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Lymphotoxin $\alpha\beta 2$ (Membrane Lymphotoxin) Is Critically Important for Resistance to *Leishmania major* Infection in Mice[1]

Guilian Xu,[2]* Dong Liu,* Yijun Fan,*† Xi Yang,*† Heinrich Korner,‡ Yang-Xin Fu,§ and Jude E. Uzonna[3]*

Although the essential role of TNF-α in the control of intracellular pathogens including *Leishmania major* is well established, it is uncertain whether the related cytokine lymphotoxin $\alpha\beta 2$ (LT$\alpha 1\beta 2$, membrane lymphotoxin) plays any role in this process. In this study, we investigated the contribution of membrane lymphotoxin in host response to *L. major* infection by using LTβ-deficient (LT$\beta^{-/-}$) mice on the resistant C57BL/6 background. Despite mounting early immune responses comparable to those of wild-type (WT) mice, LT$\beta^{-/-}$ mice developed chronic nonhealing cutaneous lesions due to progressive and unresolving inflammation that is accompanied by uncontrolled parasite proliferation. This chronic disease was associated with striking reduction in IL-12 and Ag-specific IFN-γ production by splenocytes from infected mice. Consistent with defective cellular immune response, infected LT$\beta^{-/-}$ mice had significantly low Ag-specific serum IgG1 and IgG2a levels compared with WT mice. Although administration of rIL-12 to *L. major*-infected LT$\beta^{-/-}$ mice caused complete resolution of chronic lesions, it only partially (but significantly) reduced parasite proliferation. In contrast, blockade of LIGHT signaling in infected LT$\beta^{-/-}$ mice resulted in acute and progressive lesion development, massive parasite proliferation, and dissemination to the visceral organs. Although infected LT$\beta^{-/-}$ WT bone marrow chimeric mice were more resistant than LT$\beta^{-/-}$ mice, they still had reduced ability to control parasites and showed defective IL-12 and IFN-γ production compared with infected WT mice. These results suggest that membrane lymphotoxin plays critical role in resistance to *L. major* by promoting effective T cell-mediated anti-*Leishmania* immunity. *The Journal of Immunology,* 2007, 179: 5358–5366.

Members of the TNF superfamily of cytokines, which include lymphotoxin (LT)[4] α, LTβ, TNF-α, and LIGHT (ligand homologous to LT, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes) and their cognate receptors, TNFR1, TNFR2, LTβR, and herpes virus entry mediator (HVEM), play important roles in the development of the immune system, immune regulation, and inflammation (reviewed in Refs. 1–5) by initiating signaling cascades that regulate cell death, survival, and differentiation. TNF-α is the most studied member of this family, and its role in resistance to many pathogens, including *Leishmania major*, is well documented (6–10). In contrast, the functions of other members of this family in disease pathogenesis caused by infectious agents are still not well defined.

LTα and LTβ form three distinct ligands: a secreted homotrimer (LT$\alpha 3$), and two membrane-bound heterotrimers, LT$\alpha 1\beta 2$ (the predominant form) and LT$\alpha 2\beta 1$, collectively called membrane lymphotoxin (mLT). The LTβ subunit provides the membrane anchor of the heterotrimers and hence is more important for signaling purposes. LT$\alpha 3$ binds to the TNF-α receptors (TNFR1 and TNFR2) and HVEM (11). In contrast, LT$\alpha 1\beta 2$ signals exclusively via the LTβR, a receptor it shares with LIGHT (11, 12). Although LIGHT can signal via the LTβR, it also interacts with its specific receptor, HVEM. This shared use of ligands and receptors by members of this family may suggest functional redundancy. However, gene deletion studies are beginning to reveal unique as well as cooperative roles for each ligand-receptor pair in both the development and function of the immune system and in disease pathogenesis (13).

The LTs are primarily expressed by activated T, B, and NK cells and play an important role in the development of the immune system (5, 14–16). Both LT$\alpha^{-/-}$ (which are deficient in both soluble LT$\alpha 3$ and membrane LT$\alpha 1\beta 2$), LT$\beta^{-/-}$ (which are deficient only in membrane-associated LT$\alpha 1\beta 2$), and LTβR$^{-/-}$ mice have profoundly defective development of the peripheral lymphoid organs (14, 16–18). These mice lack peripheral lymph nodes and their spleen architecture is structurally dysregulated. Whereas LT$\alpha 3$ has been shown to play an important role in resistance to many pathogens including *Mycobacterium tuberculosis*, *Leishmania donovani*, and *Toxoplasma gondii* infection (19–21), the role of membrane LT in infectious disease is unclear.

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[4] Abbreviations used in this paper: LT, lymphotoxin; BMDM, bone marrow-derived macrophage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; mLT, membrane LT; HVEM, herpes virus entry mediator; LIGHT, ligand homologous to LT, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes; SLA, soluble *Leishmania* Ag; WT, wild type.

Cutaneous leishmaniasis caused by *L. major* is an important human disease. The outcome of *L. major* infection in mice is dependent on the type of $CD4^+$ Th cell subset that is induced (22–24). Healing in resistant mice is associated with IL-12-dependent development of IFN-γ-producing $CD4^+$ Th1 cells, which activate macrophages to produce NO, an effector molecule for killing intracellular parasites. In contrast, susceptible mice produce early IL-4, which promotes the development and expansion of Th2 cells that produce IL-4 and IL-10, cytokines that deactivate macrophages and inhibit intracellular parasite killing. The cytokine and chemokine signals that modulate these processes are still poorly understood.

To investigate the role of mLT in *L. major* infection, we have assessed the outcome of *L. major* infection in LT$\beta^{-/-}$ mice. The data presented here show that a deficiency of mLT led to chronic nonhealing infection in the usually resistant B6 mice. This chronic disease was associated with a reduction in IL-12- and Ag-specific IFN-γ production by splenocytes from infected LT$\beta^{-/-}$ mice. Although administration of rIL-12 to *L. major*-infected LT$\beta^{-/-}$ mice caused complete resolution of cutaneous lesion, it only partly (but significantly) reduced parasite proliferation. In contrast, blockade of LIGHT signaling in infected LT$\beta^{-/-}$ mice resulted in acute and progressive lesion development and massive parasite proliferation and visceralization. Similar to LT$\beta^{-/-}$ mice, LT$\beta^{-/-}$chimeric mice infected with *L. major* had defective IL-12 and IFN-γ production and impaired ability to control parasite replication, indicating that mLT plays an important role in resistance to this intracellular protozoan parasite by regulating T cell-mediated immunity.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 (B6) mice were purchased from Charles River Laboratories. The generation of LTβ-deficient mouse (on B6 background) has been previously described (25). Mice were housed at the University of Manitoba Central Animal Care Services facility under specific pathogen-free conditions. All experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council on Animal Care.

Parasites and infection

L. major parasites (MHOM/IL/80/Friedlin) were grown in Grace's insect medium (Invitrogen Life Technologies) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For infection, 7-day stationary-phase promastigotes were washed three times in PBS and counted. Mice were infected by injecting 5 million parasites suspended in 50 μl of PBS into the hind footpad. After infection, the development and progression of footpad lesions were monitored weekly by measuring the diameter of footpads with calipers.

Injection of LTβR-Ig, HVEM-Ig fusion protein and rIL-12 treatment

One day before infection, some mice were injected with LTβR-Ig or HVEM-Ig fusion protein (consisting of extracellular region of murine LTβR or HVEM linked to the Fc region of human IgG1; 100 μg/mouse) or control Ig (human IgG1, 100 μg; Sigma-Aldrich) i.p. followed by one injection weekly for another 5 wk. Some infected LT$\beta^{-/-}$ mice were injected (intralesionally) with rIL-12 (0.3 μg/mouse; PeproTech) three times a week for 2 wk. In some experiments, the injection of rIL-12 was continued to 5 wk postinfection.

Creation of LT$\beta^{-/-}$ chimeras

Wild-type (WT; C57BL/6) and LT$\beta^{-/-}$ mice were lethally irradiated (10 Gy, given in two split doses, 5.0 Gy on 2 consecutive days) and reconstituted with bone marrow cells (10^7 cells/mouse) obtained from the femur of WT (B6) and LT$\beta^{-/-}$ mice. The peripheral blood of the chimeras was monitored weekly for the level of T cell engraftment by FACS (by staining with anti-CD3 Ab). Chimeric mice were infected with *L. major* 5–6 wk after bone marrow reconstitution when the level of T cell engraftment was $\geq$75% of the unmanipulated (control) B6 mice.

In vitro recall response and intracellular cytokine staining

At various times after infection, spleens were harvested and made into single-cell suspensions. Cells were washed, resuspended at 4 million/ml in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin), and plated at 1 ml/well in 24-well tissue culture plates (Falcon; VWR). Cells were stimulated with soluble *Leishmania* Ag (SLA; 50 μg/ml) for 72 h and the culture supernatant fluids were collected and stored at –20°C until assayed for cytokines using ELISA. After 72 h, some cells were stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml) for 4–6 h and used for intracellular cytokine staining as previously described (26).

Measurement of total serum and Leishmania*-specific Ab levels*

Serum levels of *Leishmania*-specific IgG1 and IgG2a Abs in infected mice were determined by ELISA. Briefly, ELISA plates were coated with freeze-thawed *L. major* (stationary-phase promastigotes, 10^6/well) overnight. The next morning, the plates were washed twice with PBS/Tween 20 (PBST), blocked with PBS containing 10% FBS (at 37°C for 2 h), and serial dilutions of serum samples from infected mice were added to the well in triplicates. The plates were further incubated for 2 h, washed four times with PBST, and 100 μl of HRP-goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates) was added. After another 2-h incubation, the plates were washed six times with PBST, developed by adding 100 μl of substrate (ABTS; Mandel), and the OD was read at 405 nm. To determine the total serum IgG level, ELISA plates were first coated overnight with rabbit anti-mouse IgG, washed, and the various dilutions of serum were added. Thereafter, the above protocol was followed.

Bone marrow-derived macrophages (BMDM), dendritic cells (BMDC) and in vitro infection

Bone marrow cells were isolated from the femur and tibia of WT and LT$\beta^{-/-}$ mice as described previously (27). Briefly, after depletion of erythrocytes with ACK lysis buffer (150 mM NH_4Cl, 0.1 mM $KHCO_3$, 0.01 mM Na_2 EDTA (pH 7.2)) the cells were resuspended in macrophage medium (complete DMEM containing 30% L929 cell supernatant), seeded in petri dishes at 2×10^5/ml (10 ml/petri dish), and allowed to differentiate at 37°C in a CO_2 incubator. The culture medium were changed at day 3 and adherent macrophages were harvested by gentle scraping on day 7, washed, resuspended in complete medium (10^6/ml) and used for in vitro experiments. For BMDC, bone marrow cells were differentiated in petri dishes in the presence of rGM-CSF (20 ng/ml; PeproTech) as previously described (28). Immature DCs were harvested on day 8 washed, resuspended in complete medium (10^6/ml), and used for in vitro experiments.

For infection, aliquots (500 μl) of BMDM and BMDC in 5-ml polypropylene tubes were incubated with parasites for 5 h at a ratio of 1:10 (cell: parasite). After 6 h, the free parasites were washed away by low-speed centrifugation (three times at 500 rpm for 5 min) and infected cells were cultured in complete medium in the presence or absence of varying concentrations of IFN-γ, LPS, and anti-CD40 mAb. In some experiments, BMDM were first infected with *L. major* for 24 h before being stimulated with IFN-γ or LPS. At different times after infection, cytospin preparations were made, stained with Giemsa, and infection was determined by microscopy. The supernatant fluids were collected and assayed for NO (BMDM) or IL-12 (BMDC) as described.

Cytokine ELISAs and measurement of nitrite

IL-4, IL-10, IL-12, and IFN-γ concentrations in culture supernatant fluids were determined by sandwich ELISA using the following Ab pairs from BioLegend: IL-12p40, C15.6 and C17.8; IL-10, JES5-16E3 and JES5-2A5; IFN-γ, R4-6A2, and XMG1.2; and IL-4, 11B11, and BVD6-24G2. NO concentration in culture supernatant fluids was determined indirectly by measuring the levels of nitrite, a stable by-product of NO, using the Griess assay as previously described (29).

Estimation of parasite burden

Parasite burden in the footpads and spleens of infected mice was quantified by limiting dilution analysis as previously described (30).

Histological assessment

After sacrifice, the infected feet were fixed in 10% neutral-buffered formalin, decalcified in HNO_3, routinely processed, and embedded in paraffin.

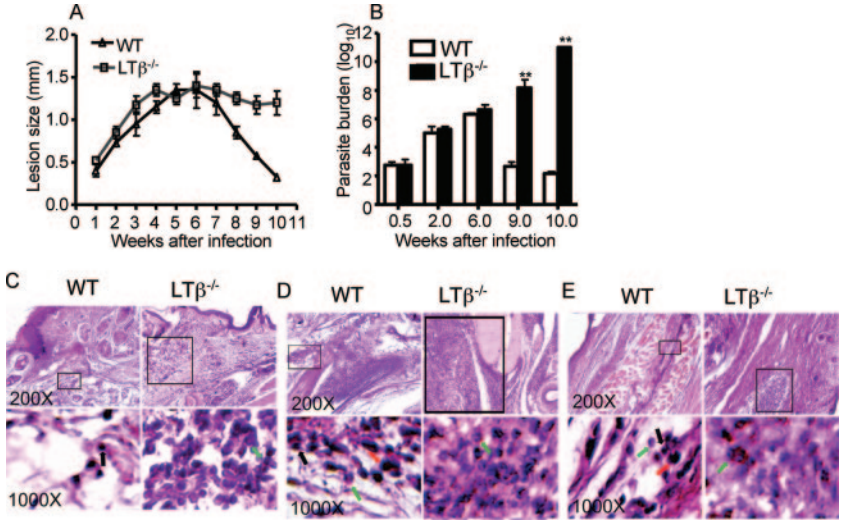

FIGURE 1. *Leishmania major*-infected LT$\beta^{-/-}$ mice develop chronic nonhealing cutaneous lesions characterized by unresolving inflammation. WT and LT$\beta^{-/-}$ mice were infected with 5 million *L. major* and the footpads of infected mice were measured weekly with calipers to determine lesion size (*A*). At the indicated times, parasite burden in the footpads of sacrificed mice was determined by limiting dilution (*B*). *C–E,* H&E staining of sections from infected footpads of WT and LT$\beta^{-/-}$ mice at 3 days (*C*), 2 wk (*D*), and 9 wk (*E*) after infection. Note the early onset of (day 3 post-infection) and more severe and persistent (9 wk) inflammation in the footpads of infected LT$\beta^{-/-}$ mice. Arrows, One representative cell in each category (black, lymphocytes; green, neutrophils; and red, macrophages). Squares, The inflammatory areas. *Upper panels*: original magnification, ×200, and *lower panels*: original magnification, ×1000. The results presented are representative of three different experiments (n = 5–8 mice/group) with similar results. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

Four-micrometer sections were stained with H&E before microscopic evaluation at ×200 and ×1000 magnification.

RT-PCR

LTα, LTβ, LTβR, and β-actin mRNA transcripts in the draining lymph nodes of infected mice were analyzed by RT-PCR. Briefly, total RNA was extracted from lymph node tissue homogenates using an RNA extraction kit (TRIzol; Invitrogen Life Technologies) according to the manufacturer's suggested protocols. To prevent RNA contamination by genomic DNA, total RNA was treated with DNase (Sigma-Aldrich) and 2 μg of total RNA was reverse transcribed into cDNA using a Superscript RT kit (Invitrogen Life Technologies) according to the manufacturer's specifications. PCR was conducted using a TaqPCR core kit (Qiagen according to the manufacturer's instruction in a final volume of 25 μl. PCR primers used for amplification of LTα, LTβ, LTβR, and β-actin (Operon) were as follows: LTα forward primer, 5′-AGGGGCCCAGGGACTCTCT-3′; LTα reverse primer, 5′-ACGATCCGTGCTTGCTCTC-3′; LTβ forward primer, 5′-GAGACAGTCACACCTGTTG-3′; LTβ reverse primer, 5′-CCTGTAGTCCACCATGTCG-3′; LTβR forward primer, 5′-GAGCAGAACCGGACACTAGC-3′; LTβR reverse primer, 5′-GAAGGTAGGGATGAGCACC-3′; β-actin forward primer 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, and β-actin reverse primer, 5′-AAAACGCAGCTCAGTAACAGTCCG-3′. PCR conditions were optimized for each primer sets, and were performed at different cycles to ensure that amplification occurred in the linear range. The PCR products were resolved in 2% agarose gel and stained with ethidium bromide for visualization.

Statistical analysis

A two-tailed Student t test was used to compare means of lesion sizes, parasite burdens, and cytokine production from different groups of mice. Significance was considered if $p < 0.05$.

Results

L. major-*infected LT$\beta^{-/-}$ mice developed chronic nonhealing cutaneous lesions*

Although TNF-α and its receptors (TNFR1 and TNFR2) have been shown to play important roles in resistance to *L. major* (6–10), the role of the related cytokines, LTα and mLT, in this process is not clear. Because we found that the expression of LTβ and its receptor (LTβR) was increased in the draining lymph nodes of infected C57BL/6 mice (data not shown), we hypothesized that this cytokine plays an important role in resistance to *L. major*. Therefore, we infected WT and LT$\beta^{-/-}$ mice with *L. major* and monitored the development and progression of cutaneous lesion and parasite burden at various times after infection. As shown in Fig. 1*A*, the onset of development and progression of cutaneous lesion were similar in both WT and LT$\beta^{-/-}$ mice during the early phase of the disease. However, by 6 wk post-infection when WT mice began to resolve their lesions, infected LT$\beta^{-/-}$ mice developed chronic nonhealing cutaneous lesions that persisted for 12 wk post-infection (when the experiment was terminated). Analysis of parasite burden revealed that LT$\beta^{-/-}$ mice had uncontrolled parasite proliferation (Fig. 1*B*) beyond 6 wk post-infection. Furthermore, although differences in cutaneous lesions were not grossly evident between infected WT and LT$\beta^{-/-}$ mice until 6 wk post-infection, histological examination revealed that LT$\beta^{-/-}$ mice developed more severe and early inflammation in the infected footpad than WT mice. For instance, as early as 3 days after infection when no visible gross lesion was present in WT mice, LT$\beta^{-/-}$ mice already had clear and significant inflammation in the infected footpads (Fig. 1, *C–E*). Whereas WT mice had only a few inflammatory cells (Fig. 1*C*, mainly lymphocytes, black arrow) in the s.c. area, LT$\beta^{-/-}$ mice had much heavier inflammatory cell infiltration, which consisted mainly of neutrophils (green arrow) and very few lymphocytes (Fig. 1*C*). By 2 wk post-infection, WT mice show patchy focal infiltration of inflammatory cells, mainly neutrophils (green arrow) with few lymphocytes (black arrow) and macrophages (red arrow) in the shallow s.c. areas (Fig. 1*D*). In contrast, LT$\beta^{-/-}$ mice showed large areas of neutrophilic infiltration extending into the deep s.c. area and reaching to the surface of bone tissues (Fig. 1*D*). Furthermore, at 9 wk after infection when tissue structure in WT mice was almost close to normal (very few neutrophils and macrophages), LT$\beta^{-/-}$ mice still had severe inflammation associated with extensive neutrophilic infiltration necrosis and abscess formation in some areas of the infected footpad (Fig. 1*E*). These observations indicate that LT$\beta^{-/-}$ mice are very susceptible to *L. major* infection.

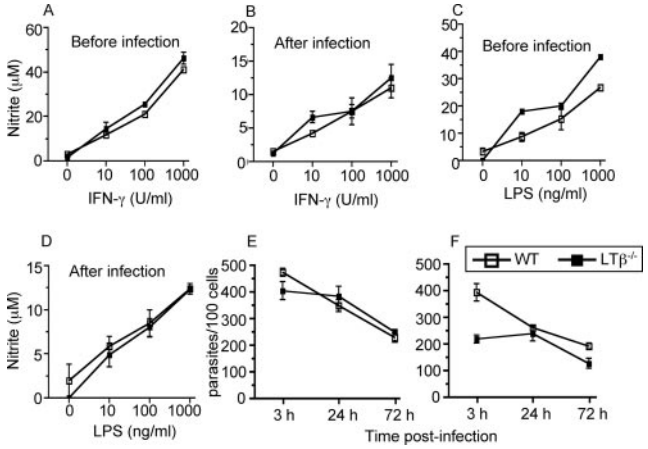

FIGURE 2. Intact NO production and control of *L. major* proliferation in vitro by LT$\beta^{-/-}$ macrophages. BMDMs were differentiated in vitro from LT$\beta^{-/-}$ and WT bone marrow cells and stimulated with various concentrations of rIFN-γ or LPS before or after (24 h) infection with *L. major*. The cells were further incubated for 48 h (before infection, *A* and *C*) or 24 h (after infection, *B* and *D*), and nitrite levels were assessed by the Greiss assay. Some cells stimulated with IFN-γ or LPS were infected with *L. major* for 72 h and cytospins were prepared, stained with Giemsa, and the number of parasites per 100 cells was enumerated under light microscopy (*E* and *F*). The results presented are representative of two different experiments with similar results.

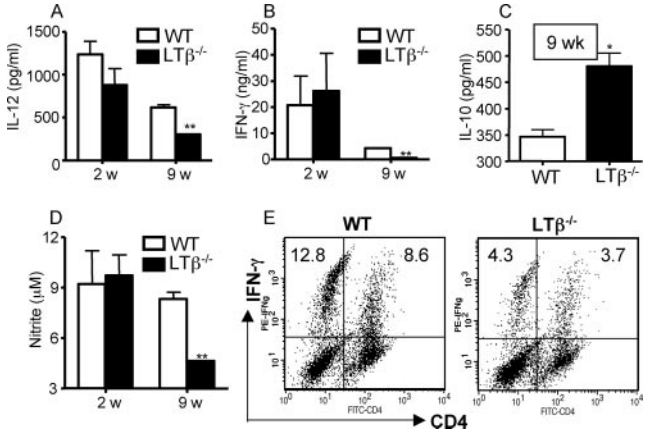

FIGURE 3. Impaired IL-12p40 and IFN-γ production by spleen cells from *L. major*-infected LT$\beta^{-/-}$ mice. At 2 and 9 wk after infection, WT and LT$\beta^{-/-}$ mice were sacrificed and their splenocytes were stimulated in vitro with SLA for 72 h and the levels of IL-12p40, IL-10 (9 wk only), and IFN-γ (*A–C*) and nitrite concentration (*D*) in the supernatant fluids were determined by ELISA and Griess assay, respectively. At 72 h, some cells from mice infected for 9 wk were stimulated with PMA, ionomycin, and brefeldin A for an additional 5 h, stained for intracellular expression of IFN-γ, and the percentage of IFN-γ-secreting CD4$^+$ and non-CD4$^+$ cells was determined by flow cytometry (*E*). Numbers in the box represent the percentage of IFN-γ- positive cells. The results presented are representative of three experiments (n = 4–5 mice/group) with similar results. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

Intact macrophage function in LT$\beta^{-/-}$ mice

Previous studies show that members of the TNF-α superfamily of cytokines (particularly TNF-α and LTα), play important roles in NO production by macrophages and killing of intracellular *Leishmania* (6, 7, 31, 32). Therefore, we investigated whether LT$\beta^{-/-}$ macrophages were defective in NO production and hence in their ability to control parasite proliferation in vitro. Following stimulation with different concentrations of IFN-γ or LPS, uninfected and *L. major*-infected BMDM from WT and LT$\beta^{-/-}$ mice produced comparable amounts of NO at all of the times tested (Fig. 2, *A–D*, and data not shown). Consistent with this, no significant differences were observed in the parasite killing ability of WT and LT$\beta^{-/-}$ BMDM following stimulation with IFN-γ or LPS (Fig. 2, *E* and *F*). Together, these results show that given the right stimuli, macrophages from LT$\beta^{-/-}$ mice are able to produce NO and control the growth of intracellular *L. major*. They further suggest that the chronic disease observed in LT$\beta^{-/-}$ mice infected with *L. major* may not be due to intrinsic defects in the ability of their macrophages to produce NO and kill intracellular parasites.

Late impaired production of IL-12, IFN-γ, and NO in L. major*-infected LT$\beta^{-/-}$ mice*

Resistance to *L. major* is mediated by IFN-γ-producing CD4$^+$ Th1 cells whose induction is dependent on IL-12 produced by dendritic cells (DCs) (24, 33–35). Given that deficiency of mLT results in the resistant mice becoming sensitive to *L. major*, we determined whether this was due to impaired IL-12 induction and defective Th1 cell response. At the time of sacrifice, single-cell suspensions from spleens of infected mice were cultured alone or stimulated with SLA and the production of IL-12 and IFN-γ was assessed by ELISA. Unstimulated and SLA-stimulated cells from uninfected mice did not produce any detectable amounts of IL-12 or IFN-γ (data not shown). In contrast, the production of these cytokines by cells from infected LT$\beta^{-/-}$ mice early during infection was increased but comparable to those of infected WT mice. However during the late phase of infection (beyond 6 wk), cells from LT$\beta^{-/-}$ mice produced lower amounts of these cytokines than those from infected WT mice (Fig. 3, *A* and *B*). This impaired cytokine response corresponds to the period of chronic lesion development in infected LT$\beta^{-/-}$ mice. Intracellular cytokine analysis at 9 wk postinfection further confirmed the defect in IFN-γ production (Th1 cell response) and showed that the percentages of IFN-γ-producing CD4$^+$ and non-CD4$^+$ cells following in vitro restimulation with SLA, PMA, and ionomycin (Fig. 3*E*) was lower in infected LT$\beta^{-/-}$ mice. However, the failure of LT$\beta^{-/-}$ mice to control infection was not due to the development of a nonprotective Th2 response, because the production of IL-4 by cells from infected LT$\beta^{-/-}$ mice was either lower or similar to WT cells (data not shown). Interestingly, cells from infected LT$\beta^{-/-}$ mice produced significantly more IL-10 than those from WT mice (Fig. 3*C*). Furthermore, the production of NO by spleen cells from infected LT$\beta^{-/-}$ mice was significantly lower than WT cells only during the late phase of infection (Fig. 3*D*), suggesting that this

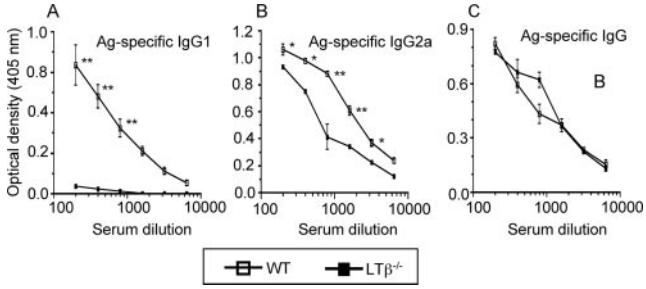

FIGURE 4. Impaired Ag-specific IgG1 and IgG2a Ab production in *L. major*-infected LT$\beta^{-/-}$ mice. Nine weeks after infection, mice were sacrificed and sera were collected and the levels of *Leishmania*-specific IgG1 (*A*) and IgG2a (*B*) and total (nonspecific) IgG (*C*) in the serum were determined by ELISA. The results presented are representative of two experiments (n = 4–5 mice/group) with similar results. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

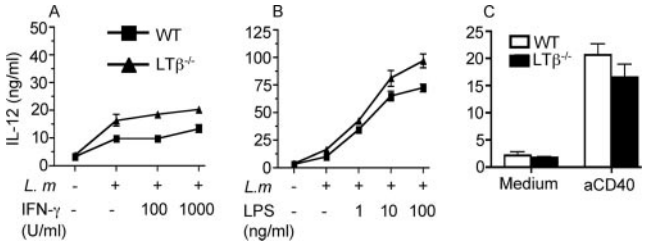

FIGURE 5. Unimpaired IL-12p40 production by BMDC from LT$\beta^{-/-}$ mice. BMDCs were differentiated in vitro from LT$\beta^{-/-}$ and WT bone marrow cells with recombinant GM-CSF (20 ng/ml), infected with *L. major* at a 1:10 (BMDC:parasite) ratio, and stimulated with various concentrations of rIFN-γ (*A*) or LPS (*B*). After 48 h, the supernatant fluids were collected and assayed for IL-12p40 by ELISA. Some uninfected cells were also stimulated with anti-CD40 (10 μg/ml) for 48 h and the supernatant fluids were assayed for IL-12p40 by ELISA (*C*). The results presented are representative of two different experiments with similar results.

impairment may be related to the defective IFN-γ production during this time.

Defective Ag-specific Ab response in LT$\beta^{-/-}$ mice

The role of Abs in resistance to *L. major* is controversial. Although previous reports suggest Abs enhance disease leading to susceptibility (36–39), a recent report suggests the opposite and shows that a strong B cell response promotes parasite clearance and resistance (40). Cytokines produced by T cells influence the quality of Ab response by regulating isotype switching in B cells. Consistent with the observed defective cytokine (Th1 and Th2) response at 9 wk postinfection, both Ag-specific IgG1 (Fig. 4*A*) and IgG2a (Fig. 4*B*) Ab levels were significantly low in infected LT$\beta^{-/-}$ mice. However, the total serum IgG level was relatively normal (Fig. 4*C*). Taken together, these results suggest that deficiency in mLT severely affects anti-*Leishmania* humoral immune response.

Intact DC function in LT$\beta^{-/-}$ mice

Given that IL-12 production and Ag-specific IFN-γ response were impaired in LT$\beta^{-/-}$ mice, we investigated whether intrinsic defects in IL-12 production by LT$\beta^{-/-}$ DCs are responsible for this impaired response in vivo. We stimulated BMDC from WT and LT$\beta^{-/-}$ mice with different concentrations of IFN-γ and LPS and determined their IL-12p40 production after 48 h. Both uninfected and *L. major*-infected BMDC from WT and LT$\beta^{-/-}$ mice produced comparable levels of IL-12 following stimulation with IFN-γ and LPS in vitro (Fig. 5, *A* and *B*, and data not shown). Furthermore, there was no difference in IL-12 production by uninfected BMDC from WT and LT$\beta^{-/-}$ mice following stimulation with anti-CD40 mAb (Fig. 5*C*). Taken together, these results suggest that some other host factors may be responsible for the impaired IL-12 production in vivo following *L. major* infection.

Administration of rIL-12 cures L. major*-infected LT$\beta^{-/-}$ mice*

The development of CD4$^+$ Th1 cells and production of IFN-γ in mice infected with *L. major* is influenced by IL-12 production by APCs (33, 41–43). At 9 wk post-infection, spleen cells from LT$\beta^{-/-}$ mice produced significantly less IL-12 than those from infected WT mice (Fig. 3*A*). Therefore, we investigated whether defective IL-12 production in LT$\beta^{-/-}$ mice was responsible for the impaired IFN-γ (Th1) response and susceptibility of these mice to *L. major*. We treated LT$\beta^{-/-}$ mice with recombinant murine IL-12 (rIL-12) and assessed the outcome of *L. major* infection. LT$\beta^{-/-}$ mice treated with rIL-12 exhibited enhanced resistance to *L. major* as evidenced by minimal lesion development (Fig. 6*A*) and effective parasite control in infected footpads (Fig. 6*B*) and spleens (Fig. 6*C*). The parasite burden in the footpads of WT mice treated with rIL-12 was similar to untreated WT controls, suggesting that the antiparasitic effect observed in LT$\beta^{-/-}$ mice was not simply due to excessive and unphysiologic effects of high-dose rIL-12 administration (data not shown). Interestingly, although LT$\beta^{-/-}$ mice treated with rIL-12 completely resolved their cutaneous lesions, they still harbored significantly more parasites than infected WT mice (Fig. 6, *B* and *C*), suggesting that other LTβ-dependent events distinct from its effect on IL-12 production may also be important for optimal resistance to *L. major*. Prolonged treatment with rIL-12 (up to 5 wk post-infection) resulted in a better parasite control in LT$\beta^{-/-}$ mice, although parasite burden in this group was still significantly higher than in WT mice (data not shown). These results strongly indicate that the susceptibility of LT$\beta^{-/-}$ mice to *L. major* is due, in part, to impaired IL-12 production, which results in defective Th1 response. Furthermore, because rIL-12 treatment did not result in complete parasite control, the results also suggest that other factor(s) contribute to the susceptibility of LT$\beta^{-/-}$ mice to *L. major*.

Blockade of LIGHT signaling in infected LT$\beta^{-/-}$ mice leads to acute and progressive lesion development and massive parasite proliferation

We previously showed that LIGHT, another related member of the TNF-α superfamily of cytokines, is important for optimal IL-12 production by DCs, the development of Th1 cells in vivo and resistance to *L. major* (G. Xu, D. Liu, I. Okwor, S. P. Kung, H. Korner, Y.-X. Fu, and J. E. Uzonna, manuscript in preparation). Although LIGHT binds to its unique receptor, HVEM, it also binds to LTβR, a receptor it shares with LT$\alpha\beta$2 (11, 12). Because there were no differences in immune response and parasite control between WT and LT$\beta^{-/-}$ mice during the early phase of infection, we speculated that the LIGHT-LTβR interaction has a compensatory effect on early IL-12 production, leading to Th1 development and prevention of acute disease in infected LT$\beta^{-/-}$ mice. To investigate this, we injected infected LT$\beta^{-/-}$ mice with fusion proteins, LTβR-Ig or HVEM-Ig, to block LIGHT binding to LTβR

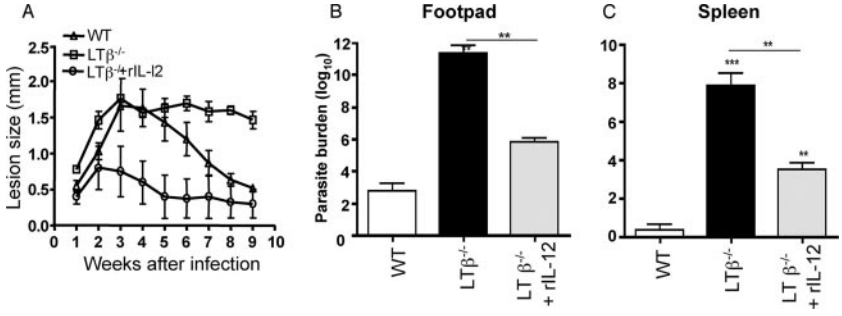

FIGURE 6. Administration of rIL-12 cures *L. major*-infected LT$\beta^{-/-}$ mice. LT$\beta^{-/-}$ mice infected with *L. major* were treated intralesionally with rIL-12 (0.5 μg/mouse, three times a week) for 2 wk and lesion size was monitored weekly (*A*). Nine weeks after infection, mice were sacrificed to estimate parasites load in infected footpads (*B*) and spleens (*C*). The results presented are representative of three experiments (n = 3–4 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

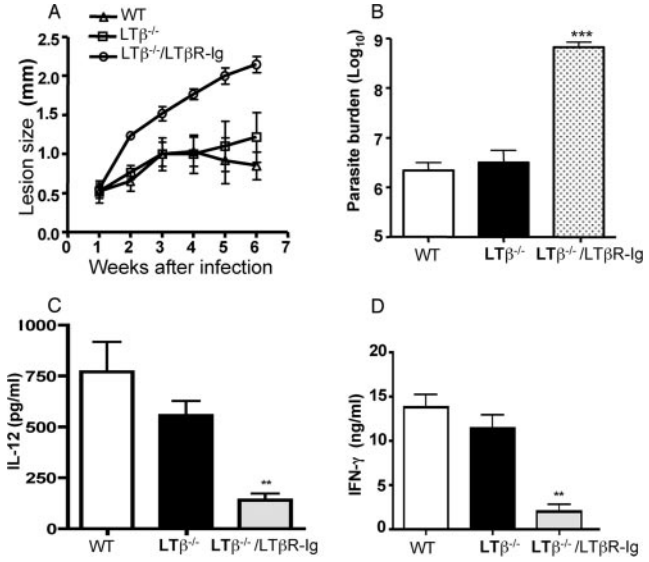

FIGURE 7. Blockade of LIGHT causes acute and progressive lesion development and massive parasite proliferation in *L. major*-infected LT$\beta^{-/-}$ mice. LT$\beta^{-/-}$mice were treated with LTβR-Ig (100 μg/mouse) or control human Ig and infected with *L. major* the next day. Infected mice were further treated weekly (for 5 wk) with LTβR-Ig and the footpad lesion size was measured with calipers (*A*). Six weeks after infection, mice were sacrificed and parasite burden in the footpads was estimated by limiting dilution (*B*). Single-cell suspensions were made from spleens of infected mice, stimulated with SLA for 72 h, and the levels of IL-12p40 (*C*) and IFN-γ (*D*) in supernatant fluids were determined by ELISA. The results presented are representative of two different experiments (n = 3–4 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

and assessed the outcome of the infection. LT$\beta^{-/-}$ mice injected with LTβR-Ig (Fig. 7) or HVEM-Ig (data not shown) became highly susceptible to *L. major* and developed acute uncontrolled progressive lesions, which became ulcerative after 6 wk, necessitating sacrifice and termination of the experiment (Fig. 7*A*). Analysis of parasite burden in the infected footpad revealed that LTβR-Ig-treated LT$\beta^{-/-}$ mice contained significantly higher parasite numbers than WT and LT$\beta^{-/-}$ mice (Fig. 7*B*). Whereas the levels of IL-12 and IFN-γ produced by cells from infected LT$\beta^{-/-}$ and WT mice were similar, the production of these cytokines in LT$\beta^{-/-}$ mice treated with LTβR-Ig were significantly impaired (Fig. 7*C*). Furthermore, LTβR-Ig-treated LT$\beta^{-/-}$ mice infected with *L. major* produced significantly more IL-10 than infected WT and LT$\beta^{-/-}$ mice (data not shown). Together, these results indicate that the early normal IL-12 production, Th1 response, and parasite control in LT$\beta^{-/-}$ mice is due to compensatory signals generated by LIGHT binding to its receptors. They further suggest that although this signal is enough for early parasite control, it is not sufficient for resistance to *L. major*.

LT$\beta^{-/-}$ WT chimeras infected with L. major *have impaired IL-12 and IFN-γ production and reduced ability to control parasite proliferation*

Members of the TNF-α superfamily of cytokines and their receptors play a crucial role in lymphoid organogenesis and structural integrity. Mice with targeted deletion of the LTβ gene do not have peripheral lymph nodes and their spleens are structurally disorganized (5, 14, 16, 17, 44). Thus, one possible explanation for the susceptibility of LT$\beta^{-/-}$ mice to *L. major* infection is that the absence of lymph nodes or the aberrant splenic architecture associated with deficiency of this cytokine impede the generation of effective immunity (13, 17, 45). To address this, we generated WT $\rightarrow$ LT$\beta^{-/-}$ and LT$\beta^{-/-}$ $\rightarrow$ WT bone marrow chimeras and infected them with *L. major*. As shown in Fig. 8*A*, although LT$\beta^{-/-}$ $\rightarrow$ WT chimeras could partially control their cutaneous lesion and parasite replication compared with LT$\beta^{-/-}$ mice, these mice still had significantly higher parasite burden compared with WT mice. Furthermore, although infected WT $\rightarrow$ LT$\beta^{-/-}$ mice were unable to fully resolve their cutaneous lesions, they had a better parasite control than infected LT$\beta^{-/-}$ mice. Analysis of cytokine response at sacrifice showed that cells from infected WT $\rightarrow$ LT$\beta^{-/-}$ and LT$\beta^{-/-}$ $\rightarrow$ WT chimeric mice produced significantly low IL-12

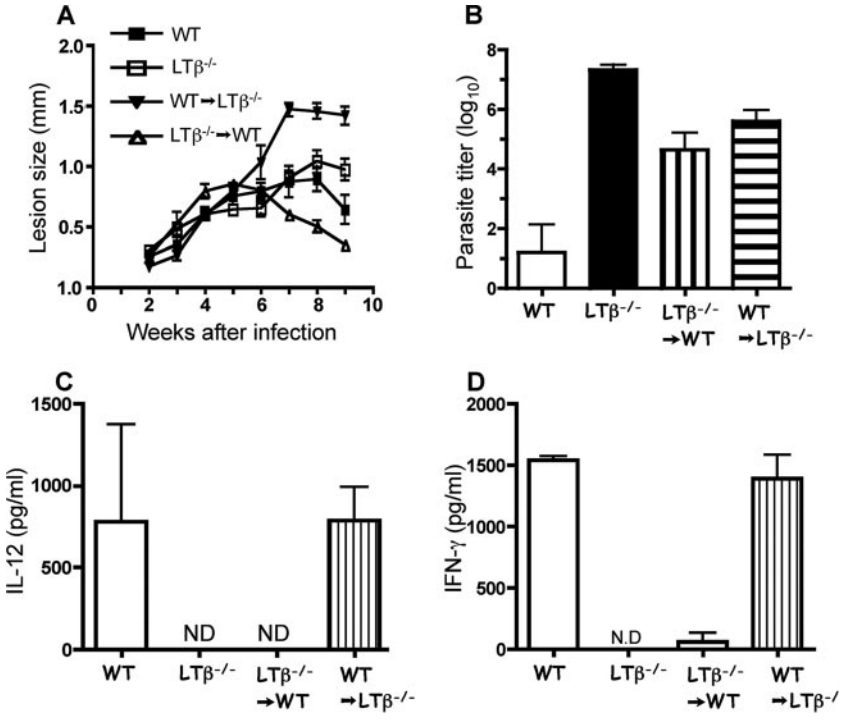

FIGURE 8. Absence of peripheral lymph nodes and/or dysregulated splenic architecture contributes to susceptibility of LT$\beta^{-/-}$ mice to *L. major*. WT $\rightarrow$ LT$\beta^{-/-}$, and LT$\beta^{-/-}$ $\rightarrow$ WT chimeras were generated by i.v. transfer of bone marrow cells (10^7/mouse) from WT and LT$\beta^{-/-}$ mice into irradiated LT$\beta^{-/-}$ and WT mice, respectively. Five weeks post-transfer, the resulting chimeras were infected with *L. major* and the cutaneous lesion was measured weekly (*A*). At 9 wk postinfection, mice were sacrificed and parasite burden in the infected footpads was determined by limiting dilution (*B*). Single- cell suspension of the spleens of infected mice were stimulated with SLA for 72 h and the levels of IL-12p40 (*C*) and IFN-γ (*D*) in the culture supernatant fluids were determined by ELISA. The results presented are representative of two experiments (n = 3–4 mice/group) with similar results. *, $p < 0.05$; **, $p < 0.01$.

(Fig. 8*B*) and IFN-γ (Fig. 8*C*) compared with those from infected WT mice. These results support our findings that impaired IL-12 production and the consequent defective Th1 response due to disruption of LTβ-LTβR signaling play a critical role in the susceptibility of LTβ$^{-/-}$ mice to *L. major*.

Discussion

In this study, we report that mLT is critically important for immunity to *L. major*. We show that mice with targeted deletion of the mLT gene develop chronic nonhealing cutaneous lesions and uncontrolled parasite proliferation, including metastasis to visceral organs. This inability to control parasite proliferation was associated with impaired IL-12 production late during infection leading to a profound defect in Ag-specific Th1 cell (IFN-γ) response. This impaired resistance of LTβ$^{-/-}$ to *L. major* was partially but significantly reversed following treatment with rIL-12, suggesting that the observed impairment in IL-12 production contributes in part to the susceptibility of LTβ$^{-/-}$ mice to *L. major*. Using bone marrow chimeras, we critically confirmed the effect of IL-12 and subsequent Th1 cell impairment in the susceptibility of LTβ$^{-/-}$ mice to *L. major*. Taken together, our results show that mLT is important for optimal IL-12 production in vivo, Th1 cell development, and resistance to *L. major*.

The impairment in IL-12 production was only evident during the late phase of infection (after 6 wk), corresponding to the period of impaired IFN-γ response. Interestingly, BMDC from LTβ$^{-/-}$ mice were not impaired in their ability to produce IL-12 following in vitro stimulation with IFN-γ, and LPS (Fig. 5). Therefore, deficiency of mLT does not lead to intrinsic defects in IL-12 production by DCs, suggesting that the impaired IL-12 response in vivo following infection of LTβ$^{-/-}$ mice with *L. major* is highly specific to the pathogen. Furthermore, the fact that IL-12 and IFN-γ production and parasite control in infected LTβ$^{-/-}$ mice were similar to WT mice up to 6 wk postinfection suggests that other signaling pathway(s) may be important for IL-12 production and Th1 cell development in LTβ$^{-/-}$ mice early during the infection process. In line with this, we recently found that the interaction of LIGHT with its receptors (particularly HVEM and LTβR) is a major pathway involved in IL-12 production by APCs leading to effective CD4^{+} Th1 cell development in vitro and in vivo (G. Xu, D. Liu, I. Okwor, S. P. Kung, H. Korner, Y.-X. Fu, and J. E. Uzonna, manuscript in preparation). Indeed, injection of HVEM-Ig or LTβR-Ig into LTβ$^{-/-}$ mice (to block LIGHT signaling) resulted in the development of acute and fulminating disease associated with dramatically impaired early IL-12 and IFN-γ production. Because mLT and LIGHT signal via the LTβR, it is conceivable that in the absence of mLT (as seen in LTβ$^{-/-}$ mice), signals generated via LIGHT-LTβR interaction may be sufficient for IL-12 production and Th1 response early during infection leading to parasite control. However, because *L. major*-infected LTβ$^{-/-}$ mice eventually develop chronic disease (despite intact LIGHT signaling), our results strongly suggest that optimal resistance to *L. major* requires intact LTα1β2 and LIGHT signaling in vivo.

We confirmed the susceptibility of LTβ$^{-/-}$ mice to *L. major* was due in part to the observed impairment in IL-12 production in these mice. Thus, LTβ$^{-/-}$ mice injected with rIL-12 completely resolved their otherwise chronic lesions and significantly controlled parasite replication. Interestingly, despite restoration of IFN-γ production and effective lesion resolution in infected LTβ$^{-/-}$ mice by rIL-12 treatment, the parasite burden in these mice remained significantly high compared with infected WT mice. This suggests that although impairment in IL-12 and IFN-γ production contributed to the susceptibility of LTβ$^{-/-}$ mice to *L. major*, other events also are involved in this process. Consistent with this, it has been shown that signals generated via the LTβR on DCs is critical for DC homeostasis, expansion, and maturation in vivo into competent APCs (46). Although these effects were mediated by LIGHT, it is conceivable that mLT could mediate similar effects by virtue of its capacity to bind to LTβR with high affinity (similar to LIGHT).

A recent report showed that intact mLT and LTβR (47) are important for proper macrophage activation and generation of NO leading to efficient macrophage antibacterial activity, including *Mycobacterium tuberculosis* and *Listeria monocytogenes* (47). Although we found impaired NO production in LTβ$^{-/-}$ mice infected with *L. major*, this only became evident during the late phase (after 6 wk) of infection, a period when IFN-γ production was also severely impaired. This late impairment in NO production is more likely to be a consequence of impaired IFN-γ response and may not be related to direct effects of LTα1β2 on macrophage activation. Our finding that uninfected and *L. major*-infected BMDMs from WT and LTβ$^{-/-}$ mice produced similar amounts of NO following in vitro stimulation with IFN-γ and LPS (Fig. 2) supports this conclusion. However, it is possible that in addition to the effects of impaired IFN-γ production, failure of macrophage inducible NO synthase activation due to disruption of LTα1β2-LTβR interaction (47) could contribute to the observed impairment in NO production in infected LTβ$^{-/-}$ mice.

Previous reports using bone marrow chimeras suggest that the susceptibility of LTβ-deficient mice to *L. major* (13) and during viral infection (45) is related to the absence of peripheral lymph nodes and/or dysregulated splenic architecture. However, several reports indicate that mLT plays a crucial role (independent of its function in peripheral lymphoid structural integrity) in host defense against several infectious agents including bacteria, *L. monocytogenes* (21, 47) and *M. tuberculosis* (47, 48), viruses, (49, 50), and parasites (19, 51, 52). We found that the ability of LTβ$^{-/-}$ mice treated with rIL-12 to control parasite proliferation was far much better than LTβ$^{-/-}$ WT chimeric mice. Furthermore, IL-12-treated LTβ$^{-/-}$ mice mounted strong *Leishmania*-specific IFN-γ response whereas this was severely impaired in infected LTβ$^{-/-}$ WT chimeric mice. Taken together, these observations suggest that impaired Th1 cell response due mainly to impaired IL-12 production is a major contributing factor to the susceptibility of LTβ$^{-/-}$ mice to *L. major*. Thus, our studies indicate that the role of LTα1β2 in resistance to *L. major* is not only due to its role in maintaining the integrity of peripheral lymphoid architecture, but is also related to its role in enhancing optimal T cell-mediated immunity. In line with this conclusion, we found in separate studies that male LTβ$^{-/-}$ mice infected with *L. major* develop acute and fulminating disease with uncontrolled parasite proliferation necessitating sacrifice only after 6 wk (G. Xu and J. E. Uzonna, unpublished data). This acute disease was associated with early impairment in IL-12 and IFN-γ production. If dysregulated peripheral lymphoid tissue architecture is the main cause of enhanced susceptibility of LTβ$^{-/-}$ mice to *L. major*, the outcome of infection in male and female LTβ$^{-/-}$ mice would be similar. Furthermore, we have found that LTβ$^{-/-}$ mice are highly resistant to *Trypanosoma congolense* (J. E. Uzonna, unpublished data), an extracellular protozoan pathogen in which pathology and death are caused by excessive Th1 cell-mediated cytokine responses (53, 54). Thus, although peripheral lymphoid tissue insufficiency may be a contributing factor, our data strongly suggest that impaired T cell-mediated immune response (due to deficiency of LTα1β2) also contributes to the enhanced susceptibility of LTβ$^{-/-}$ mice to *L. major* infection. However, because we did not determine the effect of T cell depletion on resistance to *L. major* in our bone

marrow chimeras, it is possible that mLT mediates its effect by enhancing the activity of other cells (non-T cells). In line with this, we found that the Ag-specific Ab response is severely impaired in *L. major*-infected LT$\beta^{-/-}$ mice (Fig. 4, *A* and *B*).

How does mLT influence the development of effector CD4$^+$ Th1 response and optimal resistance to *L. major*? Our study suggests that this may in part be due to its role in mediating optimal IL-12 production by DCs in vivo. In vitro, we found that BMDC from LT$\beta^{-/-}$ mice were not impaired in their ability to produce IL-12 in response to several stimuli including IFN-γ, LPS, CpG, and anti-CD40 mAb (Fig. 5). This suggests that the observed impaired IL-12 response in infected mice is not related to inherent defects in DCs from LT$\beta^{-/-}$ mice to produce this cytokine. mLT could be critical for optimal DC function in vivo such as migration to and clustering in the draining lymph node, expression of costimulatory molecules, proper positioning for optimal DC-T cell interaction, and IL-12 production (55, 56). In this scenario, the role of local draining lymph nodes would be to facilitate optimal DC-T cell interaction, leading to efficient effector T cell development at close proximity to the site of insult. In the absence of peripheral lymph nodes and dysregulated splenic architecture (as occur in LT$\beta^{-/-}$ mice), these events (DC activation, IL-12 production, and optimal Th1 response) will be impaired, resulting in defective T cell-mediated immunity. Indeed, DCs have been shown to express LTβR (57), which signals via NF-κB (58), and blockade of LTβR signaling impairs DC expansion and activation (59). It is possible that the interaction of LTα1β2 on T cells with its receptor, LTβR, on DCs potentiates DC activation (including IL-12 production) in the same way that CD40 costimulation enhances DC function (60). In support of this, a recent report published while this manuscript was in revision show that the expression of LT$\alpha\beta$2 on CD4$^+$ T cells is obligatory for this cytokine to enhance DC activation, leading to effective T cell-mediated immunity (61). Interestingly, we found that *L. major*-infected LT$\beta^{-/-}$ mice produced significantly more IL-10 than WT mice during the chronic disease phase of the disease (Fig. 3*C*). IL-10 has been shown to regulate disease outcome in mice infected with *L. major* (62, 63). It is conceivable that the high production of IL-10 in infected LT$\beta^{-/-}$ mice contributes to their susceptibility to *L. major*. Blockade of IL-10 signaling with anti-IL-10R mAb is necessary to conclusively determine the contribution of this cytokine to susceptibility of LT$\beta^{-/-}$ mice to *L. major*.

The susceptibility of LT$\beta^{-/-}$ mice to *L. major* is surprising given that previous reports showed similar susceptibility in TNF-α- and TNFR-deficient mice (8–10). However, unlike TNF-α- or TNFR-deficient mice, which develop acute progressive and fatal leishmaniasis or resolve primary lesion but harbor persistent parasites depending on the *L. major* strain (8–10), LT$\beta^{-/-}$ mice infected with *L. major* only develop chronic disease, with differences in lesion size and parasite burden becoming apparent after 6 wk postinfection. Acute progressive lesion and parasite metastasis (similar to those of TNF-α-deficient mice) were only observed following additional blockade of LIGHT signaling in LT$\beta^{-/-}$ mice. Because TNF-α-deficient mice had normal levels of mLT and vice versa, our results suggest that mLT, LIGHT, and TNF-α are required for effective control of *L. major* infection in nonredundant ways. Thus, a complex regulatory network involving the TNF-α superfamily of cytokines and their receptors act individually or in groups to regulate the outcome of infection with *L. major* in mice.

In conclusion, we have investigated the role of mLT in resistance to *L. major* infection in mice. Our data show that the susceptibility of LT$\beta^{-/-}$ mice to *L. major* is related in part to impaired IL-12 production, which leads to impairment in effector T cell development. We hypothesize that the role of peripheral lymph nodes in resistance to *L. major* is related to its function in DC clustering and positioning, which facilitates optimal DC-T cell interaction, DC activation, and IL-12 production and subsequent development of CD4$^+$ Th1 development in vivo.

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Disclosures

The authors have no financial conflict of interest.

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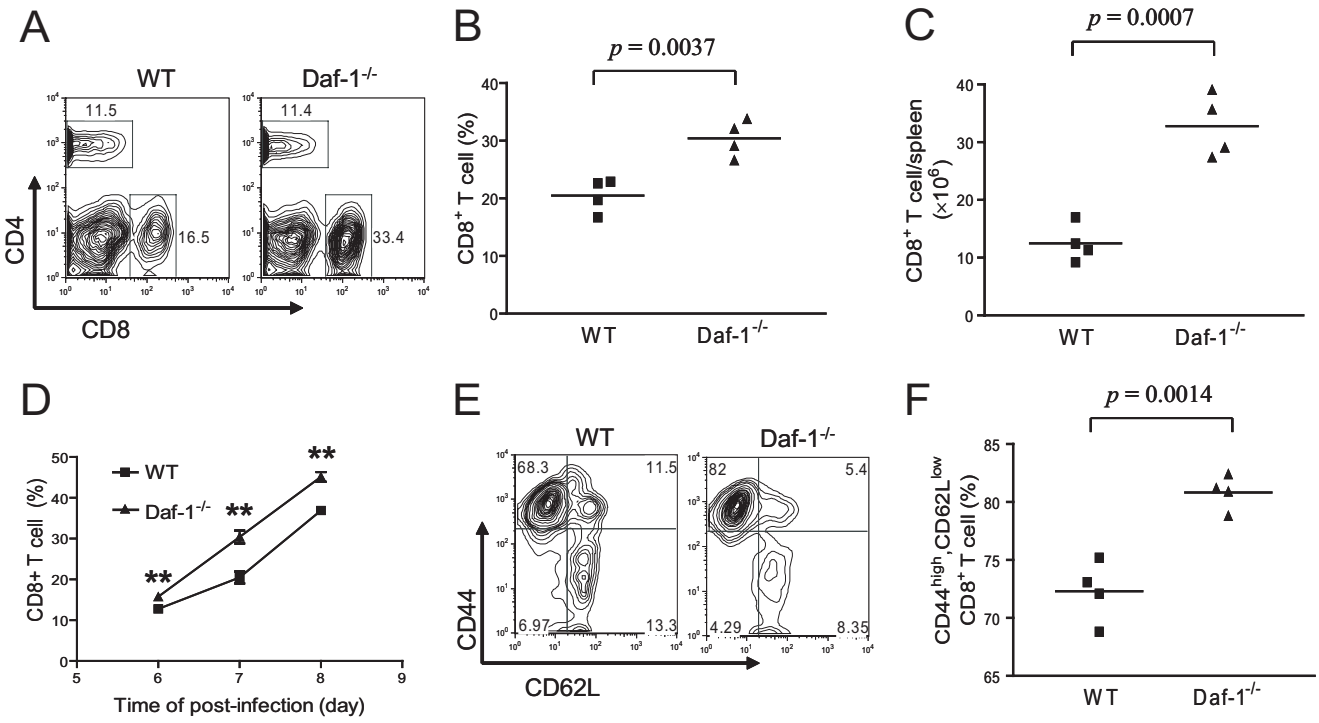
In Fig. 1*F* the data for WT and Daf-1$^{-/-}$ were reversed. The corrected figure is shown below.



Shibata, T., K. Nagata, and Y. Kobayashi. 2007. Cutting edge: a critical role of nitrogen oxide in preventing inflammation upon apoptotic cell clearance. *J. Immunol.* 179: 3407–3411.

In the title, "Nitrogen" is incorrect. The corrected title is shown below.

Cutting Edge: A Critical Role of Nitric Oxide in Preventing Inflammation upon Apoptotic Cell Clearance

Xu, G., D. Liu, Y. Fan, X. Yang, H. Korner, Y.-X. Fu, and J. E. Uzonna. 2007. Lymphotoxin $\alpha\beta$2 (membrane lymphotoxin) is critically important for resistance to *Leishmania major* infection in mice. *J. Immunol.* 179: 5358–5366.

In **Footnotes**, the current address for the first author is incorrect. The corrected footnote is shown below.

[2] Current address: Department of Immunology, Third Military Medical University, Chongqing 400038, People's Republic of China.