SDC, Material and methods

Material and Methods

Animals

Twelve- to sixteen-week-old female Sprague-Dawley rats (Harland, Netherlands) and eight- to twelve-week-old female C57BL/6 (H-2^b, B6, CD45.2), BALB/c (H-2^d), CB6F1 mice (offspring of B6 × BALB/c, H-$2^{b/d}$) were bred at the animal facility of the University of Leon (Spain). Age- and sex-matched wild-type homozygous littermates control (LIGHT $^{+/+}$) and LIGHT-deficient mice (LIGHT $^{-/-}$) were also used. LIGHT-deficient mice were backcrossed more than ten times onto C57BL/6 background and PCR genotyping of LIGHT mutation was performed with the following set of primers: 5′- ACG CAT GTG TCC TGC GTG TGG - 3′, (mLIGHT type 1); 5′- CGA CAG ACA TGC CAG GAA TGG - 3′, (mLIGHT type 2); and 5′- GAC GTA AAC TCC TCT TCA GAC - 3′ (pneo1) (12).

Ethics statement

All experiments with rodents were performed in accordance and specifically approved for the Ethical Committee for Animal Research of the School of Veterinary Medicine (University of Leon) and the European Guidelines for Animal Care and Use of Laboratory Animals.

Recombinant fusion proteins and cell transfection

Flag-tagged soluble human LIGHT (hereafter, Flag-shLIGHT) and Flag-Foldon-tagged soluble mouse LIGHT (from now on, FF-mouse LIGHT) were kindly provided by Dr. Carl Ware, La Jolla, CA, USA and Dr. Shintani, Osaka, Japan respectively (7,14). Mouse HVEM linked to the Fc fragment of mouse IgG_{2a} (HVEM-Ig) was produced in insect cells by Genentech Inc. Mouse LTβR (amino acids 1-217) linked to human IgG1 Fc fragment (LTβR.Ig) cloned into a pcDNA3 vector was prepared essentially as described (51). The extracellular domain of the receptor DcR3 linked to human IgG1 cloned into a pcDNA3 plasmid was obtained from Dr. Hsieh (Taipei, Taiwan) (52).

Full-length mouse HVEM gene was cloned into a modified pcDNA3.1 (+) vector (Invitrogen), upstream of the gene encoding monster Green Fluorescent Protein (mGFP, Clontech) as previously described (53). Full-length mouse LIGHT gene was cloned into an ecotropic retroviral vector pMIG-IRES-eGFP. Human LIGHT gene kindly provided by Dr. Koji Tamada. Mouse LTβR-GPI (LTβR attached to membrane through a glycolipid anchor) and human DcR3-GPI have been described previously (31). HEK-293T cells were expanded and seeded on 6-well plates at 2.5×10^5 cells per well in complete RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μg/ml gentamycin and 5×10^{-5} M β-mercaptoethanol, and allowed to grow until they reached 60-70% confluence (54). All genetic constructs were purified using endotoxin-free Maxi-prep kit (Qiagen) and were then transiently transfected in HEK 293T cells for the expression of membrane-bound proteins or recombinant soluble proteins. PlatE cells were transfected with pMIG-mouse LIGHT-IRES-GFP for the production of viral particles that were used for the transduction of mouse NIH-3T3 cells and the generation of a stable membrane LIGHT

cell line. For transfection experiments, 2 μg DNA/well of each construct/liposome complex (lipofectamine, Invitrogen) were added for 6 to 16 h (55).

Flag-tagged recombinant human or mouse LIGHT recombinant fusion proteins were purified through an immunoaffinity chromatography column that used anti-Flag mAb (clone M2, Sigma Aldrich) as ligand linked to agarose.

Monoclonal antibodies against mouse LIGHT

Female Sprague Dawley rats were immunized intraperitoneally (i.p.) with 0.5 ml of a 1:1.2 mixture of 5-10 × 10^6 mouse NIH-3T3 cells transduced with the complete mouse LIGHT gene and coexpressing GFP in Freund's Incomplete Adjuvant (Sigma). Six weeks after the first immunization, the animals received a final booster intravenously with 10 × 10^6 of mouse LIGHT-transduced NIH-3T3 cells. Rat splenocytes were immortalized with X63.Ag8.653 myeloma cells using the polyethylene glycol cell fusion technique, as previously described (56,57). Nine days after the fusion, culture supernatants from growing rat-mouse heterohybridomas were collected from 96-well plates and tested by flow cytometry against mouse LIGHT-GFP-transduced or control GFP-transduced NIH-3T3 cells after incubation for 2 h at 37 ºC. Then, cells were washed and subsequently incubated with an optimal dilution of Cy5-labeled polyclonal mouse anti-rat IgG (H+L) (Jackson Immunoresearch). For the *in vivo* experiments, hybridomas secreting anti-LIGHT mAbs or isotype control rat IgG_{2a} (anti-plant cytokinin, clone AFRC-MAC-157) were produced in spinner flasks and the supernatant was purified through a protein G-Sepharose affinity chromatography column, quantified, filtered through 0.45 μm and kept frozen at 1 mg/ml in Dulbecco´s PBS.

Binding specificity of anti-LIGHT monoclonal antibody

10F12 was labeled with 100 µg of EZ-link Sulfo-NHS-LC-biotin (Pierce) per mg of antibody for 2 h at room temperature in 0.1 M Na-Borate pH 8.8. The reaction was quenched by addition of an excess of NH_4Cl. Buffer was exchanged for PBS by repeated concentrations in an Amicon Ultra centrifugal device with 10 kDa cutoff (Millipore). ELISA plates were coated with 5 µg/ml goat anti-human IgG antibody (Jackson Immuno Research) in PBS, and blocked. Fc-ligands in conditioned supernatants of transfected 293T cells (31) were added and revealed either with biotinylated 10F12 at 0.5 µg/ml followed by peroxidase-coupled streptavidin, or with peroxidase-coupled donkey anti-human IgG (Jackson Immuno Research).

Fab fragment preparation and affinity determination by surface plasmon resonance (SPR)

An IgG1 Fab preparation kit was used according to the manufacturer's instructions (Pierce). Briefly, purified 10F12 was digested for 16 h at 37 °C with or without immobilized ficin, after which time 10 µg of antibody was analyzed by SDS-PAGE and Coomassie Blue staining to monitor cleavage efficiency. The digested antibody was concentrated and applied onto a Superdex-200 gel permeation chromatography column eluted in PBS, with absorbance recording at 280 nm, and relevant fractions (containing co-eluting Fab and Fc fragments) were concentrated in an Amicon concentrator with 10 kDa cutoff (Millipore). The concentration of the Fab fragment was calculated assuming an absorption coefficient of 1.4 for the mixture and a Fab to Fc ratio of 2 to 1.

The BIACORE 3000 system, sensor chip CM5, surfactant P20 and amine coupling kit containing N-hydroxysuccinimide (NHS), N-Ethyl-N'-

dimethylaminopropyl carbodiimide (EDC) and 2-(2-pyridinyl-dithioethaneamine) (PDEA) were from GE-Healthcare (Upsala, Sweden). All biosensor assays were performed with Hepes-buffered saline (HBS-P) as running buffer (10 mM Hepes, 150mM sodium acetate, 3mM magnesium acetate and 0.005% surfactant P20, pH 7.4). The different compounds were dissolved in the running buffer.

Immobilization of FF-mouse LIGHT was performed by injecting, onto the activated surface by EDC/NHS of a sensor chip CM5, 50 µl of FF-mouse LIGHT (68 µg/ml in sodium acetate 10mM buffer, pH 4.9) followed by 20 µL of ethanolamine hydrochloride, pH 8.5, to saturate the free activated sites of the matrix, which gave a signal of approximately 5000 RU.

All binding experiments were carried out at 25°C with a constant flow rate (20 µl/min). Different concentrations of Fab anti-mouse LIGHT mAb (clone 10F12) were injected for 3 min, followed by a 3 min dissociation phase (1.25nM to 40nM). The sensor chip surface was regenerated after each experiment injecting 10 µl of 10 mM HCl.

The kinetic parameters were calculated using the BIAeval 4.1 software on a personal computer. Global analysis was performed using the simple Langmuir binding model 1:1. The specific binding profiles were obtained after subtracting the response signal from the channel control (activated/desactivated) and from a blank-buffer injection. The fitting to each model was judged by the reduced chi square and randomness of residue distribution.

Flow cytometry ligand/receptor binding and competition assays

The selection of the anti-LIGHT mAbs with blocking activity was performed as follows: LIGHT-transduced NIH-3T3 cells were incubated with a saturating amount of isotype control or anti-mouse LIGHT mAbs. In the presence of these antibodies as control and experimental inhibitors, cells were then incubated with either HVEM-Ig or LTβR-Ig and the reaction was developed with the appropriate biotinylated conjugates. After a washing step, cells were finally incubated with phycoerythrin-coupled streptavidin.

We performed saturation curves of soluble HVEM-Ig and LTβR-Ig binding to membrane anchored mouse LIGHT. Graded concentrations of recombinant soluble LIGHT receptors were added to LIGHT transduced cells in binding buffer pH 7.2 (Hank´s balanced salt solution, 2.5% FBS, 5mM disodium EDTA, 0.1% bovine serum albumin, phenol red and 0.02% sodium azide) for 1 h at 37ºC. After washing the cells, the reaction was detected with either a biotinylated rat anti-mouse IgG_{2a} isotype specific mAb (R19-15, BD Biosciences) or biotinylated mouse anti-human IgG Fc fragment (Jackson ImmunoResearch). The reverse incubation of graded concentrations of soluble mouse Flag-Foldon-smLIGHT recombinant protein to monitor the binding with membrane-bound mouse HVEM or mouse LTβR-transfected cells was also performed. After 2 h incubation at 37ºC, the reaction was washed and incubated with biotinylated anti-Flag monoclonal antibody. Phycoerythrin- or allophycocyanin-coupled streptavidin were used for the detection of biotinylated secondary antibodies in all binding experiments. The mean fluorescence intensity of soluble receptors or ligands binding to their counterparts was calculated by subtracting the background fluorescence staining of untransfected cells or isotype matched control antibody from the experimental group

and plotted against Log [soluble fusion proteins] using PRISM version 5 (GraphPad, San Diego, CA).

To measure the relative affinities of HVEM and LTβR for mouse LIGHT, a competition assay was performed using HVEM-Ig (tagged with mouse IgG_{2a}), LTβR.Ig (tagged with human IgG1). HVEM-Ig and LTβR.Ig were detected with biotinylated rat anti-mouse IgG_{2a} or biotinylated polyclonal anti-human IgG antibody, respectively, followed by allophycocyanin-coupled streptavidin.

To monitor the ability of anti-LIGHT antibodies to prevent the binding of soluble HVEM-Ig or LTβR.Ig to membrane-bound mouse LIGHT transduced cells, each unlabeled anti-LIGHT mAb was first incubated with LIGHT transduced cells at saturating concentrations (10 µg of antibody per 2.5×10^5 cells) for 30 min at room temperature. Then, in the presence of each anti-LIGHT antibody as competitor, an optimal saturating concentration of each soluble recombinant fusion protein was added to the cells and incubated for 2 h at 37 ºC. The supernatant was then washed out and the immunostaining was detected with either a biotinylated rat anti-mouse IgG_{2a} isotype specific mAb (R19-15, BD Biosciences) or mouse anti-human IgG Fc fragment (Jackson ImmunoResearch), followed by allophycocyanin-coupled streptavidin.

For competition assays, an optimal concentration of 10F12 antibody was preincubated for 1 hour at 37 ºC with graded concentrations of soluble FF-mouse LIGHT and the binding of remaining free 10F12 was monitored on mouse transduced LIGHT cells. Staining was performed for 30 minutes at 37ºC, and 10F12 binding was revealed with biotinylated mouse anti-rat IgG_{2a} and SA-PE

Graded concentrations of anti-LIGHT antibody were pre-incubated for 1 hour at 37 ºC with a fixed amount of FF-mouse LIGHT (1µg/ml). The complex was then added

for 30 minutes at 37ºC to HVEM-transfected CHO cells or LTβR-GPI transfected HEK293T cells. Binding of residual FF-LIGHT was revealed with biotinylated anti-Flag mAb (clone BioM2) and SA-PE.

In vitro polyclonal T cell and NK cell activation

C57BL/6 WT or LIGHT-deficient splenocytes were polyclonally activated *in vitro* with PMA (100 ng/ml) plus ionomycin (500 ng/ml) at 37ºC for 5 h. To detect LIGHT expression on polyclonal activated T lymphocytes, splenocytes (2×10^5 cells / well) were incubated for 5 h in the presence of 0.2 µg/well of Hylite 647-labeled anti-LIGHT mAb (clone 10F12) or Hylite 647-labeled isotype-matched rat IgG_{2a} control. Mouse LIGHT expression was analyzed by flow cytometry on lineage-negative ($CD19^+$ $CD11b^+$ $CD11c^+$) resting and activated CD4 and CD8 T cells. The expression of LIGHT was also analyzed on resting and activated NK cells of WT and LIGHT KO (as control) after gating out $CD19^+$ and $CD11c^+$ cells.

In vivo cytotoxicity assay

Spleen cell suspensions from mice of three different MHC haplotypes B6 ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$) and CB6F1 ($H\text{-}2^{b/d}$) were prepared in complete medium as a source of target cells. After a washing step in Dulbecco's PBS, target cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) at 10 µM (F1), 2 µM (BALB/c) or 0.4 µM (B6) for 10 min at 37 ºC. 30×10^6 of each target cells (B6, BALB/c and F1 cells) differentially labeled with CFSE were mixed at a 1:1:1 ratio and were subsequently injected i.v. into naive 8-12 week-old B6 WT mice or B6 LIGHT

KO recipient mice. At the same time, 0.5 mg of anti-LIGHT 10F12 or of a rat IgG_{2a} isotype control was injected i.p. (~20 mg/kg). The half-life of 10F12 in serum was found to be about 15 days. Three days later, recipient B6 mice were euthanized and target cells were analyzed in spleen and peripheral lymph nodes. The percentage of specific target lysis was calculated by comparing the survival of each target population (BALB/c or F1) to the survival of the syngeneic population (58,59).

Dead cells were excluded by DAPI nuclear staining. Flow cytometry acquisition was carried out on a CyAn 9 cytometer (Beckman Coulter, USA) running on Summit software. Data analysis was performed using the WinList 3D Version 7 (Verity Software House, Topsham, ME, USA).

In vivo proliferative assay of donor alloreactive T cells

$70x10^6$ of B6 WT or LIGHT-deficient splenocytes were labeled with 5 μM CFSE and subsequently adoptively transferred to non-irradiated F1 recipients, which were treated with isotype-control (rat IgG_{2a}) or 10F12 mAb (a single dose of 0.5 mg of antibody at the day of the adoptive transfer). Three days later, the ratio of the absolute number of non-divided/divided donor CFSE-labeled CD4 T cells and CD8 T cells was calculated. Similarly, identical number of splenocytes from B6 WT and LIGHT KO were injected to F1 recipients and the expression of the alpha chain of IL-2R was monitored on donor alloreactive T cells at day 5 after the adoptive transfer.

Statistical analysis

Collected data was organized in Graph Pad prism Version 5 worksheets with mean ± SEM, along with other statistical parameters. Then, individual data from experimental and control groups were plotted and statistically analyzed using prism software and the parametric Student's *t* test and non-parametric Mann-Whitney test. The Welch´s correction was applied when the groups compared exhibited distinct variance. A value of $p< 0.05$ was considered statistically significant.