Targeted Disruption of LIGHT Causes Defects in Costimulatory T Cell Activation and Reveals Cooperation with Lymphotoxin β in Mesenteric Lymph Node Genesis

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Abstract

The recently described tumor necrosis factor (TNF) family member LIGHT (herpes virus entry mediator [HVEM]-L/TNFSF14), a ligand for the lymphotoxin (LT)β receptor, HVEM, and DcR3, was inactivated in the mouse. In contrast to mice deficient in any other member of the LT core family, LIGHT$^{-/-}$ mice develop intact lymphoid organs. Interestingly, a lower percentage of LIGHT$^{-/-}$LTβ$^{-/-}$ animals contain mesenteric lymph nodes as compared with LTβ$^{-/-}$ mice, whereas the splenic microarchitecture of LIGHT$^{-/-}$LTβ$^{-/-}$ and LTβ$^{-/-}$ mice shows a comparable state of disruption. This suggests the existance of an additional undiscovered ligand for the LTβ receptor (LTβR) or a weak LTα$_3$–LTβR interaction in vivo involved in the formation of secondary lymphoid organs. LIGHT acts synergistically with CD28 in skin allograft rejection in vivo. The underlying mechanism was identified in in vitro allogeneic MLR studies, showing a reduced cytotoxic T lymphocyte activity and cytokine production. Detailed analyses revealed that proliferative responses specifically of CD8$^+$ T cells are impaired and interleukin 2 secretion of CD4$^+$ T cells is defective in the absence of LIGHT. Furthermore, a reduced ^{3}[H]-thymidine incorporation after T cell receptor stimulation was observed. This for the first time provides in vivo evidence for a cooperative role for LIGHT and LTβ in lymphoid organogenesis and indicates important costimulatory functions for LIGHT in T cell activation.

Key words: TNF • lymphotoxin • HVEM • lymphoid organogenesis • transplantation

Introduction

Members of the TNF superfamily are involved in a broad range of biological functions such as cell proliferation and differentiation, apoptosis, and lymphoid organogenesis (1–3). TNF, lymphotoxin (LT)*α, and LTβ together with the recently identified TNF ligand family member LIGHT (4) can be defined as a core group within the larger TNF superfamily. The counterpart to the core family of ligands is formed by four TNF receptor (TNFR) superfamily members: TNFRp55; TNFRp75; LTβ receptor (LTβR); and the herpes virus entry mediator (HVEM), that share overlapping but distinct ligand-binding patterns. TNF$_3$ and LTα$_3$ engage the TNFRp55 and TNFRp75 as homotrimers. In combination with the exclusively membrane bound LTβ, LTα binds to the LTβR predominantly as LTα$_1$β$_2$ heterotrimer (5, 6).

LIGHT (HVEM-L, TNFSF14) is expressed as a homotrimer on activated T cells (4, 7) and also on immature dendritic cells (DCs; reference 8). Three receptors with distinct cellular expression patterns are described to interact with LIGHT. LTβR, found on follicular DCs (FDCs) and stromal cells (9, 10) binds LIGHT and LTα$_1$β$_2$. HVEM, in contrast, is detected on immature DCs (11, 12), T and B lymphocytes, NK cells, monocytes, and endothelial cells (7, 13, 14) and signals upon engagement of LIGHT or LTα$_3$. DcR3 (TR6), a TNFR family member lacking a transmembrane region competes with LTβR and HVEM for LIGHT engagement, thereby acting as a nega-

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**Abbreviations used in this paper.* DC, dendritic cell; ES, embryonic stem; FDC, follicular DC; GC, germinal center; HVEM, herpes virus entry mediator; LT, lymphotoxin; MLN, mesenteric LN; HSV-TK, herpes simplex virus thymidine kinase; PP, Peyer's Patch; VSV, vesicular stomatitis virus; WT, wild-type.

tive regulator. In addition to LIGHT, DcR3 also binds to FasL (15, 16).

Overlapping but also nonredundant biological functions have been assigned to ligands and receptors of the LT/TNF core family using gene targeting techniques and treatment with receptor fusion proteins or agonistic antibodies (17–19). TNF, TNFRp55, and TNFRp75 are dispensable for LNs formation, though TNF- and TNFRp55-deficient mice fail to form germinal centers (GCs) and fully developed Peyer's Patches (PP) (20–23). A more severe phenotype is observed in the absence of $LT\alpha_1\beta_2$-LTβR signaling leading to a lack of all LNs, defects in T and B cell segregation, and FDC networks formation within the spleen (24). This phenotype is largely shared by LTα-deficient mice (25, 26), despite the fact that occasional lymphoid aggregates appear in the mesentery of these animals (25). In contrast, mice deficient for LTβ retain mesenteric LNs (MLNs) and in some cases cervical LNs (27, 28). Furthermore, the splenic microarchitecture in these animals appears less severely disturbed as compared with $LT\alpha^{-/-}$ or $LT\beta R^{-/-}$ mice. In the search for an explanation for the nonoverlapping phenotypes of mice deficient for LTα, LTβ, or LTβR it was speculated on the involvement of LIGHT–LTβR or LIGHT–HVEM interactions during lymphoid organogenesis.

So far, in vitro studies suggested a role of LIGHT as a costimulatory molecule providing T cells with the "second signal" in addition to "signal one" delivered by the T cell receptor. For some other members of the TNF superfamily such costimulatory functions in T cell activation have been established (29). LIGHT induces T cell proliferation, NF-κB translocation, and secretion of IFN-γ in vitro (8, 30). In vivo blockade of LIGHT and $LT\alpha_1\beta_2$ by administration of LTβR-Fc leads to reduced T cell responses in a graft-versus-host disease model (30). Recent studies demonstrate that LIGHT cooperates with CD40 ligand in DC maturation presumably by signaling via HVEM expressed on immature DCs (12).

Here we report the generation and characterization of LIGHT-deficient mice. Functional analysis of the phenotype of $LIGHT^{-/-}$ mice indicates an essential costimulatory role for LIGHT in supporting T cell activation. With respect to lymphoid organogenesis, LIGHT cooperates with LTβ in formation of MLNs as demonstrated by the comparison of LTβ singly deficient with LIGHT/LTβ doubly deficient mice.

Materials and Methods

Cells. E14.1 embryonic stem (ES) cells from 129/Ola mice were grown in DMEM medium (GIBCO BRL) supplemented with L-glutamine (2 mM; Seromed), leukemia inhibitory factor, penicillin/streptomycin (100 μg/ml; Seromed), 2-mercaptoethanol (0.05 mM; GIBCO BRL), and 15% heat-inactivated FCS (Boehringer).

P815 ($H\text{-}2^d$) mouse mastocytoma and EL-4 ($H\text{-}2^b$) mouse T cell lymphoma cell lines were purchased from the American Type Culture Collection (Rockville) and maintained in complete RPMI 1640 medium (Biochrom) supplemented with 10% heat-inactivated FCS (Boehringer), 0.05 mM 2-ME, and penicillin/streptomycin (100 μg/ml; Seromed).

Mouse splenocytes were cultured in RPMI 1640 medium (Biochrom) supplemented with 5% heat-inactivated FCS (Boehringer), 0.05 mM 2-ME, Hepes-Buffer (10 mM; GIBCO BRL), and Pen/Strep (100 μg/ml; Seromed).

Targeting the LIGHT Genomic Locus by Homologous Recombination. A murine genomic ES-129 BAC library (Genome Systems Inc.) was screened by hybridization with a 314-bp murine *light* cDNA fragment as a probe. The resulting BAC clone was mapped by Southern blot hybridization using murine *light* cDNA fragments. Two adjacent BAC fragments of 4.0 kb and 5.8 kb containing the complete coding sequence for the *light* locus were cloned into pBluescript (Stratagene) and fully sequenced. The targeting vector was constructed in pBluescript in a way that a 4.5-kb fragment of the genomic *light* locus encoding the complete ORF of the LIGHT protein was replaced by a neomycin resistance cassette, and a herpes simplex virus thymidine kinase (HSV-TK) cassette was inserted 2.8 kb upstream of the targeted sequence (Fig. 1 A). The neomycin resistance cassette was inserted in antisense to the transcriptional direction of LIGHT. RT-PCR for CD27-L, the TNF superfamily member shown to be located in the same human genomic region next to *light* (31), revealed undisturbed transcriptional regulation in untreated and PMA/ionomycin stimulated $LIGHT^{-/-}$ splenocytes (data not shown). E14.1 ES cells were electroporated with the linearized targeting vector as described previously (32). G418- and gancyclovir-resistant ES cell colonies were picked. Homologous recombination was detected by PCR and subsequently confirmed by genomic Southern blot hybridization with a 3′ flanking probe (see Fig. 1 A) after digestion of ES cell DNA with SpeI (see Fig. 1 B). Single integration of the targeting vector was verified by probing the Southern blot with the neomycin resistance cassette (data not shown). Correctly targeted ES cell clones were injected into C57BL/6 blastocysts, which were transferred into pseudopregnant foster mothers. Resulting chimeric mice were backcrossed to C57BL/6 mice, and germline transmission of the targeted allele was confirmed by Southern blot analysis (see Fig. 1 B).

Generation and Screening of $LIGHT^{-/-}$ ($H\text{-}2^b$), $LIGHT^{-/-}$ ($H\text{-}2^d$), $LIGHT^{-/-}CD28^{-/-}$, $LIGHT^{-/-}LT\beta^{-/-}$, and $LT\beta R^{-/-}$ Mice. The LIGHT mutation was moved into a C57BL/6 background by at least three successive backcrosses, initiated with (C57BL/6 × 129/Ola) F1 $LIGHT^{+/-}$ mice. The resulting heterozygotes were intercrossed to establish C57BL/6 $LIGHT^{-/-}$ ($H\text{-}2^b$) mice. Genotyping for the LIGHT mutation was performed by PCR with the following primers: 5′-ACG CAT GTG TCC TGC GTG TGG-3′ (mLIGHT type1); 5′-CGA CAG ACA TGC CAG GAA TGG-3′ (mLIGHT type2); and 5′-GAC GTA AAC TCC TCT TCA GAC-3′ (pneo1).

To obtain mice deficient for LIGHT on a $H\text{-}2^d$ background, C57BL/6 $LIGHT^{+/-}$ mice were backcrossed once with BALB/c mice and resulting $LIGHT^{+/-}$ mice were mated with each other. Progeny was FACS® analyzed for the $H\text{-}2^d$ haplotype on both alleles (staining for $H\text{-}2D^d$ and $I\text{-}A^d$) and typed for the LIGHT mutation.

To obtain mice deficient for CD28 and LIGHT, homozygous single knockout mice on the C57BL/6 background (at least four times backcrossed) were bred, F1 littermates intercrossed, and progeny was genotyped. Genotyping for the CD28 mutation (33) was performed by PCR using the following primers: 5′-CCT GAG TCC TGA TCT GTC AGA CT-3′ (979–54); 5′-CTG

CTT GTG GTA GAT AGC AAC GA-3′ (979–55); and 5′-ATT CGC CAA TGA CAA GAC GCT GG-3′ (HSV-TK).

For the generation of LIGHT/LTβ doubly deficient mice homozygous single knockout mice (27) on the C57BL/6 background (at least two times backcrossed) were bred to LIGHT$^{-/-}$ mice and F1 littermates crossed to LTβ$^{-/-}$ mice to obtain LIGHT$^{+/-}$LTβ$^{-/-}$ mice. These were backcrossed to C57BL/6 mice and the resulting doubly heterozygous mice were interbred to obtain LIGHT and LTβ singly deficient and LIGHT/LTβ doubly deficient mice as littermates. Genotyping for the LTβ mutation was performed by PCR with the following primers: 5′-CGG GTC TCC GAC CTA GAG ATC-3′ (gtype1); 5′-CCA CAA CAG GTG TGA CTG TCT C-3′ (gtype2); and 5′-GAG GTG GGT GGA TTG GAA AGA G-3′ (gtype3).

LTβR$^{-/-}$ mice were at least six times backcrossed to the C57BL/6 background, bred, and genotyped as described previously (24). Mice were kept according to German guidelines for animal care in a SPF animal facility.

Northern Blot Analysis. Mouse splenocytes were stimulated for 4 h with PMA (10 ng/ml) and Ca^{2+}-Ionophor (200 ng/ml). Total RNA was prepared, run on an 1% formamide gel, and blotted on a Gene Screen membrane (Dupont). mRNAs for LIGHT and TNF-α were detected using ^{32}[P]-labeled cDNA probes containing the whole ORF coding for the respective protein.

Analysis of MLN Formation. Mice were dissected and mesenteric regions were examined and documented using a Leica MZ APO photomicroscope in combination with a JVC KY-F70 digital camera unit.

Histology. Tissue samples were embedded in tissue-freezing medium (Leica) and snap-frozen in 2-methylbutane (Merck) prechilled by liquid nitrogen. Cryostat sections (7 μm) were fixed for 8 min in acetone (Merck). Frozen sections were thawed, rehydrated, blocked, and incubated with biotinylated and FITC-conjugated Abs in a dark humidified atmosphere for 1 h at RT. After washing, PE-conjugated streptavidin (Becton Dickinson) was added for 1 h; slides were washed and mounted with Fluoromount-G (Southern Biotech). Analysis was performed 2–24 h later with a laser scanning microscope (Zeiss).

Proliferation Assays. Lectin-induced T cell activation was performed on total splenocytes (2.5×10^4 cells per well) cocultured with titrated amounts of ConA (Sigma-Aldrich) in 96-well, round-bottomed plates for 32 h. For TCR-mediated T cell activation total splenocytes (10^5 cells per well) or purified T cells (4×10^5 cells per well) were stimulated with titrated amounts of soluble or plate-bound monoclonal anti-CD3 Ab (BD PharMingen), respectively, in 96-well round-bottomed plates for 72 h. T cells were positively selected by Thy1.2-microbeads in a magnetic field according to the manufacturer's instructions (Miltenyi Biotec). The purity of isolated T cells was >95%. ^{3}[H]-thymidine incorporation of T cells was assessed by addition of 1 μCi per well ^{3}[H]-thymidine (Amersham Pharmacia Biotech) during the last 6–9 h of the culture. Incorporation of ^{3}[H]-thymidine was measured with a Matrix™ 96 direct β counter (Packard Instrument Co.). For cell cycle analysis, splenocytes were labeled with 5 μM CFSE (Molecular Probes) and stimulated with 2 μg/ml of soluble monoclonal anti-CD3 Ab (BD PharMingen) in 24-well plates.

Vesicular Stomatitis Virus Infection. Vesicular stomatitis virus (VSV) (Indiana serotype; Mudd-Summers isolate) was originally obtained from D. Kolakofsky (University of Geneva, Geneva, Switzerland), and was grown on BHK cells in MEM with 5% FCS to virus stocks containing 10^9 PFU/ml. Mice were immunized intravenously on day 0 with 2×10^6 PFU of VSV. For determination of neutralizing Ab titers serial twofold dilutions of serum samples (previously diluted 1:40) were mixed with equal volumes of VSV containing 500 PFU/ml, and the mixtures were incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. 100 μl of the mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. Monolayers were overlaid with 100 μl of DMEM containing 1% methylcellulose, incubated for 24 h at 37°C, then the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet dissolved in 5% formaldehyde, 50% ethanol, and 4.25% NaCl. The dilution reducing the number of plaques by 50% was taken as titer. To determine IgG titers undiluted serum was treated with an equal volume of 0.1 M 2-ME in MEM medium for 1 h at RT (34). CTL activity against VSV was measured using splenocytes on day 6 after infection in a standard ^{51}[Cr]-release assay on VSV-infected MC57 target cells.

Skin Grafting. Skin grafting was performed on anesthetized recipients according to a technique of Davis et al. (35). Briefly, a full thickness of tail skin (0.5 cm^2) was grafted on the lateral flank. Grafts were observed daily after removal of the bandage (day 8) and considered rejected when no viable donor skin was present. LIGHT$^{+/\pm}$ CD28$^{+/\pm}$, LIGHT$^{-/-}$ CD28$^{+/\pm}$, LIGHT$^{+/\pm}$ CD28$^{-/-}$, and LIGHT$^{-/-}$CD28$^{-/-}$ littermate mice, were transplanted with allogeneic tailskin of LIGHT$^{-/-}$ (H-2^d) mice, to exclude influences of LIGHT, possibly brought into the system via the graft. Recipients were backcrossed at least four times into the C57BL/6 background.

CTL Assay. ^{51}[Cr]-release assays were performed with effector cells from allogeneic MLRs on day 5. EL-4 or P815 target cells (10^6) were labeled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech) for 1 h at 37°C and washed. 100 μl (10^3 cells) of these target cells were added to the same volume of replicate serial dilutions of effector CTLs as indicated. After 4 h of incubation at 37°C, 100 μl of culture supernatant were removed and radioactivity was measured. Specific lysis was calculated according to the formula: percentage of specific lysis = (cpm [sample] − cpm [spontaneous release])/(cpm [maximal release] − cpm [spontaneous release]) × 100. Spontaneous release was 4–10%.

Allogeneic MLR and Measurement of Proliferative Responses and Cytokines in the Supernatant. For induction of MLR, allogeneic splenocytes (H-2^b versus H-2^d) were cocultured at concentrations of 2×10^6 cells/ml for stimulator cells and total splenocytes as responders, 1.5×10^6 cells/ml for CD4-depleted responders, or 1.7×10^6 cells/ml for CD8-depleted responders. Depletion was performed using anti-CD4 or anti-CD8 microbeads, respectively, according to the manufacturer's instructions (Miltenyi Biotec). Depletion efficiency was >99%. The stimulator population was irradiated with 30Gy. Proliferation was measured after 72 h of MLR activation by addition of 1 μCi/well ^{3}[H]-thymidine (Amersham Pharmacia Biotech) during the last 9 h of the culture. After 5 d, culture supernatants were harvested and cytokine concentration was assessed by sandwich ELISA for IFN-γ, IL-2, IL-4, IL-10, and IL-12p40 according to the manufacturer's instructions (R&D Systems). Detection limits are 2 pg/ml for IL-4 and IFN-γ, 3 pg/ml for IL-2, and 4 pg/ml for IL-10 and IL-12p40.

Flow Cytometric Analysis. Aliquots of the allogeneic MLRs were harvested on days 0, 4, and 5. After pretreatment with Fc-block (anti-CD16/32; BD PharMingen), cells were stained for 10 min at 4°C with the indicated Abs. The following Abs were used for staining of T lymphocytes: anti–CD4-bio (L3T4); anti–CD8α-PE (Ly-2); anti–CD3ε-Cy5 (145–2C11); and anti–H-2K^b-FITC (KH95). Biotinylated Abs were detected using streptavidin-PerCP (Becton Dickinson). Splenocytes were gated

on $CD3^+$ H-$2K^b$ lymphocytes for naive T cells on day 0 and on $CD3^+$ H-$2K^b$ high forward side scatter lymphocytes for MLR-activated T cells on days 4 and 5. Fluorescence was analyzed using FACSCalibur™ flow cytometry and CELLQuest™ software (both Becton Dickinson).

Results

Inactivation of the Murine LIGHT Gene. To evaluate the functions of LIGHT in vivo, we generated LIGHT-deficient mice by homologous recombination in ES cells. A targeting vector was designed to replace the complete genomic region coding for the open reading frame by a *neomycin* resistance cassette (Fig. 1 A). Two targeted mutant ES cell clones were identified by Southern blot analysis and injected into C57BL/6 blastocysts. The resulting chimeric mice transmitted the disrupted *light* allele through the germline. Homozygously mutant mice were identified by Southern blot analysis of genomic tail DNA. *Spe*I bands of 5.8 and 2.6 kb were detected as the wild-type (WT) and the mutated alleles, respectively (Fig. 1 B). $LIGHT^{-/-}$ mice were born healthy and proved fertile. Successful inactivation of LIGHT was demonstrated by the absence of LIGHT mRNA in PMA/Ionomycin activated splenocytes as determined by Northern blot analysis (Fig. 1 C). $LIGHT^{-/-}$ mice derived from both ES cell clones showed the same phenotype. Furthermore, in all assays performed no significant differences between WT and $LIGHT^{+/-}$ mice were observed indicating that a single *light* allele is sufficient for function.

Cooperation of LIGHT with LTβ in MLN Formation. Members of the TNF ligand and receptor superfamilies are involved in lymphoid organogenesis. LTβR-deficient mice lack all peripheral and MLNs and PP and display a largely destroyed splenic architecture (24). $LT\beta^{-/-}$ mice which are deficient for the $LT\alpha_1\beta_2$-heterotrimeric ligand of the LTβR retain organized MLNs (27, 28). Furthermore, in the spleen of $LT\beta^{-/-}$ mice T and B cell compartmentalization is largely intact. Because of this incomplete developmental defect one can speculate on LIGHT, as a second ligand for LTβR, to be involved in the formation of secondary lymphoid tissues and their microarchitecture. Yet, $LIGHT^{-/-}$ mice develop a complete set of primary and secondary lymphoid organs including peripheral and MLNs and PP with unaltered microarchitecture (data not shown). Flow cytometry analysis of primary and secondary lymphoid organs revealed normal cell counts for all major cell populations (data not shown). In the spleen, all major cellular compartments, such as B cell follicles with FDCs and GCs, T cell areas including DCs, and the marginal zones are present and properly organized (data not shown). To

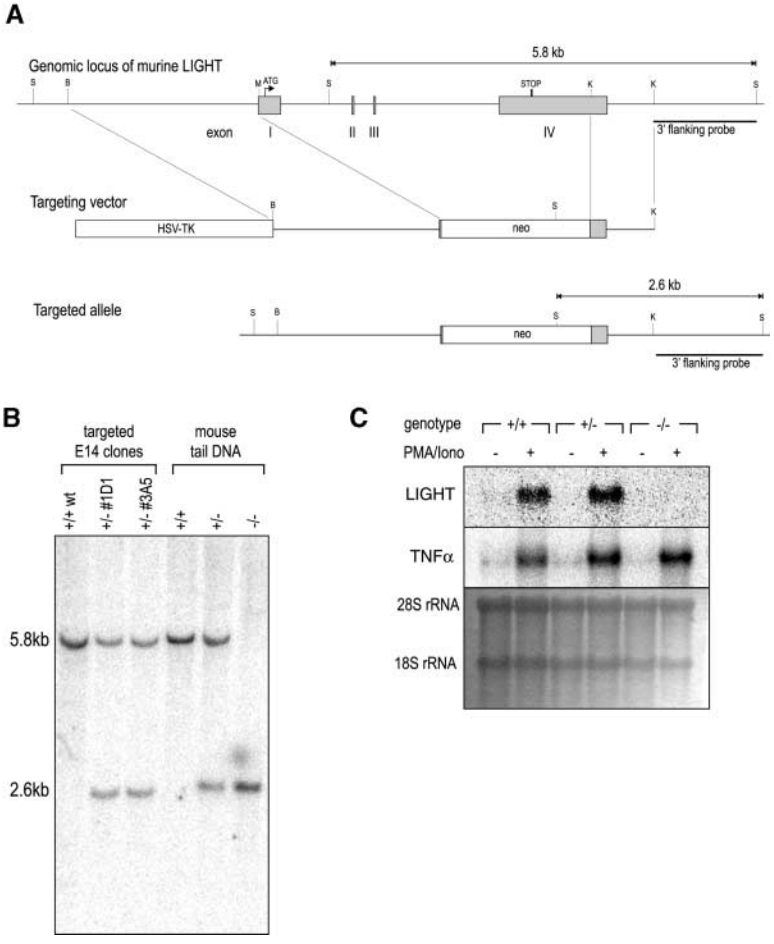

Figure 1. Generation of $LIGHT^{-/-}$ mice. (A) The restriction map of the murine *light* genomic locus (top), the targeting vector (middle), and the targeted allele (bottom) are shown. Locations of translation start, stop codon, and flanking probe are indicated. B, BamHI; K, KpnI; M, MfeI; S, SpeI. (B) Southern blot hybridization of SpeI-digested genomic DNA from targeted ES cell clones and mouse tail biopsies with the 3′ flanking probe yields a 5.8-kb fragment for the WT allele and a 2.6-kb fragment for the targeted allele. (C) The absence of LIGHT mRNA in mice homozygous for the targeted *light* allele is verified by Northern blot analysis. Total RNA was prepared from untreated or PMA/ionomycin–activated splenocytes from $LIGHT^{+/+}$, $LIGHT^{+/-}$, and $LIGHT^{-/-}$ mice and hybridized with cDNA probes containing the whole ORF for murine LIGHT or TNF-α (activation control). Methylene blue staining of 28S and 18S rRNA serves as loading control.

verify functional integrity of secondary lymphoid organs and intact GC function, high affinity Ab responses to T cell–dependent (4-hydroxy-3-nitrophenyl-acetyl)-chicken γ globulin (NP-CG) immunization were assessed and found unaltered in LIGHT$^{-/-}$ mice with respect to IgM and all IgG subclasses (data not shown). This demonstrates functional class switch and affinity maturation and suggests that sufficient LTβR-signaling is accomplished by the presence of the $LT\alpha_1\beta_2$ heterotrimer.

Mice deficient for LIGHT and LTβ were intercrossed to analyze lymphoid organogenesis in the absence of both known ligands for the LTβR. *light* and the *ltβ-tnfα-ltα* locus are located on murine chromosome 17 (36). However, the distance of ~10 cM between the two loci allowed for the breeding of LIGHT/LTβ doubly deficient mice (unpublished data). No PP or brachial, axillary, inguinal, popliteal, paraaortic, or parapancreatic LNs could be detected in LIGHT$^{-/-}$LTβ$^{-/-}$ mice and LIGHT$^{+/-}$LTβ$^{-/-}$ mice (data not shown). However, as compared with LTβ$^{-/-}$ (27, 28) or LIGHT$^{+/-}$LTβ$^{-/-}$ mice, there was a marked reduction in the presence of MLNs (Fig. 2 A) in LIGHT$^{-/-}$LTβ$^{-/-}$ animals. Upon careful microscopic inspection, MLNs were found in all LIGHT$^{+/-}$LTβ$^{-/-}$ mice, whereas only in four out of 16 LIGHT$^{-/-}$LTβ$^{-/-}$ animals MLNs were present which was additionally verified by preparation of cryosections of the mesenteric regions. The splenic microarchitecture of LIGHT$^{-/-}$LTβ$^{-/-}$ and LIGHT$^{+/-}$LTβ$^{-/-}$ mice resembles that one described for LTβ$^{-/-}$ mice (27, 28) with T cells accumulating around the central arteriole surrounded by a wall of B cells. In contrast, T and B cell areas were completely intermixed in LTβR-deficient mice (24) (Fig. 2 B). In the spleen and LNs of LIGHT$^{-/-}$LTβ$^{+/-}$ mice no significant differences compared with LIGHT$^{+/+}$LTβ$^{+/+}$ mice were observed in histological analysis.

Thus, it appears that, though LIGHT and LTβ are able to substitute for each other in morphogenesis of MLNs, $LT\alpha_3$ (via HVEM?) or a yet undiscovered ligand for LTβR is responsible for the relatively conserved splenic T and B cell segregation and the incomplete absence of MLNs in LIGHT/LTβ doubly deficient mice.

Proliferative Responses of LIGHT$^{-/-}$ T Cells In Vitro. Studies using exogenous addition/overexpression or, alternatively, blockade of LIGHT by receptor Fc-fusion proteins have suggested LIGHT as a costimulatory molecule which enhances T cell proliferation presumably through HVEM (8, 30, 37). Total splenocytes or purified T cells from LIGHT-deficient mice reproducibly showed reduced amounts of ^{3}[H]-thymidine incorporation induced by plate bound or soluble anti-CD3 mAb, respectively, as compared with those of WT or heterozygous littermate controls (Fig. 3 B and C). This emphasizes the importance of LIGHT as a costimulatory molecule in TCR-mediated T cell responses, whereas its presence is not required to achieve optimal proliferation in lectin-mediated T cell activation (Fig. 3 A). When cell cycle activity of TCR-stimulated T lymphocytes was measured using the fluorescent dye CFSE no differences between WT or LIGHT$^{-/-}$ T cells either of the CD4$^+$ or the CD8$^+$ subpopulations could be detected (Fig. 3 D) rather suggesting a function of LIGHT in supporting T cell survival.

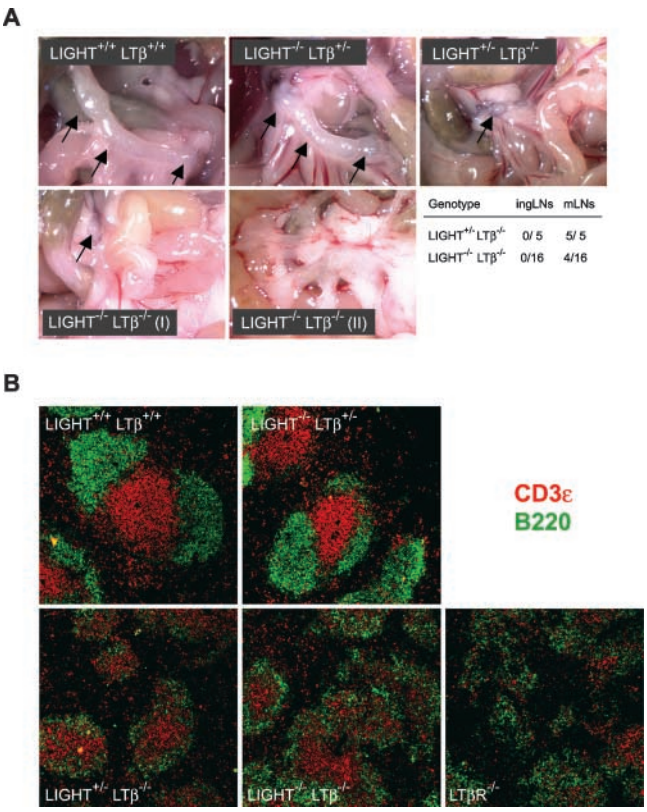

Figure 2. Reduced frequency of MLNs and relatively conserved splenic architecture in LIGHT/LTβ doubly deficient mice. (A) Analysis of the mesenteric region of LIGHT$^{+/+}$LTβ$^{+/+}$, LIGHT$^{-/-}$LTβ$^{+/-}$, LIGHT$^{+/-}$LTβ$^{-/-}$, and LIGHT$^{-/-}$LTβ$^{-/-}$ animals. After dissection of the abdominal wall the area of the mesenteric origin was prepared. MLNs are indicated by arrows. Frequency of inguinal (ing) and MLNs in LIGHT/LTβ doubly deficient and LTβ singly deficient littermates are given. Five LIGHT$^{+/-}$LTβ$^{-/-}$ mice and 16 LIGHT$^{-/-}$LTβ$^{-/-}$ mice were microscopically analyzed. (B) Confocal microscopy of spleen microarchitecture. Splenic sections from mice of the indicated genotype were stained with fluorescence labeled anti-CD3 Abs (red) and anti-B220 Abs (green) to detect T and B cells, respectively.

VSV Infection in LIGHT-deficient Mice. To assess the consequences of LIGHT deficiency for host resistance to viral infections LIGHT$^{-/-}$ and littermate control mice were inoculated with 2×10^6 PFU VSV. After VSV infection, LIGHT$^{-/-}$ mice and littermate controls mounted similar neutralizing T cell–independent IgM responses around day 4 after infection, followed by a T cell–dependent switch to the IgG subclass between days 6 and 8 (38, 39) (Fig. 4 A). VSV-specific, ex vivo CTL activity showed no significant differences between VSV-infected LIGHT$^{-/-}$ and LIGHT$^{+/+}$ effector cells (Fig. 4 B). Similar results were obtained when mice were infected with a lower dose of 2×10^4 PFU VSV (data not shown). Thus, LIGHT does not play a crucial role in the induction of a primary anti-VSV immune response.

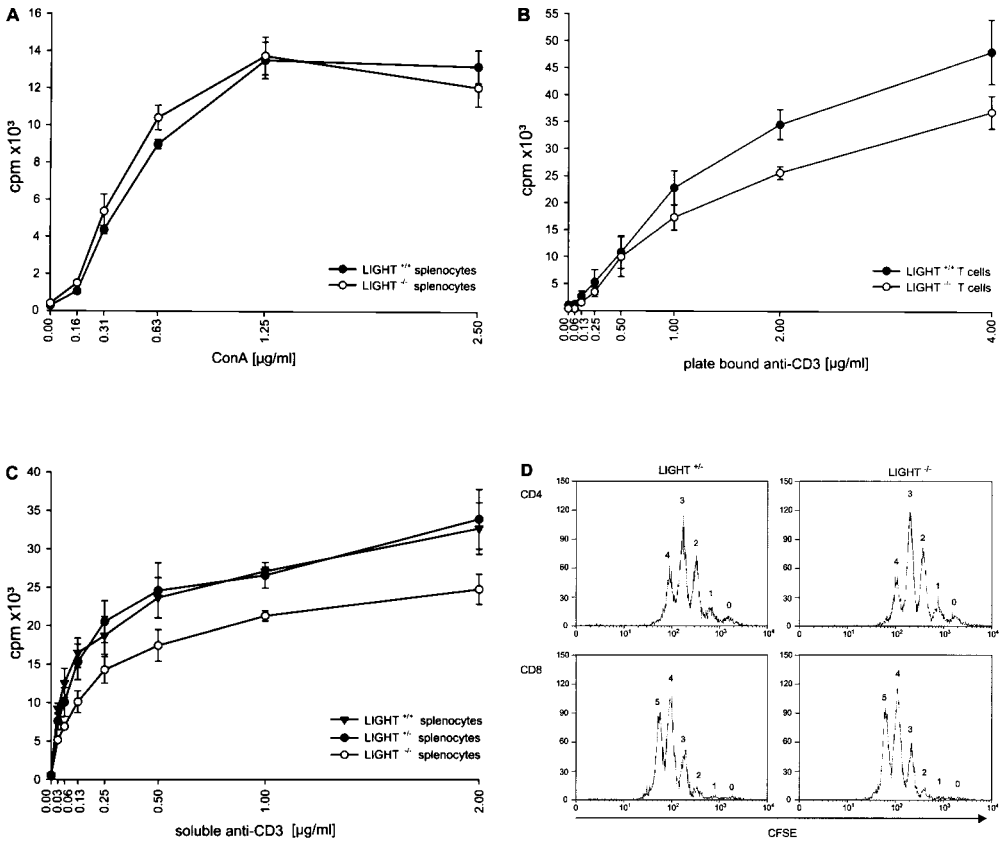

Figure 3. T lymphocyte proliferation in vitro in the absence of LIGHT. (A) Normal proliferation of LIGHT$^{-/-}$ T cells in response to ConA stimulation. Splenocytes from LIGHT-deficient and WT mice were incubated with titrated amounts of ConA for 2 d. (B and C) Reduced ^{3}[H]-thymidine incorporation of LIGHT$^{-/-}$ splenocytes upon TCR-mediated stimulation. Purified T cells from LIGHT$^{-/-}$ and WT littermates (B) or total splenocytes from LIGHT$^{-/-}$, LIGHT$^{+/-}$, and WT littermates (C) were cultured for 72 h with the indicated doses of plate-bound (B) or soluble (C) anti-CD3 mAb. Cells were pulsed with 1 μCi for the last 6–9 h (B and C) or the last 4 h (A) of the culture. Shown is one representative result of four independent experiments, respectively. (D) Unaltered cell cycle activity of LIGHT-deficient T lymphocytes. Splenocytes were labeled with CFSE and incubated with 2 μg/ml soluble anti-CD3 mAb for 72 h. After that cells were harvested and FACS® analyzed after gating on live CD4^{+} or CD8^{+} cells, respectively.

Skin Allograft Survival in LIGHT$^{-/-}$CD28$^{-/-}$ Mice. To address the role of LIGHT in antiallogeneic immune responses in vivo, allogeneic skin graft rejection was investigated in LIGHT$^{-/-}$ mice. A first set of experiments showed, that LIGHT-deficient mice exhibited no significant differences in their ability to acutely reject skin allografts. The same was true for CD28$^{-/-}$ recipient mice as described previously (40). Since CD28-mediated signals work in synergy with some other TNF superfamily members (e.g., 4–1BB, reference 41), LIGHT$^{-/-}$ animals were crossed to a CD28$^{-/-}$ background, to be able to assess the importance of LIGHT as an alternative costimulatory molecule in the absence of a possibly complementing coactivating pathway. In this setting, LIGHT$^{-/-}$CD28$^{-/-}$ mice showed a skin graft survival of up to 19 d, i.e., 6 d longer than singly deficient or WT mice (Fig. 5). This indicates

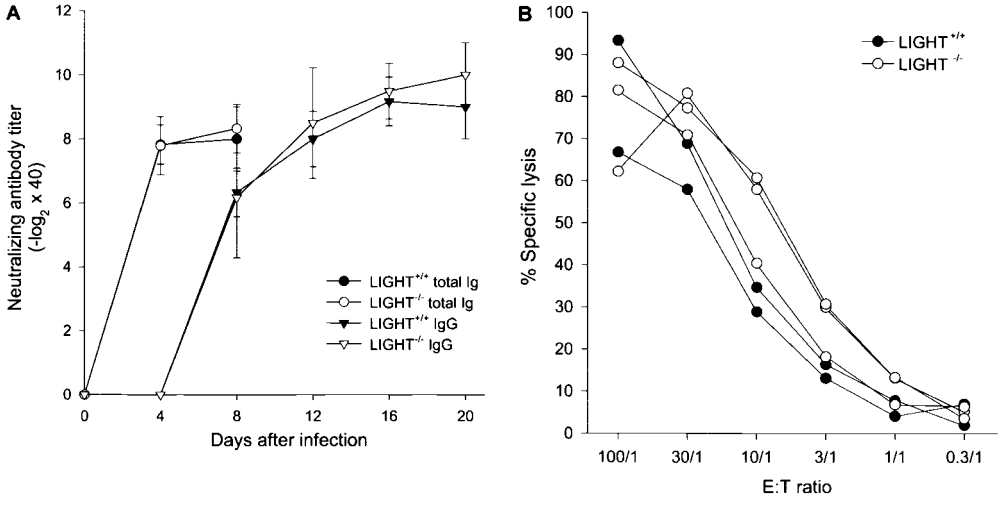

Figure 4. Humoral and cellular immune responses of LIGHT$^{-/-}$ mice to VSV infection. (A) Groups of three LIGHT$^{-/-}$ (white symbols) and WT littermate control mice (black symbols) were inoculated with 2 × 10^{6} PFU VSV intravenously. Blood was taken at the indicated time points. Sera were separated and prediluted 40-fold, and neutralizing VSV-specific total Ig (circles) and IgG (triangles) responses were analyzed. (B) Splenocytes from LIGHT$^{+/+}$ (black circles, two mice) and LIGHT$^{-/-}$ (white circles, three mice) animals 6 d after infection with 2 × 10^{6} PFU VSV were cocultured with ^{51}[Cr]-labeled VSV-infected target cells and specific lysis was assessed. Control assays with unchallenged animals or uninfected target cells showed no significant specific lysis (data not shown).

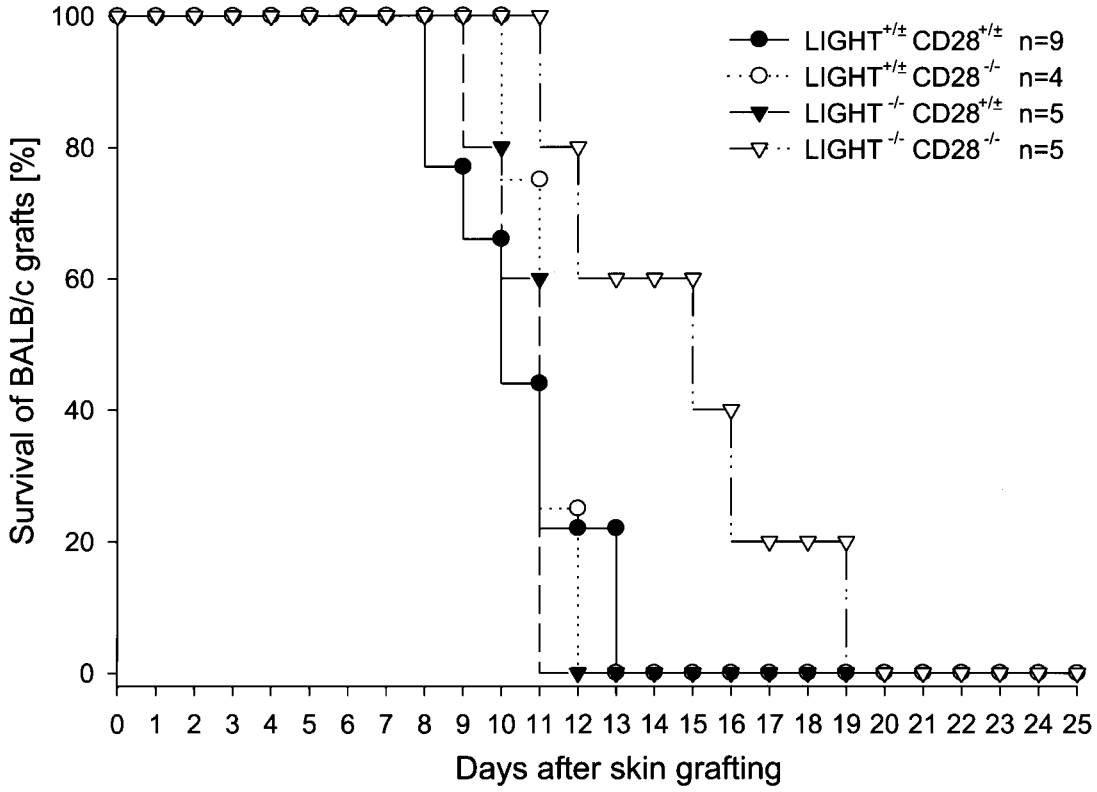

Figure 5. Delayed allogeneic skin graft rejection in LIGHT$^{-/-}$CD28$^{-/-}$ mice. Groups of 4–9 mice were given tail skin transplants from fully allogeneic LIGHT$^{-/-}$ (H-2^{d}) mice. Grafts were monitored daily. Black circles, WT animals or mice heterozygous for either the *cd28* or the *light* allele ($n = 9$); white circles, CD28 singly deficient mice, WT, or heterozygous for *light* ($n = 4$); black triangles, LIGHT singly deficient mice, WT, or heterozygous for *cd28* ($n = 5$); white triangles, LIGHT CD28 doubly deficient mice ($n = 5$).

that LIGHT together with CD28 plays an important role in allo-graft rejection.

MLR-induced CTL Activity and Cytokine Production in the Absence of LIGHT. In search for the underlying cellular mechanisms of the observed delay in skin graft rejection, it was tested whether LIGHT is required for the generation of efficient T cell effector functions in vitro, induced by an allogeneic MLR. Possible influences of soluble LIGHT shed from the cell surface by matrix metalloproteinases (7) were excluded by comparing MLRs with LIGHT-deficient effector as well as stimulator populations to those with both populations heterozygous or WT for the *light* allele. Interestingly, when tested for their ability to kill allogeneic target cells, LIGHT$^{-/-}$ effector splenocytes showed a marked reduction in specific target cell lysis as compared with LIGHT$^{+/-}$ effectors (Fig. 6 A). MLR supernatants from LIGHT-deficient splenocytes contained remarkably reduced amounts of IFN-γ and IL-2, as well as IL-4 and IL-10. In contrast, IL-12p40 concentrations, a cytokine predominantly produced by DCs, were similar in all MLR setups (Fig. 6 B). CD8/CD4 ratios of MLR-activated responder T cells were determined using FACS® analysis (Fig. 6 C). On day 5, we found the ratio of CD8$^+$ versus CD4$^+$ activated responder T cells significantly reduced by 40% in the LIGHT-deficient MLRs as compared with those with LIGHT$^{+/-}$ or WT splenocytes. The CD8/CD4 ratio of naive splenocytes was comparable in WT, LIGHT$^{+/-}$, and LIGHT$^{-/-}$ mice. To elucidate the differential effects of LIGHT on CD8$^+$ versus CD4$^+$ T cell subpopulations, MLRs were performed after depletion of CD4$^+$ T cells or CD8$^+$ T cells from the LIGHT$^{-/-}$ or LIGHT$^{+/-}$ responder cells (Fig. 7). LIGHT$^{-/-}$ CD4-depleted MLRs showed drastically reduced proliferation as compared with the corresponding LIGHT$^{+/-}$ MLRs, whereas in MLRs with CD8-depleted responder populations no significant differences in proliferation could be found (Fig. 7 A). Supernatants from CD8-depleted LIGHT$^{-/-}$ MLRs showed drastically reduced amounts of IL-2 as compared with LIGHT$^{+/-}$ MLRs, clearly indicating LIGHT as a crucial cytokine for the induction of IL-2 secretion by CD4$^+$ T cells. In accordance with published results, only very small amounts of IL-2 could be measured after CD4-depletion independent of LIGHT expression (Fig. 7 B). Concentrations of IFN-γ and IL-4 in supernatants from MLRs with CD4- or CD8-depleted T cell subpopulations were below the detection limit (data not shown). This is in line with previously made observations which describe no detectable IFN-γ or IL-4 production of separated CD4$^+$ or CD8$^+$ T cells after primary alloantigeneic stimulation (42, 43).

These results clearly indicate, that LIGHT is essential for efficient activation of cytotoxic T lymphocytes in an allo-

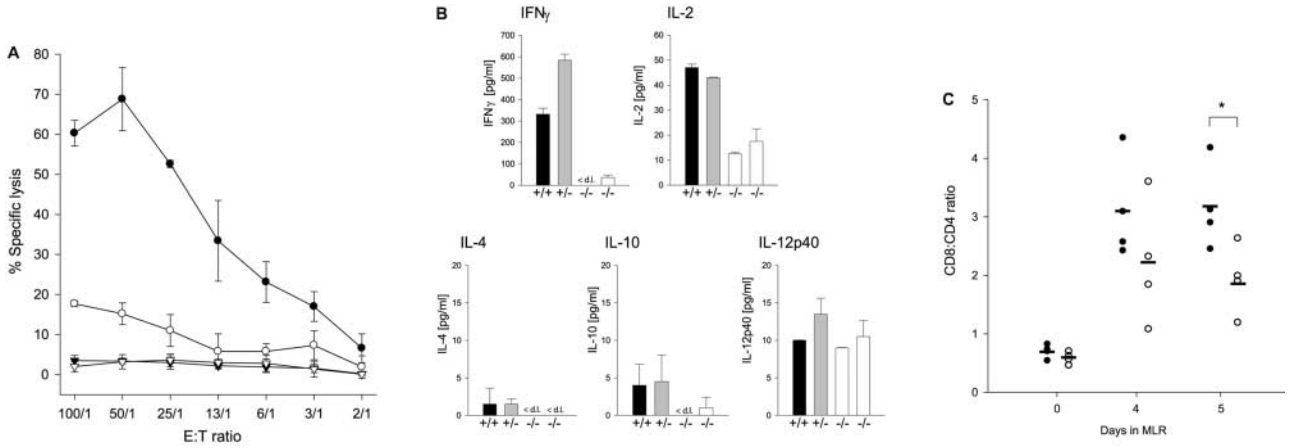

Figure 6. Impaired CTL responses and cytokine production after allogeneic MLR activation. (A) Splenocytes from LIGHT$^{+/-}$ (black symbols) or LIGHT$^{-/-}$ (white symbols) H-2^b mice were cultured with irradiated LIGHT$^{+/-}$ or LIGHT$^{-/-}$ H-2^d splenocytes. On day 5, CTL activity of effector splenocytes was assessed against the H-2^d target P815 (circles) or the H-2^b target EL-4 (triangles) in a standard ^{51}[Cr]-release assay. One representative of five independent experiments is shown. (B) On day 5, cytokine amounts in the supernatant of the MLR were measured by sandwich ELISA. < d.l.: below detection limit. Two representatives of five independent experiments are shown. (C) FACS® analysis was performed on allogeneic MLRs on days 0, 4, and 5. Shown is the ratio of CD8$^+$ versus CD4$^+$ cells in MLRs with LIGHT$^{+/-}$ or LIGHT$^{+/+}$ (black circles) or LIGHT$^{-/-}$ (white circles). Each circle represents an independent MLR. Means of experimental groups are shown as bars. Asterisk indicates statistically significant results ($P = 0.0025$).

geneic MLR in vitro. Interestingly, the accompanying decrease in cytokine production by LIGHT-deficient splenocytes encompassed not only Th cell type 1 responses represented by IFN-γ and IL-2 but also the typical Th cell type 2 cytokines IL-4 and IL-10. Detailed analyses revealed that LIGHT has direct influence on the proliferative activity of CD8$^+$ T cells and the IL-2 production of the CD4$^+$ T cell subset.

Discussion

The biological functions of the individual members of the TNF ligand and receptor families are highly complex and span from developmental processes to innate and adaptive immune responses (1, 29, 44). Insight into the underlying molecular mechanisms was gained by gene targeting or administration of receptor Fc-fusion proteins. However, when receptors are engaged by several distinct ligands, as is the case for HVEM and LTβR (4, 45), or if ligands bind to more than one receptor, as is the case for LIGHT (4, 16), only gene targeting can reveal the individual developmental and immunological roles of a defined molecule. In this study, LIGHT was inactivated in the germline of the mouse. We demonstrate that the recently described TNF/LT core family member LIGHT is implicated in the costimulatory activation of T cells and, in cooperation with LTβ, in formation of MLNs.

Secondary lymphoid organs including peripheral and MLNs, spleen, and PP are present with a properly developed and functional intact microarchitecture in LIGHT singly deficient animals, suggesting that in the absence of LIGHT-sufficient signaling activity through the LTβR is provided by the $LT\alpha_1\beta_2$ heterotrimer. However, one can still not completely exclude a possibly additional role of HVEM signaling in lymphoid organogenesis since $LT\alpha_3$-HVEM interactions are intact in LIGHT-deficient animals.

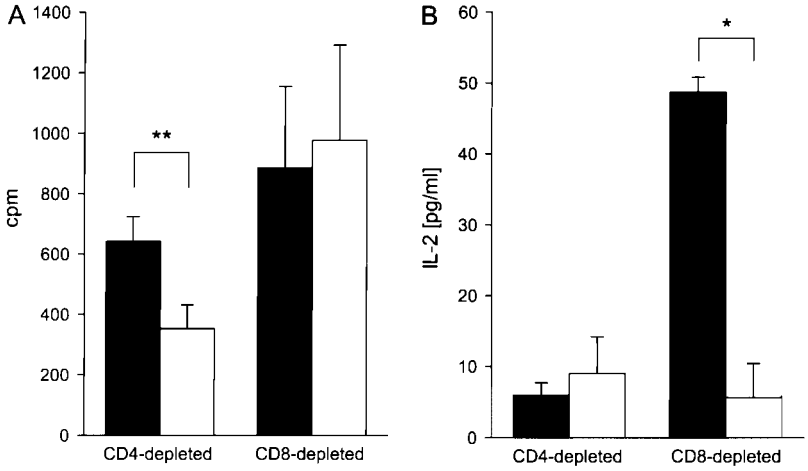

Figure 7. Proliferation and IL-2 production of CD4- or CD8-depleted LIGHT$^{-/-}$ splenocytes after allogeneic MLR activation. Splenocytes from LIGHT$^{+/-}$ (black bars) or LIGHT$^{-/-}$ (white bars) H-2^b mice were either depleted for CD4$^+$ or CD8$^+$ cells, followed by coculture with irradiated LIGHT$^{+/-}$ or LIGHT$^{-/-}$ H-2^d splenocytes, respectively. (A) For the measurement of proliferation in the absence of CD4$^+$ or CD8$^+$ cells, ^{3}[H]-thymidine was added for the last 9 h of a 4-d culture period. One representative of three independent experiments is shown. Please note that direct β-counting was used. (B) Amounts of IL-2 on day 5 in the supernatant of MLRs. Cytokine concentrations were measured by ELISA. One representative of two independent experiments is shown. *$P = 0.007$; **$P = 0.002$.

Next, the putatively redundant roles of LIGHT and $LT\alpha_1\beta_2$ heterotrimers were addressed by intercrossing $LIGHT^{-/-}$ and $LT\beta^{-/-}$ mice. Our studies with $LIGHT^{-/-}$ $LT\beta^{-/-}$ mice revealed a cooperative role for LIGHT with LTβ in MLN development. Generally, LTβ singly deficient animals retain MLNs, with frequencies of LTβ-deficient animals containing MLNs ranging from 100 to 60% in different laboratories (27, 28, 46, 47). The basis of the differences reported in frequency of MLNs in LTβ strains is not clear but might relate to influences of background or housing conditions. Compared with these data and to our own $LIGHT^{+/-}LT\beta^{-/-}$ littermates, animals doubly deficient for LIGHT and LTβ exhibited a markedly reduced frequency of mice containing MLNs, indicating that in the absence of LTβ, LIGHT provides alternative signaling via the LTβR, the latter being indispensable for MLN development (24). The presence of MLNs in 25% of the LIGHT/LTβ doubly deficient animals hints at a yet undiscovered ligand signaling via LTβR or a weak binding activity of the $LT\alpha_3$ homotrimer to the LTβR in vivo, though in vitro data do not support the latter possibility (45).

The relatively conserved splenic microarchitecture of LTβ single knockout mice lacking $LT\alpha_1\beta_2$ heterotrimers with largely intact T and B cell segregation is retained in LIGHT/LTβ double knockout animals which contrasts to the more disturbed situation found in LTβR- as well as LTα-deficient mice (24–26). Thus, LIGHT does not seem to contribute to the organization of the splenic microenvironment, suggesting again a yet unknown third ligand for the LTβR. Alternatively, one can argue for a functional role of LTα beside its interaction with the LTβR as member of the $LT\alpha_1\beta_2$ heterotrimer. The three members of the TNFR family described to engage LTα as homotrimer are TNFRp55, TNFRp75, and HVEM. Since LTβ/TNF doubly deficient animals have profound defects in T and B cell segregation (46), the TNFRp55 and the TNFRp75 cannot be excluded from participating in splenic T and B cell segregation. The role of HVEM in lymphoid organogenesis, however, has still to be elucidated. To this aim HVEM deficient animals are currently being generated in our lab.

Previous studies focused on LIGHT as a costimulatory molecule involved in T cell proliferative responses and cytokine secretion (8, 30, 37, 48). However, $LIGHT^{-/-}$ splenocytes responded normally to ConA-mediated activation, thereby questioning conclusions drawn from experiments where LTβR-Fc or HVEM-Fc efficiently blocked ConA induced proliferation of WT splenocytes (37, 48). These discrepancies are explainable either by the existence of an undiscovered alternative ligand for LTβR involved in T cell activation directly via HVEM or by redundant functional roles of $LT\alpha_3$, blocked by HVEM-Fc, or of $LT\alpha_1\beta_2$, blocked by LTβR-Fc. In contrast, $LIGHT^{-/-}$ T cells and $LIGHT^{-/-}$ total splenocytes showed a reduction in ^{3}[H]-thymidine incorporation after anti-CD3 stimulation yet normal cell cycle activity in both $CD4^+$ and $CD8^+$ T cell subpopulations as measured in a CFSE assay, a situation quite similar to the one found in $CD27^{-/-}$ mice (49). The amount of incorporated ^{3}[H]-thymidine is influenced by the number of cell divisions as well as the number of cells partaking in the cell cycle activity, with the latter being also affected by the cell survival. Thus, one can assume an involvement of LIGHT in T cell survival after TCR triggering.

Ex vivo CTL responses even to low infection doses of VSV are unaffected in the absence of LIGHT. This is probably due to the ability of other accessory molecules as for example CD28 (50) to provide sufficient costimulation in a cytolytic or humoral immune response to VSV, masking a possible involvement of LIGHT. Preliminary experiments using LIGHT/CD28 doubly deficient animals, however, show an almost complete lack of VSV-specific CTL activity, whereas the $CD28^{-/-}$ littermate controls retain a certain degree of lytic activity (data not shown).

CD28 as well as e.g., 4–1BB signaling alone have been shown to be dispensable for allograft rejection (40, 41). Lack of both CD28 and 4–1BBL leads to a delay of allograft rejection of up to 14 d. In our studies, combined deficiency for LIGHT and CD28 resulted in an allograft survival time of up to 19 d. This observation most likely reflects the early requirement of LIGHT-HVEM signaling during T cell activation in the course of which other costimulatory molecules as e.g., 4–1BB/4–1BBL, CD40/CD154, CD27/CD70, and OX40/OX40L (13, 29, 51) are upregulated and then able to substitute for the missing LIGHT-HVEM or LIGHT-LTβR interactions.

To further elucidate the underlying mechanisms, additional in vitro studies were performed. Strikingly, LIGHT was shown to be indispensable for mounting an effective allogeneic CTL response in vitro. The basis for this lies in the reduced frequency of activated $CD8^+$ T cells observed in the allogeneic MLR. In addition, cytokines reduced in the supernatant of MLRs from LIGHT-deficient splenocytes included both Th cell type 1 and 2 cytokines contradicting earlier observations that blockade of LIGHT costimulation predominantly decreases Th1 cytokines (30). By analyzing MLR responses after $CD4^+$ or $CD8^+$ T cell depletion it could be demonstrated that the reduced frequency of activated $CD8^+$ T cells was caused by a significantly lower proliferation of the $LIGHT^{-/-}$ $CD8^+$ T cells, whereas proliferative responses of $CD4^+$ T cells appeared not be affected by the absence of LIGHT. Additionally, LIGHT is essential for sufficient IL-2 production by $CD4^+$ T cells in an allogeneic MLR.

The more dramatic defects found in the absence of LIGHT in allogeneic immune responses in vitro as compared with in vivo might be explained by the virtual absence of tissue resident DCs as professional APCs in the splenocyte preparations used for in vitro MLRs. Thus, the generation of primary CTLs under these conditions could be more dependent on the presence of LIGHT produced by T cells as compared with in vivo situations after allogeneic skin transplantation or VSV infection, where fully activated professional APCs expressing various costimulatory molecules could substitute for a single LIGHT deficiency.

In the future, crossing the LIGHT deficiency into MHC class I– or II–restricted TCR transgenic backgrounds should allow for a detailed analysis of the contribution of LIGHT-mediated costimulation for either the $CD4^+$ or $CD8^+$ T cell subpopulations. Interestingly, chronically increased expression of LIGHT results in autoimmune disorders (37, 48, 52).

Recent observations illuminate that the absence of LIGHT leads to defects in DC maturation (12). This may in turn result in decreased allogeneic CTL activity and delayed skin graft rejection. However, bone marrow–derived DCs from LIGHT-deficient mice, maturated in the presence of CpG, LPS, or poly I:C, show unimpaired upregulation of surface MHC class II, CD40, B7–1, and B7–2 as well as intracellular IL-12 and TNF-α production (data not shown). The expression patterns of both, LIGHT and HVEM, suggest that ligand and receptor may function during the earlier stages of the adaptive immune response, to initially enforce or bias T cell activation before CD28 as well as other costimulatory molecules take over. In conclusion, our findings define a contribution of LIGHT in the organogenesis of secondary lymphoid tissues and an important involvement in the costimulation of T cell–mediated immune responses, identifying LIGHT as a target molecule in transplantation, vaccination, or cancer therapy.

The authors like to thank A. Fütterer, K. Mink, E. Schaller, and S. Hausmann for excellent technical assistance and J. Meinecke and U. Huffstadt for blastocyst injection. Thanks to I. Förster, R.M. Vabulas, C. Brettl, H. Flaswinkel, R. Endres, and A. Schaub for critical reading of the manuscript and discussion. The continuous and generous support of H. Wagner is highly appreciated.

This work was supported by the Deutsche Forschungsgemeinschaft (grants Pf459/2-4/SFB 391, 455, and 576).

Submitted: 8 February 2002
Revised: 18 April 2002
Accepted: 6 May 2002

Note added in proof. During the period of revision of the manuscript a publication appeared that also indicates a role for LIGHT in allogeneic solid organ rejection (Ye et al., *J. Exp. Med.* 2002. 195:795–800).

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