

# Targeted Disruption of LIGHT Causes Defects in Costimulatory T Cell Activation and Reveals Cooperation with Lymphotoxin $\beta$ 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) $\beta$  receptor, HVEM, and DcR3, was inactivated in the mouse. In contrast to mice deficient in any other member of the LT core family, LIGHT<sup>-/-</sup> mice develop intact lymphoid organs. Interestingly, a lower percentage of LIGHT<sup>-/-</sup>LT $\beta$ <sup>-/-</sup> animals contain mesenteric lymph nodes as compared with LT $\beta$ <sup>-/-</sup> mice, whereas the splenic microarchitecture of LIGHT<sup>-/-</sup>LT $\beta$ <sup>-/-</sup> and LT $\beta$ <sup>-/-</sup> mice shows a comparable state of disruption. This suggests the existence of an additional undiscovered ligand for the LT $\beta$  receptor (LT $\beta$ R) or a weak LT $\alpha_3$ -LT $\beta$ R 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<sup>+</sup> T cells are impaired and interleukin 2 secretion of CD4<sup>+</sup> T cells is defective in the absence of LIGHT. Furthermore, a reduced <sup>3</sup>[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 LT $\beta$  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) $\alpha$ , and LT $\beta$  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; LT $\beta$  receptor (LT $\beta$ R); and

the herpes virus entry mediator (HVEM), that share overlapping but distinct ligand-binding patterns. TNF $_3$  and LT $\alpha_3$  engage the TNFRp55 and TNFRp75 as homotrimers. In combination with the exclusively membrane bound LT $\beta$ , LT $\alpha$  binds to the LT $\beta$ R predominantly as LT $\alpha_1\beta_2$  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. LT $\beta$ R, found on follicular DCs (FDCs) and stromal cells (9, 10) binds LIGHT and LT $\alpha_1\beta_2$ . 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 LT $\alpha_3$ . DcR3 (TR6), a TNFR family member lacking a transmembrane region competes with LT $\beta$ R 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 $\alpha_1\beta_2$ -LT $\beta$ R 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 LT $\alpha$ -deficient mice (25, 26), despite the fact that occasional lymphoid aggregates appear in the mesentery of these animals (25). In contrast, mice deficient for LT $\beta$  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 LT $\alpha$ , LT $\beta$ , or LT $\beta$ R it was speculated on the involvement of LIGHT-LT $\beta$ R 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- $\kappa$ B translocation, and secretion of IFN- $\gamma$  *in vitro* (8, 30). *In vivo* blockade of LIGHT and LT $\alpha_1\beta_2$  by administration of LT $\beta$ R-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 LT $\beta$  in formation of MLNs as demonstrated by the comparison of LT $\beta$  singly deficient with LIGHT/LT $\beta$  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  $\mu$ g/ml; Seromed), 2-mercaptoethanol (0.05 mM; GIBCO BRL), and 15% heat-inactivated FCS (Boehringer).

P815 (H-2<sup>d</sup>) mouse mastocytoma and EL-4 (H-2<sup>b</sup>) 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  $\mu$ g/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  $\mu$ g/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-2<sup>b</sup>), LIGHT $^{-/-}$  (H-2<sup>d</sup>), 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  $\times$  129/Ola) F1 LIGHT $^{+/-}$  mice. The resulting heterozygotes were intercrossed to establish C57BL/6 LIGHT $^{-/-}$  (H-2<sup>b</sup>) 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-2<sup>d</sup> background, C57BL/6 LIGHT $^{+/-}$  mice were backcrossed once with BALB/c mice and resulting LIGHT $^{+/-}$  mice were mated with each other. Progeny was FACS<sup>®</sup> analyzed for the H-2<sup>d</sup> haplotype on both alleles (staining for H-2D<sup>d</sup> and I-A<sup>d</sup>) 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/LT $\beta$  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 LT $\beta$  $^{-/-}$  mice to obtain LIGHT $^{+/-}$ LT $\beta$  $^{-/-}$  mice. These were backcrossed to C57BL/6 mice and the resulting doubly heterozygous mice were interbred to obtain LIGHT and LT $\beta$  singly deficient and LIGHT/LT $\beta$  doubly deficient mice as littermates. Genotyping for the LT $\beta$  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).

LT $\beta$ R $^{-/-}$  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 a 1% formamide gel, and blotted on a Gene Screen membrane (Dupont). mRNAs for LIGHT and TNF- $\alpha$  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) pre-chilled by liquid nitrogen. Cryostat sections (7  $\mu$ m) 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 ( $2.5 \times 10^4$  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 ( $4 \times 10^5$  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  $\mu$ Ci 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<sup>TM</sup> 96 direct  $\beta$  counter (Packard Instrument Co.). For cell cycle analysis, splenocytes were labeled with 5  $\mu$ M CFSE (Molecular Probes) and stimulated with 2  $\mu$ g/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  $2 \times 10^6$  PFU of VSV. For de-

termination 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  $\mu$ l 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  $\mu$ l 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 $^{+/-}$  CD28 $^{+/-}$ , LIGHT $^{-/-}$  CD28 $^{+/-}$ , LIGHT $^{+/-}$  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  $\mu$ Ci Na $_2$  $^{51}$ CrO $_4$  (Amersham Pharmacia Biotech) for 1 h at 37°C and washed. 100  $\mu$ l ( $10^3$  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  $\mu$ l 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])  $\times$  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  $2 \times 10^6$  cells/ml for stimulator cells and total splenocytes as responders,  $1.5 \times 10^6$  cells/ml for CD4-depleted responders, or  $1.7 \times 10^6$  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  $\mu$ Ci/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- $\gamma$ , 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- $\gamma$ , 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-CD8 $\alpha$ -PE (Ly-2); anti-CD3 $\epsilon$ -Cy5 (145-2C11); and anti-H-2K $^b$ -FITC (KH95). Biotinylated Abs were detected using streptavidin-PerCP (Becton Dickinson). Splenocytes were gated

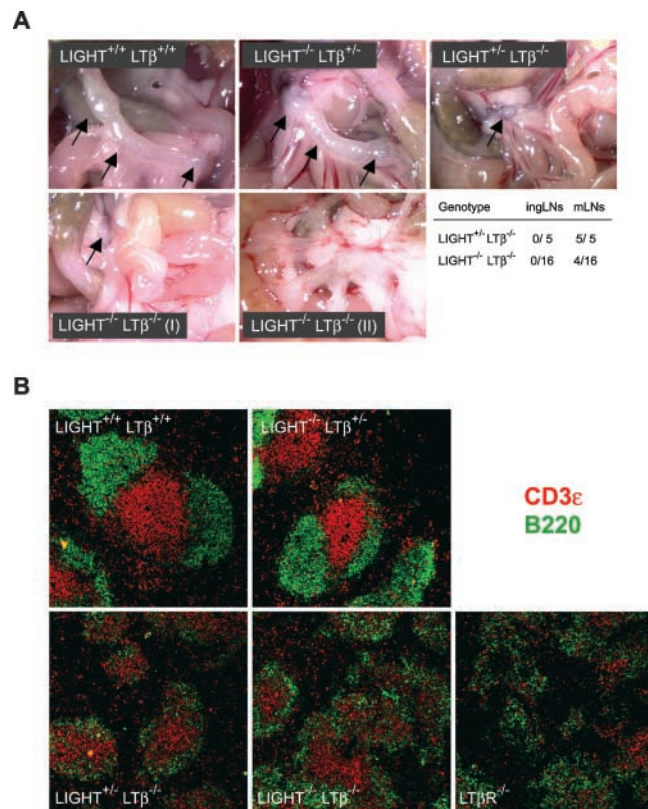


verify functional integrity of secondary lymphoid organs and intact GC function, high affinity Ab responses to T cell-dependent (4-hydroxy-3-nitrophenyl-acetyl)-chicken  $\gamma$  globulin (NP-CG) immunization were assessed and found unaltered in  $LIGHT^{-/-}$  mice with respect to IgM and all IgG subclasses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

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

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

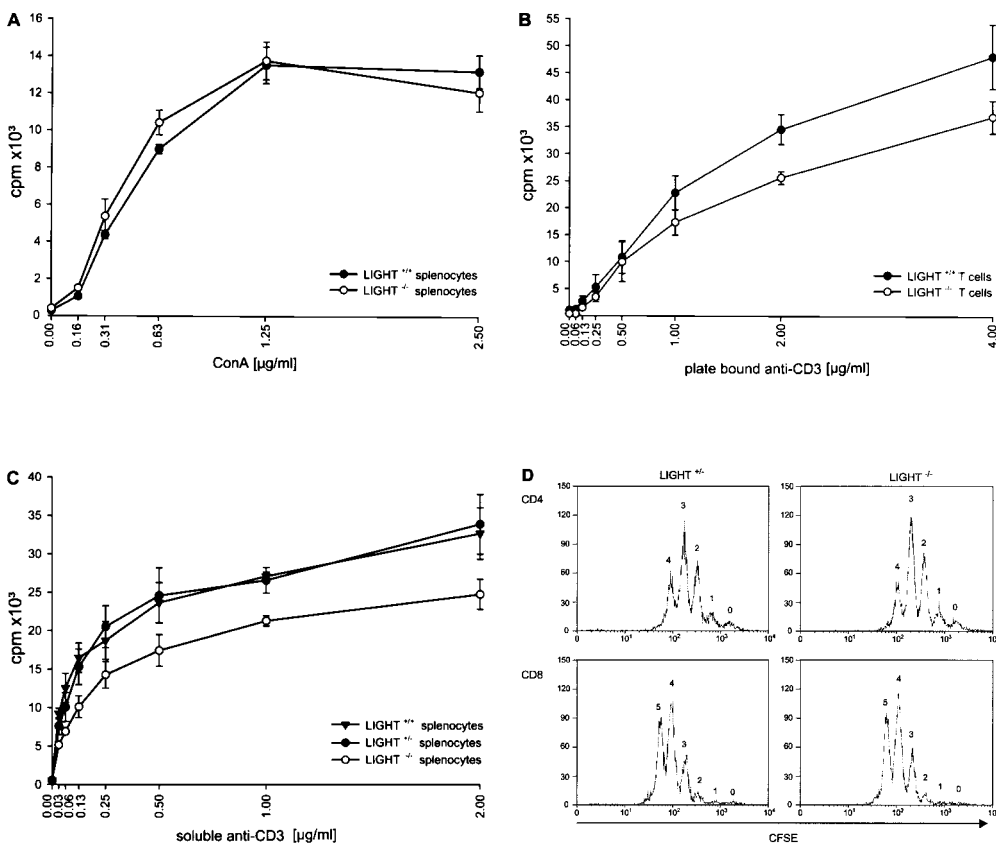
**Proliferative Responses of  $LIGHT^{-/-}$  T Cells In Vitro.** Studies using exogenous addition/overexpression or, alternatively, blockade of  $LIGHT$  by receptor Fc-fusion proteins have suggested  $LIGHT$  as a costimulatory molecule which enhances T cell proliferation presumably through HVEM (8, 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



**Figure 2.** Reduced frequency of MLNs and relatively conserved splenic architecture in  $LIGHT/LT\beta$  doubly deficient mice. (A) Analysis of the mesenteric region of  $LIGHT^{+/+}LT\beta^{+/+}$ ,  $LIGHT^{-/-}LT\beta^{+/-}$ ,  $LIGHT^{+/-}LT\beta^{-/-}$ , and  $LIGHT^{-/-}LT\beta^{-/-}$  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  $LIGHT/LT\beta$  doubly deficient and  $LT\beta$  singly deficient littermates are given. Five  $LIGHT^{+/-}LT\beta^{-/-}$  mice and 16  $LIGHT^{-/-}LT\beta^{-/-}$  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.

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.

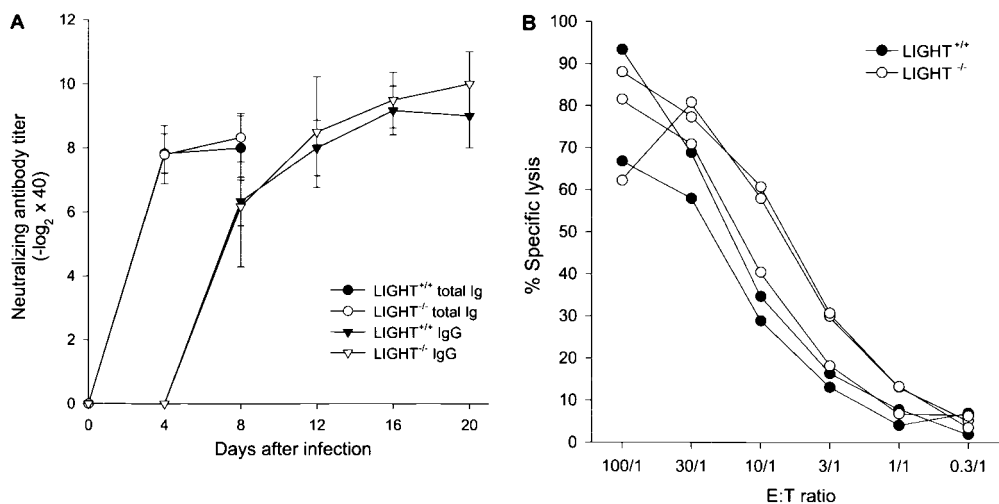
**VSV Infection in  $LIGHT$ -deficient Mice.** To assess the consequences of  $LIGHT$  deficiency for host resistance to viral infections  $LIGHT^{-/-}$  and littermate control mice were inoculated with  $2 \times 10^6$  PFU VSV. After VSV infection,  $LIGHT^{-/-}$  mice and littermate controls mounted 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  $2 \times 10^4$  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  $^3\text{H}$ -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  $\mu\text{Ci}$  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  $\mu\text{g/ml}$  soluble anti-CD3 mAb for 72 h. After that cells were harvested and FACS<sup>®</sup> analyzed after gating on live  $\text{CD4}^+$  or  $\text{CD8}^+$  cells, respectively.

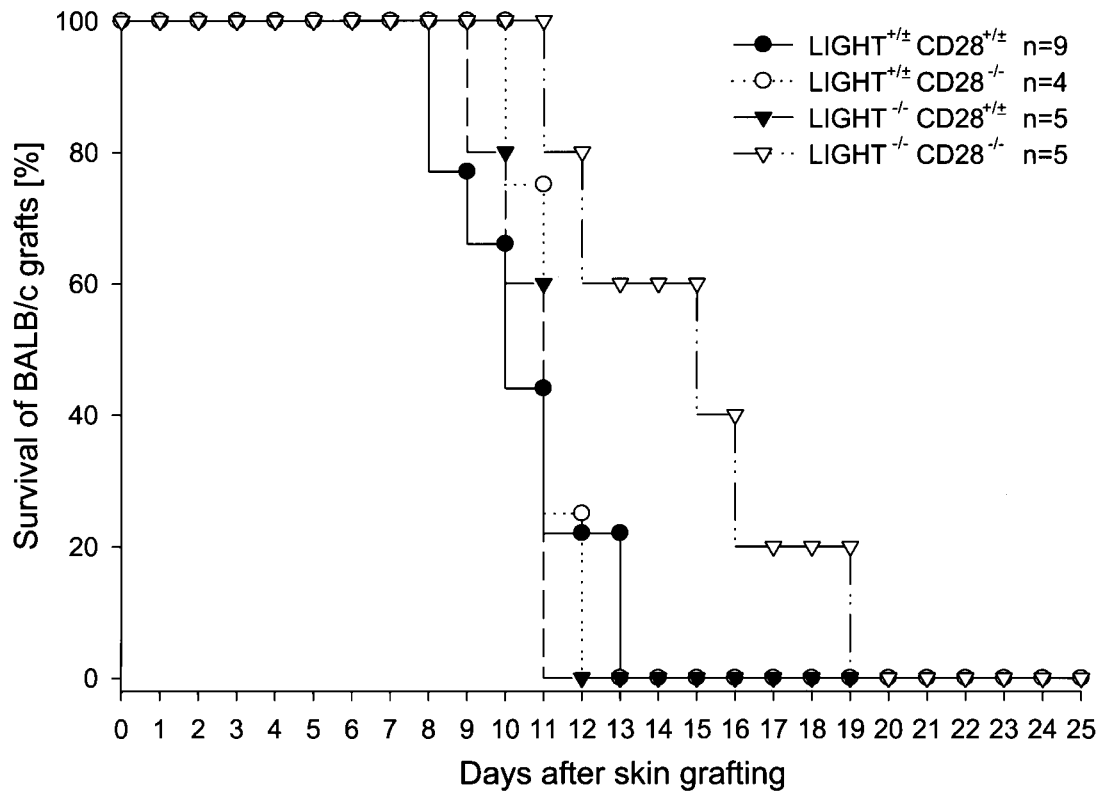
**Skin Allograft Survival in  $LIGHT^{-/-}$   $\text{CD28}^{-/-}$  Mice.** To address the role of LIGHT in antiallogeneic immune responses in vivo, allogeneic skin graft rejection was investigated in  $LIGHT^{-/-}$  mice. A first set of experiments showed, that LIGHT-deficient mice exhibited no significant differences in their ability to acutely reject skin allografts. The same was true for  $\text{CD28}^{-/-}$  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  $\text{CD28}^{-/-}$  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,  $LIGHT^{-/-}$   $\text{CD28}^{-/-}$  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  $2 \times 10^6$  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

$2 \times 10^6$  PFU VSV were cocultured with  $^{51}\text{Cr}$ -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).



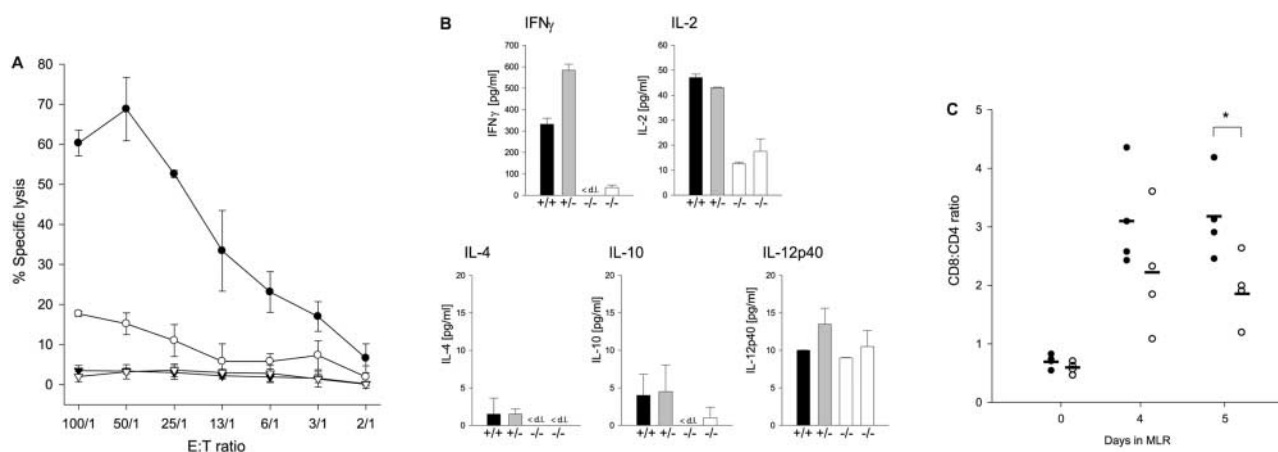
**Figure 5.** Delayed allogeneic skin graft rejection in LIGHT<sup>-/-</sup>CD28<sup>-/-</sup> mice. Groups of 4–9 mice were given tail skin transplants from fully allogeneic LIGHT<sup>-/-</sup> (H-2<sup>d</sup>) mice. Grafts were monitored daily. Black circles, WT animals or mice heterozygous for either 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<sup>-/-</sup> effector splenocytes showed a marked reduction in specific target cell lysis as compared with LIGHT<sup>+/-</sup> effectors (Fig. 6 A). MLR supernatants from LIGHT-deficient splenocytes contained remarkably reduced amounts of IFN- $\gamma$  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<sup>®</sup> analysis (Fig. 6 C). On day 5, we found the ratio of CD8<sup>+</sup> versus CD4<sup>+</sup> activated responder T cells significantly reduced by 40% in the LIGHT-deficient MLRs as compared with those with LIGHT<sup>+/-</sup> or WT splenocytes. The CD8/

CD4 ratio of naive splenocytes was comparable in WT, LIGHT<sup>+/-</sup>, and LIGHT<sup>-/-</sup> mice. To elucidate the differential effects of LIGHT on CD8<sup>+</sup> versus CD4<sup>+</sup> T cell subpopulations, MLRs were performed after depletion of CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells from the LIGHT<sup>-/-</sup> or LIGHT<sup>+/-</sup> responder cells (Fig. 7). LIGHT<sup>-/-</sup> CD4-depleted MLRs showed drastically reduced proliferation as compared with the corresponding LIGHT<sup>+/-</sup> 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<sup>-/-</sup> MLRs showed drastically reduced amounts of IL-2 as compared with LIGHT<sup>+/-</sup> MLRs, clearly indicating LIGHT as a crucial cytokine for the induction of IL-2 secretion by CD4<sup>+</sup> 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- $\gamma$  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- $\gamma$  or IL-4 production of separated CD4<sup>+</sup> or CD8<sup>+</sup> T cells after primary alloantigenic 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<sup>+/-</sup> (black symbols) or LIGHT<sup>-/-</sup> (white symbols) H-2<sup>b</sup> mice were cultured with irradiated LIGHT<sup>+/-</sup> or LIGHT<sup>-/-</sup> H-2<sup>d</sup> splenocytes. On day 5, CTL activity of effector splenocytes was assessed against the H-2<sup>d</sup> target P815 (circles) or the H-2<sup>b</sup> target EL-4 (triangles) in a standard <sup>51</sup>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<sup>®</sup> analysis was performed on allogeneic MLRs on days 0, 4, and 5. Shown is the ratio of CD8<sup>+</sup> versus CD4<sup>+</sup> cells in MLRs with LIGHT<sup>+/-</sup> or LIGHT<sup>+/+</sup> (black circles) or LIGHT<sup>-/-</sup> (white circles). Each circle represents an independent MLR. Means of experimental groups are shown as bars. Asterisk indicates statistically significant results ( $P = 0.0025$ ).

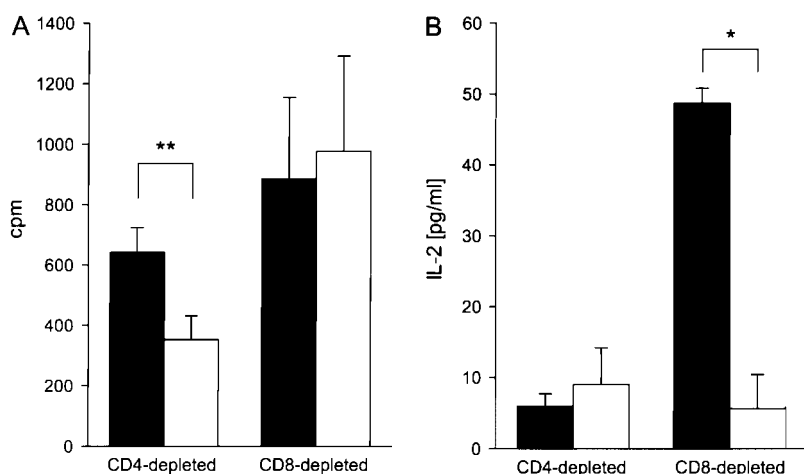
genic MLR in vitro. Interestingly, the accompanying decrease in cytokine production by LIGHT-deficient splenocytes encompassed not only Th cell type 1 responses represented by IFN- $\gamma$  and IL-2 but also the typical Th cell type 2 cytokines IL-4 and IL-10. Detailed analyses revealed that LIGHT has direct influence on the proliferative activity of CD8<sup>+</sup> T cells and the IL-2 production of the CD4<sup>+</sup> 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 LT $\beta$ R (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 LT $\beta$ , 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 LT $\alpha$  $\beta$  heterotrimer. However, one can still not completely exclude a possibly additional role of HVEM signaling in lymphoid organogenesis since LT $\alpha$  $\beta$ -HVEM interactions are intact in LIGHT-deficient animals.



**Figure 7.** Proliferation and IL-2 production of CD4- or CD8-depleted LIGHT<sup>-/-</sup> splenocytes after allogeneic MLR activation. Splenocytes from LIGHT<sup>+/-</sup> (black bars) or LIGHT<sup>-/-</sup> (white bars) H-2<sup>b</sup> mice were either depleted for CD4<sup>+</sup> or CD8<sup>+</sup> cells, followed by coculture with irradiated LIGHT<sup>+/-</sup> or LIGHT<sup>-/-</sup> H-2<sup>d</sup> splenocytes, respectively. (A) For the measurement of proliferation in the absence of CD4<sup>+</sup> or CD8<sup>+</sup> cells, <sup>3</sup>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  $\beta$ -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  $LT\beta$  in MLN development. Generally,  $LT\beta$  singly deficient animals retain MLNs, with frequencies of  $LT\beta$ -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  $LT\beta$  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  $LT\beta$  exhibited a markedly reduced frequency of mice containing MLNs, indicating that in the absence of  $LT\beta$ , LIGHT provides alternative signaling via the  $LT\beta R$ , the latter being indispensable for MLN development (24). The presence of MLNs in 25% of the  $LIGHT/LT\beta$  doubly deficient animals hints at a yet undiscovered ligand signaling via  $LT\beta R$  or a weak binding activity of the  $LT\alpha_3$  homotrimer to the  $LT\beta R$  in vivo, though in vitro data do not support the latter possibility (45).

The relatively conserved splenic microarchitecture of  $LT\beta$  single knockout mice lacking  $LT\alpha_1\beta_2$  heterotrimers with largely intact T and B cell segregation is retained in  $LIGHT/LT\beta$  double knockout animals which contrasts to the more disturbed situation found in  $LT\beta R$ - as well as  $LT\alpha$ -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  $LT\beta R$ . Alternatively, one can argue for a functional role of  $LT\alpha$  beside its interaction with the  $LT\beta R$  as member of the  $LT\alpha_1\beta_2$  heterotrimer. The three members of the TNFR family described to engage  $LT\alpha$  as homotrimer are TNFRp55, TNFRp75, and HVEM. Since  $LT\beta/TNF$  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  $LT\beta R$ -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  $LT\beta R$  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  $LT\beta R$ -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- $LT\beta R$  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 to 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<sup>+</sup> or CD8<sup>+</sup> 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, matured 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- $\alpha$  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.

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