally dysfunctional, such as in the athymic *nu/nu* mouse in which bacterial counts reach $\geq 10^{10}$ AFB.³⁸ Yet, the granulomatous responses that developed in the infected FP, and the cytokine and chemokine profiles generated, were quite distinct among the various knockouts. *M. leprae*-infected IFN γ ^{-/-} mice developed a large, unorganized cellular infiltration in the FP and a Th2-type cytokine profile.²⁹ In contrast, NOS2^{-/-} FP exhibited a granulomatous inflammation that infiltrated the musculature of the foot and a Th1-type cytokine and chemokine response.²⁸ Of note, as shown here, *M. leprae*-infected TNF-deficient mice generated an intense and diffuse T cell inflammation and growth of the bacilli was augmented throughout infection, phenomena quite different from those observed in the LT α -deficient mice. These data indicate that TNF and LT α exert dramatically different effects in the execution of CMI in experimental leprosy. Our results also suggest that signaling via TNFR1 is primarily a function of TNF rather than LT α , as LT α did not compensate for the lack of TNF in the TNF^{-/-} mice. These findings are currently being explored further.

Cytokines also influence chemokine gradients, which facilitate the temporal and spatial coordination of antigen-presenting cells and Ag-specific T cells in granulomas. In humans, CCL-3 and CCL-4 are chemoattractants for CD4⁺ and CD8⁺ lymphocytes, respectively.⁴¹ CCL2 promotes macrophage migration, and CCL5 and CXCL10 induce activated T cell migration to the granulomas.¹ The contribution of TNF especially to the regulation of *M. tuberculosis*-induced granuloma structural organization and maintenance via the induction of chemokines has been extensively studied⁴²⁻⁴⁴; LT α_3 can also mediate chemokine expression.⁴⁵ In addition, LT α_3 induces the expression of CAMs, such as VCAM and ICAM, on endothelial cells,⁴⁵ which are up-regulated by IFN γ .⁴⁶ Moreover, because *in vivo* administration of a transgenic LT preparation induced ICAM and VCAM expression, it was postulated that LT is an initiating factor in chronic inflammation.⁸ Therefore, LT α is not only critically involved in the early steps of effector T cell generation via modulation of cytokine induction, but also in the effective recruitment of macrophage and T cells by regulating chemotactic signals and CAM expression, all of which could significantly alter appropriate granuloma formation. In our model, *M. leprae*-infected LT α -deficient mice generated a markedly depressed cytokine, chemokine, and CAM response and were unable to optimally develop effector CD4⁺ T cells and maintain an inflammatory ac-

cumulation around this persistent, virtually indestructible organism.

The poor granulomatous response in the FP of *M. leprae* infected cLT $\alpha^{-/-}$ mice is reminiscent of that in the lungs of *M. tuberculosis* infected cLT $\alpha^{-/-}$ mice.¹³ Although CD4⁺ cells migrated to the lung, they collected in the perivascular and peribronchial regions and not in the well-defined granulomas characteristic of wild-type mice. Lung lesions in the *M. tuberculosis*-infected cLT $\alpha^{-/-}$ mice, however, consisted of neutrophils and necrotic material; in contrast, we saw little neutrophil accumulation in the *M. leprae*-infected FP. This difference may reflect the rather innocuous nature of the leprosy bacillus compared with the more proinflammatory tubercle bacillus. In addition, growth of *M. tuberculosis* in the lungs was enhanced >3 log₁₀ in cLT $\alpha^{-/-}$ while *M. leprae* growth in the FP was only enhanced late in infection. It is noteworthy that despite the poor infiltration of mononuclear cells into the site of infection, the bacilli survived and multiplied in the cLT $\alpha^{-/-}$ FP suggesting that a large macrophages response is not necessary to support the growth or persistence of *M. leprae*. This is not unreasonable as a single macrophage can accommodate more than 100 viable *M. leprae* and remain physiologically normal and functional.^{47,48} Furthermore, in immunocompetent mouse FP as well as in some KO mouse models, which on *M. leprae* infection develop an enlarged FP with a strong macrophage influx (eg, IFN $\gamma^{-/-}$, NOS2 $^{-/-}$), a large percentage of these cells are, in fact, uninfected (unpublished data).

These findings are especially interesting in light of a recent study in human leprosy, which, using whole genome based genetic approaches and positional cloning, identified a polymorphism, LTA + 80 located in the promoter region of the gene encoding LT α , that was significantly associated with an increase in leprosy risk.¹⁹ This association was seen in patients of three different ethnic populations (Brazilian, Vietnamese, and Indian) and was strongest in persons <16 years of age. Importantly, this study provided strong evidence that susceptibility to this complex disease is dependent on a genetic predisposition in the host.⁴⁹ This genetic susceptibility could influence the early responses to *M. leprae*, a pathogen extremely dependent on host cellular processes for its survival, and control key immunoregulatory events that determine the long term outcome of this slowly progressing infection. Our results correlate this clinically identified susceptibility gene for leprosy with a significantly reduced granulomatous response and growth control in an experimental model of chronic infection. Studying the immunoregulatory mechanisms of leprosy, both in the subclinical and unstable borderline areas of the spectrum, should contribute to our understanding of the complex interactions between pathogen and host.

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