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*J Immunol* 2008; 181:8544-8551; doi: 10.4049/jimmunol.181.12.8544

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Leukotrienes Are Potent Adjuvant during Fungal Infection: Effects on Memory T Cells

Alexandra I. Medeiros,* Anderson Sá-Nunes,* Walter M. Turato,* Adriana Secatto,* Fabiani G. Frantz,* Carlos A. Sorgi,* Carlos H. Serezani,† George S. Deepe, Jr.,‡ and Lúcia H. Faccioli2*

Leukotrienes (LTs) are potent lipid mediators involved in the control of host defense. LTB₄ induces leukocyte accumulation, enhances phagocytosis and bacterial clearance, and increases NO synthesis. LTB₄ is also important in early effector T cell recruitment that is mediated by LTB₄ receptor 1 (BLT1), the high-affinity receptor for LTB₄. The aims of this study were to evaluate whether LTs are involved in the secondary immune response to vaccination in a murine model of Histoplasma capsulatum infection. Our results demonstrate that protection of wild-type mice immunized with cell-free Ags from H. capsulatum against histoplasmosis was associated with increased LTB₄ and IFN-γ production as well as recruitment of memory T cells into the lungs. In contrast, cell-free Ag-immunized mice lacking 5-lipoxygenase−/−, a critical enzyme involved in LT synthesis, displayed a marked decrease in recruitment of memory T cells to the lungs associated with increased synthesis of TGF-β as well as IL-10. Strikingly, these effects were associated with increased mortality to 5-lipoxygenase−/−-infected mice. These data establish an important immunomodulatory role of LTs, in both the primary and secondary immune responses to histoplasmosis. The Journal of Immunology, 2008, 181: 8544–8551.

Infection with the dimorphic fungus Histoplasma capsulatum ranges from a mild asymptomatic illness to a progressive and disseminated disease. The effective immune response to H. capsulatum relies on adaptive immunity and the collaboration between infected macrophages with CD4⁺ and CD8⁺ T cells (1, 2). This interaction results in increased amounts of Th1 and Th1-related cytokines, including IL-12, IFN-γ, TNF-α, and GM-CSF, which are critically involved in the protective immune response in H. capsulatum-infected mice (3–7). In opposition is the anti-inflammatory cytokine TGF-β, which counterregulates Th1 cell differentiation. TGF-β blockade of Th1 cell differentiation is associated with reduced IL-12Rβ2, T-bet, and STAT4 expression, whereas T-bet expression inhibits IFN-γ production during recall responses (8–10).

In addition to cytokines, another prominent class of molecules involved in the control of host defense are leukotrienes (LTs), lipids derived from arachidonic acid (AA) metabolism. LTs are derived from the metabolism of AA through the enzyme 5-lipoxygenase (5-LO), in concert with its helper protein, 5-LO-activating protein (FLAP). The 5-LO oxygenates AA to the intermediate 5-hydroperoxyeicosatetraenoic acid, which is either enzymatically reduced by 5-LO to the unstable epoxide LTA₄ or alternatively is reduced to 5-hydroxyeicosatetraenoic acid. LTA₄ can be hydrolyzed to form LTB₄ or can be conjugated with glutathione to form the cysteinyl LTs, LTC₄, LTD₄, and LTE₄ (11). The role of endogenous LTs in host defense during microbial infection was first demonstrated by Bailie et al. (12), who reported that 5-LO deficiency impaired survival and pulmonary bacterial clearance in a model of Klebsiella pneumoniae pneumonia. Subsequent studies have documented a protective function of endogenous LTs in several experimental models, including bacterial peritonitis (13), tuberculosis (14), and parasitic infection (15, 16). We have recently demonstrated an important role for LTs in the primary immune response to H. capsulatum (17). Reduction in LT synthesis used by administration of MK886, an inhibitor of the FLAP, resulted in 100% mortality in sublethally H. capsulatum-infected mice, higher numbers of lung and spleen CFU, and impaired NO production.

The effector functions involved in innate immune responses that are influenced by LTs include direct effects on leukocyte accumulation (18–21), microbial phagocytosis and killing (15, 22–24), and indirect effects mediated by elaboration of other inflammatory molecules (15, 17, 24). For example, the effects of LTs on the secretion of cytokines have been reported in vitro and in vivo. We demonstrated that inhibition of LT synthesis in animals infected with H. capsulatum resulted in lower IL-2, IL-5, IL-12, and IFN-γ levels (17). Moreover, in vitro, pharmacological blockade of LTB₄ receptor 1 (BLT1) resulted in increased IL-10 production (25). DiMeo et al. (26) demonstrated that LPS-stimulated spleen cells from 5-LO-deficient mice produced less IL-12p70, IL-6, TNF-α, and IFN-γ. LTB₄ has also been shown to exhibit potent chemotactic

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Received for publication June 16, 2008. Accepted for publication October 6, 2008.

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1 This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (02/07849-7; 02/12856-2), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), from Brazil and American Lung Association.

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3 Abbreviations used in this paper: LT, leukotriene; 5-LO, 5-lipoxygenase; AA, arachidonic acid; BHI, brain-heart infusion; BLT1, LTB₄ receptor 1; CF, cell-free Ag; DTH, delayed-type hypersensitivity; FLAP, 5-LO-activating protein; Hsp, heat shock protein; i., intratracheal; KC, keratinocyte-derived chemokine; neg, negative; p.o., perorally; WT, wild type.

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activity for in vitro activated effector CD4+ and CD8+ lymphocytes, which is mediated by BLT1, the high-affinity receptor for LTB4 (27, 28).

Because LTs enhance both phagocyte function and T cell function, we hypothesized that LTB4 might be an important mediator involved in the protective response to vaccination designed in a murine model of H. capsulatum infection. By using a standardized preparation of extracts containing H. capsulatum cell-free Ags (CFAgS), we observed that immunization was able to protect mice against a lethal inoculum of H. capsulatum and significantly reduce fungal burden in the lungs and spleens of infected mice (29). In the present study, we demonstrate that LTs play a key role in the secondary immune response induced by CFAg immunization through modulation of cytokine synthesis and generation or migration of memory T cells against H. capsulatum.

Materials and Methods

Mice

Six- to 8-wk-old 5-LO−/− (129-Alox5tm1Kem) and strain-matched wild type (WT) were obtained from Jackson ImmunoResearch Laboratories, and male C57BL/6 mice were bred in the Faculdade de Ciências Farmacêuticas de Ribeirão Preto (Universidade de São Paulo, Brazil). All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of the University. Infected animals were kept in biohazard facilities and housed in cages within a laminar flow safety enclosure under standard conditions.

Preparation of H. capsulatum and infection of mice

The clinical isolate of H. capsulatum obtained from a patient at the Hospital das Clínicas de Faculdade de Medicina de Ribeirão Preto (Universidade de São Paulo, Brazil) was grown on Sabouraud-dextrose-agar at room temperature for 2 mo, and conidia morphology was observed. Later, mycelia were grown in brain-heart infusion (BHI)-agar-blood at 37°C for 15 days to convert them into the yeast form, and morphology was also confirmed. After this characterization, preparation of H. capsulatum yeast cells was performed, as previously described (17). Yeast cells were used when fluorescein diacetate and ethidium bromide staining (30) revealed their viability to be ≥99%.

Lethal challenges of 107 yeasts intratracheal (i.t.) in C57BL/6 mice and 3 × 106 yeasts in 5-LO−/− (129-Alox5tm1Kem) and strain-matched WT mice were used to evaluate mortality rates and cellular immunity in subsequent experiments. The LD50 for both strains were previously determined (data not shown).

Preparation of CFAgS

CFAgS were produced according to Camargo et al. (31), with minor modifications (29). Briefly, ~300 mg of H. capsulatum yeasts growth on BHIAgar-blood slants were collected and suspended in 1 ml of PBS, mixed on a vortex-meter, and centrifuged at 10,000 × g for 60 s. The supernatant fluid was collected, and the procedure was repeated. This material was filtered, and its protein content was estimated using Coomassie Protein Assay Reagent Kit (Pierce), using albumin standard curves prepared according to the manufacturer’s instructions.

Immunization with CFAgS

CFAg preparation was emulsified in CFA (v/v) at a final concentration of 200 μg/ml. Mice were injected s.c. with 50 μg/0.25 ml emulsified CFAgS. One week later, mice received the same amount of the preparation emulsified in IFA. After 2 wk, mice received an i.v. booster of 5 μg of CFAg preparation in 0.1 ml. The control group received an equal amount of BSA suspended in the same adjuvants during the immunizations and a booster of 5 μg of BSA in 0.1 ml.

Treatment of mice with MK 886

C57BL/6 mice were divided into two groups. Group 1 animals were treated with MK 886 perorally (p.o.) by gavage (5 mg/kg/0.5 ml) 1 h before immunization s.c. with CFAgS, and again every 24 h until day 21 (1 day before rechallenge with H. capsulatum). This group is denominated CFAgS + MK886immunization. Group 2 animals were treated with MK 886 p.o. by gavage (5 mg/kg/0.5 ml) 1 h before rechallenge with H. capsulatum, and again every 24 h until day 60. This group is denominated CFAgS + MK886postinfection. Control animals were inoculated i.t. with PBS and were given water p.o. for 60 days.

Measurement of LTB4 and cytokines

For cytokine and LTB4 measurement, lungs were removed on day 7 postinfection. Tissues were homogenized (Mixier Homogenizer; Laborteknik) in 2 ml of RPMI 1640, centrifuged at 1500 × g, filtered, sterilized, and stored at −70°C until assayed. Quantification of LTB4 in the samples was performed by specific enzyme immunoassay (Amersham Biosciences) following the supplier’s instructions. The OD of samples was determined at 412 nm in a microplate reader (µ Quant Bio; TEK Instruments), and concentrations of eicosanoids were calculated based on a standard curve. The sensitivity for LTB4 was <4 pg/ml.

Commercially available ELISA Abs were used to measure TNF-α, IL-1α, IL-4, IL-10, IL-12 (p70), active TGF-β1, and IFN-γ (BD OptEIA ELISA sets; BD Pharmingen). Keratinocyte-derived chemokine (KC) was measured by sandwich ELISA (R&D Systems). The cross-reactivity with murine TGF-β was >90%. The sensitivity for cytokines was >30 pg/ml. Reactions were performed on 96-well ELISA plates (Ultra-High Binding EIA Plates; Corning Glass), and the ODs were read on a microplate reader (µ Quant Bio; TEK Instruments) at a wavelength of 450 nm. The cytokine concentration in each sample was estimated by interpolation of sample ODs with the cytokine standard by a four-parameter curve-fitting program.

Quantitation of NO

NO production was assessed by measuring the amount of nitrite (NO2−) in lung homogenates, obtained as described above, using the Griess reaction, as previously described (17). Values were determined using a standard curve with serial dilutions of NaNO2 (Sigma-Aldrich).

Organ culture for H. capsulatum

Recovery of H. capsulatum was performed, as previously described by Sá-Nunes et al. (32). Two hundred-microliter samples from each suspension were collected, and serial dilutions (10-fold) were plated on BHI-agar-blood slants. After incubation at 37°C for 21 days, the burden of H. capsulatum was assessed. Results are expressed as CFU/g lung or CFU/spleen.

Flow cytometry

Lung leukocytes were adjusted to a concentration of 1 × 106 cells/0.1 ml and washed with staining buffer (consisting of PBS (pH 7.4), 2% BSA, and 0.02% sodium azide). FcRs were blocked by the addition of unlabeled anti-CD16/32. The proportion of cells bearing a memory phenotype was determined by staining with appropriate concentrations of fluorochrome-conjugated Abs specific for murine CD4+ CD44hiCD62L− and CD8+ CD44hiCD62L− (BD Pharmingen). The samples were washed and fixed in 2% paraformaldehyde. The results were calculated by percentage of CD4+ or CD8+ T cells with memory phenotype (CD4+CD44hiCD62L−low/neg or CD8+CD44hiCD62L−low/neg).

Statistics

The log-rank test was used to assess differences in survival. ANOVA, with Bonferroni’s multiple comparison as a post test, was used to analyze differences in delayed-type hypersensitivity (DTH) responses, cytokine, LTs, and memory T cells. Value of p < 0.05 was considered statistically significant.

Results

LTs play an essential role in the survival and protection induced by CFAg immunization against H. capsulatum

To determine whether LTs are required for the generation of the protective immune response, 5-LO-deficient mice were immunized with CFAgS and challenged with a lethal inoculum of H. capsulatum. All WT and 5-LO−/− mice that had been immunized with BSA (irrelevant Ag) succumbed to infection by 17 days postinfection (Fig. 1A). However, 5-LO−/− BSA-immunized mice succumbed to infection 5 days before WT-BSA. Similarly, only 17% of the 5-LO−/− CFAg mice survived until 30-day postinfection. Conversely, 83% of WT CFAgS survived until the end of the observation period.

In another set of experiments, lungs and spleen were harvested from infected animals at day 7 postinfection and examined for fungal burden. We found significant increase in the CFU numbers in WT and 5-LO−/− BSA-immunized mice.
(\(p < 0.001\) and \(p < 0.05\)) when compared with WT CFAg mice (Fig. 1, B and C). Fungal burden in the lungs and spleen of 5-LO\(^{-/-}\) CFAg mice was significantly higher from that in WT CFAg mice on day 7 (\(p < 0.001\)) (Fig. 1, B and C). Moreover, the number of yeasts recovered in spleen at day 7 was higher in 5-LO\(^{-/-}\) BSA-immunized mice when compared with WT BSA- or CFA-immunized mice (Fig. 1C).

The 5-LO deficiency abolished the increased LTB\(_4\) and NO\(_2\)\(^{-}\) production induced by CFAg.

Because the lack of 5-LO abrogated the protective immune response induced by CFAg, we measured LTB\(_4\) in the lung after \(H.\) capsulatum infection. LTB\(_4\) levels in the lungs from WT BSA mice were \(\sim 2\)-fold higher than either PBS-injected or 5-LO\(^{-/-}\) BSA mice. Moreover, CFAg immunization in WT mice induced a 10-fold increase in LTB\(_4\) production when compared with both groups of BSA-immunized mice and infected with \(H.\) capsulatum (WT or 5-LO\(^{-/-}\) BSA). In contrast, the levels of LTB\(_4\) in lungs of 5-LO\(^{-/-}\) mice immunized with either CFAg or BSA were not altered when compared with uninfected mice (Fig. 2A).

One of the most important microbicidal molecules involved in the control of intracellular pathogens is NO. In addition, it has been shown that LTB\(_4\) induces NO secretion and inducible NO synthase expression (15). Our results demonstrated that \(H.\) capsulatum infection in WT CFAg mice induced the increase of NO\(_2\)\(^{-}\) production by \(\sim 4\)-fold when compared with WT and 5-LO\(^{-/-}\) BSA mice. However, the deficiency of 5-LO abolished the increased NO\(_2\)\(^{-}\) in the lung induced by CFAg immunization (Fig. 2B).

Effects of 5-LO deficiency on pulmonary cytokines

Production of Th1-related cytokines is critically important in the host resistance to \(H.\) capsulatum (33). Therefore, we examined cytokine production in lung homogenates from BSA-immunized or CFAg-immunized mice after 7 days of infection. \(H.\) capsulatum infection in CFAg WT mice induced a 2-fold increase in TNF-\(\alpha\) production when compared with BSA-immunized mice (WT or 5-LO\(^{-/-}\)-BSA). However, the levels of TNF-\(\alpha\) in lungs of 5-LO\(^{-/-}\) CFAg mice were not different from those of WT CFAg mice (Fig. 3A).

KC levels in the lungs of infected mice were increased in all groups when compared with those of uninfected animals. However, \(H.\) capsulatum infection in CFAg WT mice induced an increase in KC production when compared with BSA-immunized mice (WT or 5-LO\(^{-/-}\) BSA). Moreover, the levels of KC in lungs

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\(\text{FIGURE 1. Effect of endogenous LT synthesis inhibition on mouse survival rate and on fungal recovery.} \) Survival of CFAg- and BSA-immunized mice rechallenged with lethal dose of \(H.\) capsulatum. Mice were monitored for 30 days (\(n = 5–6\)). Fungal burden in lungs (B) and spleen (C). Tissue samples were harvested at 7 days of \(H.\) capsulatum infection. Data are expressed as mean \(\pm\) SEM from two experiments. \(*\), \(p < 0.05\) vs WT BSA; \#, \(p < 0.05\) vs WT CFAg.

\(\text{FIGURE 2.} \) LT\(_B\(_4\) and NO\(_2\)\(^{-}\) synthesis in lung tissue. Enzyme immunoassay quantification of LT\(_B\(_4\) concentrations in lungs from mice submitted to either i.t. PBS injection (uninfected) or CFAg- and BSA-immunized mice and rechallenged with lethal dose of \(H.\) capsulatum. LT\(_B\(_4\) (A) and NO\(_2\)\(^{-}\) (B) levels were quantified in the lungs of animals at day 7 of infection, as described in \textit{Materials and Methods}. Data are presented as mean \(\pm\) SEM and are representative of one of three independent experiments (\(n = 6\)). \(*\), \(p < 0.05\) vs uninfected; \#, \(p < 0.05\) vs WT BSA; \&, \(p < 0.05\) vs WT CFAg.

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of 5-LO−/− CFAg mice were reduced when compared with WT CFAg mice (Fig. 3B).

IFN-γ levels were increased only in lung of WT CFAg mice. The deficiency of 5-LO−/− in CFAg-immunized mice impaired the production of IFN-γ in the lungs of these animals. The levels of IFN-γ in lungs of WT BSA, 5-LO−/− BSA, or 5-LO−/− CFAg mice were not different from those uninfected (Fig. 3C).

The levels of IL-10 were not different between the WT animals immunized with CFAgs or BSA. However, the deficiency of 5-LO enzyme resulted in increased levels of IL-10 when compared with WT CFAgs and WT BSA (Fig. 3D).

*H. capsulatum* infection in either WT BSA or 5-LO−/− BSA increased active TGF-β levels above that seen in uninfected mice. CFAg immunization in WT mice impaired the increase of active TGF-β levels when compared with WT BSA. However, the deficiency of 5-LO resulted in the increase of active TGF-β synthesis in the lung of 5-LO−/− CFAg mice when compared with WT CFAg mice (Fig. 3E).

Levels of IL-1, IL-12, and GM-CSF in lungs of infected WT and 5-LO−/− mice immunized with BSA or CFAgs were similar (data not shown). IL-4 in the lung was undetectable 7 days postinfection (data not shown).

**Effects of 5-LO deficiency on generation of memory T cells**

The generation of memory cells is an essential component of adaptive immunity and mediates protection in peripheral tissues during a secondary immune response to pathogens. We then investigated whether the increased susceptibility of 5-LO-deficient animals could be attributed to altered proportions of memory cells. The results were calculated by percentage of CD4+ or CD8+ T cells with memory phenotype. WT and 5-LO−/− mice were immunized with CFAgs and BSA and challenged with a lethal dose of *H. capsulatum*. At day 7 following infection with *H. capsulatum*, the lungs were analyzed for the presence of memory T cells (Table I and Fig. 4).

5-LO−/− CFAg mice infected with 5-LO−/− CFAg mice (20% CD4+ memory T cells) and 5-LO−/− BSA mice (19.8% CD4+ memory T cells) infected with *H. capsulatum* (Fig. 4). Similar results were observed with CD8+ CD44highCD62Llow/neg cells (Table I). Thus, these data suggest that LTs are involved in the recruitment or generation of memory T cells.

**Effect of LT synthesis blockade during immunization and secondary immune response**

To determine whether the pharmacologic inhibition of LT biosynthesis affected the generation/activation/migration of memory T cells to the infection site, C57Bl/6 mice were treated with MK886 (FLAP inhibitor) only during the immunization or only during
postinfection periods. Consistent with previous results (29), BSA-immunized mice succumbed by 14 days postinfection, whereas 72% of those immunized with CFAs survived until day 60 postinfection.

Inhibition of LT biosynthesis by MK 886 during immunization (CFAs + MK 886 immunization) only partially affected the generation of the adaptive immune response. However, when the animals received MK 886 postinfection (CFAs + MK 886 postinfection), the activation and/or migration of cells to the site of infection were abolished, resulting in 100% mortality (Fig. 5). Thus, these data suggest that in addition to impairment of the generation of memory T cells, the inhibition of LT synthesis abolished the migration and/or activation of memory CD4+ and CD8+ T cells.

In another set of experiments, lungs and spleen were harvested from immunized mice treated or not with MK 886 on days 7 and 14 after infection, and the fungal burden was determined. We did not observe significant differences in the lung and spleen CFU 7 days postinfection (data not shown). However, on day 14 after infection, we found a significant decrease in the lung (p < 0.001) and spleen (p < 0.05) CFUs of CFAg-immunized mice when compared with BSA-immunized mice (Fig. 5, B and C). Fungal burden in lungs and spleen of CFAg-immunized animals that received only MK 886 during immunization (CFAs + MK 886 immunization) was similar to CFAg-immunized mice. However, the treatment of CFAg-immunized mice with MK 886 only after challenge with H. capsulatum (CFAs + MK 886 postinfection) resulted in significant increase in the lung CFU (p < 0.01) and spleen (p < 0.001) when compared with BSA-immunized mice (Fig. 5, B and C).

To determine whether the difference in the susceptibility is associated with the production of the cytokines, TNF-α, IFN-γ, GM-CSF, IL-4, and IL-10, we evaluate its production in the lungs of WT and 5-LO−/− mice immunized with BSA and CFAs and infected for 7 days with H. capsulatum.

<table>
<thead>
<tr>
<th>T Cells</th>
<th>WT BSA</th>
<th>5-LO−/− BSA</th>
<th>WT CFAs</th>
<th>5-LO−/− CFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+CD44hi/CD62Llo/neg/CFUs</td>
<td>13.8 ± 2.24%</td>
<td>18.29 ± 1.68%</td>
<td>47.50 ± 6.19%</td>
<td>23.02 ± 6.01%</td>
</tr>
<tr>
<td>CD8+CD44hi/CD62Llo/neg/CFUs</td>
<td>9.17 ± 1.38%</td>
<td>7.7 ± 0.82%</td>
<td>37.8 ± 7.1%</td>
<td>12.03 ± 0.78%</td>
</tr>
</tbody>
</table>

*WT and 5-LO−/− mice were immunized with CFAs and BSA following the challenge with a lethal dose of H. capsulatum. The results were calculated as percentage of CD4+ or CD8+ T cells with memory phenotype (CD4+CD44hi/CD62Llo/neg or CD8+CD44hi/CD62Llo/neg). Data represent mean ± SEM of six animals per group. Data from one of three experiments are shown.

\(^{a}\) p < 0.05 compared with WT BSA animals.
\(^{b}\) p < 0.05 compared with WT CFAg animals.
\(^{c}\) p < 0.001 compared with WT BSA animals.
Discussion

It is becoming clear that LTs are essential to the host defense of a variety of pathogens (34). However, it is still unknown whether LTs participate in the generation of a secondary immune response during microbial infections. The present study demonstrates that protection of WT mice immunized with CFAgs against *H. capsulatum* infection was associated with increased LTB4 recruitment of memory T cells, and increased production of NO and IFN-γ. Moreover, there was a decreased production of TGF-β and IL-10 in the lungs of these animals in a manner dependent on LT production.

CFAg immunization conferred protection in WT mice, but not 5-LO−/− mice (Fig. 1A). In addition, WT CFAg mice presented more than 10-fold (1.1 log_{10}) less *H. capsulatum* CFU in the lungs when compared with other groups (WT BSA, 5-LO−/− BSA, and 5-LO−/− CFAgs) (Fig. 1B). Accordingly, the lungs of WT-BSA mice presented a 3-fold increase in LTB4 production when compared with uninfected mice. These augmented levels were even higher (~4-fold) in WT CFAg mice, but not 5-LO−/− CFAgs, which correlates with the mortality data. Unexpectedly, in accordance with Bafica et al. (35), LTB4 production was detected in the 5-LO−/− groups. We do not know why such levels can be detected in our condition; we speculate that it might be some undescribed metabolic pathway or if they represent some intrinsic error on the detection kit. These results suggest that LTB4 production is up-regulated during *H. capsulatum* infection and its production is associated with the protective immunity attained by CFAg immunization.

The role of NO in host resistance during murine histoplasmosis is well known (36, 37). In fact, LT inhibition in primary histoplasmosis infection decreases NO production and increases mice susceptibility (17). Our results demonstrated that WT CFAg mice were the only ones that survived the lethal inoculum used, and this protection was associated with a 4-fold increase of NO in their
lungs (Fig. 5). However, $5\text{-LO}^{-/-}$ CFAg mice presented lower levels of NO, which correlates with a lack of protection in these animals. Serezani et al. (15) showed that LT$_B$ induces NO secretion and inductive NO synthase expression in murine macrophages and NO inhibition abolished LT$_B$ effects on macrophage leishmanicidal activity.

It is known that _H. capsulatum_ immunogens such as heat shock protein 60 (Hsp60) are protective and trigger protective immune response associated with CD4$^+$ T cells and production of IFN-$\gamma$, IL-12, and IL-10 (38). Thus, we sought to investigate whether the absence of 5-LO affects survival of _H. capsulatum_-infected mice through the modulation of cytokines involved in the process of T cell activation by measuring cytokines levels. TGF-$\beta$ and IL-10 are cytokines involved in the host immunological homeostasis, and also display potent immunosuppressive activities in both phagocytes and lymphocytes (39). We observed that the increased susceptibility of 5-LO$^{-/-}$ BSA and 5-LO$^{-/-}$ CFAg mice was associated with an up-regulation of TGF-$\beta$ and IL-10, but not protected WT CFAg mice. In our model, we suggested that CFA immunization increases LT$_B$ production, which might impair excessive TGF-$\beta$ production and leads to progressive deterioration of the protective immune response. However, the effect of LT$_B$ on TGF-$\beta$ production remains to be determined.

Memory T lymphocyte confers protection in peripheral tissues during a secondary immune response to pathogens. We have previously demonstrated that DTH in histoplasmosis is associated with the degree of the immune response (29). In this study, we demonstrated that WT mice immunized with CFAgs exhibited a strong DTH reaction, and 5-LO$^{-/-}$ mice developed a smaller reaction when compared with WT mice (data not shown). These results suggest that products of the 5-LO pathway could be important mediators involved in the generation of memory cells, and consequently to the development of a protective immune response to _H. capsulatum_. When we evaluated whether the increased susceptibility of 5-LO-deficient animals could be attributed to altered proportions of memory cells in the lung, our results demonstrated that WT CFAg mice are able to recruit at least 2-fold more memory CD4$^+$ T cells in the lungs after _H. capsulatum_ infection compared with 5-LO$^{-/-}$ mice (Fig. 4). In keeping with these results, Gomez et al. (40) demonstrated _H. capsulatum_-derived rHsp60 increases memory V$\beta$6 and V$\beta$ CD4$^+$ T cells in infected mice. In addition, the depletion of V$\beta$8/8.2$^+$ CD4$^+$ T cell subpopulation abolished protection induced by _H. capsulatum_-derived rHsp60. Although CD8$^+$ T cells appear not to be required to confer protection during murine histoplasmosis, they contribute to the optimal clearance of _H. capsulatum_ in the organs (2). In addition, a CD8$^+$-dependent immunity to _H. capsulatum_ may be achieved by vaccination of CD4$^+$-depleted mice (41). We have observed a 4-fold increase in CD8$^+$ T cells of _H. capsulatum_-infected WT CFAg mice, in comparison with 5-LO$^{-/-}$ CFAg mice (data not shown). Recently, it has been shown that LT$_B$ is a chemoattractant factor for effector T cells (27, 28), but not naive T cells or central memory CD8$^+$ T cells. However, the specific role of LT$_B$ in the recruitment of CD8$^+$ T cells during pathogen infection is unknown. Our results demonstrate that the immunization of WT CFAg mice induces the generation and recruitment of memory CD4$^+$ and CD8$^+$ T cells to the infection site. Moreover, it seems that products of 5-LO activity (e.g., LTs) may affect the establishment of a protective response conferred by the immunization as well as recruitment and activation of memory T cells during subsequent infection. Furthermore, the migration of effector cells from WT mice into the inflamed peritoneal cavity is 3 times higher than that observed in BLT1-deficient mice (27, 28). The pharmacological approach using MK886 (a FLAP inhibitor) did not affect the generation of the adaptive immune response (CFAgs + MK$_{Immunization}$), but rather the activation and/or migration of cells to the infection site. This assumption is supported by the same survival rate and fungal burden presented by WT CFAg mice as well as MK886-treated mice during the immunization period (CFAgs + MK$_{Immunization}$). However, when mice were treated with MK886 after challenge with _H. capsulatum_ (CFAgs + MK$_{Postinfection}$), all animals displayed higher fungal dissemination and dramatic reduction of protective immunity. Moreover, such as observed in 5-LO$^{-/-}$ CFAgs, the inhibition of LTs by MK 886 postinfection with _H. capsulatum_ resulted in the inhibition of protective cytokines, such as IFN-$\gamma$. These data strongly suggest that LT deficiency (both genetic and pharmacologic) during infection is more associated with impairment in the migration and/or activation of memory CD4$^+$ and CD8$^+$ T cells, than the generation of them.

The lack of 5-LO products in special LTs is associated with the inhibition of recruitment and/or activation of effector memory T cells during _H. capsulatum_ infection, resulting in mortality of immunized mice and increased CFU in the lung and spleen. LT$_B$ affects many cells involved in both the innate and adaptive immune response. LT$_B$ enhances phagocytosis, microbicidal activity, as well as generation/modulation of chemoattractants, such as chemokines and cytokines. In addition, LT$_B$ enhances dendritic and T cell migration and activation and mounting of the immune response. Because LT$_B$ acts on both arms of the immune response and because it is rapidly produced during infections or inflammatory conditions (seconds to minutes), it is an important target for adjuvant studies.

Acknowledgments

We thank Marc Peters-Golden for valuable discussions and for helpful comments, and C. Lewis for revising the manuscript. We are grateful to Merck Frosst Canada for furnishing the MK 886.

Disclosures

The authors have no financial conflict of interest.

References


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