Modulation of LIGHT-HVEM Costimulation Prolongs Cardiac Allograft Survival

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Abstract

LIGHT (TNFSF14), a tumor necrosis factor superfamily member expressed by activated T cells, binds to herpes virus entry mediator (HVEM) which is constitutively expressed by T cells and costimulates T cell activation in a CD28-independent manner. Given interest in regulating the effector functions of T cells in vivo, we examined the role of LIGHT-HVEM costimulation in a murine cardiac allograft rejection model. Normal hearts lacked LIGHT or HVEM mRNA expression, but allografts showed strong expression of both genes from day 3 after transplant, and in situ hybridization and immunohistology-localized LIGHT and HVEM to infiltrating leukocytes. To test the importance of LIGHT expression on allograft survival, we generated LIGHT$^{-/-}$ mice by homologous recombination. The mean survival of fully major histocompatibility complex–mismatched vascularized cardiac allografts in LIGHT$^{-/-}$ mice (10 days, $P < 0.05$) or cyclosporine A (CsA)-treated LIGHT$^{+/+}$ mice (10 days, $P < 0.05$) was only slightly prolonged compared with LIGHT$^{+/+}$ mice (7 days). However, mean allograft survival in CsA-treated LIGHT$^{-/-}$ allograft recipients (30 days) was considerably enhanced ($P < 0.001$) compared with the 10 days of mean survival in either untreated LIGHT$^{-/-}$ mice or CsA-treated LIGHT$^{+/+}$ controls. Molecular analyzes showed that the beneficial effects of targeting of LIGHT in CsA-treated recipients were accompanied by decreased intragraft expression of interferon (IFN)-γ, plus IFN-γ–induced chemokine, inducible protein-10, and its receptor, CXCR3. Treatment of LIGHT$^{+/+}$ allograft recipients with HVEM-Ig plus CsA also enhanced mean allograft survival (21 days) versus wild-type controls receiving HVEM-Ig (mean of 7 days) or CsA alone ($P < 0.001$). Our data suggest that T cell to T cell–mediated LIGHT/HVEM-dependent costimulation is a significant component of the host response leading to cardiac allograft rejection.

Key words: transplantation • allograft rejection • T cell activation • costimulation • TNF superfamily

Introduction

By binding to structurally related but distinct receptors, TNF cytokine family members play key roles in inflammation, immunity, and homeostasis (1). An extensive literature exists on how antigenic stimulation of T cells in the absence of costimulation induces anergy, and addition of a second or costimulatory signal such as ligation of CD28 by B7–1 (CD80) or B7–2 (CD86) on dendritic cells (DCs) promotes a primary T cell response. Various CD28-independent costimulatory pathways are also now recognized. In particular, accruing data suggest that TNF superfamily members can function as costimulatory molecules in T cell activation by delivering a second signal for T cell proliferation, cytokine production, and Th1/Th2 differentiation (2).

LIGHT, whose name is derived from homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus (HSV) glycoprotein D for herpesvirus entry mediator (HVEM/TR2), a receptor expressed by T lymphocytes (3), is a recently identified member of the human (4) and mouse (5) TNF superfamily. LIGHT is a 29-kD type II transmembrane protein produced by activated T cells (4), as well as monocytes and granulocytes (6), and immature DCs (7). Apart from its receptor on T cells, HVEM, LIGHT binds to the lympho-

toxin β receptor (LTβR) on stromal cells (4), and the DcR3/TR6 soluble protein (8).

In vitro, LIGHT expression induces potent CD28-independent costimulatory activity, leading to NF-κB activation, production of IFN-γ and other cytokines, and T cell proliferation in response to allogeneic DCs (7, 9). In vivo blockade studies show LIGHT is involved in promotion of cytolytic T cell responses to tumors (5) and the development of GVHD (5), and transgenic overexpression of LIGHT within T cells leads to T cell expansion and causes various severe autoimmune diseases (10–12). No data are yet available concerning the role of the LIGHT/HVEM pathway in solid organ transplantation. In this study, we generated LIGHT deficient mice by gene targeting and demonstrated that LIGHT is a regulator of host allogeneic T cell activation and allograft rejection.

Materials and Methods

Generation of LIGHT$^{-/-}$ Mice. A targeting vector was constructed using a 10.5-kb genomic fragment containing exons 1–4 of the LIGHT gene. A 0.8-kb sequence around exon 1, including ATG, was deleted and replaced by pMC1 neo, the targeting vector was linearized and electroporated into ES cells, and LIGHT$^{+/-}$ ES cell clones were selected in media containing G418 and Gancyclovir, as described previously (13). The correctly targeted event was screened by Southern blot analysis, and chimeric mice were derived by blastocyst injection. Offspring of LIGHT$^{+/-}$ mice were crossed to produce LIGHT$^{-/-}$ mice. Mice used in this study were on a B6/129 background. Since human LIGHT maps closely to the third complement protein (C3) (14), plasma C3 levels in LIGHT$^{-/-}$ versus LIGHT$^{+/+}$ were screened by radial immunodiffusion using goat anti–mouse C3 antibody (Cappel, ICN Pharmaceuticals).

Transplantation. Male 6-wk-old BALB/c (H-2^d), 129Sv/J (H-2^b), C57BL/6 (H-2^b), and B6/129 (H-2^b) mice were purchased from The Jackson Laboratory and maintained in our specific pathogen-free facility. Heterotopic abdominal cardiac allografting was done with the use of BALB/c donors and B6/129 recipients, as well as in some experiments, pure 129 or B6 recipients, using six allografts per experimental group (15); data reported are from BALB→B6/129. Graft function was monitored twice daily by palpation, and rejection confirmed by laparotomy and histology. At harvest at rejection or at the time indicated, midventricular samples were fixed in formalin for light microscopy or snap-frozen in liquid nitrogen and stored at −80°C for immunohistology and RNA studies.

Posttransplant Therapies. LIGHT$^{-/-}$ or LIGHT$^{+/+}$ allograft recipients were treated with cyclosporin A (CsA) (Sigma-Aldrich), dissolved in olive oil and administered daily (10 mg/kg intraperitoneally) for 14 d after transplant. Additional LIGHT$^{+/+}$ recipients were treated with a mHVEM-human IgG1 (HVEM-Ig) fusion protein, which was generated using the extracellular region of HVEM (3), as described previously (4); HVEM-Ig was purified by affinity chromatography and shown by gel electrophoresis to be free of aggregation. Mice received 100 μg of HVEM-Ig daily, from the time of transplantation until day 14, or control human IgG1 (Sigma-Aldrich); levels of endotoxin contamination of these preparations were <1 pg/ml as shown using a Limulus amebocyte lysate assay (Associates of Cape Cod).

RNA Isolation and RT-PCR Analysis of HVEM and LIGHT Expression. Total RNA was prepared from each recipient's heart and cardiac allograft using guanidine-thiocyanate, and RNA integrity was confirmed by electrophoresis (16). RNA was reverse transcribed at 45°C for 60 min, 95°C for 3 min, and placed on ice. For LIGHT detection, primers (mLIGHT1F, 5-ATGGAGAGT-GTGGTACAGCCTTC-3; mLIGHT1R, 5-GACCATGA-AAGCTCCGAAATAGG-3) were used. For HVEM, primers (mHVEM1F, 5-ATGGAACCTCTCCCAGGATGGG-3; mHVEM1R, 5-TCAGTTGGAGGCTGTCTCCTCC-3) were used. Each three-step thermal cycle for routine PCR analysis included 30 s at 95°C, 30 s at 60°C, and 60 s at 72°C; additional PCR reactions were performed for 35 cycles. PCR products were visualized by ethidium bromide staining of agarose gels and identified by size markers. A negative control containing all reagents except cDNA was included in each PCR analysis.

In Situ Hybridization (ISH). LIGHT riboprobes were synthesized from T3 (sense probe) and T7 (antisense probe) promoters, labeled with biotin-UTP (Roche Laboratories) and used to localize LIGHT mRNA expression within cardiac tissue sections by ISH as described previously (17).

RNase Protection Assays. RNA was evaluated by the Riboquant system (BD PharMingen), using mouse template sets mCK-1 and mCK-3b for cytokines, mCK5 for chemokines, and mCR5 and mCR6 for CC and CXC chemokine receptors, respectively. A riboprobe for mouse CXCR3 was prepared in house (18). Methods for in vitro transcription, riboprobe purification and use, plus densitometric analysis and normalization of data to L32 and GAPDH gene expression, were as described previously (17).

Immunopathology. Hearts were fixed in formalin, paraffin-embedded, and stained with hematoxylin and eosin. Cryostat sections fixed in paraformaldehyde-lysine-periodate were stained by immunoperoxidase using mAbs to mouse leukocytes (BD PharMingen), anti-LIGHT antibody (Santa Cruz Biotechnology), or isotype-matched controls (15).

Results and Discussion

Given the importance of LIGHT expression in the development of T cell activation in vivo, including in host T cell–dependent antitumor responses (5, 6), we undertook a serial study of LIGHT and HVEM expression during the development of cardiac allograft rejection across a full MHC disparity. Total RNA was prepared from each recipient's heterotopic transplant and own native heart after collection on days 3 and 7 after transplant, and HVEM and LIGHT mRNA expression were analyzed by RT-PCR. Negligible LIGHT or HVEM mRNA was detected in native hearts, but both LIGHT and HVEM were markedly upregulated at days 3 and 7 after transplant (Fig. 1 a). LIGHT mRNA expression was localized by ISH to cells with the morphology of small mononuclear cells (Fig. 1 b), and similar localization of HVEM mRNA, plus focal endothelial expression, was detected by ISH for HVEM (data not shown). Immunoperoxidase studies confirmed expression of LIGHT protein by infiltrating leukocytes (Fig. 1 c); labeled cells included lymphocytes, plus some inflammatory macrophages and tissue DCs.
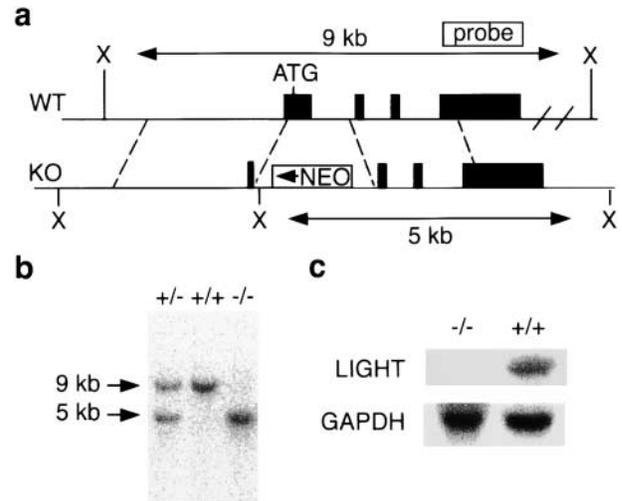
To determine the role of LIGHT expression in allograft rejection, we used homologous recombination to disrupt exon 1, containing the ATG initiation codon, of the LIGHT
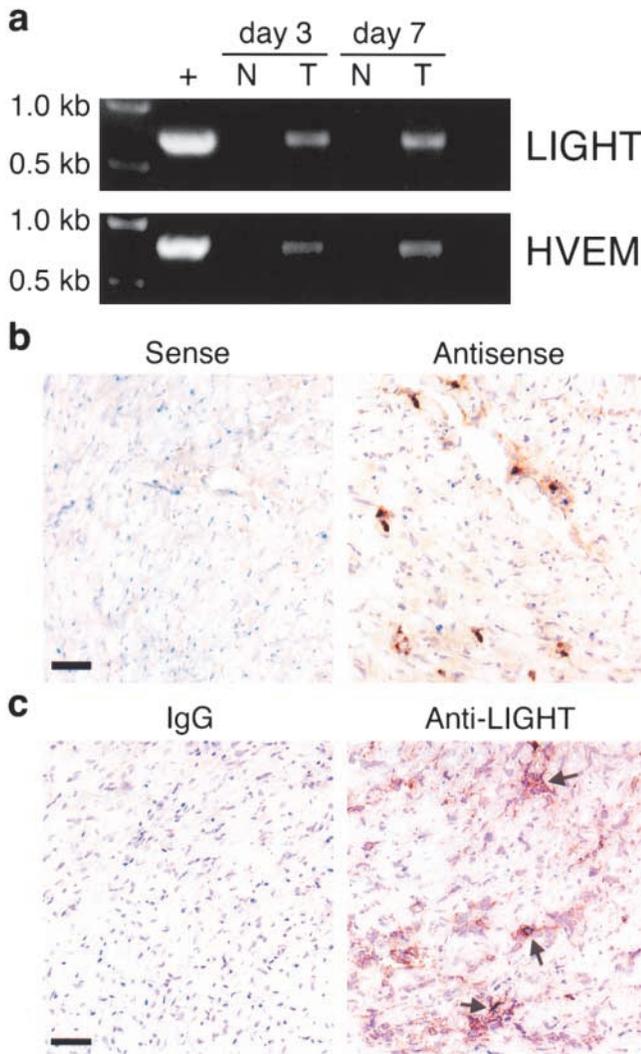
Figure 1. Analysis of LIGHT and HVEM expression during allograft rejection. (a) Northern analysis of LIGHT and HVEM in serial cardiac transplants (T) rejected by day 7 versus each recipient's native heart (N). (b) ISH analysis of graft LIGHT mRNA expression at day 7 posttransplant is restricted to focal mononuclear cells (sense control, left). (c) Immunoperoxidase staining shows LIGHT protein expression by infiltrating mononuclear leukocytes at day 7 posttransplant, including in cell clumps containing larger, branching DC-like cells (arrows) (IgG control, right). Scale bars, 100 μ. Data in each case are representative of the results from three samples per group per time point.


Figure 2. Generation of LIGHT$^{-/-}$ mice. (a) Genomic organization of the murine LIGHT locus and resulting mutation induced by the targeting event; exons are filled boxes. A LIGHT gene-targeting construct was generated to replace exon 1 of LIGHT containing the initiating methionine (ATG) with the neomycin resistance gene (NEO). (b) Southern analysis of LIGHT wild-type (+/+), heterozygous knockout (+/−), and homozygous knockout (−/−) mouse genomic DNA. Homologous recombination with the knockout construct results in the introduction of an Xba (X) site when using a flanking probe. (c) Northern analysis of LIGHT wild-type (+/+) and homozygous knockout (−/−) mouse liver RNA using a probe containing the entire coding region of LIGHT; blots were reprobed with a GAPDH probe to evaluate RNA loading.

gene (Fig. 2). Mice heterologous and homozygous for the LIGHT mutation were normal in appearance, growth and fertility, had normal numbers of T and B cells, monocytes and granulocytes, and normal lymphoid architecture. LIGHT$^{-/-}$ mice also had normal levels of plasma C3 despite the chromosomal proximity of LIGHT and C3 genes (14); detailed characterization of these mice is underway.

Homozygous LIGHT$^{-/-}$ mice were used as recipients of fully MHC-disparate cardiac allografts. Whereas LIGHT$^{+/+}$ mice rejected BALB/c cardiac allografts within 1 wk, LIGHT$-/−$ mice maintained their grafts for an extra 3–4 d (Fig. 3 a), which was about as effective as CsA (10 mg/kg/d) in mice (both $P < 0.05$ vs. untreated LIGHT$^{+/+}$ recipients).

However, use of the same regimen of CsA in LIGHT$^{-/-}$ mice led to significantly prolonged engraftment ($\sim$30 d, $P < 0.001$) (Fig. 3 a), indicating a synergistic effect of CsA and LIGHT targeting on allograft survival. Histologic analysis showed that rejecting grafts harvested at 7 d from LIGHT$^{-/-}$ mice, or LIGHT$^{+/+}$ recipients treated with CsA, were morphologically similar to allografts harvested form control untreated LIGHT$^{+/+}$ recipients (Fig. 3 b), with diffuse mononuclear cell infiltrates and focal myocyte necrosis. In contrast, allografts harvested at day 7 from LIGHT$^{-/-}$ recipients treated with CsA showed a marked absence of leukocyte infiltration and essentially normal morphology.

Given concerns that gene-targeted mice may not always reveal the role of a given gene in the normal state because of secondary effects or compensatory responses, we investigated whether targeting of LIGHT was also beneficial in wild-type allograft recipients. We constructed an HVEM–Ig fusion protein for therapeutic blockade of the effects of endogenous LIGHT on host HVEM$^+$ T cells. In line with the modest effects of LIGHT targeting by homologous recombination in this strong MHC disparity, we found neither HVEM-Ig nor control IgG1 had any significant effect on allograft survival in LIGHT$^{+/+}$ recipients ($P > 0.05$), whereas HVEM-Ig, but not control IgG1, was markedly synergistic with a subtherapeutic dose of CsA in prolonging graft survival ($P < 0.001$) (Fig. 3 c).

Expression of cytokines, chemokines, and their receptors by host leukocytes vary during graft rejection. We used RNase protection assays to examine the likely mechanisms
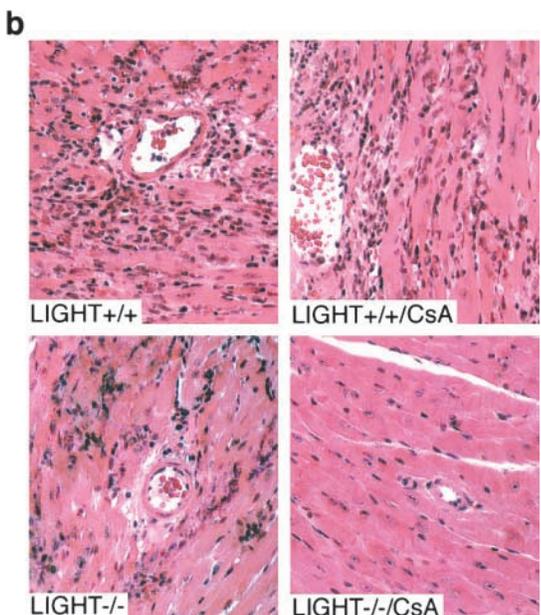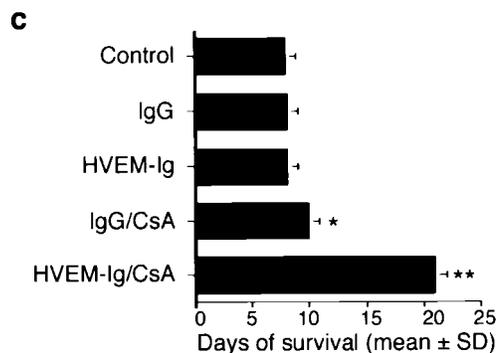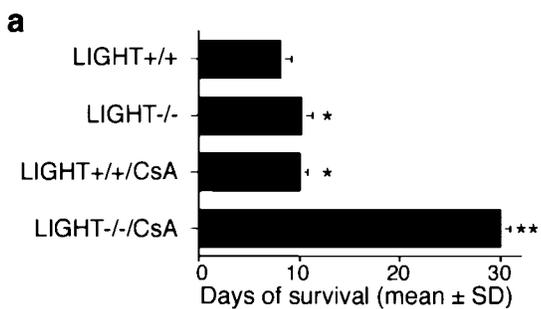
Figure 3. Effects of targeting LIGHT on the survival of fully MHC-mismatched cardiac allografts (H-2^d→H2^b). (a) Compared with untreated LIGHT$^{+/+}$ recipients, LIGHT$^{-/-}$ recipients, or LIGHT$^{+/+}$ recipients treated with a subtherapeutic course of CsA, maintained their cardiac grafts for an extra 2–3 d (*$P < 0.05$), whereas use of the same sub-therapeutic CsA regimen in LIGHT$^{-/-}$ recipients synergistically prolonged graft survival as compared with each of the other groups (**$P < 0.001$). (b) Histologic analysis of allografts harvested at day 7 showed similar mononuclear cell infiltration and myocardial injury in LIGHT$^{+/+}$, LIGHT$^{-/-}$, and LIGHT$^{+/+}$ recipients treated with CsA (day 10). By contrast, use of CsA in LIGHT$^{-/-}$ mice suppressed leukocyte recruitment and graft injury (hematoxylin and eosin, original magnifications: ×100). (c) Beneficial effects of targeting LIGHT were also seen in LIGHT$^{+/+}$ recipients since, in contrast to the effects of control IgG1 or HVEM-Ig ($P > 0.05$), or IgG/CsA alone (*$P < 0.05$), HVEM-Ig plus low-dose CsA significantly prolonged cardiac allograft survival (**$P < 0.001$ versus each of the other groups).
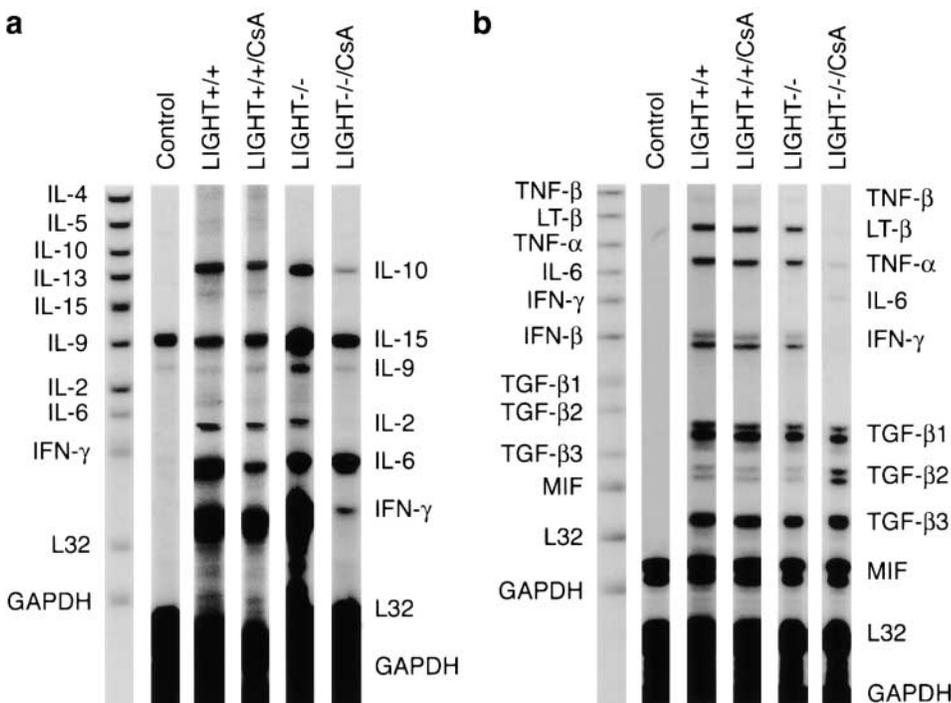
Figure 4. Mechanisms underlying beneficial effects of targeting LIGHT in allograft recipients. (a) RNase protection assay comparison of intragraft Th1- and Th2-associated cytokines in control normal heart versus allografts harvested at day 7 after transplant. Use of CsA had minimal effects on cytokine expression in LIGHT$^{+/+}$ mice, whereas marked suppression of IL-2, IL-10, and IFN-γ were seen in LIGHT$^{-/-}$ mice treated with CsA versus untreated LIGHT$^{-/-}$ recipients. (b) RNase protection assay of the same samples as in panel a, shows suppression of LT-β, TNF-α, and IFN-γ expression with use of CsA in LIGHT$^{-/-}$ recipients, and modestly enhanced expression of TGF-β2. Data are representative of three allografts per group per time-point.

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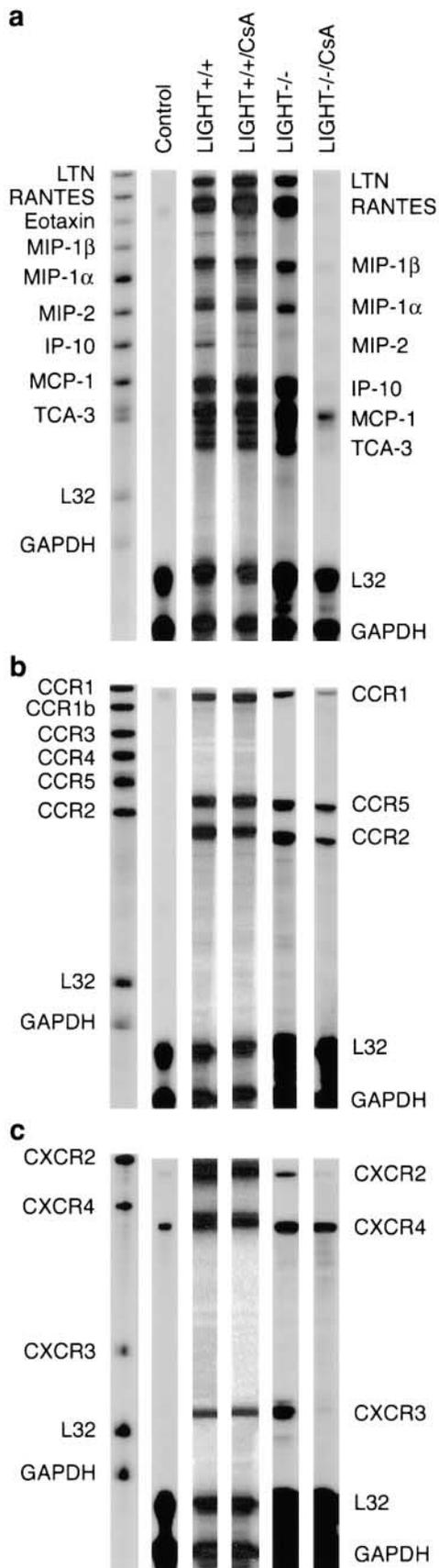
by which targeting of LIGHT, especially with concomitant CsA, induced prolonged graft survival. Use of CsA in LIGHT$^{-/-}$ mice suppressed the intragraft upregulation of multiple cytokine mRNAs, including IFN-γ, IL-2, and IL-10 (Fig. 4 a), plus LT-β and TNF-α (Fig. 4 b). Consistent with these effects and the reduction in cellularity apparent histologically, expression of several IFN-γ–induced chemokines, including RANTES, MIP-1α, MIP-1β, and IP-10 was down-regulated in LIGHT$^{-/-}$ mice treated with CsA (Fig. 5 a). Along with the decreased chemokine expression, LIGHT$^{-/-}$ mice treated with CsA had modest reductions in CC-chemokine receptor expression (Fig. 5 b) and markedly decreased expression of the chemokine receptor for IP-10, CXCR3 (Fig. 5 c), which is expressed by Th1 and Tc1 lymphocytes (13).

Our prior studies in this model showed that donor-derived IP-10 production (19) and concomitant infiltration by CXCR3$^+$ leukocytes (13) play central roles in the development of allograft rejection, such that targeting of either IP-10 production or CXCR3 expression markedly prolongs allograft survival. We also recently reported that the IP-10/CXCR3 pathway is active during development of human cardiac allograft rejection (20). IP-10 production is regulated by NF-κB (21), and LIGHT-induced costimulation causes NF-κB activation and translocation in T cells (9), leading to production of IFN-γ (7). It is likely that modulation of this pathway by inhibition of LIGHT costimulation is at least one important mechanism of action in our model, and one which is potentiated by the effects of a subtherapeutic regimen of CsA, which can also diminish NF-κB activation and IFN-γ production (22). Nevertheless, given recent evidence of several distinct molecular forms of LIGHT, which are directed to distinct cellular compartments, including the extracellular space, the membrane, and the cytosol (14), and findings that LIGHT overexpression by T cells promotes inflammation via activation of multiple pathways (10–12), there remain many additional potential mechanisms by which targeting of LIGHT in conjunction with CsA could be beneficial.

Our studies show a modest role for LIGHT costimulation by itself in promoting allograft rejection, but in contrast to some other combinations of therapeutic agents and costimulation blockade, such as the use of CD154 mAb plus CsA (23), the effects of targeting LIGHT-HVEM interactions are markedly synergistic with CsA. Their combined use prevents acute allograft rejection, modulates intragraft cytokine and chemokine production, and decreases the infiltration of host immunocompetent cells. These data suggest exploration of the role of LIGHT costimulation in less rigorous models as well as in combination with other therapeutic approaches. We conclude that the LIGHT-

Figure 5. Targeting of LIGHT is synergistic with CsA in effects at day 7 after transplant on intragraft expression of (a) the chemokines lymphotactin (LTN), RANTES, MIP-1α, MIP-1β, MCP-1, IP-10, and TCA-3; (b) the CC chemokine receptors CCR1, CCR2, and CCR5; and especially (c) the key allograft rejection-linked CXC chemokine receptor, CXCR3. Data are representative of three allografts per group per time-point.

HVEM pathway is yet another of the rapidly expanding number of costimulation pathways which require attention in efforts to promote the development of safer, less toxic therapeutic protocols which may eventually facilitate development of clinical allograft tolerance.

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