ORIGINAL ARTICLE

Modulation of cytotoxic responses by targeting CD160 prolongs skin graft survival across major histocompatibility class I barrier

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CD160 is a glycosylphosphatidylinositol-anchored protein of the immunoglobulin superfamily. It exhibits a pattern of expression coincident in humans and mice that is mainly restricted to cytotoxic cells and to all intestinal intraepithelial T lymphocytes. B- and T-lymphocyte attenuator (BTLA) and CD160 interact with cysteine-rich domain 1 of the extracellular region of Herpesvirus entry mediator (HVEM). CD160 engagement by HVEM can deliver inhibitory signals to a small subset of human CD4 T cells and attenuate its proliferation and cytokine secretion, but can also costimulate natural killer cells or intraepithelial lymphocytes. In turn, CD160 and BTLA can also function as agonist ligands being capable of costimulating T cells through membrane HVEM. Based on the restricted pattern of CD160 expression in cytotoxic cells, we postulated that CD160 may represent a suitable target for immune intervention in the setting of transplantation to modulate allogeneic cytotoxic responses. We demonstrated that in vivo administration of anti-CD160 antibody in combination with anti-CD40 L antibody to limit CD4 T-cell help modulated cytotoxic responses in a major histocompatibility complex class I mismatched model of allogeneic skin graft transplantation (bm1 donor to C57BL/6 recipient) and significantly prolonged graft survival. The implementation of this strategy in transplantation may reinforce current immunosuppression protocols and contribute to a better control of CD8 T-cell responses. (Translational Research 2016;■:1–13)

Abbreviations: HVEM = herpesvirus entry mediator; BTLA = B- and T-lymphocyte attenuator; CTLA4 = cytotoxic T-lymphocyte antigen; CD = cluster of differentiation; CRD = cysteine-rich domain; mHVEM.Ig = mouse HVEM extracellular domain bound to mouse IgG2a Fc fragment;

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mCD160.Ig = mouse CD160 extracellular domain bound to mouse IgG2a Fc fragment; NK = natural killer; NKT = natural killer T cells; TCR = T-cell receptor; MHC = major histocompatibility complex; GPI = glycosylphosphatidylinositol; WT = wild type; KO = knockout; CBA = cytometric beads arrays; Th = T helper; SPR = surface plasmon resonance; TNFSF = tumor necrosis factor superfamily; TNFRSF = tumor necrosis factor receptor superfamily; MFI = mean fluorescence intensity

AT A GLANCE COMMENTARY

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Background

CD160 is the first immunoglobulin (Ig)-like receptor recognizing major histocomaptibility complex class I described on mouse natural killer cells that exhibits a pattern of expression restricted to cytotoxic cells coincident in humans and mice.

Translational Significance

With the advent of CTLA4.Ig into the clinics, the paradigm of treating the episodes of rejection is changing toward a more molecular approach, but CD8 T–cell-mediated rejection, which is dependent and independent of T-cell help, particularly in sensitized recipients, needs better therapies to achieve an adequate control.

INTRODUCTION

CD160 is a glycosylphosphatidylinositol-anchored membrane glycoprotein of the immunoglobulin superfamily, exhibiting an immunoglobulin V (IgV)-like domain within the extracellular region with 6 cysteine residues, which permits disulfide bond formation and trimerization.[1] The predicted size for the mature human CD160 polypeptide (181 amino acids) plus 2 N-linked glycosylation sites is 25 kDa, which closely corresponds with the 27-kDa band observed under reducing conditions.[2,3] An additional band of 80 kDa was also identified under nonreducing conditions suggesting trimerization through cysteine disulfur bonds. Mouse and human CD160 follow a similar pattern of expression mainly restricted to lymphoid cytotoxic cells, such as resting natural killer (NK) cells (in humans highly cytotoxic NK cell population $CD56^{dim}$ $CD16^{+}$), natural killer T (NKT), and most of the intestinal intraepithelial TcR$\gamma\delta$ CD8$\alpha\alpha$ T lymphocytes and half of the intestinal TCR$\alpha\beta$ $CD4^{+}CD8^{-}$ T lymphocytes.[4,5] Within the population of CD8 T cells, CD160 is expressed in a minor subset of human peripheral $CD8^{+\ bright}$ $CD28^{-}$ T cells, in recently activated mouse and human $CD8^{+}$ T cells, and in most $CD8^{+}$ memory T cells suggesting that CD160 expression is associated to an activation status.[2,5-8]

Several human CD160 protein isoforms have been predicted based on alternative messenger ribonucleic acid splicing of the CD160 gene, being the most abundant, the glycosylphosphatidylinositol-anchored isoform with or without immunoglobulin V-like domain and a transmembrane isoform with an intracellular tyrosine residue that can be potentially phosphorylated. A soluble isoform composed of the extracellular domain of CD160 has also been described that it is susceptible of being released from the cell membrane by a metalloprotease.[8] CD160 is the first immunoglobulin (Ig)-like receptor recognizing major histocompatibility complex (MHC) class I described on mice NK cells, which is an exception to the general rule, because all MHC class I binding receptors in mice are lectin-like receptors of the Ly49 superfamily.[7,9] Human and mouse CD160 interact weakly with classical and nonclassical MHC class I molecules, including CD1d, triggering NK cell cytotoxicity and secretion of proinflammatory cytokines.[4,6-8,10] CD160 interacts weakly with aggregated classical and nonclassical MHC class I complexes and competes with CD8 for binding to $\alpha3$ domain of MHC class I, and consequently impairs in vitro MHC class I–restricted cytotoxic CD8 T-cell responses.[11]

CD160 and B- and T-lymphocyte attenuators (BTLAs) interact with cysteine-rich domain 1 of the extracellular region of herpesvirus entry mediator (HVEM).[1,12,13] CD160 engagement by HVEM receptor can deliver inhibitory signals to a small subset of human CD4 T cells activated in response to a polyclonal stimulus and attenuate its proliferation and cytokine secretion.[14] Paradoxically, engagement of CD160 on human NK cells by soluble HVEM.Ig costimulates their functional activity,[15] and antibody-mediated triggering of CD160 delivers costimulatory signals to human double-negative T lymphocytes and $CD8^{bright+}$ cytotoxic effector T lymphocytes lacking CD28 expression.[16] CD160 is expressed preferentially by innate intraepithelial lymphocytes that engage HVEM in epithelial cells, and this interaction confers protection to the host against infection through the release of defensins.[17] In turn, CD160 and BTLA can promote T-cell survival on engagement of membrane HVEM.[13,18]

Depending on the tissue context, immune cell type, and phase of the immune response, CD160 may play distinct roles during the course of the T-cell responses. To our knowledge, the only formal in vivo evidence in the field of transplantation implicating HVEM-CD160 pathway in modulating cytotoxic allogeneic immune responses was that reported by D'Addio et al[19] who described the use of a nondepleting mouse CD160.Ig decoy receptor protein (CD160 bound to a mutated form of mouse IgG2a Fc fragment) that was effective at preventing alloreactive $CD8^+$ T-cell proliferation and IFN-gamma production in vitro in the absence of CD28 costimulation.[19] Similarly, in vivo administration of a nondepleting CD160.Ig decoy receptor prolonged significantly a fully MHC-mismatched cardiac allograft survival in CD4-CD28 double knockout mice and in cytotoxic T-lymphocyte antigen 4 (CTLA4).Ig-treated wild type (WT) recipients suggesting that CD160 functions as a costimulatory molecule, and its blockade synergized with the blockade of the costimulatory pathway CD28-CD80-CD86 of T-cell activation.[19]

A more precise definition of the role of CD160 in vivo has been lately achieved with the development of CD160-deficient mice; although it did not show deleterious signs of T-cell function, the NK cell–mediated mechanism of tumor rejection was severely impaired due to a decreased secretion of IFN-gamma suggesting that CD160 in NK cells plays a costimulatory role.[20] Based on the restricted pattern of CD160 expression to cytotoxic cells (NK and CD8 T cells),[5,15,20] it was postulated that this receptor may represent a suitable target for immune intervention in the setting of transplantation to tackle allogeneic cytotoxic responses. To test this hypothesis, we chose a skin graft transplantation murine model across an MHC class I barrier, in which the main operative mechanism of rejection involves the participation of alloreactive CD8 T cells, although CD4 T-cell help is also required to some extent to promote a fully cytotoxic response. Humoral rejection plays no role in this mouse transplant setting.[21,22] We demonstrated that antibody targeting of CD160 combined with anti-CD40L antibody modulated host antidonor cytotoxic-mediated responses and prolonged significantly skin graft survival across an MHC class I barrier. These results suggest that CD160 is a potential suitable target on cytotoxic cells and opens new avenues in the search for improved approaches to tackle cytotoxic responses in transplantation.

MATERIAL AND METHODS

Molecular cloning and protein expression of membrane-bound mouse CD160 and soluble mouse CD160.Ig. Mouse CD160 was amplified using the proofreading *pfu* polymerase (Fermentas) with a pair of primers designed using as template the Genebank sequence with accession number #BC021596. The gene sequence encoding full-length CD160 without the stop codon was fused in frame to the monster green fluorescent protein (GFP) encoding gene (Promega) and cloned into pcDNA3.1 (+) expression vector (Invitrogen, Life Technologies).

To generate a soluble mouse CD160.Ig fusion protein, the gene sequence encoding the extracellular domain of CD160 (amino acid positions 38 to 135) was fused with the mutated Fc fragment of murine IgG_{2a} (hinge, CH2, and CH3 domains of the heavy chain; from now on mCD160.Ig) and then cloned into the pSecTag2 Hygro b expression vector (Invitrogen).

Adherent human embryonic kidney 293 cells that stably express the SV40 large T antigen (HEK 293T cells) were seeded on 6-well plates at 2.5×10^5 cells per well in complete Roswell Park Memorial Institute 1640 medium and were then transfected with 2-μg DNA per well of each construct complexed with lipofectamine (Invitrogen) and incubated overnight. For the detection of high producer cell lines of recombinant mouse CD160.Ig in the supernatant, a sandwich enzyme-linked immunosorbent assay (ELISA) was designed to screen and identify colonies of cloned cells secreting the fusion protein into the culture medium using rat antimouse IgG_{2a} mAb (clone R11–89, rat IgG_1) as capture mAb and biotinylated rat antimouse IgG_{2a} (clone R19–15, rat IgG_1) as detector antibody, followed by horseradish peroxidase-streptavidin, as previously reported.[23] The extracellular region of mouse HVEM bound to mouse IgG_{2a}.Fc (from now onward referred as mHVEM.Ig) was produced in insect cells and kindly provided by Genentech (South San Francisco, Calif).

Rodents. Twelve- to 16-week-old female Lewis rats (Harland, Netherlands), 8- to 12-week-old female C57BL-6J (B6) and B6.C-H-2^{bm1}-By mice (H2-K^{bm1}, hereafter bm1 mice) were the strains of rodents used in these studies. All animals were bred at the animal facility of the University of Leon (Spain), and the experiments with rodents were approved by the Ethical Committee for Animal Research of the School of Veterinary Medicine (University of Leon) and Animal Welfare Committee of University of Alcala de Henares (Madrid) and followed the European Guidelines for Animal Care and Use of Laboratory Animals.

Production and characterization of rat antimouse CD160 monoclonal antibodies. Female Lewis rats were immunized intraperitoneally (i.p.) with 0.5 mL of a 1:1.2 mixture of 100 μg of soluble mCD160.Ig protein in Freund's Incomplete Adjuvant (Sigma). Six weeks after the first immunization, the animals were inoculated intravenously with 100 μg of mCD160.Ig in saline, and 3 days later splenic B cells were

immortalized by fusion with X63.Ag8.653 mouse myeloma cell line, following a protocol described in previous reports.[23] Nine to twelve days after the fusion, stable heterohybridomas rat × mouse were selected in the presence of hypoxanthine-aminopterin-thymidine (HAT) medium, and the culture supernatants were screened by flow cytometry against mouse CD160-GFP–transfected HEK293 T cells. Antimouse CD160 antibodies and isotype-matched controls were produced in spinner flasks and further purified through a protein G-sepharose affinity chromatography, quantified, filtered through 0.45-μm, and stored frozen at 1 mg per mL.

Specificity and epitope mapping of anti-CD160 antibodies. To demonstrate the specificity of the antibodies for the extracellular region of mouse CD160, rat antimouse CD160 antibodies were first preincubated for 30 minutes in the presence or absence of soluble recombinant mCD160.Ig and then added to mouse CD160-transfected cells. The reaction was then developed with a biotinylated mouse antirat, isotype-specific monoclonal antibody.

The mapping of the potential epitopes recognized by anti-CD160 antibodies was done by flow cytometry using a competition assay, in which a saturating amount (10 μg) of each unlabeled isotype control or anti-CD160 mAbs (competitor antibody) was first incubated with 2.5×10^5 CD160-GFP–transfected HEK293 T cells. Then, in the presence of the competitor antibody, biotinylated anti-CD160 antibodies or the commercially available PE-labeled antimouse CD160 (CNX46-3) were added to the cells.

Surface plasmon resonance. The BIACORE 3000 system, sensor chip CM5, surfactant P20, amine coupling kit containing N-hydroxysuccinimide (NHS), and N-Ethyl-N'-dimethylaminopropyl carbodiimide (EDC) were from BIACORE (Upsala, Sweden). All biosensor assays were performed with Hepes-buffered saline as running buffer (10-mM Hepes, 150-mM sodium acetate, 3-mM magnesium acetate, 3.4-mM EDTA, and 0.005% surfactant P20, pH 7.4). The different compounds were dissolved in the running buffer.

FcγR were immobilized at 50 μg per mL in formate buffer, pH 4.3 by injection onto the EDC/NHS-activated surface of a CM5-type sensor chip until a signal of approximately 6000 RU was obtained, followed by 20 μL of ethanolamine hydrochloride, pH 8.5, to saturate the free activated sites of the matrix.

To test the binding affinity of the different clones of rat antimouse CD160 mAbs to mouse CD160.Ig, immobilizations were performed by injecting, onto the activated surface by EDC-NHS of a sensor chip CM5, soluble mouse CD160.Ig (100 μg per mL in formate buffer, pH 4.3), which gave a signal of approximately 5000 RU, followed by 20 μL of ethanolamine hydrochloride, pH 8.5, to saturate the free activated sites of the matrix. All binding experiments were carried out at 25°C with a constant flow rate of 30 μL per min. Different concentrations of anti-CD160 antibodies were injected for 3 minutes followed by a dissociation phase of 3 minutes. The sensor chip surface was regenerated after each experiment by injection of 20 μL of 10-mM NaOH.

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. The specific binding profiles were obtained after subtracting the response signal from the channel control (ethanolamine) and from the blank buffer injection. The fitting to each model was judged by the reduced chi square test and randomness of residue distribution.[24-26]

Skin grafting. Skin graft transplantation was performed according to a protocol previously reported.[27,28] Briefly, syngeneic (B6) or allogeneic (bm1) skin graft beds were prepared on the right side of the thorax in the proximity of the axillar region of recipient mice under ketamine-xylazine anesthesia. Grafts were covered with vaseline gauze and a sticking plaster, which was removed on day 8. Signs of onset of rejection, such as dryness, loss of hair, contraction, scaling, and necrosis were recorded for a period of time of 40 days after transplantation. Grafts were considered rejected when complete necrosis of the graft was observed.

Experimental groups. Hybridoma cell lines secreting monoclonal antibodies against mouse CD40 L (clone MR1),[29] antimouse CD160 (clone 6H8, rat IgG_{2b}, λ chain), or isotype control mAb, rat IgG_{2b} (AFRC MAC 51) monoclonal antibodies were adapted to grow under limiting amounts of immunoglobulin-depleted fetal calf serum and produced in spinner flasks with constant shaking. All antibodies were injected i.p. at the indicated time points.

The half-life of antimouse CD160 antibody (clone 6H8) in serum samples was calculated using the following sandwich ELISA: Serum samples from naïve mice or mice treated at day 0 with anti-CD160 mAb (clone 6H8, 1 mg per mouse i.p.) were collected at days 1, 4, and 7. Maxisorp ELISA plates (NUNC, Denmark) were coated overnight with 5 μg per mL of mouse antirat IgG (H + L). The plates were then blocked with 3% nonfat dry milk diluted in phosphate-buffered saline. Serial dilutions of purified rat IgG2b isotype control (clone AFRC MAC 51) mAb were added to the wells to generate a standard curve. Mouse sera collected at different times after the

injection of the anti-CD160 mAb were diluted and incubated for 2 hours at room temperature. Plates were washed 4 times with phosphate-buffered saline-Tween 20 (0.05%) and 1 μg per mL of biotinylated mouse antirat λ light chain mAb was added to the plates. After 4 washing steps, horseradish peroxidase-streptavidin was incubated, washed out, and the reaction was finally developed by adding 3,3′,5,5′-tetramethylbenzidine substrate, according to the manufacturer's instructions (Sigma). The reaction was stopped with 2M sulfuric acid, and the absorbance was measured at a wavelength of 450 nm using a multiwell spectrophotometer plate reader (BioRad).

The half-life of circulating unbound anti-CD160 mAb antibody was calculated to ensure that the soluble form of the receptor, in case it was present, was saturated over time and an excess of antibody was present in the serum of treated mice by using the following exponential decay formulas: $Nt = N0(1/2)^{t/t1/2}$, $Nt = N0e^{-t/\tau}$, and $Nt = N0e^{-\lambda t}$, where N0 is the initial quantity present in the serum 1 day after the administration of the therapeutic antibody, Nt is the quantity that still remains after a time t, t1/2 is the half-life, τ is the mean lifetime, and λ is the decay constant.

The following experimental groups were established to dissect the role of CD160 as an immunotherapeutic target in combination with other strategies aiming at attenuating the contribution of allogeneic CD4 T-cell help to CD8 T cells to reject bm1 mismatched skin allografts. Anti-CD160 antibody and rat IgG_{2b} isotype control were injected i.p. at days 0 and 7, 1 mg at each time point. Anti-CD40L antibody was injected i.p. in a single dose of 0.5 mg at the time of transplantation. The following experimental groups were established: group I: isotype control, rat IgG_{2b}; group II: anti-CD160 (clone 6H8) antibody; group III: anti-CD40L antibody (clone MR1), and group IV: anti-CD40L antibody plus anti-CD160 antibody.

Flow cytometry and antibodies. The following anti-mouse antibodies were used to monitor the expression of CD160 in distinct hematopoietic cell populations: anti-CD3 (145-2C11), anti-CD4 (L3T4), anti-CD8α (53–6.7), anti-CD62L (MEL-14), anti-CD44 (IM7), and anti-NK1.1 (PK136) antibodies were all purchased from Biolegend. Anti-CD160 antibody (clone CNX46-3) was obtained from eBiosciences (Affymetrix), whereas unlabeled or biotinylated anti-CD160 mAbs (4B4, 4D2, 9D8 and 6H8) developed in this work were prepared in house for this study. Sepharose protein G affinity chromatography–purified anti-CD160 antibodies were biotinylated in our laboratory according to the EZ-link NHS-PEO4-Biotin protocol recommended by Pierce. In all flow cytometry experiments, dead cells were excluded by propidium iodide staining. Flow cytometry acquisition was carried out on a Cyan 9 cytometer (Beckman Coulter). Data analysis was performed using the WinList 3D Version 8 (Verity Software House, Topsham, Maine).

Statistical analysis. The results from the experiments were recorded in excel spreadsheets and the mean and standard deviation were calculated for each experimental group. Comparisons of continuous variables between groups and statistical significance were assessed using the parametric unpaired 2-tailed Student's t-test. A value of $P < 0.05$ (*) was considered statistically significant and P values less than $P < 0.005$ (**) and $P < 0.0005$ (***) were denoted with 2 and 3 stars, respectively.

Skin graft survival was calculated by using the Kaplan-Meier life table method and statistical analysis for the comparison of the survival curves was performed by the log-rank test. The statistical analysis was performed using Graphpad Prism 6.0 software (Graphpad Software, Inc).

RESULTS

Generation and characterization of monoclonal antibodies against mouse CD160. A panel of 4 rat anti-mouse CD160 hybridoma cell lines secreting monoclonal antibodies with specificity for the extracellular region of mouse CD160 was raised after immunization of Lewis rats with recombinant mouse CD160.Ig. Its initial specificity was first confirmed by demonstrating efficient binding of antimouse CD160 antibodies to membrane-bound mouse CD160 transiently expressed on HEK 293T cells (Fig 1*A*, left panel). To further validate the specificity of the newly generated rat antimouse CD160 mAbs, the antibodies were preincubated with soluble mouse CD160.Ig fusion protein, which could effectively block the binding of each of the anti-CD160 antibodies to CD160-transfected HEK 293T cells, confirming their specificity (Fig 1*A*, right panel).

To determine whether anti-CD160 antibodies were recognizing the same or different epitopes, we mapped their recognition sites on the extracellular region of CD160 by using a flow cytometry competitive binding assay in which each biotinylated anti-CD160 antibody was incubated with mouse CD160-transfected cells precoated with each of the unlabeled anti-CD160 antibodies as competitors. We observed that all antibodies recognized the same or closely related epitopes because they compete each other for binding to CD160-transfected cells (data not shown). The set of anti-CD160 antibodies turned out to recognize a different epitope from that of commercially available anti-CD160 antibody (clone CNX46-3), because the

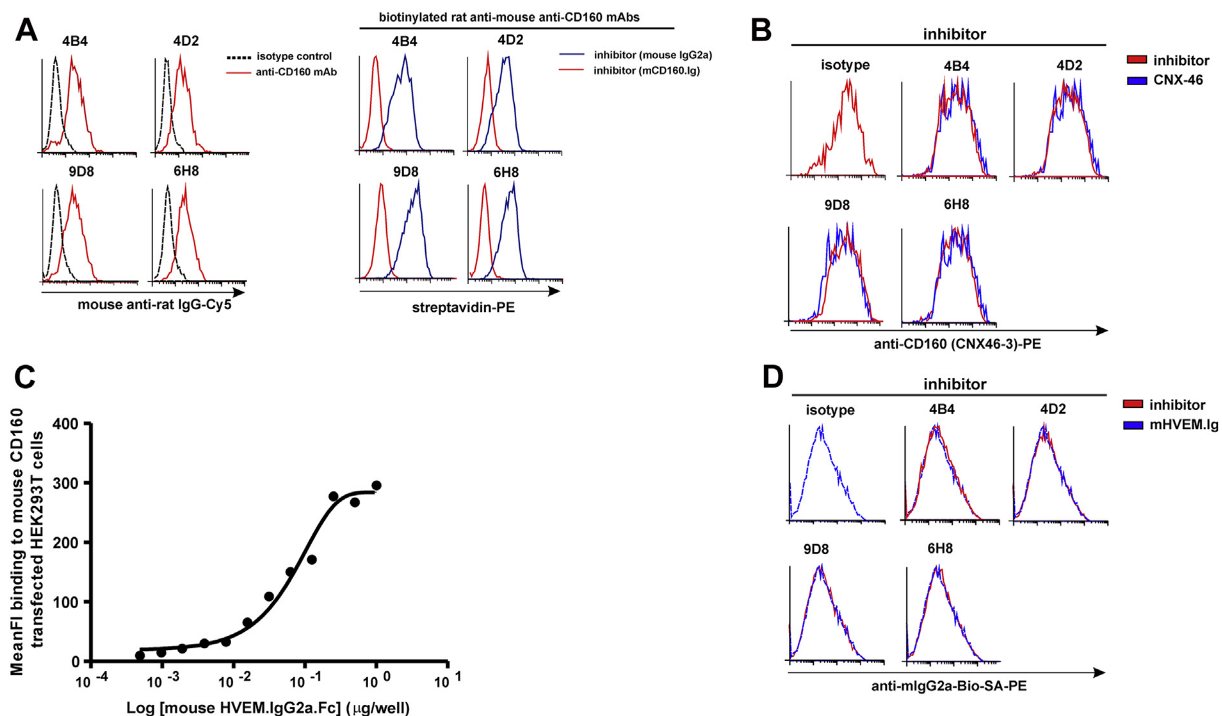


Fig 1. Characterization of a set of hybridomas secreting monoclonal antibodies against the extracellular region of murine CD160. (**A**, left panel) 2×10^5 membrane-bound mouse CD160-GFP transiently transfected HEK 293T cells were incubated with supernatants obtained from a panel of 4 rat antimouse CD160 antibodies. The antibody-antigen interaction was developed using a Cy5-labeled mouse antirat IgG secondary antibody. Black-dotted lines represent the binding of the isotype control mAb; red solid lines depict the binding of antimouse CD160 antibodies. (**A**, right panel) To further validate the specificity of the newly generated anti-CD160 antibodies, a saturating amount of 2 μg per well of mIgG2a inhibitor control (blue solid lines) or mouse CD160.Ig protein (red dotted lines) was preincubated with each biotinylated anti-CD160 antibodies for 30 minutes at room temperature before adding to mouse CD160-GFP–transfected HEK 293T cells. After a washing step, the reaction was developed using phycoerythrin-coupled streptavidin. (**B**) A competition assay was designed to dissect whether the distinct antimouse CD160 antibodies recognized the same or different epitopes from that of the commercial antimouse CD160 antibody (clone CNX46-3). To that aim, 2×10^5 mouse CD160-GFP–transfected HEK 293T cells were first incubated for 30 minutes with a saturating amount of each anti-CD160 antibody (inhibitor antibody, red solid lines). Cells were then washed and incubated with phycoerythrin-coupled antimouse CD160 antibody (CNX46-3, blue solid lines). One representative experiment of 2 performed with identical results is shown. (**C**) Mouse CD160-transfected HEK 293T cells (2×10^5) were incubated with graded concentrations of purified mouse HVEM.Ig fusion protein. The mean fluorescence intensity of sHVEM.Ig binding to membrane CD160 was calculated. (**D**) Mouse CD160-transfected HEK 293T cells (2×10^5) were incubated with saturating amounts (2 μg per well) of isotype control or rat antimouse anti-CD160 mAbs (red solid lines). In the presence of the inhibitor, mouse HVEM.Ig fusion protein (2 μg per well) was added to the cells. After a washing step, the binding of the fusion protein was developed using biotinylated rat antimouse IgG2a mAb followed by phycoerythrin-coupled streptavidin (blue dotted lines). PE, phycoerythrin

precoating with each of the unlabeled anti-CD160 mAbs did not prevent clone CNX46-3 from recognizing CD160-transfected cells (Fig 1*B*).[7]

We then evaluated whether the anti-CD160 antibodies displayed antagonist activity and prevented binding of mHVEM.Ig to CD160-transfected cells. To that aim, we first titrated HVEM.Ig binding to CD160-transfected cells and established that the reaction was saturated at 2 μg per well (Fig 1*C*). None of the antimouse CD160 antibodies described in this manuscript antagonized the binding of mHVEM.Ig to CD160-transfected cells (Fig 1*D*).

Surface plasmon resonance was applied to determine the binding avidity of anti-CD160 antibodies to activating FcγR: FcγRI (CD64), FcγRIII (CD16), and FcγRIV (CD16.2). Clone 6H8 was chosen for the in vivo studies for exhibiting the highest binding affinity for the 3 activating FcγR (Supplementary Fig 1), as all 4 antimouse CD160 antibodies tested exhibited a similar equilibrium dissociation constant affinity (K_D)

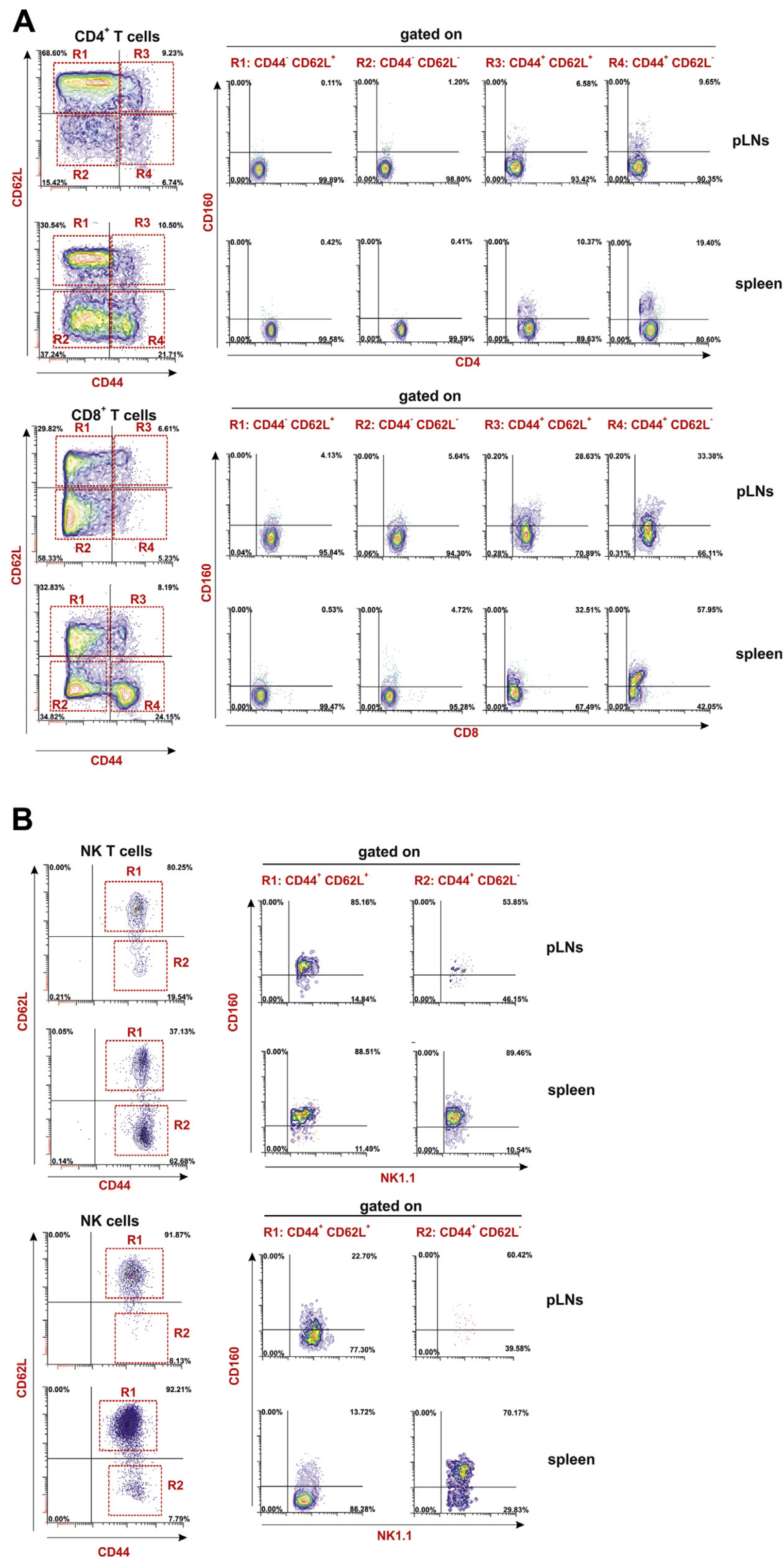


Fig 2. Pattern of expression of CD160 on T cells, NK cells, and NKT cells of spleen and peripheral lymph nodes. (**A**) Naïve ($CD62L^+CD44^-$, R1), activated ($CD62L^-CD44^-$, R2), central memory ($CD62L^+CD44^+$, R3), and effector memory ($CD62L^-CD44^+$, R4) $CD4^+$ and $CD8^+$ T cells were analyzed for the expression of surface CD160 in spleen and peripheral lymph nodes. (**B**) The expression of CD160 was also assessed on NKT cells and NK cells ($CD62L^+CD44^-$, R1 and $CD62L^-CD44^-$, R2) from spleen and peripheral lymph nodes (pLN) of naïve mice. pLNs, peripheral lymph nodes.

for immobilized mouse CD160.Ig (Supplementary Fig 2).

The half-life of anti-CD160 antibody administered i.p. to recipient B6 mice was monitored between day 1 and day 7 by sandwich ELISA and the half-life for antibody 6H8 was 1.20 days (Supplementary Table).

In summary, a set of rat antimouse CD160 antibodies was characterized with specificity for epitopes located at the extracellular region of mouse CD160 and clone 6H8 was chosen for in vivo studies because of its good binding affinity to activating FcγR receptors and to soluble, immobilized mCD160.Ig fusion protein.

Mouse CD160 expression is restricted to most NKT cells, a subset of NK cells, and a small subpopulation of memory $CD4^+$ T cells and $CD8^+$ T cells in secondary lymphoid organs. The expression pattern of a molecule under homeostatic conditions or in response to an inflammatory stimulus in a particular cell type provides information on the putative function of this molecule. For that reason, we initially proceed with the characterization of mouse CD160 expression on lymphoid cells from peripheral lymph nodes and spleen of naïve B6 mice in different subpopulations of CD4 and CD8 T cells expressing CD44 and CD62L surface markers. Mouse CD160 expression was restricted to a small subset of central memory ($CD44^+CD62L^+$) and effector memory ($CD44^+CD62L^-$) $CD4^+$ and $CD8^+$ T lymphocytes, whereas naïve T cells ($CD44^-CD62L^+$) and recently activated ($CD44^-CD62L^-$) remained negative for CD160 expression in both pLNs and spleen (Fig 2*A*). CD160 expression was also analyzed on NKT cells and NK cells of secondary lymphoid organs (pLNs and spleen). More than 85% of $CD44^+CD62L^+$ NKT cells or between 50% and 90% $CD44^+CD62L^-$ NKT cells expressed CD160. The expression of CD160 on $CD44^+CD62L^+$ NK cells ranged only from 10%–20%, whereas more than 60% of $CD44^+CD62L^-$ NK cells were positive for CD160 (Fig 2*B*).

These observations confirmed the results of previous authors[5] and indicate that the expression pattern of this molecule is restricted to cells with a cytotoxic profile.

Combined targeting of CD160 receptor with anti-CD40L antibody prolongs skin graft survival across an MHC class I mismatched barrier. Preliminary evidences showed that CD160.Ig administration was effective in preventing fully MHC-mismatched heart allograft rejection when combined with CTLA4.Ig or in CD28-CD4 double-deficient mice.[19] Based on this report, we designed an experimental skin graft model across an MHC class I barrier, in which CD8 T cells are the main effector mechanism of rejection. In this transplant context, there is no role for alloantibodies, but the contribution of CD4 T-cell help is required to a certain extent for fully CD8 T-cell differentiation toward effector T cells.[21,22]

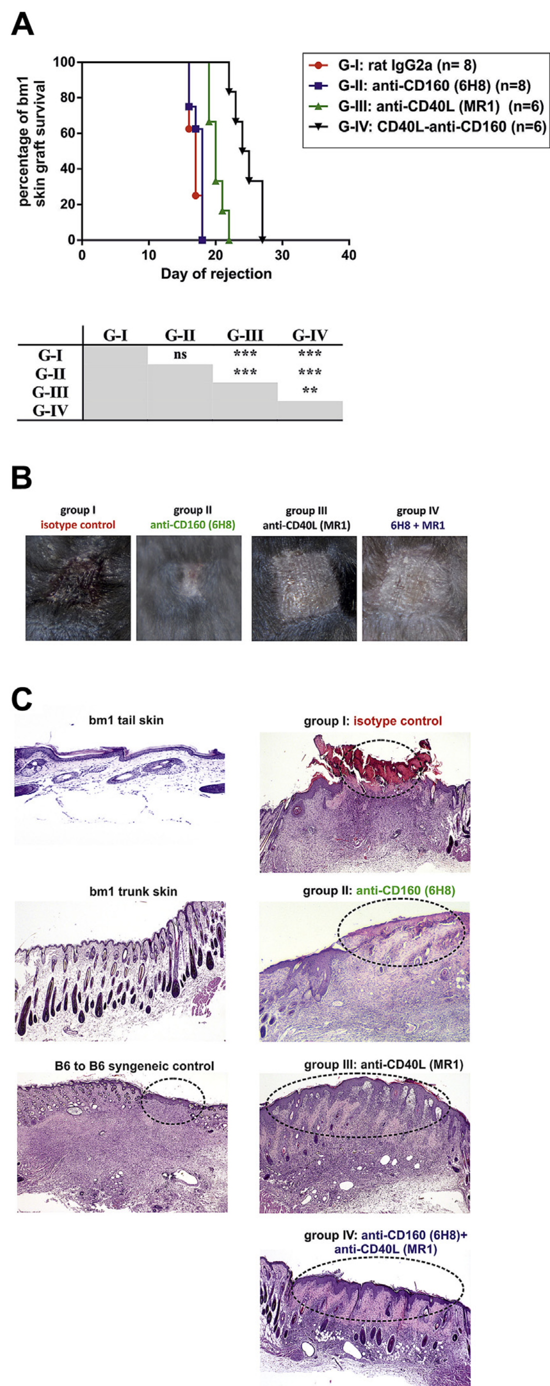
To evaluate the in vivo therapeutic consequences of targeting the surface receptor CD160 on cytotoxic cells, an anti-CD160 monoclonal antibody (clone 6H8, rat IgG_{2b}, λ chain) was selected with good binding affinity for activating FcγR. We implemented an immunosuppressive tolerogenic regimen based on the use of a single dose of anti-CD40L antibody at the time of skin graft transplantation. This immune intervention is known to deplete recently activated CD4 T cells expressing CD40L, and consequently decreases T-cell help to CD8 T cells and also prevents licensing of dendritic cells for proper stimulation of CD8 T-cell responses.[30,31] These scenarios were recreated to unmask the role of CD160 as a therapeutic target for the control of CD8 T-cell–mediated responses with limited access to CD4 T-cell help.

We first noticed that anti-CD160 antibody alone was not sufficient to enhance graft survival across an MHC class I barrier. The treatment with anti-CD40L antibody plus anti-CD160 antibody achieved a significant better protection against rejection of MHC class I skin allografts than the treatment with either anti-CD160 antibody alone ($P < 0.0005$) or anti-CD40L antibody ($P < 0.005$, respectively) (see table at the bottom of survival curves) (Fig 3*A*).

Macroscopic visual inspection at day 16 after transplantation denoted that group I (isotype control) and group II (anti-CD160 antibody) were clearly rejected, whereas group III (anti-CD40L antibody) and group IV were both apparently without signs of rejection (Fig 3*B*). We analyzed the histologic findings after hematoxilin-eosin staining and found that isotype control and anti-CD160-treated mice showed signs of complete rejection with loss of epithelium. At day 16 after transplantation, rejection was however initiated in anti-CD40L–treated mice evidenced by a prominent infiltration of mononuclear cells into the dermis and cellular attachment of the dermal-epidermal interphase of donor skin graft, while the combined treatment with anti-CD40L and anti-CD160 antibodies conferred complete protection from rejection (Fig 3*C*).

Overall, these data suggest that CD160 could serve as a target for modulating cytolytic responses in a transplant context in which T-cell help to CD8 T cells is compromised.

In vivo immunotherapeutic targeting of CD160 induces CD160 receptor downmodulation but does not deplete cytotoxic cells. To gain insight into the in vivo immunotherapeutic activity of anti-CD160 antibody and find out an explanation that accounted for the synergistic effect of anti-CD160 antibody administration along with



	G-I	G-II	G-III	G-IV
G-I		ns	***	***
G-II			***	***
G-III				**
G-IV				

Fig 3. Targeting CD160 in combination with anti-CD40L antibody enhances skin allograft survival across an MHC class I mismatched barrier. (**A**) The course of skin allograft survival across an MHC class I barrier was followed up for 40 days after placing bm1 skin allografts onto the right flank of B6 recipients. Recipient mice were treated at day 0 with isotype control, anti-CD160 antibody (6H8), anti-CD40 L antibody (MR1), or a combination of anti-CD40L antibody plus anti-CD160 antibody. Skin graft survival curves were calculated using the Kaplan-Meier life table method and the statistical analysis was performed by the log-rank test. (**B**) The composite photograph depicts skin allografts across an MHC class I barrier in recipient mice that underwent different treatments 16 days after transplantation. The survival plot is the global result of a pool of 3 experiments. (**C**) Histologic findings of bm1 skin grafts in C57BL-6 recipients 16 days after transplantation stained with hematoxilin/eosin. Left-hand side panel: upper image, normal tail skin from bm1 mice; middle image, normal trunk skin from bm1 mice with abundant pilous follicles; lower image, syngeneic skin grafting (B6 to B6) with a continuous layer of donor and host epidermis. Right-hand side panel: group I (isotype control, rat IgG2a) and group II (anti-CD160 antibody), severe rejection of donor bm1 tail skin with desquamation of donor epithelium and intense dermal mononuclear infiltration; group III (anti-CD40L antibody): initiation of skin graft rejection with a profuse dermal and subepidermal mononuclear infiltration and vacuolar alteration of basal cells at the dermal-epidermal junction. Group IV (anti-CD160 + anti-CD40L): no signs of rejection of donor bm1 skin graft were observed on day 16 after transplantation. Black-dotted ellipse highlights the epithelium and dermis underneath of donor bm1 skin graft in the tissue section. Statistical significance is indicated as follows: $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$, and ns, nonsignificant.

anti-CD40L antibody in prolonging graft survival across an MHC class I–mismatched barrier, the absolute number of cells expressing CD160 was calculated after the administration of the therapeutic antibodies. The absolute number of memory type CD4 and CD8 T cells ($CD44^+$ $CD62L^+$, central memory and $CD44^+$ $CD62L^-$, effector memory) and the absolute number of NK and NKT cells were monitored and no significant differences were seen when isotype control–treated mice was compared with anti-CD160–treated mice (Fig 4*A*).

CD160 expression was also monitored in splenocytes and peripheral lymph node cells 4 days after i.p. injection of anti-CD160 antibody with an anti-CD160 antibody (clone CNX46-3) that was not blocked by the in vivo administered anti-CD160 antibody (clone 6H8) (Fig 1*B*). CD160 expression was downmodulated due to the binding of the therapeutic antibody, particularly on NKT cells and NK cells of spleen (Fig 4*B*) and pLNs (Fig 4*C*).

In summary, antibody-mediated targeting of CD160 receptor did not lead to T-cell depletion of cytotoxic cells expressing CD160, but induced a profound down-regulation of the receptor particularly on NK cells and NKT cells.

DISCUSSION

Novel strategies aiming at targeting the process of T-cell activation and differentiation toward effector T cells may in the future contribute to improve the current protocols of immunosuppression for the treatment of episodes of graft rejection and for the long-term maintenance of graft survival under the therapeutic protocols of sustained low-dose immunosuppression.[32] The

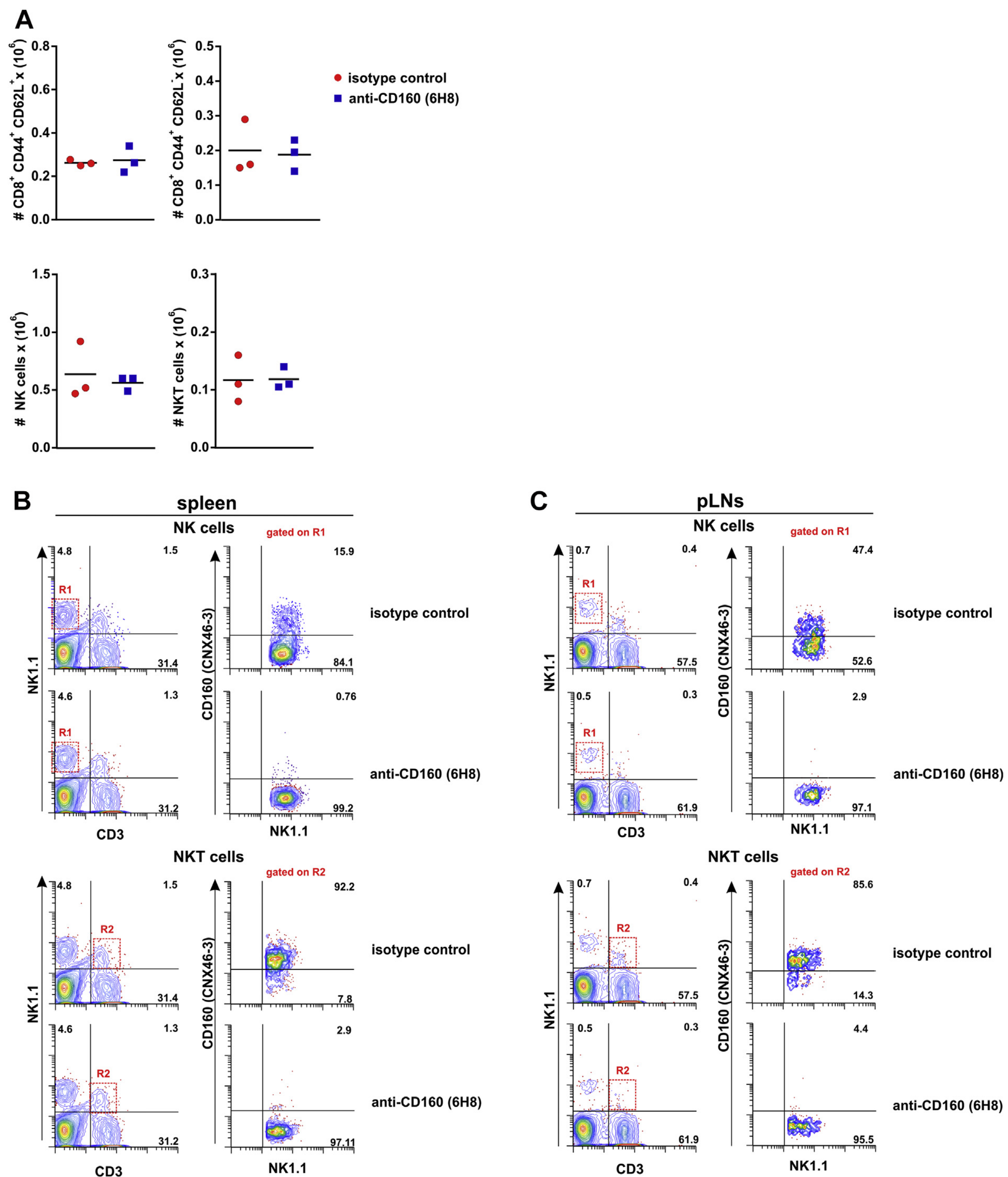


Fig 4. In vivo therapy with anti-CD160 antibody (clone 6H8) downmodulated CD160 receptor expression on NK cells and NKT cells, but neither depletes T cells nor NK cells or NKT cells. (**A**) Absolute numbers of central and effector memory CD8 T cells as well as NK cells and NKT cells from spleen of naïve mice treated on day 0 with isotype-matched control or anti-CD160 (clone 6H8) antibodies. Naïve mice were treated i.p. on day 0 with 1 mg of isotype control or anti-CD160 (6H8) antibodies. Four days after treatment, the expression of CD160 was assessed on NK cells ($CD3^{-}NK1.1^{+}$, R1) and NKT cells ($CD3^{+}NK1.1^{+}$, R2) in spleen (**B**) and pLNs (**C**) using a phycoerythrin-labeled anti-CD160 antibody (CNX46-3). The percentage of each population is indicated in the quadrants. One representative experiment of 3 performed is illustrated. pLN, peripheral lymph nodes.

control of CD8 T-cell–mediated rejection is an unmet goal awaiting better approaches.

With the advent of CTLA4.Ig into the clinics, the paradigm of the treatment of rejection is changing toward a more molecular approach to tackle the episodes of rejection, but CD8 T-cell–mediated rejection, which is to some extent independent of T-cell help, particularly in sensitized recipients, needs better therapies to achieve an adequate control.[33,34]

CD160 is a member of the immunoglobulin superfamily like CD28, CTLA4, programmed death 1, BTLA, inducible T-cell costimulator, and others.[7,16] CD160 caught our attention for its restricted pattern of expression to cytotoxic cells (NK cells, NKT cells, and memory type T cells), and that prompted us to evaluate its potential as a target for immune modulation of allogeneic responses. Herein, we provide evidence that CD160 is a promising target for the modulation of cytotoxic responses by demonstrating that in vivo administration of an anti-CD160 antibody when used in combination with anti-CD40L antibody prolongs significantly MHC class I–mismatched skin allografts, in which the mechanism of rejection is mainly mediated by alloreactive CD8 T cells. We chose an allogeneic skin graft transplantation model across an MHC class I mismatched barrier to focus the attention in allogeneic CD8 T-cell responses, although help from CD4 T cells in this mouse model is required to a certain extent to achieve full CD8 T-cell activation and differentiation toward effector cells.[22] The MHC class I H-2K^{bm1} only differs from H-2K^{b} in 7 nucleotides that cluster very close to each other and results in 3 amino acid difference.[35] These differences in amino acid sequence do not configure epitopes recognizable by B cells, and therefore the humoral response is absent in this transplant setting. Donor and host antigen presenting cell participate through the indirect pathway of antigen presentation in processing and presenting a narrow repertoire of H-2K^{bm1}–derived peptides in the context of host and donor MHC class II (IA^{b}) that mobilizes a low frequency of alloreactive CD4 T cells, which provide sufficient T-cell help for proper differentiation of CD8 T cells to effector cells.[36] Host CD8 T cells recognize directly the mutated version of allele H-2K^{bm1} on donor antigen presenting cell present in the skin grafts. The rejection response in this model is preferentially mediated by CD8 T cells, as depletion of host CD4 T cells does not substantially change the course of graft rejection. The indirect pathway of antigen presentation of H-2K^{bm1} donor–derived allopeptides is, however, necessary for the activation of CD4 T cells that in turn will help alloreactive CD8 T cells to promote their activation and differentiation toward cytotoxic cells.[21,22] The evidence that host CD4 T cells are relevant in this model comes from depleting experiments in which removal of both CD4 and CD8 T cells enhanced graft survival to a much greater extent than depletion of only CD8 T cells.[21]

The anti-CD160 antibody described in this work neither depletes nor blocks the functional activity of cells expressing this receptor. We found no evidence for depletion of cell populations expressing CD160 (NK cells, NKT cells, or CD8 T cells) despite the fact that rat antimouse CD160 antibody (clone 6H8) is a rat IgG$_{2b}$ isotype with fairly good binding affinity for activating FcγR (Supplementary Fig 1) and in principle with the potential to activate antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), which are the main mechanisms of depletion for rodent therapeutic antibodies.[37,38]

Receptor modulation may somehow condition the functional activity of NK and NKT cells expressing CD160. This is in agreement with the recent report of the phenotype of CD160-deficient mice that exhibited impaired IFN-gamma secretion. CD160 deficiency was associated with a significant reduced antitumor response, suggesting that signaling through the receptor is required for NK cell functional activity.[20] Other evidences suggest that NK cells can modulate CD8 T-cell responses. Thus, depletion of NK cells in the early phase of viral infection,[39,40] together with our own unpublished observations in the transplantation setting, have shown to augment the CD8 T-cell responses. This implies that at the initial phase of the allogeneic immune response, NK cells negatively modulate CD8 T-cell responses, and that antibody targeting the costimulatory receptor CD160 on NK cells may impact their cytotoxic activity on recently activated alloreactive CD8 T cells.[6] Our experiments indicate that CD8 T-cell–mediated responses are attenuated and graft survival was prolonged on anti-CD160 antibody administration in combination with known tolerogenic strategies aiming at targeting CD4 T-cell alloreactive responses, such as treatment with anti-CD40L antibody. When anti-CD160 antibody is coadministered with anti-CD40L antibody, a substantial increase in skin graft survival was observed. To account for the observation that the combined therapy with anti-CD160 antibody and anti-CD40L antibody prolongs graft survival longer than either antibody alone or compared with that observed in isotype-matched control, we postulated that anti-CD40L antibody administration depletes recently activated allogeneic CD4 T cells reducing their frequency and also preventing CD40-CD40L interaction leading to impaired dendritic cell maturation and subsequent defective T-cell costimulation.[30,41]

In line with the costimulatory function of CD160 expression on NK cells is the fact that this receptor is required for their activation, as demonstrated in CD160-deficient mice that exhibit decreased secretion of IFN-gamma and reduced killing of MHC class I–deficient mouse tumor cells (RMA-S cell line).[20] Previous work in humans also claimed that CD160 on NK cells functioned as a costimulatory receptor as the binding of human leukocyte antigen-C and the binding of soluble HVEM.Ig to CD160 on NK cells activate cytokine secretion and antibody-mediated receptor downmodulation may impair their function.[4,6,15] The previously reported inhibitory function of CD160 in humans[14] is not supported by the in vivo experimental data in mice as no signs of increased susceptibility to autoimmune disease have been seen in CD160-deficient mice as they aged.[20] CD160 constitutively expressed on innate lymphoid cells triggers HVEM expressed on the mucosal epithelium, and this interaction regulates the secretion of defensins by epithelial cells, providing host protection against mucosal infection. These observations are in line with the phenotype of HVEM-deficient mice that exhibit increased susceptibility to bacterial infection.[17]

The costimulatory function of CD160 on CD8 T cells is also in agreement with the studies of D'Addio et al[19] in which the administration of a nondepleting CD160.Ig recombinant protein in the absence of CD28 costimulation inhibits alloreactive $CD8^+$ T-cell proliferation and IFN-gamma production leading to the prolongation of fully MHC-mismatched heart allograft survival in CD4-CD28 double-deficient mice and also in CTLA4.Ig-treated WT recipients, but not in nontreated WT or CD8-deficient recipients.

The selective blockade of costimulatory molecules remains an appealing approach to reinforce successfully advanced clinical studies using CTLA4.Ig in renal transplant recipients and achieve clinical benefits avoiding the metabolic toxic side effects inherent to the long-term immunosuppression maintenance regimens.[42,43] This is of crucial importance in transplantation as tolerization of the CD8 T-cell compartment is much more difficult to achieve than tolerization of the CD4 T-cell compartment, particularly in sensitized recipients when CD8 T-cell differentiation is weakly dependent or independent of T-cell help.

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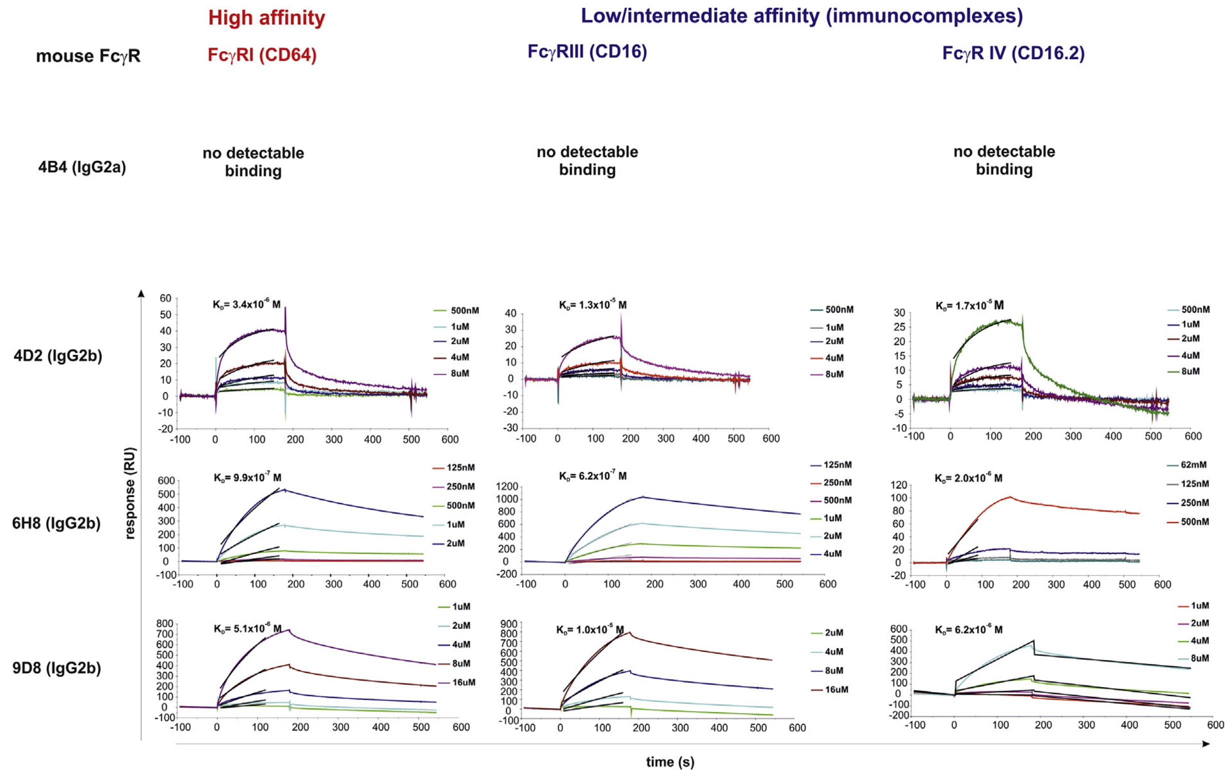
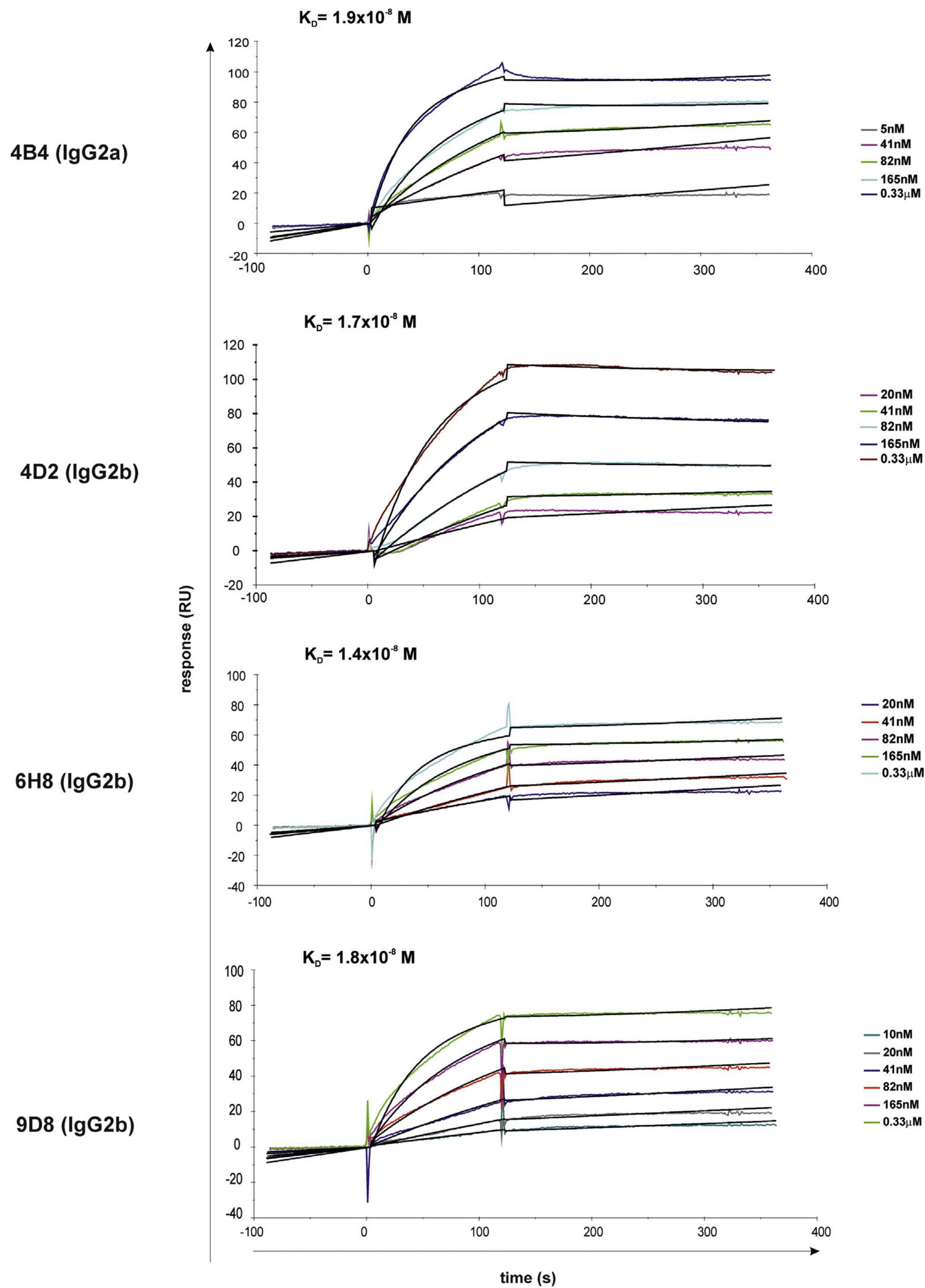
Appendix



Supplementary Figure 1. Surface plasmon resonance (SPR) analysis of antimouse CD160 antibody binding to mouse activating FcγRI (CD64), FcγRIII (CD16), and FcγRIV (CD16.2) receptors. The association and dissociation constant rates of rat antimouse anti-CD160 antibodies binding to distinct immobilized mouse FcγR were calculated by SPR. The equilibrium dissociation constant K_D for each anti-CD160 antibody is depicted. Anti-CD160 antibody, clone 4B4 did not display detectable binding affinity for FcγR. The lower the K_D value the higher the affinity of the antibody for its receptor epitope.



Supplementary Figure 2. Surface plasmon resonance analysis of antimouse CD160 antibody binding to soluble mouse CD160.Ig fusion protein. The association and dissociation constant rates of rat antimouse anti-CD160 antibodies binding to distinct immobilized mouse CD160.Ig fusion protein FcγR were calculated by SPR. The equilibrium dissociation constant K_D for each anti-CD160 antibody is represented.

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Supplementary Table. The half-life of circulating unbound anti-CD160 mAb antibody was calculated by using the following exponential decay formulas to ensure that the soluble form of the receptor, in case it was present, was saturated over time and an excess of antibody was in the serum of treated mice: $Nt = N0(1/2)^{t/t1/2}$, $Nt = N0e^{-t/\tau}$, and $Nt = N0e^{-\lambda t}$, where N0 is the initial quantity present in the serum 1 day after the administration of the therapeutic antibody, Nt is the quantity that still remains after a time t, t1/2 is the half-life, τ is the mean lifetime, and λ is the decay constant

Calculation of the half-life of therapeutic anti-CD160 antibody	t1/2 d 1→7	Mean life-time (τ)	Decay constant (λ)
anti-CD160 (6H8)	1.20	1.73	0.57