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Therapeutic blockade of LIGHT interaction with HVEM and LTβR attenuates *in vivo* cytotoxic allogeneic responses

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Abstract

Background—TNF/TNFR superfamily members conform a group of molecular interaction pathways of essential relevance during the process of T cell activation and differentiation towards effector cells and particularly for the maintenance phase of the immune response. Specific blockade of these interacting pathways, such as CD40/CD40L, contributes to modulate the deleterious outcome of allogeneic immune responses. We postulated that antagonizing the interaction of LIGHT expression on activated T cells with its receptors, HVEM and LTβR may decrease T cell-mediated allogeneic responses.

Methods—A flow cytometry competition assay was designed to identify anti-LIGHT monoclonal antibodies capable to prevent the interaction of mouse LIGHT with its receptors expressed on transfected cells. An antibody with the desired specificity was evaluated in a short-term *in vivo* allogeneic cytotoxic assay and tested for its ability to detect endogenous mouse LIGHT.

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Material and Methods (SDC, Material and methods)

Disclosure of Conflicts of Interest

The authors declare no conflicting financial interests.

Results—We provide evidence for the first time that in mice, as previously described in humans, LIGHT protein is rapidly and transiently expressed after T cell activation, and this expression was stronger on CD8 T cells than on CD4 T cells. Two anti-LIGHT antibodies prevented interactions of mouse LIGHT with its two known receptors HVEM and LTβR. *In vivo* administration of anti-LIGHT antibody (clone 10F12) ameliorated host anti-donor short-term cytotoxic response in WT B6 mice, although to a lesser extent than that observed in LIGHT-deficient mice.

Conclusions—The therapeutic targeting of LIGHT may contribute to achieve a better control of cytotoxic responses refractory to current immunosuppressive drugs in transplantation.

Keywords

HVEM (TNFRSF14); LIGHT (TNFSF14); LTβR (TNFRSF3); DcR3 (TNFRSF6b); co-stimulation; transplantation; alloreactivity; graft rejection; graft versus host disease; cytotoxicity

Introduction

Human LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes) is a member of the TNF superfamily transiently detected on human T cells upon activation (1,2) and immature dendritic cells (3,4). Mouse LIGHT is a type II transmembrane protein of 239 amino acids, with an extracellular region 74% similar in amino acid sequence to human LIGHT (1,5).

LIGHT can act as a costimulatory molecule independently of CD28 (3,4), fostering T cell proliferation in the mixed lymphocyte reaction and promoting the process of DC maturation as well (6). It can even augment antitumor activity directly (7) or indirectly through enhancing CTL activity against tumor cells (4). In line with the costimulatory activity of LIGHT, constitutive transgenic expression of LIGHT under the control of a T cell-specific promoter led to chronic inflammation of mucosal tissues (8,9). In contrast, gene deletion of LIGHT results in defective CD8 T cell proliferation and acquisition of CTL effector function, which is associated with prolonged graft survival in several allogeneic mouse models of transplantation (10-13).

One of the LIGHT receptors is HVEM (TNFRSF14), which is broadly expressed on hematopoietic and non hematopoietic cells (14,15). HVEM is a type I transmembrane molecule with an extracellular portion divided into cysteine-rich domains (CRD1-4) (16-18) with distinct binding sites for its ligands. BTLA and CD160 bind to the CRD1 and part of the CRD2 of HVEM, and so does the viral protein gD of Herpes Simplex Virus (HSV) (19,20), whereas LIGHT interacts with CRD2 and CRD3 on opposite sides of the extracellular part of HVEM (21). Furthermore, membrane LIGHT can be released by the action of a metalloprotease (22) and the soluble form of LIGHT binds to BTLA/HVEM complex and strengthens the molecular interaction, whereas engagement of membrane anchored HVEM by LIGHT in *cis* displaces BTLA from its interaction with HVEM and allows bidirectional *trans* co-stimulatory contacts between HVEM and LIGHT (1,23,24).

The other well-characterized receptor of LIGHT is the LTβR, which is expressed on follicular dendritic cells (FDCs), dendritic cells (DCs), macrophages, stromal cells and high endothelial venules (HEV) (25). LTαβ $CD4^+CD3^-$ inducer cells interact with LTβR on stromal organizer cells to guide lymphoid organogenesis during development and, later on, stroma-derived LTβR signaling is still essential for the maintenance of the lymphoid tissue structure (26,27). LTαβ expression on activated $CD4^+$ helper T cells (28) and LTβR on DCs and B cells follows a similar pattern to that of CD40L and CD40 expression on T cells and antigen presenting cells respectively, suggesting that LTαβ/LTβR pathway may regulate the exchange of information between antigen presenting cells and T cells, and therefore participate in T cell activation and differentiation. LIGHT expressed on activated T cells may provide a licensing signal upon interaction with LTβR expressed on DC (6) or on stromal cells that would in turn modify the lymphoid tissue environment to achieve proper T cell priming.

So far, there have been no reagents available capable to specifically recognize conformational epitopes on the extracellular region of the mouse LIGHT, although reagents against human LIGHT are available (6), (29). In an attempt to define the therapeutic potential of targeting LIGHT in animal model systems, and to detect and follow membrane LIGHT expression, rat monoclonal antibodies against mouse LIGHT were raised and selected based on their ability to block the binding of soluble LTβR-Ig or HVEM-Ig to LIGHT-transduced cells. Their therapeutic activity was then assessed in an *in vivo* mouse model of alloreactivity and we demonstrated that the specific blockade of LIGHT mitigated the *in vivo* cytotoxic allogeneic immune response. These observations pointed out that LIGHT/HVEM/LTβR interacting pathway is an amenable therapeutic target for the immune intervention for the control of cell-mediated cytotoxic responses.

Results

Conserved cross-interactions between mouse LIGHT receptors and mouse and human LIGHT

The TNF receptor-binding domain of LIGHT interacts with CRD2 and CRD3 on one side of membrane anchored HVEM, whereas BTLA and CD160 interact with CRD1 and CRD2 on the opposite side of HVEM (2,21,30). According to molecular modeling and previous studies, the receptors HVEM, LTβR and DcR3 share widely overlapping binding sites on LIGHT (2). DcR3 is a soluble decoy receptor that is present in human but has no known counterpart in mouse. Interactions of mouse LIGHT with its receptors are not very well documented, in part due to the difficulty of preparing active soluble mouse LIGHT (31). We indeed found that either recombinant soluble mouse LIGHT fused to an IgG_{2a} Fc fragment, or LIGHT multimerized with an isoleucine zipper, failed to bind its receptors (data not shown) (32). However, a third soluble form of mouse LIGHT, containing amino acids 72-239 linked to a Flag-Foldon tag proved to be active and efficiently reacted with mouse HVEM, mouse LTβR and human DcR3 expressed as full-length or glycolipid-anchored proteins on HEK-293T cells (Figure 1A). Human LIGHT gave a similar binding pattern, except that its binding to mouse LTβR was weaker in this particular experimental setting (Figure 1A). Similar results were obtained when cells expressing full-length mouse or

human LIGHT were stained with receptor.Ig fusion proteins. In these experiments, mouse HVEM and mouse LTβR bound to mouse and human LIGHT, whereas human DcR3 gave weaker stainings (Figure 1B).

Taken together, the binding results indicate that in the mouse system, LIGHT indeed binds the relevant receptors HVEM and LTβR, which is in agreement with and extends conclusions of previous reports describing the binding interactions between LIGHT and its natural receptors (7,31).

Differential competition of soluble forms of LTβR and HVEM for binding to LIGHT

HVEM delivers costimulatory signals to T cells when engaged by LIGHT, while LIGHT/LTβR functionally modulates dendritic cells and stromal cells to promote an adequate environment for T cell priming (33,34). Since both LTβR and HVEM bind to LIGHT, when both receptors are simultaneously expressed in *cis* (on the same cell) or in *trans* (in different cells), the advantageous competition of one of the receptors over the other would displace the less competitive receptor from interacting with LIGHT (2). Under those circumstances, binding of the receptor with the highest affinity may dominate a particular signaling pathway. Saturation LIGHT binding curves were established for mHVEM.IgG_{2a} (HVEM-Ig) and for mLTβR.IgG_1 (LTβR-Ig). Interestingly, concentrations of receptors required to achieve saturation binding was about 20-fold higher for HVEM-Ig than for LTβR-Ig (Figure 2A). LIGHT-transduced cells preincubated with a saturated amount of soluble HVEM-Ig did not prevent LTβR-Ig from binding to LIGHT (Figure 2B, left lower panel). In contrast, preincubation of LIGHT transduced cells with LTβR-Ig completely abrogated the binding of HVEM-Ig to LIGHT-transduced cells (Figure 2B, right lower panel).

These experiments suggest that HVEM, at least in its recombinant form, has a lower affinity for mouse LIGHT than for mouse LTβR and confirm that HVEM and LTβR binding sites on LIGHT overlap.

Anti-LIGHT antibodies that efficiently recognize and block mouse LIGHT

A LIGHT-transduced NIH-3T3 cell line coexpressing eGFP (clone 2B7) was obtained by limiting dilution cloning and used as immunogen in rats to produce anti-mouse LIGHT hybridomas. Hybridoma supernatants were screened for their recognition of mouse LIGHT by flow cytometry using LIGHT-GFP- or control GFP-transduced NIH-3T3 cell lines. Four rat IgG_{2a} anti-LIGHT antibodies (10F12, 3G1, 6H12 and 9B7) specifically recognized an epitope located at the extracellular region of mouse LIGHT (Figure 3A, left panels). Of note, all four antibodies cross-reacted with human LIGHT expressed on transiently transfected HEK-293T cells (Figure 3A, right panels).

Anti-LIGHT antibodies were screened for their ability to prevent the binding of HVEM-Ig and LTβR-Ig to LIGHT-transduced cells. Two anti-LIGHT antibodies (10F12 and 3G1) fully prevented the binding of HVEM-Ig to mouse LIGHT-expressing cells in a FACS-based assay, whereas antibodies 9B7 and 6H12 did not (Figure 3B). Anti-LIGHT, clone 10F12 was the most potent antibody to inhibit the binding of LTβR-Ig to mouse LIGHT,

although inhibition did not reach baseline, whereas 3G1 only partially inhibited this interaction (Figure 3B).

Anti-LIGHT mAb 10F12 was further characterized in a binding assay between membrane-bound and soluble LIGHT, in which 10F12 binding to membrane-bound LIGHT is competed by soluble Flag-Foldon (FF)-LIGHT (Figure 3C). At the EC_{50}, 10F12 and FF-LIGHT were at equimolar concentrations, indicating no obvious bias of 10F12 to recognize membrane-bound LIGHT, and therefore validating the quality of soluble recombinant FF-LIGHT. The ability of 10F12 to prevent the binding of FF-LIGHT to membrane-bound HVEM and LTβR was then investigated (Figures 3D, E). 10F12 inhibited the binding of FF-LIGHT to both full-length HVEM and GPI-anchored LTβR with a similarly good efficiency: at EC_{50}, the ratio of antibody binding sites to FF-LIGHT epitopes was stoichiometric, and a five-fold molar excess of antibody to LIGHT totally abrogated LIGHT binding to both receptors (Figures 3D, E). Of the nineteen mouse TNF family members, 10F12 only recognized LIGHT in a sandwich ELISA assay, validating its binding specificity (Figure 3F). The affinity of the interaction of FF-LIGHT with a monomeric Fab fragment of 10F12 prepared by ficin digestion (Fig. 3G) was measured by surface plasmon resonance. The association rate constant (k_a) was 4.2×10^5 M^{-1} s^{-1}, the dissociation rate constant (k_d)) was 2.3×10^{-3} s^{-1} and the affinity (K_D) was 5.4 nM (Rmax: 176, Chi^2: 3.4) (Figure 3H). For comparison, a panel of Fab from agonist anti-EDAR antibodies with *in vivo* activity had k_d ranging from 9.6 to 0.24 $\times10^{-3}$ s^{-1} (the smallest the number, the better the antibody sticks to its antigen once bound) and affinities from 0.5 - 40 nM (35).

In summary, we have identified both blocking and non-blocking monoclonal antibodies recognizing surface-exposed mouse LIGHT that cross-reacted with human LIGHT. One of these antibodies had decent binding parameters and showed efficient and specific blockade of the interaction of LIGHT with its two receptors, making it possible for the first time to monitor the expression of mouse LIGHT at protein level and to conduct LIGHT-blocking therapeutic experiments in mice.

Mouse LIGHT is transiently expressed on activated CD8 T cells and NK cells, and to a lesser extent in CD4 T cells

Anti-LIGHT antibodies were tested for their ability to detect endogenous LIGHT. Considering that in humans, LIGHT protein is not detectable on resting T cells, fresh mouse B6 WT and B6 LIGHT KO splenocytes were stimulated *in vitro* with PMA plus ionomycin for 5 h in the presence of either HiLyte-647 labeled anti-LIGHT (10F12) or HiLyte-647-labeled rat IgG_{2a} isotype control. This strong polyclonal T cell activation prompted a transient expression of LIGHT that was readily detected in virtually all CD8 T cells and to a lower extent in a subset of CD4 T cells of WT B6 mice, but not in LIGHT-deficient T cells (Figure 4A), in line with the previous description of LIGHT expression in human T cells (29), (1). Polyclonal activation with PMA plus ionomycin also induced transient expression of LIGHT on NK cells, but not in similarly stimulated LIGHT KO NK1.1 cells (Figure 4B). Because of the transient expression of LIGHT in activated T cells, it is noteworthy to mention that the labeled antibody needs to be present during the course of activation in order to achieve successful detection.

In vivo allogeneic cytotoxic activity is significantly reduced after antibody-mediated blockade of the LIGHT/HVEM/LTβR pathway, although to a lesser extent than in LIGHT-deficient mice

To elucidate whether antibody blockade of the LIGHT/HVEM/LTβR pathway could modulate allogeneic cytotoxic responses, B6 recipient mice were injected with an identical number of B6, BALB/c and F1 target cells labeled with different amounts of CFSE, as mentioned in the Material and Methods section. As shown in figure 5, the percentage of killing of allogeneic BALB/c and F1 target cells in host B6 spleen (Figure 5A) and peripheral lymph nodes (Figure 5B) was significantly reduced in LIGHT-deficient mice compared to B6 WT mice (BALB/c target cells: spleen and pLNs, $p<0.0005$ and F1 target cells: spleen and pLNs, $p<0.005$). In line with these results, antagonist anti-LIGHT monoclonal antibody 10F12 significantly mitigated host anti-donor cytotoxic responses against BALB/c target cells (spleen, $p<0.005$ and pLNs, $p<0.05$), and showed a trend towards protection of F1 targets that however did not reach statistical significance (Figure 5A and 5B).

As the cytolytic response was diminished in LIGHT KO mice and also to a certain extent in anti-LIGHT-treated WT mice, we evaluated whether donor alloreactive T cell proliferation would be altered in semiallogeneic F1 recipients. Donor LIGHT KO CD4 and CD8 T cells proliferated significantly less efficiently than isotype control or anti-LIGHT treated donor WT T cells in the spleen of adoptively transferred F1 recipients (Figure 5C). Moreover, this observation correlated with a diminished frequency of LIGHT KO donor alloreactive CD4 T cells and CD8 T cells (Figure 5D) expressing IL-2Rα when compared to isotype- or anti-LIGHT treated WT donor T cells adoptively transferred into semiallogeneic F1 recipients.

Therefore, this short-term *in vivo* cytotoxic assay reveals that *in vivo* administration of anti-LIGHT mAb ameliorated the host anti-donor short-term cytotoxic response in WT B6 mice although to a lesser extent than that observed in LIGHT-deficient mice.

Discussion

The development of biologics aimed at preventing receptor / ligand interactions between TNF/TNFR molecules are alternative therapeutic arms of interest to conventional immunosuppression for the control of alloreactivity (14), (36). One of such therapeutic targets is the process of T cell activation and differentiation that drives the acquisition of effector T cell function. The molecules involved in the exchange of information between DC/T, B/T and T/T cell interactions belong to two major families of proteins, the Immunoglobulin (Ig) superfamily and the Tumor Necrosis Factor / Tumor Necrosis Family Receptor Superfamily (TNF/TNFRSF), the latter exhibiting cysteine-rich domains in the extracellular region of the molecule (31,37).

In this work, we provide for the first time a monoclonal antibody capable to detect LIGHT expression on mouse T cells and NK cells, and that additionally blocks the receptor binding site of LIGHT. We also demonstrated that therapeutic intervention with this antagonist anti-LIGHT antibody protected to some extent against rejection, although it did not fully recapitulate the attenuated cytotoxic immune response seen in LIGHT-deficient mice.

Although numerous reports in mouse models of inflammatory diseases have provided indirect experimental evidence that LIGHT may be involved in the pathogenesis of these immune-related diseases (10,38-42), this information contrasts with the lack of appropriate reagents for the detection of endogenous mouse LIGHT. The reason for this gap was the difficulty to engineer a genetic construct that produced a bioactive mouse LIGHT molecule with binding affinity for membrane-bound LIGHT receptors. We and others have evaluated a classical approach of fusing the extracellular region of mouse LIGHT either to Flag, the Fc fragment of the immunoglobulin heavy chain, or to an isoleucine zipper for the multimerization of the molecule (32), but none of these engineered genetic constructs succeeded in generating a protein with detectable binding affinity for mouse HVEM and LTβR. This was possible through a strategy that consisted of fusing the extracellular region of mouse LIGHT to a Flag-Foldon tag at the C-terminal site of this type II transmembrane protein (7).

Human LIGHT is constitutively expressed on intestinal mucosal CD8 T cells, CD4 T cells and NK cells and this expression is inducible by CD2-mediated signaling, which correlates with the typical activated state of resident lymphoid cells populating the intestinal mucosa (43), (44). In contrast to mucosal sites, in peripheral blood, LIGHT is inducible after exposure to PMA/ionomycin in CD8 T cells and to a similar extent in $CD4^+$/CD45RO memory T cells and in CD4 Th1 IFN-γ producer cells and this expression was higher than that detected on naive CD4 T cells (44). The differential expression of LIGHT in humans, which is constitutive on lymphocytes of mucosal tissues and inducible on peripheral blood lymphocytes, indicates that regulation of LIGHT expression is associated with an activation and responsive status of the lymphoid cell. These results are in agreement with the observed transient expression of LIGHT on mouse peripheral lymphoid cells upon exposure to PMA/Ionomycin for 5 hours, indicating a similar regulation of inducible expression on peripheral blood of both human and mouse. This suggests that mice models of disease may contribute to unravel the physiology of LIGHT and its role during the course and in the context of an allogeneic response.

LIGHT (TNFSF14) and CD40L (also named CD154, TNFSF5) are both members of the TNF ligand superfamily. CD40L expression is not detectable on naive T cells (45) and the same holds true for the expression of human LIGHT (29), which is agreement with the observation in our work that LIGHT expression is neither detectable on naïve T cells and NK cells and was only seen transiently upon T cell activation with a strong polyclonal stimulus. As a matter of fact, mouse LIGHT protein detection needed the presence of the fluorescently-conjugated antibody against LIGHT during *in vitro* stimulation of T cells and NK cells. This is probably due to the fact that LIGHT is rapidly internalized after transient surface exposure. Contrary to CD40L, which is mainly expressed on CD4 T cells, mouse LIGHT presents a more pronounced expression on CD8 T cells than on CD4 T cells, suggesting a more predominant functional role on this T cell subset.

TcR recognition of a foreign peptide in the context of MHC along with costimulation drives T cell activation, IL-2 secretion and up-regulation of IL-2Rα chain (CD25), which associates with beta and gamma chain of IL-2 to configure the high affinity IL-2R. This permits IL-2-mediated autocrine CD4 T cell proliferation and clonal expansion and provides

help for CD8 T cell clonal expansion and differentiation to effector CD8 T cells. *In vitro* studies with LIGHT KO CD4 T cells evidenced a deficiency in IL-2 secretion compared to WT CD4 T cells in response to polyclonal activation with anti-CD3/CD28 (11), which was also noticeable in the mixed lymphocyte reaction (12). The lack of secreted IL-2 likely contributes to the impaired CD8 T cell proliferation and differentiation to effector T cells, which also express less CD25 and therefore would proliferate less efficiently in response to IL-2 (12), (11). In agreement with this defective *in vitro* functional activity, our data also reflected similar defects *in vivo* such as a lower frequency of donor alloreactive CD4 and CD8 T cells expressing the IL-2Rα chain and decreased proliferative rate observed in F1 recipients receiving LIGHT KO semiallogeneic splenocytes compared to F1 recipients receiving semiallogeneic B6 WT splenocytes either treated with isotype control or with anti-LIGHT mAb.

The treatment of mice with soluble decoy receptors administered as recombinant fusion proteins disrupts various ligand/receptor interactions simultaneously. Despite the indirect evidences collected from the use of these decoy receptors on different mouse models of allogeneic transplantation, these approaches do not provide clear evidence on whether LIGHT could be a potential target for immune intervention. For example, the administration of decoy receptors, such as HVEM-Ig, LTβR-Ig or sDcR3-Ig attenuates alloreactivity in murine models of disease, but it is difficult to conclude whether the observed outcome is the result of inhibiting LIGHT interaction with its receptors and to which extent the observed effects might be due in part to inhibition of other ligands such as lymphotoxin or FasL (4,46-48). Therefore, assignment of the most significant ligand/receptor pathway responsible for the observed *in vivo* effect is inherently difficult. Another consideration is that most of the decoy receptors used in preclinical rodent models of transplantation are composed of the extracellular region of the receptor bound to human IgG1 Fc fragment. It is well known that human IgG1 binds efficiently to mouse FcγRIV, the main receptor in mice involved in ADCC-mediated depletion by myeloid cells and NK cells (49). This means that many claims in the literature stating that these decoy receptors function as blocking reagents can be biased if depletion of ligand-expressing cells indeed may occur.

In contrast to recombinant fusion proteins, selective antibody-based approaches targeting one particular ligand/receptor interaction will likely provide more relevant information than the use of soluble decoy receptors. The selective antibody-mediated blockade of the LIGHT/HVEM/LTβR pathway prevented the *in vivo* host anti-donor cytotoxic alloresponse, although to a lesser extent than that seen in LIGHT-deficient mice. The relative lack of efficacy of the antibody *in vivo* was unexpected given the stoichiometric inhibition of LIGHT by the antibody *in vitro* and the favorable antibody to ligand ratio that can be achieved *in vivo*. Perhaps recombinant and over-expressed proteins used *in vitro* underestimated the binding affinity of LIGHT for its receptors *in vivo*, and 10F12 only partially blocked endogenous LIGHT. In this case, we predict that residual signaling may be preferentially delivered through LTβR, for which LIGHT has a higher affinity. Alternatively, LIGHT may engage its receptors *in vivo* in the immunological synapses established between cells that may exclude extracellular medium containing the antibody. Finally, it is also conceivable that, because of a life-long deficiency of LIGHT, LIGHT-ko

mice are intrinsically hyporesponsive, a phenotype that could not be reproduced by an acute, even full inhibition of LIGHT. Although LIGHT deficiency has been associated with impaired lymphocyte migration to lymph nodes, this only occurred under strong inflammatory conditions, which are not present in our experimental *in vivo* cytotoxic setting. This rules out the possibility that the observed reduction of cytotoxicity in LIGHT deficient mice or anti-LIGHT treated mice was a consequence of reduced migration of the target cells to these secondary lymphoid organs (50).

In summary, we report for the first time specific blocking and non-blocking monoclonal antibodies against mouse LIGHT as new reagents in the field of TNF/TNFR interactions to follow LIGHT protein expression and explore the preclinical consequences of interrupting LIGHT interactions with its receptors. We also demonstrated that targeting LIGHT may offer novel avenues for the control of cytotoxic responses in the setting of transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

LIGHT	homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to herpesvirus entry mediator, a receptor expressed on T lymphocytes
mAb	monoclonal antibody
GvHR	Graft versus host reaction
CD	Cluster of differentiation
HSV	Herpesvirus
HVEM	Herpesvirus entry mediator
CRD	Cysteine rich domain
APC	Antigen presenting cells
FDC	Follicular dendritic cell
TNF	Tumor necrosis factor
LTβR	Lymphotoxin beta receptor

DC	Dendritic cell
CTL	Cytotoxic T lymphocyte
NK	Natural killer
WT	Wild type
KO	Knock-out
mGFP	monster green fluorescent protein
eGFP	enhanced green fluorescent protein
APC	Allophycocyanin
PE	Phycoerythrin
MHC	Major Histocompatibility Complex
GPI	Glycosylphosphatidylinositol
PMA	Phorbol myristate acetate
CFSE	Carboxyfluorescein succinimidyl ester
Flag-shLIGHT	Flag-tagged soluble human LIGHT
FF-LIGHT	Flag-Foldon-tagged soluble mouse LIGHT
HVEM-Ig: HVEM.mIgG$_{2a}$.Fc	Herpesvirus entry mediator bound to mouse IgG$_{2a}$ Fc fragment.
LTβR-Ig: LTβR.huIgG$_1$.Fc	Lymphotoxin beta receptor bound to human IgG1 Fc fragment

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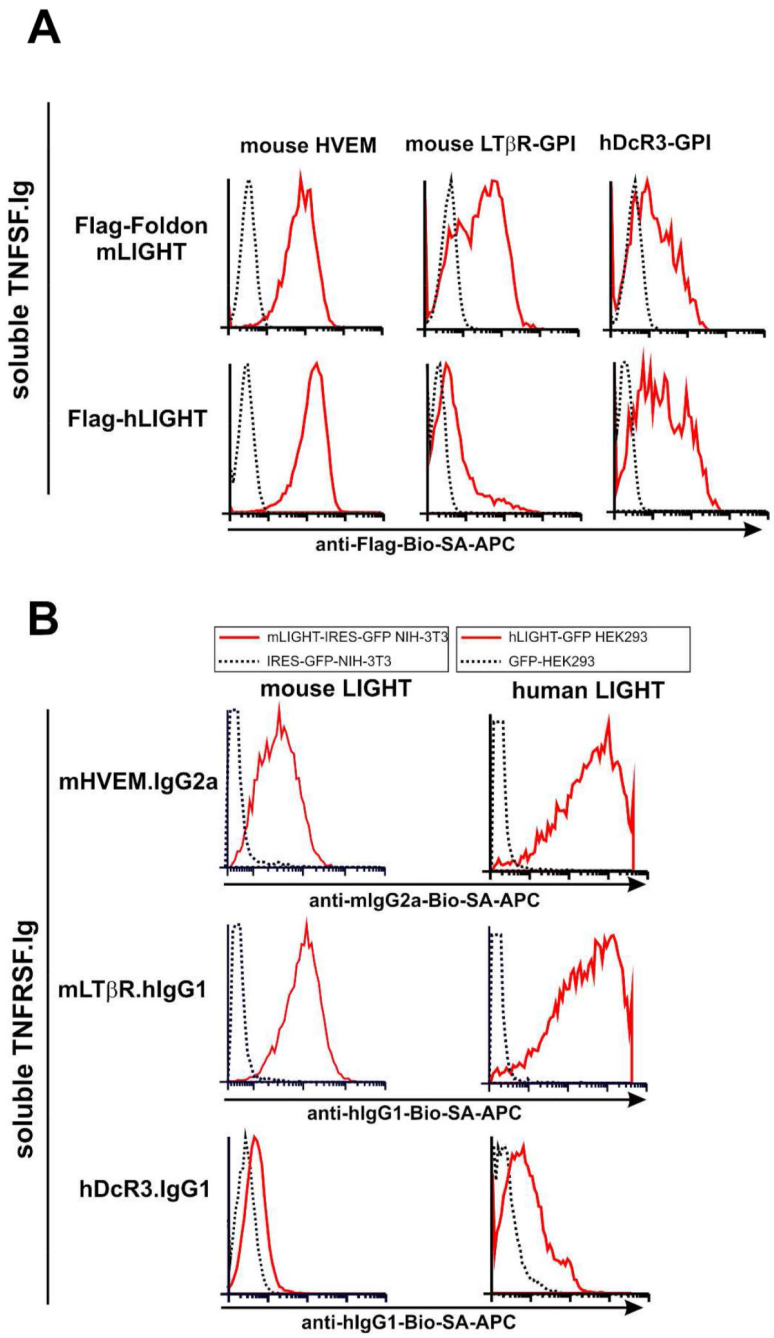

Figure 1. Cross-interaction binding assays of soluble TNFRSF and TNFSF recombinant proteins of the LIGHT/HVEM/LTβR/DcR3 system

(A) The complete gene encoding mouse HVEM molecule fused to GFP, mouse LTβR-GPI or human DcR3-GPI were transfected into HEK-293T cells (red solid lines) and stained with FF-mouse LIGHT (upper panel) or Flag-tagged human LIGHT (lower panel). HEK-293T cells transfected with the empty plasmid were used as controls (black dotted lines). Receptor/ligand interactions were detected with biotinylated anti-Flag mAb (clone M2) plus allophycoerythrin-coupled streptavidin.

(B) 2×10^5 NIH-3T3 cells transduced with pMIG-mouse LIGHT-IRES-GFP (left panel, red solid lines) or HEK-293T cells transfected with human LIGHT bound to GFP (right panel, red solid lines) were incubated with mHVEM-Ig, mLTbR-Ig or hDcR3-Ig. As negative controls, NIH-3T3 or HEK-293T cells were transduced or transfected with the empty expression vector (black dotted lines). The ligand/receptor interactions were detected using biotinylated anti-mouse IgG_{2a} or anti-human IgG_1 followed by allophycocyanin-coupled streptavidin.

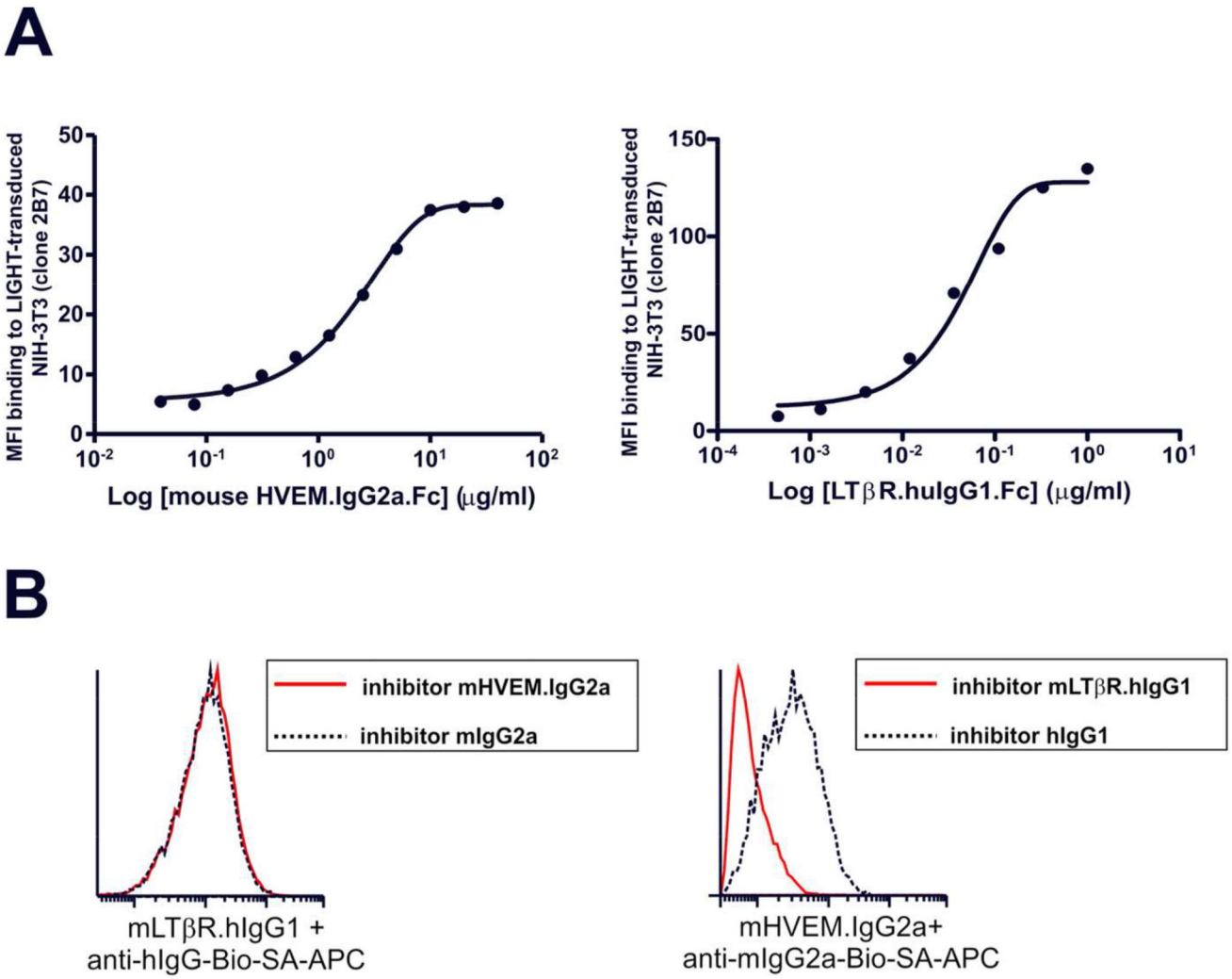

Figure 2. LTβR competes with HVEM for binding to the TNFR binding site of LIGHT

(A) LIGHT-transduced NIH-3T3 (2×10^5) cells were incubated with graded concentrations of purified sHVEM.IgG_{2a} (left panel) or LTβR.$hIgG_1$ (right panel). The binding of HVEM and LTβR to membrane LIGHT was evaluated by flow cytometry with appropriate anti-mouse and anti-human secondary antibodies. The mean fluorescence intensity (MFI) binding of LIGHT receptors to membrane-bound LIGHT was calculated and plotted against graded concentrations of HVEM.$mIgG_{2a}$ (left side panel) and LTβR.$hIgG_1$ (left side panel). (**B**) To test whether mouse HVEM-Ig and LTβR-Ig bind to the same or overlapping regions on mouse LIGHT, 2×10^5 NIH-3T3 transduced cells expressing mouse LIGHT on the cell surface were incubated with sHVEM.$mIgG_{2a}$ at 50 μg/ml (left panel) or LTβR.$hIgG_1$ at 10 μg/ml (right panel) (red solid lines) or with the controls $mIgG_{2a}$ or $hIgG_1$ (black dotted lines). In the presence of the inhibitors, either LTβR.$hIgG_1$ at 10 μg/ml (left side panel) or sHVEM.$mIgG_{2a}$ at 50 μg/ml (right side panel) was added to the reaction and detected with biotinylated anti-human IgG or anti-mouse IgG_{2a} followed by SA-APC.

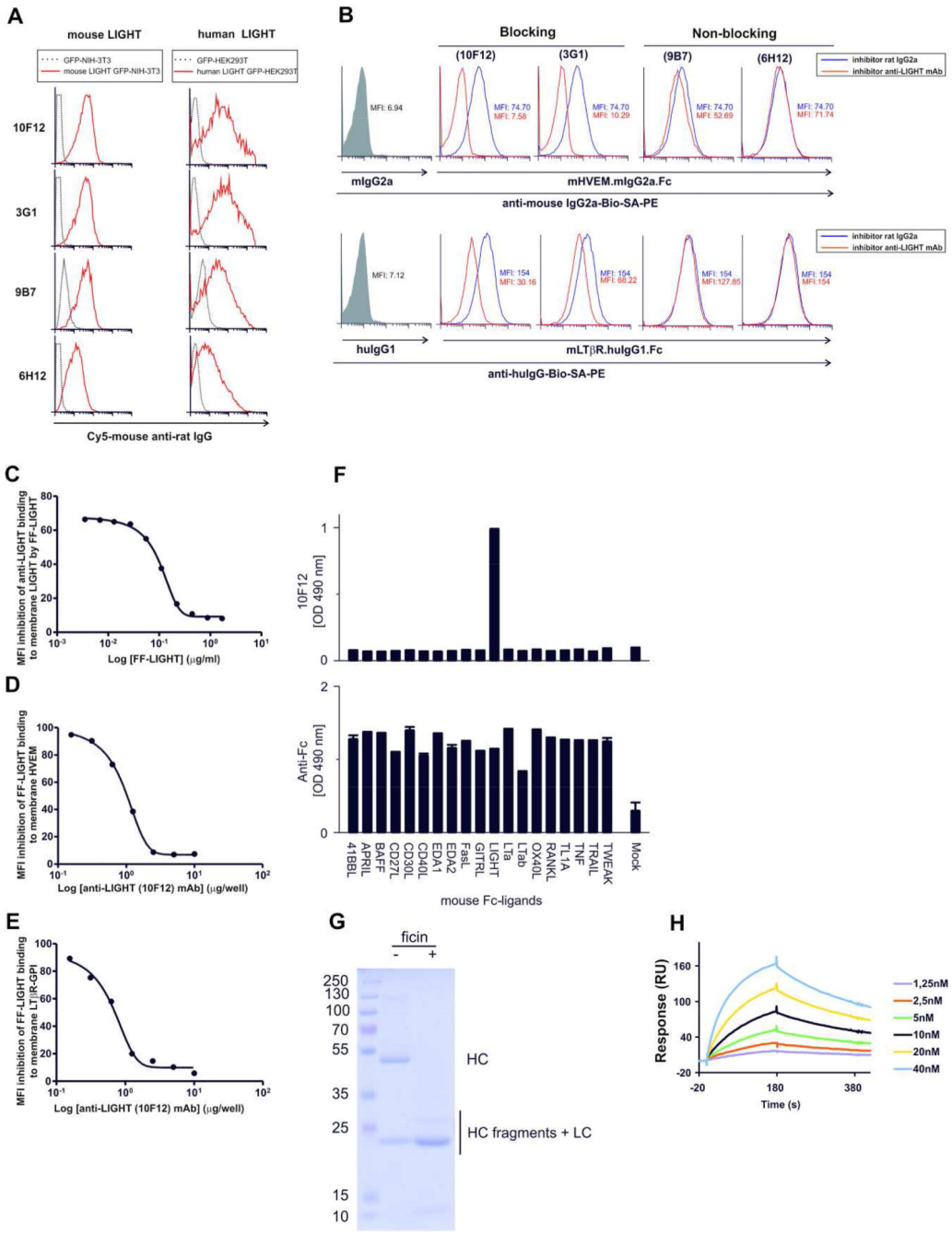

Figure 3. Specificity, cross-reactivity and antagonist activity of anti-LIGHT mAbs

(A, left panels) NIH-3T3 cells (2×10^5) were transduced with either pMIG-mouse LIGHT-IRES-GFP (red solid lines) or control plasmid pMIG-IRES-GFPs (black dotted lines) and were incubated with the indicated anti-LIGHT mAbs followed by Cy5-labeled mouse anti-rat IgG secondary antibody.

(A, right panels) Cross-reactivity of anti-mouse LIGHT mAbs with human LIGHT was tested with HEK-293T cells (2×10^5) cotransfected with either pcDNA3.1 plasmid harboring human LIGHT fused to GFP (red solid lines) or empty plasmid pcDNA3.1 - GFP

(black dotted lines). Binding was detected with Cy5-labeled mouse anti-rat IgG secondary antibody.

(B) Mouse LIGHT-transduced NIH-3T3 cells were incubated for 1 h at 37°C with anti-LIGHT mAbs (clones 10F12, 3G1, 9B7 and 6H12, red solid lines) or isotype-matched control rat IgG_{2a} (black dotted lines). Grey shaded histograms represent LIGHT-transduced cells incubated with either $mIgG_{2a}$ or $hIgG_1$ as control for the recombinant proteins. In the presence of saturating amounts of anti-LIGHT antibodies, the reaction was incubated with HVEM-Ig (upper panel) or LTβR-Ig (lower panel) and the binding of receptor.Ig was detected with appropriate biotinylated secondary antibodies followed by phycoerythrin-coupled streptavidin. The reduction of the mean fluorescence intensity (MFI) shown in each plot indicates the antagonist functional activity of anti-LIGHT antibodies.

(**C**) Graded concentrations of soluble recombinant mouse LIGHT were preincubated with a saturated amount of anti-mouse LIGHT monoclonal antibody (clone 10F12) for 1 hour at 37 °C. The antigen/antibody complex was added to LIGHT-transduced cells for 30 minutes at 37°C, then the reaction was washed and developed with biotinylated mouse anti-rat IgG_{2a} and SA-PE. The MFI inhibition of anti-LIGHT binding to membrane LIGHT in the presence of graded concentrations of FF-LIGHT is shown.

Graded concentrations of anti-LIGHT mAb were preincubated for 1 hour at 37 °C with an amount of mouse FF-mouse LIGHT recombinant protein sufficient to saturate binding to membrane-expressed receptors. Then, the antigen/antibody complex was added to HVEM-transfected CHO cells (**D**) or LTβR-GPI transfected HEK293T (**E**) cells for 30 minutes at 37°C. The reaction was washed and developed with biotinylated mouse anti-Flag mAb (anti-Flag, clone BioM2) and SA-PE. The MFI inhibition of soluble FF-mouse LIGHT binding to HVEM transfected cells or LTβR transfected cells is plotted.

F) Nineteen mouse TNF family ligands fused to the Fc portion of human IgG1 were captured in an ELISA plate and revealed with biotinylated 10F12 anti-mLIGHT mAb (top panel) or with an anti-human IgG antibody to reveal the presence of the various ligands (bottom panel).

G) Preparation of anti-LIGHT (10F12) Fab fragments. 10 μg of 10F12 mAb digested with or without immobilized ficin was analyzed by SDS-PAGE and Coomassie blue staining. HC: heavy chain. LC: light chain.

H) The Fab fragment of 10F12 mAb at the indicated concentrations was analyzed by surface plasmon resonance onto immobilized FF-mouse LIGHT. Fab solutions were applied for 180 s, and subsequently washed with buffer. The equilibrium dissociation constant K_D was 5.8×10^{-9} M.

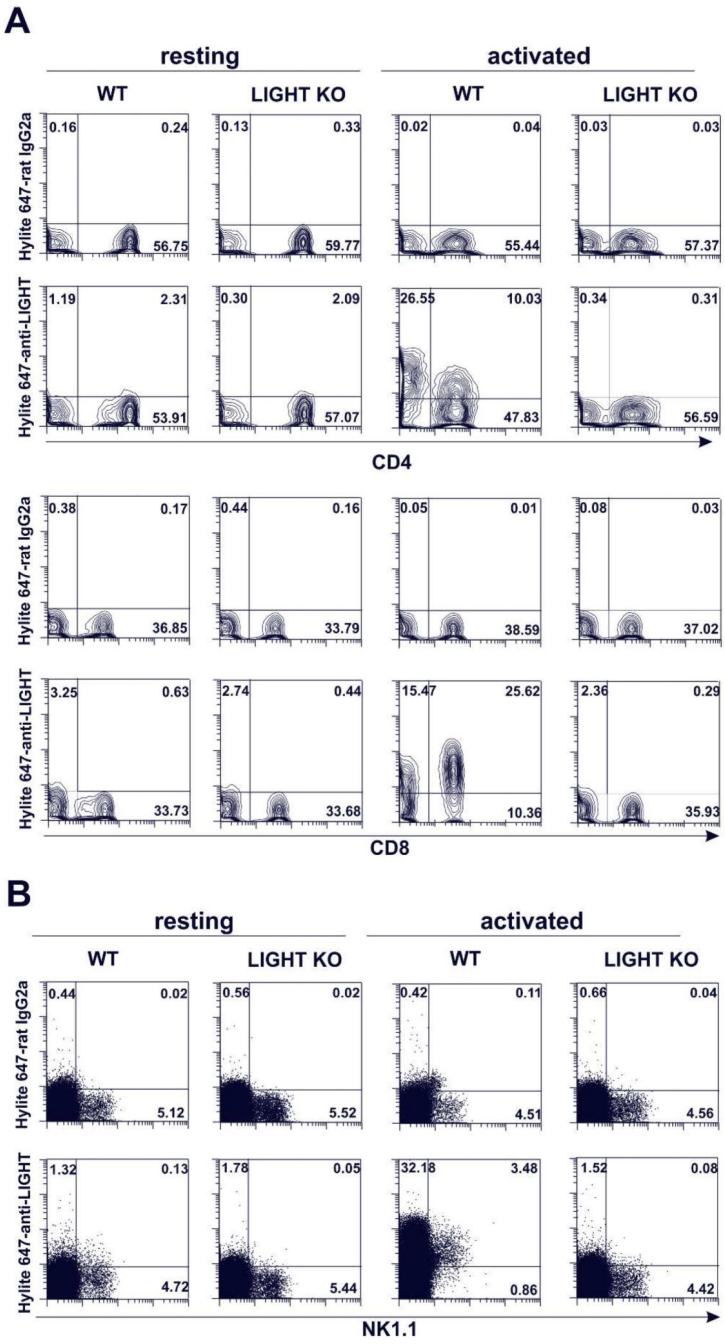

Figure 4. Mouse LIGHT is expressed upon activation more prominently on CD8 T cells than on CD4 T cells and it is also expressed on activated NK cells

C57BL/6 WT or LIGHT-deficient splenocytes were plated at 2×10^5 cells per well in 96-well plates and were left untreated or stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 5 h. Hylite 647-labeled anti-LIGHT mAb (clone 10F12) or Hylite 647-labeled isotype control (rat IgG_{2a}) were added to the cells during the incubation. The expression of mouse LIGHT was analyzed on living lineage-negative cells for CD19, CD11b and CD11c resting and activated $CD4^+$ (upper panel) and $CD8^+$ (lower panel) T cells **(A)** and on resting

and activated NK cells (NKT cells and non-T NK cells) of WT and LIGHT KO after gating out $CD19^+$ and $CD11c^+$ cells **(B)**.

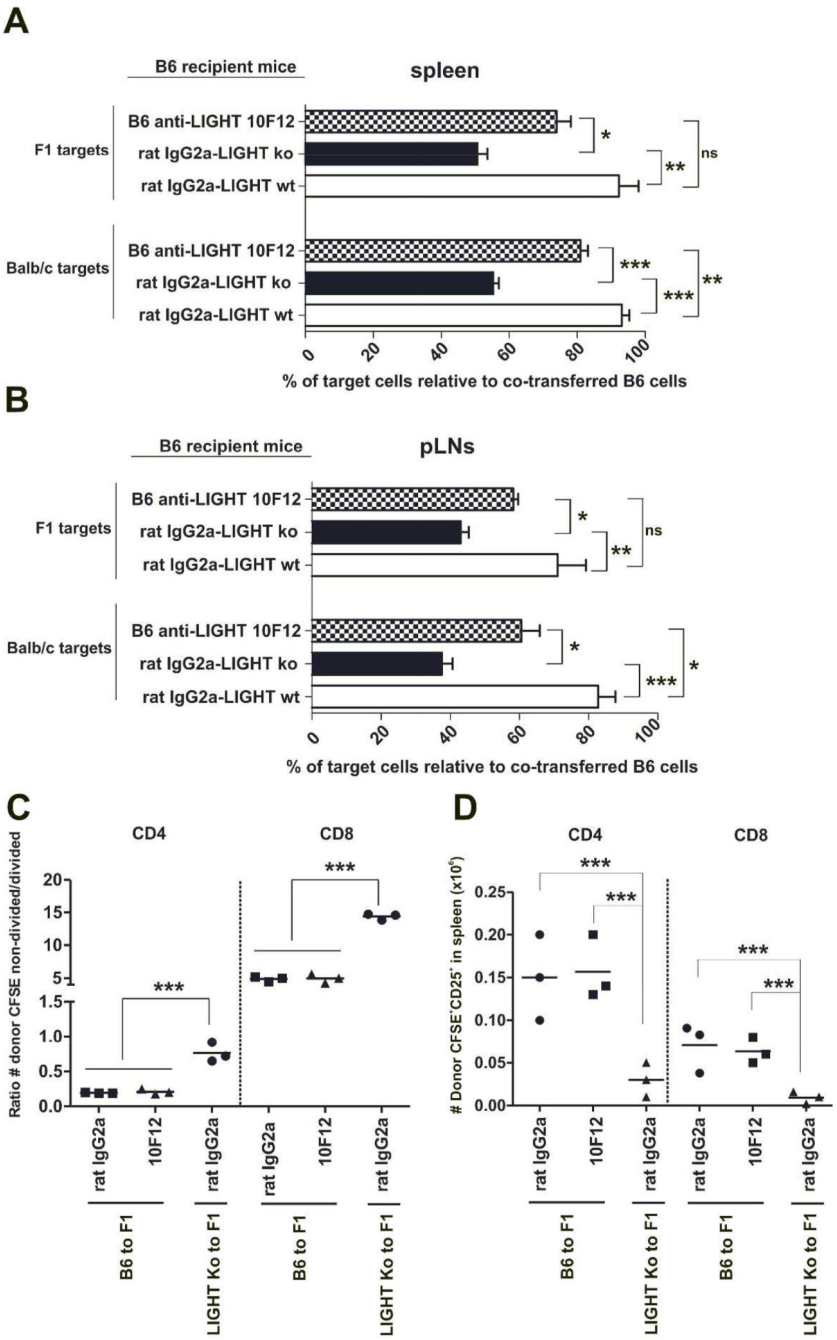

Figure 5. Decreased host anti-donor short-term cytotoxic response *in vivo* after antibody-mediated blockade of LIGHT

Isotype control (white bars) and anti-LIGHT-treated (shaded striped bars) WT B6 mice and isotype control-treated B6 LIGHT-deficient mice (black bars) received 30×10^6 splenocytes of each B6, BALB/c and F1 differentially labeled with CFSE. The percentage of specific lysis of the BALB/c and F1 populations relative to the B6 population in spleen **(A)** and peripheral lymph nodes (pLNs) (inguinal plus axillar) **(B)** was monitored 72 h after cell transfer. Data are representative of two independent experiments with three to four mice per

group. Bars indicate mean ± SEM and *t* test was used to compare differences between groups.

70×10^6 splenocytes from B6 WT or LIGHT-deficient mice labeled with 5 μM CFSE were injected into F1 recipients, which were treated with isotype control or anti-LIGHT 10F12 mAb. Three days later, the ratio of absolute number of donor CFSE labeled non-divided/divided CD4 and CD8 T cells **(C)** as well as the absolute number of donor CD4 and CD8 T cells expressing CD25 was calculated **(D)**. Statistical significance is indicated as follows: *, $p< 0.05$; **, $p< 0.005$; ***, $p< 0.0005$ and ns, non-significant.