LIGHT contributes to early but not late control of Mycobacterium tuberculosis infection

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Abstract

The TNF superfamily member, LIGHT, contributes to optimal T-cell activation in vitro through co-stimulation of dendritic cell cytokine production; however, its role in T-cell-mediated control of intracellular bacterial infections is unknown. Protective immunity against Listeria monocytogenes and Mycobacterium tuberculosis infection requires both antigen-specific CD4⁺ and CD8⁺ T cells. Using LIGHT-deficient mice we determined that LIGHT was necessary for optimal re-stimulation of anti-listerial CD8⁺ T cells in vitro. By contrast, LIGHT⁻/⁻ mice infected with L. monocytogenes generated equivalent T-cell responses and controlled the infection as effectively as normal C57BL/6 mice. Following M. tuberculosis infection, LIGHT⁻/⁻ mice showed a significant increase in bacterial replication in the lungs at 4 weeks, but by 6 weeks had controlled the infection. Analysis of T-cell responses in vivo revealed that LIGHT was dispensable for the activation of primary T-cell responses and the production of IL-12 and IFN-γ. In addition, LIGHT was not required for the induction of memory T-cell responses to anti-mycobacterial DNA or BCG vaccines and for subsequent protection against tuberculosis challenge. Therefore, LIGHT contributes to the optimal co-stimulation of anti-listerial CD8⁺ T-cell responses in vitro and to the early control of M. tuberculosis infection; however, other mechanisms compensate for LIGHT deficiency in the control of these pathogens in vivo.

Keywords: cytokines, inflammation, intracellular pathogens, LIGHT, T-cell co-stimulation

Introduction

The TNF superfamily (TNFSF) of ligands and receptors are critical regulators of numerous aspects of pathogen-induced immune responses. TNFSF14 LIGHT [Lymphotoxin-like, exhibits Inducible expression, competes with Herpes Simplex Virus Glycoprotein D for Herpesvirus Entry Mediator (HVEM) receptor, expressed by T lymphocytes] is expressed by activated T cells, NK cells and immature dendritic cells (DCs). In vitro LIGHT demonstrates an array of co-stimulatory activities, including driving a ‘type I’ immune response (1–4), primarily through interaction with the HVEM receptor (5), and lymphotoxin-β receptor (LTβR). LIGHT up-regulates CD86 expression on DCs via NF-κB signal transduction. Furthermore, LIGHT stimulates T-cell activation and the preferential induction of the Tₘ₁ profile of cytokines, including increased expression of IFN-γ, again through stimulation of the NF-κB pathway (2–5). Stimulation of DCs with LIGHT increases secretion of IL-12, IL-6 and TNF, and this effect is enhanced by co-stimulation with CD40L, which increases DC maturation (1, 6). LIGHT has also been found to induce optimal CD8⁺ T-cell activation and proliferation (3, 7), and the differentiation of naive CD8⁺ T cells into cytolytic (1, 7–10) and cytokine-secreting lymphocytes (9). The immunostimulatory effects of LIGHT in potentiating T-cell activation, proliferation and cytokine secretion are generally stronger for CD8⁺ T cells than CD4⁺ T cells, although increased IFN-γ production has been reported in both T-cell subsets following LIGHT stimulation. Moreover, LIGHT was required for the reactivation of secondary Tₘ₁ responses (11, 12). In contrast, there have been few studies that have examined the requirement for LIGHT in the immune response to infections in vivo (13–15). Therefore, we have examined the capacity of LIGHT-deficient mice to mount an immune response and control intracellular bacterial infections, specifically acute infection induced by Listeria monocytogenes and chronic disease resulting from Mycobacterium tuberculosis infection. In addition, we have investigated the role of LIGHT in the expression of optimal memory T-cell responses to infection, as well as examining its role in the expression of optimal memory responses to intracellular bacterial infection and immunization.
Methods

Animals
C57BL/6 mice at 6–10 weeks of age were purchased from the Animal Resource Centre (Perth, Australia). LIGHT-gene-deficient mice (LIGHT\(^{-/-}\)), generated as previously described (9), were the kind gift of Klaus Pfeffer and were backcrossed 10 times onto a C57Bl/6 background. All mice were kept in specific pathogen-free conditions at the Centenary Institute Animal Facility. All experiments were undertaken with the approval of the University of Sydney Animal Ethics Committee.

Experimental infection and immunization
Listeria monocytogenes infection. C57Bl/6 wild-type (WT) and LIGHT\(^{-/-}\) mice received 2000 c.f.u. of Listeria EGD (16) intravenously, and at specified time points, listerial numbers were determined by incubating serial dilutions of organ homogenates on Tryptic Soy agar (Difco, Detroit, MI, USA) at 37°C for 24 h.

Mycobacterium tuberculosis infection. For vaccination experiments WT and LIGHT\(^{-/-}\) mice received either two doses of 100 μg per DNA vector by intramuscular injection in the tibialis anterior muscle 2 weeks apart or a single dose of 5 \(\times\) 10⁵ c.f.u. of M. bovis BCG Pasteur by subcutaneous injection at the base of the tail. The DNA vaccine pcDNA-85B, expressing antigen-85 (Ag85) of M. tuberculosis was produced as previously described (17). Six weeks after immunization, naive and immunized mice were infected with 100 c.f.u. of M. tuberculosis H37Rv by aerosol in a Middlebrook airborne infection apparatus (Glascol, Terre Haute, IN, USA). Mycobacterial loads were assessed by culture of serial dilutions of organ homogenates on 7H11 agar at 37°C for 3 weeks (Difco). Inflammatory involvement in the lung was determined using Adobe Photoshop to calculate the percentage of the whole lung with inflammatory cell involvement after subtracting the background cellularity in normal lung.

In vitro infection. DCs were generated from bone marrow as previously described (18). DCs were infected with L. monocytogenes at a 1:1 ratio in RPMI media with 10% foetal calf serum (Trace, Sydney, Australia), 2 mM l-glutamine and 0.5 μM 2-mercaptoethanol (Sigma) and then antibiotics (100 U ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin were added to the media to prevent bacilli overgrowth. Splenic CD8\(^{+}\) T cells, from WT and LIGHT\(^{-/-}\) mice, infected 4–6 weeks previously with 2000 c.f.u. of Listeria, were purified by positive selection using an automated magnetic cell sorter (Miltenyi Biotec). CD8\(^{+}\) T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with either Listeria-infected DCs or in wells coated with αCD3 and αCD28. T-cell proliferation was determined by flow cytometry using a FACS Canto (Becton Dickinson, Sydney, CA, USA).

Cytokine production. Mediastinal lymph node or lung cells (2 \(\times\) 10⁵ cells per well), isolated from M. tuberculosis-infected mice, were stimulated with M. tuberculosis Ag 85 complex or culture filtrate protein (kindly provided by Dr J. Belisle under the National Institute of Health TB research material and vaccine testing contract NO1-AI-75320) as previously described (19). Splenocytes from Listeria-infected mice were cultured with live or heat-killed (80°C for 2 h) L. monocytogenes for 72 h before supernatants were harvested and IFN-γ and IL-12 measured by ELISA (19).

RNA purification and real-time quantitative PCR
Liver tissue was homogenized in RNAzol tri-reagent (Sigma) and stored at −70°C. Total RNA extraction, RNA purification, reverse-transcription–PCR and real-time quantitative PCR were performed as previously described (19). Primers for all target genes, listed in Table 1, were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA, USA) and made by Proligo (Sydney, Australia).

Statistical analysis
Statistical analysis of the results from immunological assays and log-transformed bacterial counts were conducted using analysis of variance or Student’s t-test. Fisher’s least protected significance difference post hoc test was used for pairwise comparison of multi-grouped data sets. Differences with \(P < 0.05\) were considered significant.

Results
LIGHT is required for optimal in vitro CD8\(^{+}\) T-cell proliferation in Listeria infection
LIGHT co-stimulation has been reported to be necessary for optimal proliferation of T lymphocytes (8, 20), in particular CD8\(^{+}\) T cells (3, 7), stimulated with polyclonal activators such as staphylococcal enterotoxin B and anti-CD3. The proliferative capacity of CD8\(^{+}\) T cells from LIGHT-deficient mice was assessed following L. monocytogenes infection. Mice were infected for 4–6 weeks before CD8\(^{+}\) T cells were isolated, labelled with CFSE and cultured with either Listeria-infected DCs or with anti-CD3 and anti-CD28. After 72 h culture, CD8\(^{+}\) T cells from WT mice had proliferated more extensively to CD3 and CD28 than CD8\(^{+}\) T cells from LIGHT mice (Fig. 1B). T cells from WT mice had also proliferated more extensively following stimulation with Listeria-infected DCs from either WT or LIGHT\(^{-/-}\) mice, than T cells from LIGHT mice had proliferated (Fig. 1A and C). Therefore, LIGHT was required for the optimal activation of CD8\(^{+}\) L. monocytogenes-specific T cells in vitro.

LIGHT is dispensable for protection against primary Listeria infection
Protective immunity to Listeria infection requires CD8\(^{+}\) T cells. In order to establish whether the advantage provided by LIGHT co-stimulation observed in vitro translated into a role in protection against Listeria infection in vivo, WT and

Table 1. RTQ-PCR primer sets

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Direction</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>Murine GAPDH</td>
<td>Forward</td>
<td>CTCCACCTCACGGCAAATTCA</td>
</tr>
<tr>
<td>Murine IFN-γ</td>
<td>Reverse</td>
<td>CGCTCTGGAAGATGTTGAT</td>
</tr>
<tr>
<td>Murine IL-12</td>
<td>Forward</td>
<td>CAGCAACACAGCAGGGGAAA</td>
</tr>
<tr>
<td>Murine IL-12</td>
<td>Reverse</td>
<td>GCTGGATTCCGGCAACAG</td>
</tr>
<tr>
<td>Murine IL-12</td>
<td>Forward</td>
<td>CAGAAAGCTAACCATCTCCTG</td>
</tr>
<tr>
<td>Murine IL-12</td>
<td>Reverse</td>
<td>CGGAGTAATTGGGTCTTCA</td>
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LIGHT−/− mice were infected with *L. monocytogenes* and the course of infection was determined. The pattern of bacterial growth was similar in both WT and LIGHT−/− mice, with the *Listerial* burden peaking between days 3–5 post-infection in both strains of mice (Fig. 2). Bacilli cleared from the spleen between days 7 and 11. Clearance from the liver was slower and while there was a trend for the bacilli to clear more slowly from the livers of the LIGHT−/− mice, this difference did not reach significance.

As LIGHT has been reported to play a role in stimulating type I immune responses (1–4), the transcription and production of the cytokines, IFN-γ and IL-12, were assessed in *ex vivo* cultured splenocytes from *Listeria*-infected WT and LIGHT−/− mice (Fig. 2B). IL-12 mRNA production followed a similar pattern in WT and LIGHT−/− mice, peaking in both...
by day 7 post-infection (Fig. 2B), with no significant differences in IL-12 protein production detected in cultured splenocytes from WT and LIGHT−/− mice (Fig. 2C). The peak of IFN-γ mRNA occurred in both strains of mice between days 3 and 7 post-infection (Fig. 2B); interestingly, the LIGHT−/− mice transcribed greater amounts of IFN-γ mRNA on days 5 and 7. In two of three experiments, higher levels of IFN-γ protein were detected at day 5, when cells were cultured in the live or heat-killed Listeria (Fig. 2C); however, this did not lead to enhanced clearance of the bacilli.

LIGHT deficiency is associated with a delay in control of primary tuberculosis infection

In parallel to our studies to determine the function of LIGHT in immunity to acute L. monocytogenes infection, we analysed the requirement for LIGHT in the generation and expression of protective immunity to chronic M. tuberculosis infection. WT and LIGHT−/− mice were infected with a low-dose aerosol of M. tuberculosis bacilli (Fig. 3). By 4 weeks post-infection, LIGHT−/− mice displayed ~0.5log10 (P < 0.006) higher bacterial burden in the lungs compared with the WT mice in five independent experiments. This effect, however, was transient, and by 6 weeks post-infection, there were no significant differences in bacterial burden between the two groups (Fig. 3A). This delay in control of M. tuberculosis infection was not associated with a delay in the induction of an antigen-specific T-cell response, as at 4 weeks post-infection, T cells from the lungs and lymph nodes of WT and LIGHT−/− mice exhibited a similar pattern of T-cell activation as evidenced by CD62L and CD44 expression (data not shown) and produced antigen-specific IFN-γ following ex vivo stimulation (Fig. 3C). Indeed, the lung cells from LIGHT−/− mice produced more IFN-γ than lung cells from WT mice (reaching statistical significance in two of three experiments). This may be caused by the higher bacterial loads in the lungs at this time stimulating the recruitment and/or proliferation of more antigen-specific T cells. The absence of LIGHT, however, did not affect granuloma formation as both strains of mice showed similar inflammatory involvement in the lungs at 4 weeks post-infection (WT lung 13.86 ± 3.34% and LIGHT 13.74 ± 3.78%) and analysis of cellular composition revealed similar numbers of T and B cells, macrophages and neutrophils in the lungs of WT and LIGHT−/− M. tuberculosis-infected mice.

LIGHT is not required to generate a memory T-cell response to Mycobacterium tuberculosis

LIGHT has also been reported to have a role in the generation and execution of an optimal memory response (11, 12). Therefore, WT and LIGHT−/− mice were immunized with either the M. tuberculosis vaccine pcDNA-85B (17) or M. bovis BCG and 6 weeks later challenged with aerosolized M. tuberculosis H37Rv (Fig. 4A). Analysis of antigen-specific IFN-γ production by splenocytes of vaccinated WT and LIGHT−/− mice demonstrated similar levels of IFN-γ following ex vivo stimulation with Ag 85B in mice vaccinated with pcDNA-85B (WT: 390 ± 96 pg per 10^6 cells and LIGHT−/−: 261 ± 85 pg per 10^6 cells). As described above, the LIGHT-deficient mice receiving the control DNA vaccine had a significantly higher bacterial load in the lungs at 4 weeks after the M. tuberculosis challenge than WT control-vaccinated mice (Fig. 4A). Nevertheless, the absence of LIGHT did not affect the ability of the mice to generate and express a protective immune response to pcDNA-85B or BCG. Following immunization, both WT and LIGHT−/− mice displayed significant reductions in M. tuberculosis load compared with unvaccinated mice, at 4 weeks post-infection. The protective effect of pcDNA-85B and BCG were similar in the spleens of LIGHT−/− and WT mice (Fig. 4B), as too were the levels of IFNγ produced by lymph node cultures (Fig. 4C). Therefore, although LIGHT is required for optimal control of M. tuberculosis infection at 4 weeks, it is not required for the modulation of protective immune response to BCG and DNA vaccination.
Discussion

Numerous studies have demonstrated that LIGHT is required in vitro for the optimal induction of Type 1 T-cell immune responses. Through binding to both HVEM and LTβR, LIGHT induces the maturation of immature DCs, resulting in the production of IL-12, IL-6 and TNF (1, 6, 12) and increased activation of both CD4+ and CD8+ T cells (2–4, 8, 20, 21). The current study confirmed that LIGHT is required for optimal proliferation of CD8+ T cells in vitro, as CD8+ T cells from Listeria-infected LIGHT−/− mice did not proliferate as rapidly as T cells from WT mice when stimulated with either anti-CD3 and anti-CD28 or with DCs infected with Listeria (Fig. 1). However, the importance of LIGHT for stimulating protection against intracellular pathogens in vivo has not been resolved. Transgenic mice, which constitutively express LIGHT, developed T cells with an activated phenotype and enhanced Tc1 cytokine activity (2, 21). Further, these mice displayed increased inflammation and potential for autoimmune-mediated organ damage. In parallel, the absence of LIGHT led to diminished T-cell responses, particularly in CD8+ T cells (7, 9). In the current study, LIGHT was required for optimal control of M. tuberculosis but not of L. monocytogenes infection.

In in vitro models, LIGHT functions to enhance the activity of DCs for stimulating primary and memory T-cell responses, up-regulating the co-stimulatory molecule CD86 and increasing IL-12 secretion (11, 12). Following Listeria infection, analysis of mRNA levels revealed that both WT and LIGHT−/− mice demonstrated increased expression of IL-12 transcript (Fig. 2B). Furthermore, ex vivo culture of splenocytes from infected WT and LIGHT−/− mice produced equivalent amounts of IL-12 protein (Fig. 2C). In two immunization studies, with a DNA vaccine expressing either M. tuberculosis Ag 85 or M. bovis BCG, the deficiency of LIGHT had no effect on the efficacy of DC activation of naive T cells and their subsequent production of IFN-γ. Moreover, although naive LIGHT−/− mice showed increased bacterial load at 4 weeks post-challenge, both LIGHT and WT mice responded to M. tuberculosis DNA or BCG vaccination in a comparable protective manner, indicating that in vivo compensatory mechanisms in LIGHT−/− mice lead to the generation of an equivalent T-cell response.

LIGHT has also been implicated in stimulating CD4+ T-cell responses by increasing expression of pro-inflammatory cytokines, particularly IL-2 (9, 22). Indeed, control of pulmonary tuberculosis, which depends strongly on a vigorous antigen-specific CD4+ T-cell response (23), was delayed in the absence of LIGHT, in the lungs at 4 weeks post-infection in five independent experiments. This reduced protective immunity was resolved by 6 weeks. Ehlers et al. (13) also found no difference in bacterial loads between WT and LIGHT−/− mice at 6 weeks post M. tuberculosis infection, but they had not examined the effect at 4 weeks. Thus, LIGHT aids control of M. tuberculosis early in infection, but this defect is compensated for by other mechanisms later in infection.

Several studies have identified the activity of LIGHT as a co-stimulatory molecule for increasing the activation and differentiation of naive CD8+ T cell into cytokine-producing cytolytic T cells (1, 3, 7–10). Clearance of L. monocytogenes infection requires a strong CD8+ T-cell response (24); however, mice deficient in LIGHT were equally capable of controlling the Listeria dose as their WT counterparts. Analysis of IFN-γ mRNA levels in the liver demonstrated that LIGHT was not necessary for optimal expression of cytokine transcripts (Fig. 2B). Furthermore, ex vivo culture of LIGHT−/− splenocytes confirmed that they produced equivalent antigen-specific IFN-γ as splenocytes from WT mice (Fig. 2C). These results are consistent with a recent study reporting that LIGHT was not required to control Influenza A virus (15). Although the clearance of Influenza A virus from the lungs requires strong CD8+ T-cell responses, control of influenza viral replication and the anti-influenza CD8+ T-cell responses were comparable in WT and LIGHT−/− mice.

Interestingly, in our experimental models LIGHT was not essential for the generation or expression of the inflammatory response and subsequent protective granuloma formation that developed in response to both M. tuberculosis and Listeria infection. This contrasts with a report describing an essential role for LIGHT in Listeria-induced hepatitis (14). In that study, LIGHT functioned as a pro-inflammatory cytokine
and the absence of LIGHT provided a significant survival advantage to mice given twice the LD<sub>50</sub> for <i>Listeria</i>. The variation in requirement for LIGHT in the two models may be due to differences in the bacterial dose used. It is possible that, when extremely high antigenic stimulation is used, as in the <i>Listeria</i>–hepatitis study or in <i>vitro</i> studies, the co-stimulatory and pro-inflammatory functions of LIGHT are accentuated. In contrast, when non-lethal doses of bacteria are used, more in keeping with natural infection, other pro-inflammatory molecules may signal through the same, or similar, receptors and are able to compensate for the absence of LIGHT. One possible molecule is lymphotoxin-α, which signals through common receptors to LIGHT, and is essential for protective immunity to both <i>M. tuberculosis</i> and <i>L. monocytogenes</i> infection (25, 26). A number of other members of the TNFSF are also required for optimal protective immunity against both <i>M. tuberculosis</i> and <i>Listeria</i> infection (reviewed in ref. 27) and signalling through one or more of these molecules may also provide additional compensatory stimulation in the absence of LIGHT.

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