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The CD200 Receptor Is a Novel and Potent Regulator of Murine and Human Mast Cell Function[1]

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CD200R is a member of the Ig supergene family that is primarily expressed on myeloid cells. Recent in vivo studies have suggested that CD200R is an inhibitory receptor capable of regulating the activation threshold of inflammatory immune responses. Here we provide definitive evidence that CD200R is expressed on mouse and human mast cells and that engagement of CD200R by agonist Abs or ligand results in a potent inhibition of mast cell degranulation and cytokine secretion responses. CD200R-mediated inhibition of FcεRI activation was observed both in vitro and in vivo and did not require the coligation of CD200R to FcεRI. Unlike the majority of myeloid inhibitory receptors, CD200R does not contain a phosphatase recruiting inhibitory motif (ITIM); therefore, we conclude that CD200R represents a novel and potent inhibitory receptor that can be targeted in vivo to regulate mast cell-dependent pathologies. *The Journal of Immunology,* **2005, 174: 1348–1356.**

Mast cells are a subpopulation of leukocytes that are derived from hemopoietic progenitors and are found in most connective tissues throughout the body (1–3). Engagement of FcεRI on mast cells triggers a series of biochemical events culminating in the secretion of inflammatory mediators, which has directly implicated these cells in a variety of inflammatory disorders (3–6). In recent years, a number of studies have delineated the mechanisms of FcεRI-dependent mast cell activation as well as the cell surface receptors that regulate mast cell biological responses.

CD200 (formerly OX2) is a member of the Ig supergene family that is widely expressed on variety of cell types (7–10). Recently, a receptor for CD200 (CD200R) has been identified that is primarily expressed on leukocytes of the myeloid linage (11–13). In vivo studies of mice lacking the CD200 gene have suggested that engagement of the CD200R constitutively inhibits myeloid functions (14). In the present investigation, we have studied the expression and function of CD200R on murine and human mast cells. The results presented in this study provide the first direct evidence that CD200R is an important mast cell inhibitory receptor.

Materials and Methods

Cells and Abs

Human mast cells were derived from freshly isolated cord blood mononuclear cells and were cultured in Yssel's medium (Gemini Bioproducts) containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.3 mg/ml L-glutamine (Invitrogen Life Technologies), human stem cell factor (50 ng/ml; DNAX) and human IL-6 (50 ng/ml; DNAX). After 7–9 wk of culture, human IL-4 (5 ng/ml; DNAX) and human IgE (1 μg/ml; derived from U266 tumor cells; American Type Culture Collection) was added to the culture medium and the cells were plated onto monolayers of human fibroblasts (neonatal human dermal fibroblasts; Clonetics). After 3–4 wk, 96–98% of the cells expressed typical mature mast cell markers including CD117, high surface expression of FcεRI and tyrptase/chymase.

Mouse C57BL/6 mast cells were derived from bone marrow of 4–6 wk old mice as previously described (15). Mouse mast cells overexpressing murine CD200R (mCD200R)[3] were generated by infection of normal bone marrow-derived mast cells with a retrovirus vector containing the mCD200R. A cDNA containing the CD8-leader segment followed by the c-*myc* epitope tag (EQKLISEEDL) and joined to the extracellular, transmembrane, and cytoplasmic regions of mCD200R (12) was subcloned into the pMXneo retroviral vector (16). Plasmid DNA was transfected into Pheonix ecotropic retrovirus packaging cells (a gift from G. Nolan, Stanford University, Stanford, CA) and viruses obtained were used to infect mouse mast cells, which were subsequently selected in medium containing 1 mg/ml G418 (Roche Molecular Biochemicals). Cells were sorted for mCD200R expression using the anti-myc Ab 9E10 and will be referred to as mouse mast cells overexpressing mCD200R.

PE conjugated anti-mouse CD117 (clone 2B8); mouse IgE, (TNP specific, clone C38-2), mouse IgE-FITC (clone IgE-3), anti-mouse CD11b (MAC-1), anti-human CD11b, anti-human CD200 (MRC-OX104), and isotype control Abs for both mouse and rat were obtained from BD Biosciences. Additional Abs used in this study: anti-mouse CD200 (OX2; Serotec) and anti-c-*myc* Ab (clone 9E10; Covance). Anti-mouse CD200R mAbs (DX109 and DX110) and anti-human CD200R (DX107, DX136, and DX183) were generated in rats as previously described (12). Anti-human CD117 (DX80) and anti-human FcεRI (DX68) were generated by immunizing mice and rats with human mast cells, respectively. Polyclonal Abs were also used in this study: anti-human IgE (Kirkegaard & Perry Laboratories); and goat $F(ab')_2$ anti-rat IgG and sheep $F(ab')_2$ anti-mouse IgG (Jackson ImmunoResearch Laboratories).

To murinize and mutate rat anti-mCD200R mAb, DX109 hybridoma cells were collected and RNA was prepared following the Qiagen RNeasy protocol (Qiagen). Using the BD SMART RACE cDNA Amplification kit (BD Biosciences Clontech), 1 μg of total RNA was reacted to synthesize cDNA following the 5′ RACE protocol. The heavy and light variable domains of DX109 were then PCR amplified using Qiagen TaqPCR Master Mix kit. BD SMART RACE 5′ universal primer mix primer mix along with 3′ constant domain primer specific for rat/murine IgG1: 5′-GTCACT GGCTCAGGGAAATAGCCCTTGACCAGGCATC-3′ were used to amplify the H chain variable domain. Similarly, universal primer mix (10×) and rat/murine κ constant domain primer, 5′-GGATGGTGGGAAGATGGATA CAGTTGGTGCAGCAT-3′,were used to amplify the variable region of the L chain. The PCR products were gel purified and sequenced on an ABI Prism

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[3] Abbreviations used in this paper: mCD200R, murine CD200R; hCD200R, human CD200R; KLH, keyhole limpet hemocyanin; PCA, passive cutaneous anaphylaxis; HSA, human serum albumin.

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3100 according to ABI protocol (AME Bioscience). After obtaining the variable domain sequences, cloning primers were designed to introduce unique sites for insertion into the mouse H and L chain constant domain expression vectors. The L chain variable domain was PCR amplified and cloned into the *Hind*III and *Bss*HII sites of the pULS-RML vector containing the mouse κ constant domain. The H chain variable domain was cloned between *Xho*I and *Esp*I in the puCMV-RML vector containing the mouse IgG1 constant domain. The H chain chimera expression plasmid was subjected to site-directed mutagenesis using primers designed to mutate Fc binding residues. The rat/mouse chimeric open reading frames of DX109 L chain, H chain, and H chain Fc mutant were subcloned into the pQB1-AdCMV5-GFP adenovirus transfer vector (14) for recombinant protein production. Mammalian cotransient transfection of expression plasmids containing the L chain and H chains of chimeric rat/mouse DX109 were performed using the FreeStyle 293 Expression System as per the manufacturer's protocol (Invitrogen Life Technologies). Supernatants were collected and clarified, and the NaCl concentration was adjusted to 2.5 N before affinity purification. The supernatants were run on a 5-ml Hi Trap Protein A FF affinity column (Amersham Biosciences) at a flow rate of 5 ml/min, then washed with PBS. Recombinant Ab was eluted with 0.1 M glycine, pH 3.0, immediately neutralized to pH 7.5 with 2 M Tris base, then dialysed vs PBS. To verify that the mutated murinized DX109 was incapable of binding FcRs, intact rat IgG1 or mutated DX109 was incubated with the FcR-expressing cell line, J774, at a concentration of 10 μg/ml for 30 min at room temperature. The cells were then washed, stained with a FITC conjugated goat anti-rat IgG, and analyzed by flow cytometry. Although intact rat IgG1 strongly stained J774, the FcR-mutated murinzed DX109 showed no significant staining (data not shown).

Soluble CD200 Ig fusion proteins

A fusion protein consisting of the extracellular domain of hCD200 and mCD200 (12) was fused to the Fc domain of mouse IgG1 mutated in the constant heavy chain domain 2 domain (D265A) to inhibit binding to FcRs. The CD200 and modified Fc cDNAs were assembled into a modified pCDM8 expression plasmid (Invitrogen Life Technologies), which provided the SLAM signal sequence (17). The resultant SLAM-CD200-Fc was then subcloned into a modified pQB1-Ad CMV5-GFP adenovirus transfer vector (Quantum Biotechnologies). Protein was produced following transfection or infection of 293F or 293FT cells (Invitrogen Life Technologies) and was purified over HiTrap protein A columns (Amersham Biosciences) and eluted with 0.1 M glycine, pH 3, into fraction collector tubes containing 1/10 fraction volume 1 M Tris, pH 8, to neutralize. Fractions were pooled and concentrated using Amicon Ultra 10-kDa cutoff centrifugal filters (Millipore) then dialyzed overnight against PBS. Control mIg was prepared using an identical protocol except no proteins were fused to the mutated Fc domain. The inability of these proteins to bind mouse FcR was analyzed by flow cytometry on a mouse cell line (J774; American Type Culture Collection) expressing high levels IgG FcR and compared with nonmutated wild-type control Igs (data not shown). All protein preparations contained <1 ng of LPS per milligram of protein.

Immunoprecipitations

The cell surface of mouse and human mast cells was labeled with biotin using EZ-link sulfo-N-hydroxy-succinimide-biotin (Pierce). Cells were washed with D-PBS then lysed in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris base, pH 8.0, containing protease inhibitors (complete protease inhibitor mixture; Roche Molecular Biochemicals). Lysates were spun 12,000 × *g* for 20 min at 4°C. Immunoprecipitations were done by incubating precleared lysates with 1–2 μg of anti-CD200R mAbs (DX109 for mouse and DX107 for human) or isotype control Ab and protein A and protein G beads. Beads were washed, and eluted proteins were resolved on 4–20% Tris-glycine gels (Invitrogen/Novex) and transferred to Immobilon-P (Millipore). Membranes were blocked in 5% BSA, 0.1% Tween-20 in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) then blotted with strepavidin-HRP (Amersham Biosciences); washed in TBS, 0.1% Tween-20; and visualized after incubating the membrane for in Super Signal West dura chemiluminescent substrate (Pierce) and then exposing to film.

Mast cell degranulation assays

Normal mouse mast cells or mouse mast cells overexpressing mCD200R were incubated overnight with 2 μg/ml anti-TNP-specific IgE. Cells were plated 2×10^5 cells/well into 96-well, Falcon flat-bottom plates (BD Labware) in RPMI 1640 medium containing 1% BSA. Anti-mCD200R Ab DX109, isotype control Ab (rat IgG1), a mCD200-Ig fusion protein consisting of the extracellular domain of mCD200 fused to the Fc region of mouse IgG1, or a control-Ig fusion protein (see above) were added for final concentrations of 2 μg/ml. Cells were incubated for 30 min at room temperature. Cells were washed two times to remove excess primary Ab or fusion protein. FcεRI expression on mast cells remained unchanged after anti-CD200R Ab and CD200-Ig pretreatements. Experiments in which the mCD200R was cross-linked but not coligated to the FcεR, a sheep anti-mouse $F(ab')_2$ Ab that binds both mouse and rat Ig but does not recognize the bound IgE (no. 515-006-071; Jackson ImmunoResearch Laboratories) was used at 20 μg/ml to cross-link DX109, rIgG1, mCD200-Ig, and control mIg. Degranulation was then subsequently induced with varying concentrations of the TNP-keyhole limpet hemocyanin (KLH) Ag (provided by S. Mauze, DNAX) and the incubation was continued for 1 h at 37°C and 5% CO_2. The magnitude of degranulation was directly proportional to the concentration of Ag used to trigger the receptor bound IgE. Maximium degranulation (90% of total) was observed at highest concentrations of TNP-KLH Ag. Experiments in which the FcεR was coligated with mCD200R, a goat anti-mouse $F(ab')_2$ that binds to IgE, DX109, and mCD200-Ig (no. 115-006-062; Jackson ImmunoResearch Laboratories) was added at 20 μg/ml and the incubation continued at 37°C for 1 h. To assess the extent of degranulation in each case, 20 μl of supernatant was removed and was added to 60 μl of the β-hexosamindase substrate, *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich), 1.3 mg/ml in 0.1 M citric acid, pH 4.5. After 3–4 h at 37°C, 100 μl of stop solution was added (0.2 M glycine, 0.2 M NaCl, pH 10.7) and $OD_{405-650}$ was measured using a microplate reader (Molecular Devices).

Human mature mast cells were gently harvested from fibroblast monolayers and washed in unsupplemented Yssel's medium. Cells (3×10^6) were then incubated for 30 min at 4° C in control Ab or anti-huCD200R (DX183) Ab at 20 μg/ml. Cells were then washed in Yssel's medium and plated in 96-well plates at 1×10^5 cell per well. The FcεRI was then triggered with the rat anti-hFcεRI mAb (DX68) at varied concentrations and incubated for 1 h at 37°C. The magnitude of mast cell degranulation was directly proportional to the concentration of anti-FcεRI mAb used to trigger degranulation. Maximium degranulation (90% of total) was observed at highest concentrations of Ab. For human CD200R (hCD200R) cross-linking experiments, before the addition of DX68, the cells were incubated in 2 μg/ml goat $F(ab')_2$ anti-rat IgG for 25 min at 4°C, washed three times in Yssels with 50% rat serum (to block unreacted goat anti-rat IgG) and then triggered with DX68. For hCD200R coligation to FcεRI experiments, DX68 (at various concentrations) was added to the wells containing the anti-hCD200R-stained cells, incubated for 25 min at 4°C, washed in medium three times, and then co-cross-linked with a goat $F(ab')_2$ anti-rat IgG at 2 μg/ml. Supernatants were harvested after 1 h of incubation at 37°C, and degranulation was quantified by measuring the release of tryptase into the supernatants as previously published (18).

ELISA

Mouse TNF and IL-13 present in the supernatants of stimulated mouse mast cells were measured using cytokine-specific ELISA kits as per the manufacturer's instructions (R&D Systems). Supernatants were collected after 18–30 h of stimulation.

Flow cytometry

Cells were incubated in isotype control IgGs or Ag-specific Abs at 1 μg/10^6 cells for 25 min at 4°C. After three washes in PBS containing 0.02% sodium azide, the cells were incubated for 25 min with PE conjugated, $F(ab')_2$ of either goat anti-mouse IgG or goat anti-rat IgG (Caltag Laboratories). After three washes in PBS, the cells were fixed in 1% paraformaldehyde and analyzed on a BD Biosciences FACScan using logarithmic fluorescent amplification.

Immunohistochemistry

Depilated back skin was harvested from 6- to 8-wk-old BALB/c mice and snap frozen in OCT freezing medium (Tissue-Tek) by liquid nitrogen flotation. Normal human abdominal skin biopsies were obtained from five female patients (aged 20–45 years) during plastic surgery as approved by the Human Studies Review Committee of the DNAX Research Institute. Subcutaneous fat was removed by blunt dissection and 1- to 3-mm fragments were snap frozen in OCT by liquid nitrogen flotation. All frozen tissues were stored at −80°C. Cryostat sections (5–8 μm) were fixed in cold 80% acetone and 20% methanol, air dried, then blocked with 15% normal goat serum for 30 min at room temperature. Sections were then incubated in primary Abs (3 μg/ml) for 2 h at room temperature, extensively washed in PBS, and then incubated 1 h in Alexa 594 conjugated goat anti-rat IgG or Alexa 594 goat anti-mouse IgG (Molecular Probes). Sections were then incubated with FITC labeled avidin (Vector Laboratories) at 10 μg/ml for 30 min, washed three times in PBS and wet mounted for fluorescent microscopy. Sections were examined under a Nikon E800 fluorescence microscope equipped with epi-fluorescence filters for FITC and Alexa 594.

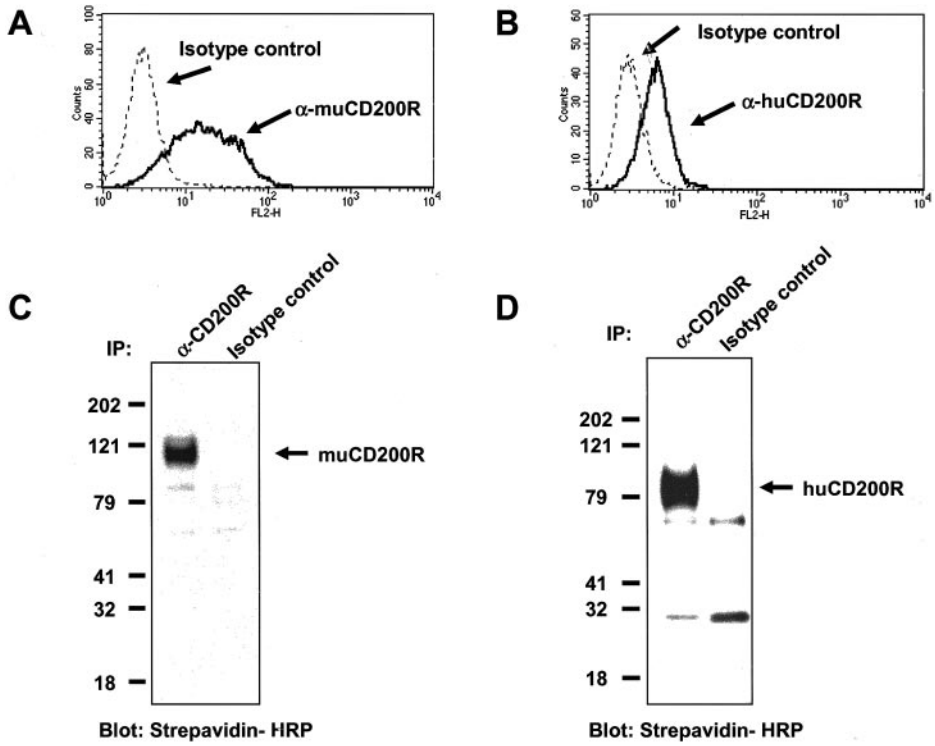

FIGURE 1. Expression of CD200R on mouse and human cultured mast cells. Cell surface expression of CD200R was analyzed by flow cytometry on mouse bone marrow-derived mast cells (*A*) and human cord blood-derived mast cells (*B*) using the CD200R-specific Abs DX109 and DX136, respectively. Immunoprecipitation of mast cell CD200R demonstrates that mCD200R migrates at ~100 kDa (*C*) and the hCD200R migrates at ~90 kDa (*D*).

Passive cutaneous anaphylaxis (PCA)

Female CD-1 (ICR)BR mice (12–16 wk) were obtained from Charles River Laboratories and maintained under specific pathogen-free conditions until used. Groups of three to five mice were injected i.v. with 1, 10, or 100 μg of rat anti-mCD200R mAb (DX109,IgG1) or isotype control in 100 μl of PBS. Mice were then anesthetized with a mixture of isofluorane and oxygen and their backs were shaved. An injection of 10, 20, or 40 ng of DNP-specific mouse IgE (Sigma-Aldrich) was then given intradermally into the mouse back at discrete sites in 20 μl of PBS. Sixteen to 24 h later, 50 μg of DNP-human serum albumin (HSA) (Sigma-Aldrich) and 0.5% Evan's blue in PBS was injected i.v. in a final volume of 100 μl. Fifteen minutes later, mice were sacrificed by CO_2 asphyxiation and the dorsal skin was reflected for analysis and photography. Identical experimental procedures were performed using the murinized, Fc domain-mutated form of anti-mCD200R (anti-mCD200RmFcmut.).

For the quantitation of the PCA reaction, skin punch biopsies (10 mm diameter) were taken at the sites of IgE injection (blue reaction site) and placed in 500 μl of formamide (Sigma-Aldrich) for 72 h to extract the Evan's blue. Evan's blue content was measured using a spectrophotometer

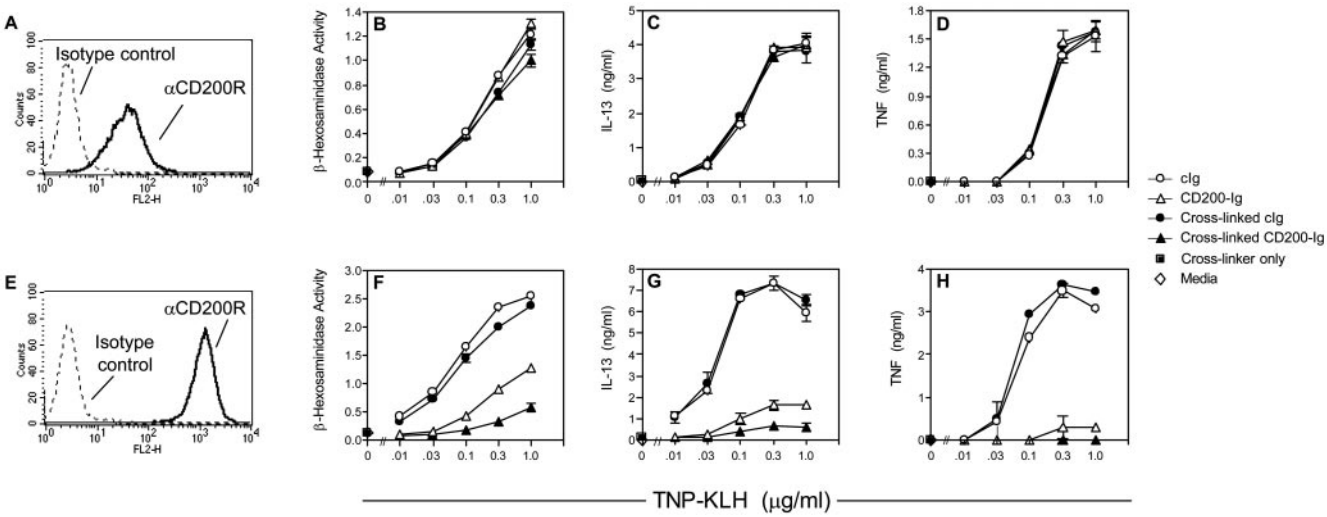

FIGURE 2. Effects of mCD200-Ig on mouse mast cells and mouse mast cells overexpressing mCD200R. Mouse mast cells loaded with TNP-specific IgE were added to 96-well plates containing mCD200-Ig or control mIg. The fusion proteins were subsequently cross-linked using a sheep anti-mouse $F(ab')_2$ that does not recognize the bound IgE (■). Degranulation was then induced by the addition of varying concentrations of TNP-KLH to cross-link the bound IgE. Supernatants were removed after 1 h at 37°C and added to the β-hexosaminidase substrate to quantitate the extent of degranulation (*B* and *F*), or after 24 h to measure IL-13 (*C* and *G*) and TNF (*D* and *H*) by ELISA. mCD200-Ig was able to inhibit degranulation and cytokine production of mast cells overexpressing mCD200R (*F–H*, △) but not on mast cells with endogenous levels of the receptor (*B–D*, △). Cross-linking the mCD200-Ig enhanced the inhibition of cells overexpressing mCD200R (*F–H*, ▲) but had little effect on cells with endogenous levels of the receptor (*B–D*, ▲). The levels of mCD200R on normal mast cells and mast cells overexpressing CD200R are shown in *A* and *E*, respectively, using the mAb, DX109. Data of a representative experiment is presented as the means of triplicate samples ± SEMs.

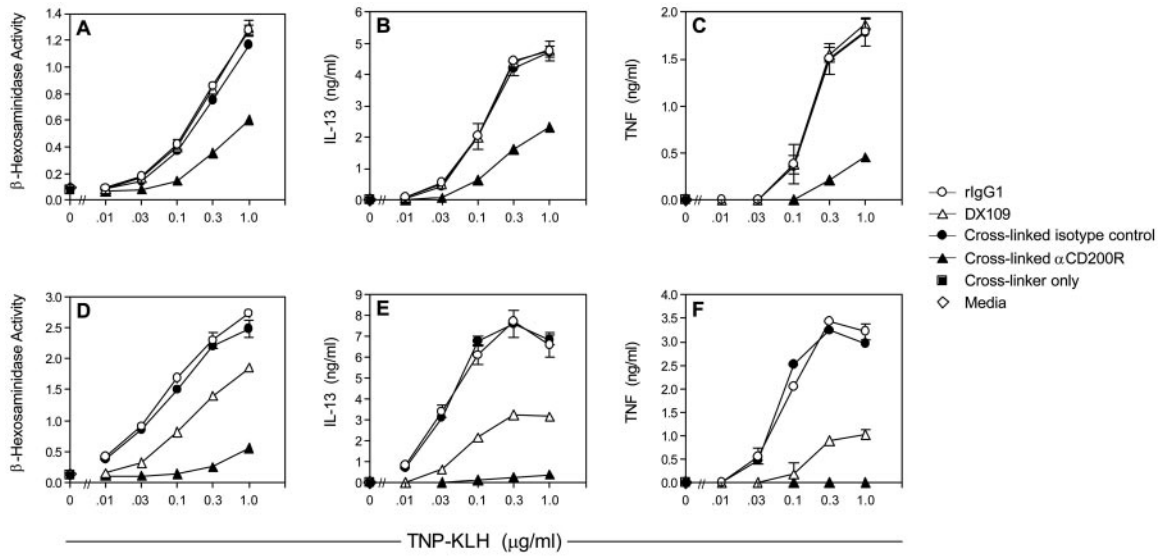

FIGURE 3. Effects of anti-mCD200R Ab on mouse mast cell degranulation and cytokine secretion. Cells were stimulated as described in the legend to Fig. 2 except that anti-mCD200R Ab DX109 and control rat IgG1 were used instead of soluble ligand to investigate the effects of triggering mCD200R in mouse mast cells (*A–C*) and mouse mast cells overexpressing mCD200R (*D–F*). Anti-mCD200R was able to inhibit degranulation (*D*, △), IL-13 (*E*, △), and TNF (*F*, △) in mast cells overexpressing mCD200R in the absence of receptor cross-linking, but not in cells expressing endogenous levels of the receptor (*A–C*, △). Cross-linking the anti-mCD200R enabled inhibition of degranulation (*A*, ▲), IL-13 (*B*, ▲), and TNF (*C*, ▲) in cells expressing endogenous levels of mCD200R, and enhanced the inhibition in cells overexpressing mCD200R (*D–F*, ▲). Data of a representative experiment is presented as the means of triplicate samples ± SEMs. Similar results were observed for at least five independent experiments.

at $\lambda = 620$ nm. The amount of Evan's blue per IgE injection site was calculated using known standards.

Results

Cultured mast cells express CD200R

Mouse and human mast cells, derived from progenitors in vitro, demonstrated cell surface expression of CD200R (Fig. 1, *A* and *B*). Biochemical analysis revealed a single band from both mouse and human mast cells of ~100 and 90 kDa respectively (Fig. 1, *C* and *D*). These molecular mass species were also observed in CD200R-transfected cell lines (data not shown) and were substantially larger than the expected framework proteins of ~36 kDa due to the high degree of CD200R glycosylation, as previously reported (11, 12).

CD200R is an inhibitory receptor for mouse mast cells

TNP-specific IgE coated mast cells were incubated with saturating amounts of a soluble form of the mCD200R ligand, which consisted of the extracellular domain of mCD200 fused to the FcR binding domain of mouse IgG (mCD200-Ig). Degranulation was induced by cross-linking the FcεRI-bound IgE with various amounts of TNP-KLH. Although mCD200-Ig bound to the mCD200R on normal mouse mast cells (data not shown), saturating concentrations of the soluble ligand did not give reproducible inhibition of mast cell degranulation or cytokine secretion, even in the presence of additional cross-linking (Fig. 2, *B–D*). Interestingly, coligation of receptor-bound CD200-Ig to the FceRI showed strong inhibition of normal mast cell degranulation (data not shown). Because the functional capabilities of inhibitory receptors are often dependent upon receptor density, the expression of mCD200R on mouse mast cells was increased using retroviruses containing mCD200R (Fig. 2*E*). Potent inhibition of mast cell FcεRI-induced degranulation and cytokine secretion was observed when mCD200R was triggered by mCD200-Ig in cells over-expressing CD200R (Fig. 2, *F–H*). Although additional cross-linking of the mCD200R/mCD200R complex with a goat anti-mouse IgG

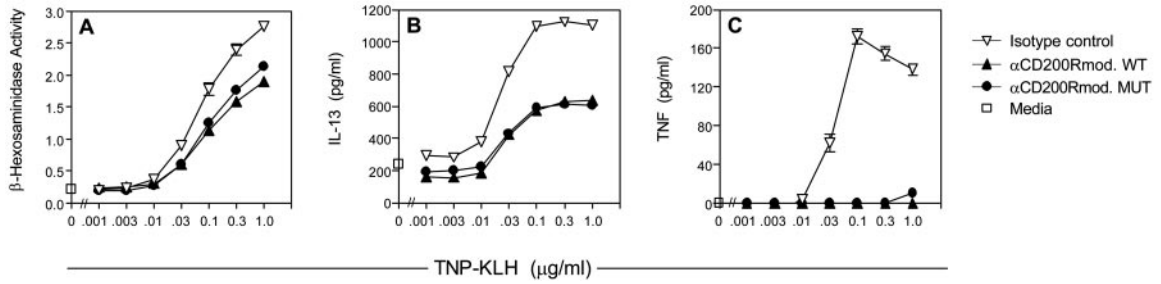

FIGURE 4. Effects of murinized/Fc domain-mutated anti-mCD200R Ab on mouse mast cell degranulation and cytokine secretion. Mouse mast cells overexpressing mCD200R loaded with TNP-specific IgE were added to 96-well plates containing rat anti-mCD200R Ab (DX109) engineered to contain a mouse Fc domain (anti-mCD200RmFc, ▲) or the same Ab further mutated not to bind to FcR (anti-mCD200RmFcmut., ●). An intact mouse IgG1 was used as an isotype control (△). Degranulation was then induced by the addition of varying concentrations of TNP-KLH to cross-link the bound IgE. Supernatants were removed after 1 h at 37°C and added to the β-hexosaminidase substrate to quantitate the extent of degranulation (*A*), or after 24 h to measure IL-13 (*B*) and TNF (*C*) by ELISA. Mutation of the FcR binding capability did not alter the extent of inhibition of degranulation and cytokine secretion triggered by anti-mCD200R. Data of a representative experiment is presented as the means of triplicate samples ± SEM.

increased the magnitude of the inhibition, coligation of the mCD200R/mCD200 complex to FcεRI was not required to inhibit degranulation or cytokine secretion.

Engagement of mCD200R by agonist anti-receptor Abs on normal mast cells did not cause significant inhibition of FcεRI-induced degranulation (Fig. 3*A*). Modest inhibition of degranulation was observed; however, if the anti-mCD200R mAb was cross-linked with a goat anti-rat-specific Ab that did not coligate the FcεRI-bound mouse IgE (Fig. 3*A*). Cross-linking the mCD200R also potently blocked the secretion of TNF and IL-13, measured 18 h after triggering the FcεRI (Fig. 3, *B* and *C*). Enhanced inhibition was also observed when the two receptors were coaggregated (data not shown). Mast cells overexpressing the mCD200R showed pronounced inhibition of FcεRI-dependent degranulation and cytokine secretion when mCD200R was triggered with anti-mCD200R Abs alone (Fig. 3, *E* and *F*). Cross-linking the anti-mCD200R Abs or coligating the mCD200R and FcεRI substantially increased the magnitude of the inhibitory responses (Fig. 3*D* and data not shown).

Because mouse mast cells express the ITIM-containing isoform of CD32, it was possible that the anti-mCD200R-induced inhibition of mast cell degranulation was manifested through IgG FcR interactions. The rat anti-mCD200R mAb was murinized to express the mouse IgG1 isotype and the Fc domain was further molecularly engineered not to bind FcR. The Fc-mutated anti-mCD200R Ab was equally effective, as the intact Ab, in inhibiting FcεRI-induced mast cell degranulation and cytokine secretion (Fig. 4).

CD200R is an inhibitory receptor for human mast cells

Unlike normal mouse mast cells, in vitro-derived human mast cells showed a slight but consistent inhibition in degranulation when CD200R was independently triggered by the anti-hCD200R Abs (Fig. 5*A*). This inhibition was more pronounced at the lower concentrations of the anti-hFcεRI Ab and was significantly enhanced by cross-linking the anti-hCD200R Ab (Fig. 5*B*). Although coligation of the anti-hCD200R to FcεRI was not required to inhibit degranulation, enhanced inhibition was observed when the two receptors were coaggregated (Fig. 5*C*).

Normal mouse and human skin mast cell express CD200R

Immunofluorescent microscopy was performed on frozen sections of normal mouse and human skin (Figs. 6 and 7). Mast cells were readily identified by the prominent staining of cytoplasmic granules with fluorescent (FITC) avidin (19–21). Staining of frozen mouse skin sections with an anti-mCD200R mAb and FITC avidin clearly demonstrated that the majority of mouse skin mast cells expressed CD200R (Fig. 6, *E* and *F*) without coexpression of the macrophage/granulocyte marker, CD11b (Fig. 6, *C* and *D*). CD200R was also expressed on a significant percentage of non-mast cell leukocytes in the skin, which were identified as macrophages, dendritic cells, and T cells (data not shown). Interestingly, CD200R was not expressed on any cells of the epidermis including Langerhans cells (Fig. 6).

Frozen sections of human skin were also analyzed by two-color immunofluorescence using anti-hCD200R Abs and FITC avidin. The majority of human skin mast cells strongly expressed CD200R without coexpression of the macrophage marker, CD11b (Fig. 7, *E* and *F*). Melanocytes of the stratum basale region of the epidermis did not express CD200R nor was CD200R observed on the Langerhans cells of the epidermis. CD200R was strongly expressed on the majority of dermal infiltrating leukocytes. Two-color immunofluorescence studies revealed that the majority of CD200R-positive cells in human skin were dermal macrophages and dendritic cells (data not shown).

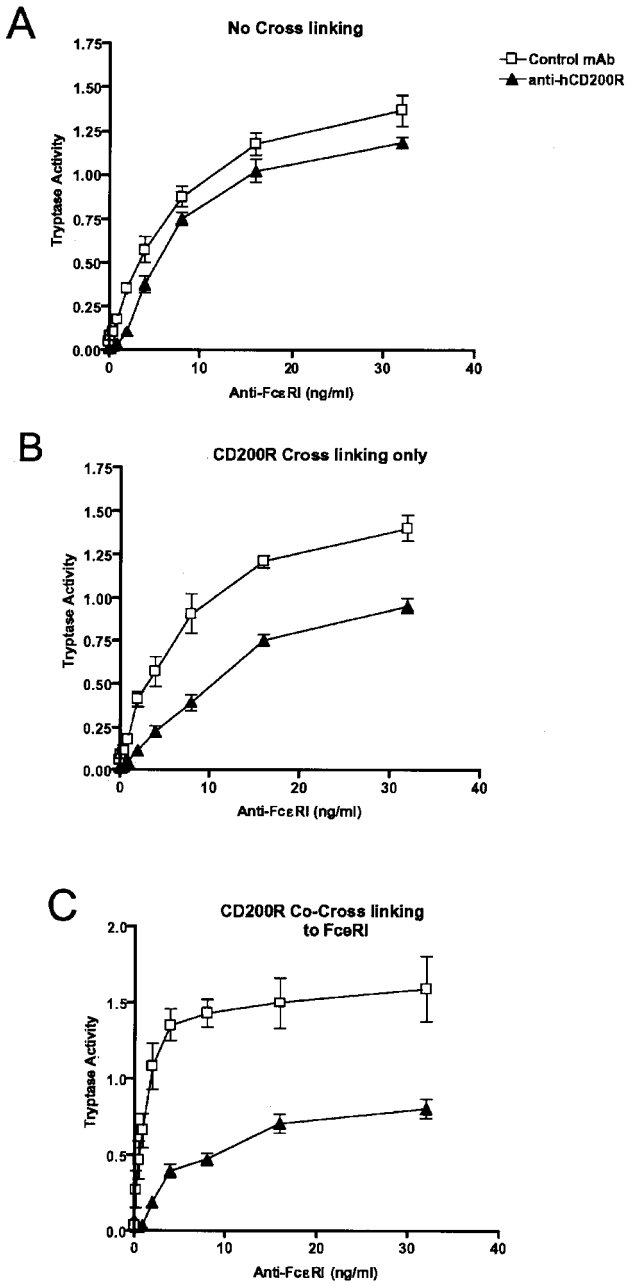

FIGURE 5. Effects of anti-hCD200R Ab on human mast cell degranulation. IgE-loaded human mast cells were stained with a saturating concentration (50 μg/ml) of anti-hCD200R mAb (DX183) or isotype control mAb. After washing, the mast cells were triggered with various concentrations of an anti-hFcεRI mAb (DX68) without any additional cross-linking (*A*), with cross-linking (XL) of the anti-hCD200R only (*B*) and with coligating (CoXL) the anti-hCD200R Ab to the anti-hFcεRI Ab (*C*). Degranulation was assessed by measuring the release of tryptase into the supernatant at OD readings of 405–450. Data of a representative experiment is presented as the means of triplicate samples ± SEM. Similar results were observed for five mast cell preparations each from different normal cord blood donors.

Cellular distribution of CD200 in mouse and human skin

Frozen sections of mouse and human skin were analyzed by simultaneous two-color immunofluorescence for the localization of CD200 (the natural ligand for CD200R) and mast cells. In both mouse and human skin, CD200 was strongly expressed on some dermal fibroblasts, endothelial cells, dermal nerve bundles, hair

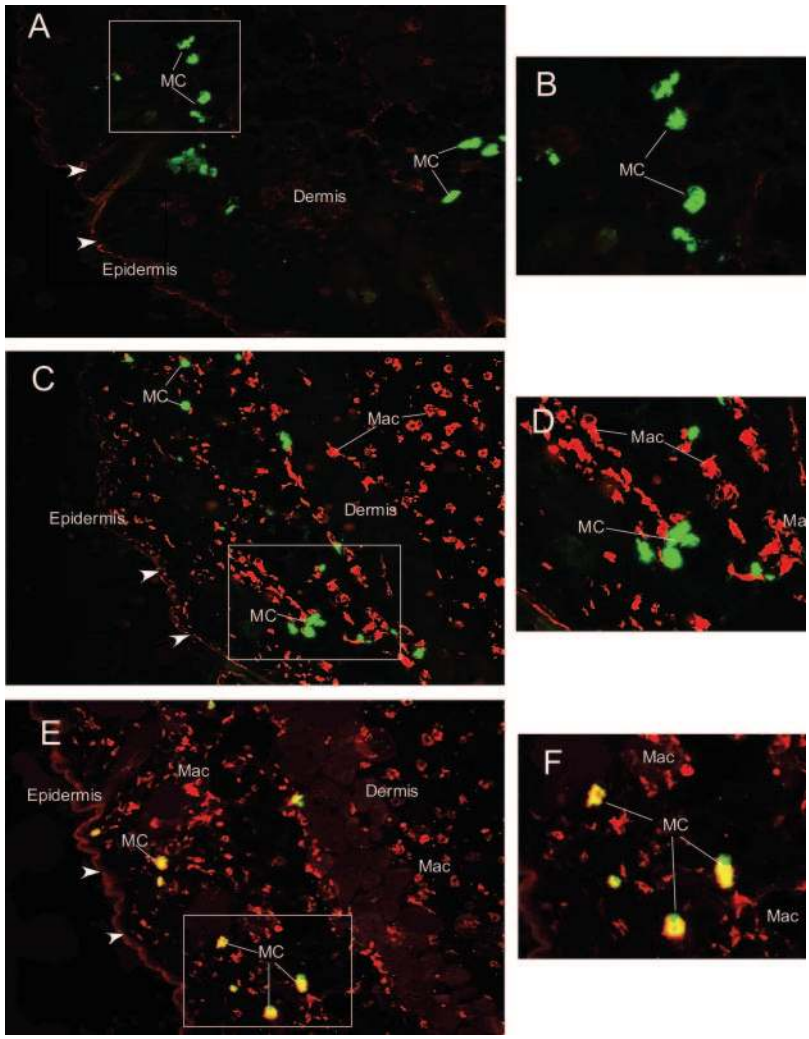

FIGURE 6. Mouse skin mast cells express CD200R. *A,* Frozen sections of normal BALB/c mouse skin (original magnification, ×10) were fixed in methanol and stained with an isotype control mAb (in red) and FITC avidin (in green). Because of the strong ionic interactions with mast cell granules, FITC avidin readily identifies mouse skin mast cells (MC). *B,* Higher magnification (original magnification, ×40) of the area indicated in *A*. *C,* Frozen sections of mouse skin (original magnification, ×10) were stained with anti-mCD11b mAb (red) and FITC avidin (green). *D*, Mouse dermal macrophages (Mac) strongly express CD11b; however, mast cells (MC) were negative for CD11b expression. *E,* Frozen sections of mouse skin (original magnification, ×10) were stained with anti-mCD200R mAb, DX109 (red), and FITC avidin (green). *F*, Mouse skin mast cells (MC) strongly express mCD200R as do most dermal infiltrating leukocytes. Sections were analyzed by standard fluorescent microscopy. Boxed regions are represented at higher magnifications (*B*, *D*, and *F*; original magnification, ×40). Arrows indicate the epidermal region of the skin.

follicles, and subsets of glandular epithelial cells (Fig. 8). Interestingly, CD200 was not expressed on keratinocytes, Langerhans cells, or the majority of mast cells and macrophages of the dermis in both mouse and human skin (Fig. 8). Although most mouse skin mast cells were not observed in direct contact with CD200-expressing cells, occasionally mast cells, followed through serial sections, were seen in close proximity to CD200-expressing cells or nerve fibers in mouse skin (Fig. 8*B*). Human skin demonstrated significantly less CD200 expression than that observed in mouse skin (Fig. 8*C*) and only rarely were mast cells observed in close proximity to CD200-expressing cells (Fig. 8, *D* and *E*).

CD200R is an inhibitory receptor for mouse mast cells in vivo

To determine the ability of the CD200R to regulate mast cell activity in vivo, we tested the effects of anti-mCD200R mAbs on PCA in mouse skin (22–24). Mice were injected i.v. with anti-mCD200R mAb or isotype control mAb followed by localized intradermal injections of various concentrations of DNP-specific mouse IgE. Twenty-four hours after IgE injections, the mice received i.v. injections of DNP-albumin in 0.5% Evan's blue. Engagement of FcεRI-bound IgE by specific ligand results in mast cell activation and the subsequent increase in vascular permeability that is readily visualized by the accumulation of tissue localized Evan's blue and quantified in formamide-extracted skin punches. Treatment of mice with anti-mCD200R mAb, 24 h before mast cell triggering, inhibited IgE-dependent PCA skin reactions in a dose-dependent fashion (Fig. 9, *A* and *B*). CD200R-mediated inhibition of PCA was observed at all IgE concentrations and was detectable with as little as 1 μg of anti-mCD200R mAb per mouse (0.04 mg/kg). A murinized version of the rat anti-mCD200R mAb that was mutated to prevent FcR binding interactions gave identical results to the intact rat anti-muCD200R Ab (Fig. 9*C*).

Discussion

Mast cells have long been considered one of the primary sentinel of peripheral tissue inflammatory immune responses. The tissue localization of mast cells particularly in connective tissues, and beneath epithelial layers of the skin and mucosa have implicated mast cells in a variety of severe atopic disorders as well as a variety of autoimmune diseases (25–35). In an effort to define molecular mechanisms that negatively regulate mast cells functions, we have analyzed the expression patterns of several potential inhibitory receptors on murine and human mast cells. One such receptor, CD200R, is prominently expressed at the mRNA level in mouse bone marrow-derived mast cells and human cord blood-derived mast cells (12).

A variety of in vivo studies have strongly suggested that CD200R is an inhibitory receptor that negatively regulates myeloid functions, particularly cells of the macrophage/dendritic cell lineage (14, 36). Although in vivo studies presented strong circumstantial evidence that CD200R was an inhibitory receptor, the

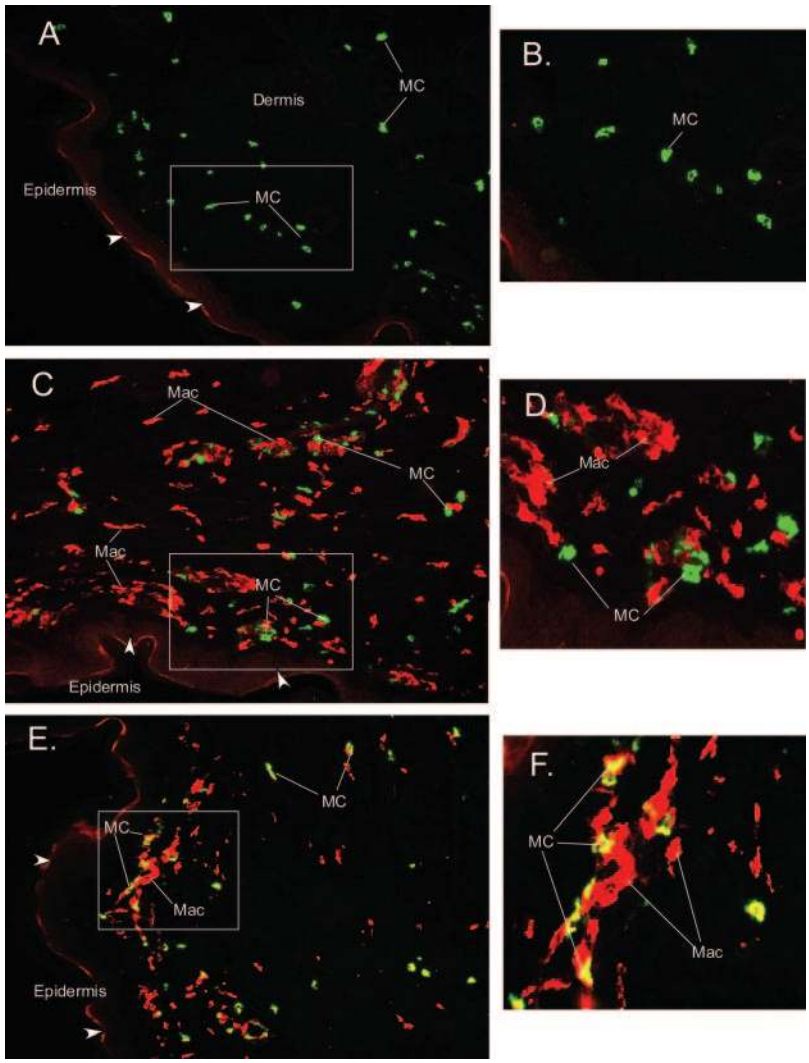

FIGURE 7. Human skin mast cells express hCD200R. *A,* Frozen sections of normal human skin (original magnification, ×10) were fixed in methanol and stained with a mixture of IgG1 and IgG2 rat isotype control mAbs (in red) and FITC avidin (in green) to identify mast cells (MC). *B,* Higher magnification (original magnification, ×40) of the area indicated in *A*. *C,* Frozen sections of human skin (original magnification, ×10) were stained with anti-hCD11b mAb (red) and FITC avidin (green). Human skin mast cells (MC) do not express the macrophage marker, CD11b. *D*, However, CD11b is strongly expressed on dermal macrophages (Mac). *E,* Frozen sections of human skin (original magnification, ×10) were stained with anti-hCD200R mAb, DX136 (red), and FITC avidin (green). *F*, Human skin mast cells (MC) and macrophages (Mac) strongly express hCD200R. Sections were analyzed by standard fluorescent microscopy. Boxed regions are represented at higher magnifications (*B*, *D*, and *F*; original magnification, ×40)). Arrows indicate the epidermal region of the skin.

expression of CD200R on mast cells allowed us to directly investigate the effects of this receptor on well characterized mast cell biological responses. In this study, we show that soluble mCD200 did not inhibit mast cell degranulation in normal in vitro-derived mouse mast cells, but overexpression of mCD200R made these cells extremely sensitive to mCD200-mediated inhibition. The direct triggering of mCD200R by ligand without coligation to the FcεR is the first direct evidence that mCD200R functions through the generation of inhibitory signals in mast cells. Pretreatment of mouse and human mast cells with saturating amounts of agonist anti-CD200R Abs likewise inhibited FcεRI-dependent responses, particularly if the anti-CD200R Abs were cross-linked to themselves. Indeed, preliminary studies have shown that CD200R is rapidly tyrosine phosphorylated after engagement of the receptor with ligand or anti-CD200R Abs (data not shown). The increased sensitivity to inhibition of FcεRI activation in mast cells overexpressing the CD200R and the enhanced inhibition observed with cross-linking the CD200R likewise indicates that CD200R expression density may regulate the magnitude of the inhibitory response.

The ability of anti-CD200R Abs to inhibit mast cell degranulation without coligation to an activating receptor suggests a molecular mechanism different from the majority of immune inhibitory receptors. In recent years, a variety of inhibitory receptors have been associated with the in vitro regulation of mast cell degranulation responses. Among the most investigated mast cell inhibitory receptors are FcγRIIB (37–39), gp49B1 (mouse (40–32)) PIR-B (mouse (19, 20, 43, 44)) MAFA (rat (21–24, 45)), and Sirp-α (humans (46, 47)), all of which contain at least one cytoplasmic inhibitory domain referred to as an ITIM. ITIM-containing receptors inhibit activating ITAM receptors by recruiting phosphatases to the phosphorylated ITIMs (48–54). Studies using Abs against the inhibitory receptors have demonstrated that most ITIM receptors require coaggregation to FcεRI to induce inhibition of mast cell degranulation responses (48–51, 53). MAFA and FcγRIIB are the only two receptors that inhibit FcεRI-dependent degranulation without artificial coaggregation (21, 23, 24, 37–39, 55). Recent studies have shown that these inhibitory receptors can be colocalized with FcεRI in mouse mast cells suggesting functional regulatory interactions. CD200R does not contain an ITIM sequence in its cytoplasmic domain; nevertheless, it can clearly inhibit degranulation and cytokine secretion without coaggregation to the FcεRI. The mechanism of CD200R inhibition of FcεRI is presently not known, however, because anti-CD200R Abs lacking FcR binding capabilities inhibited mast cell function to the same degree as intact Abs, the inhibitory mechanism does not involve Fc domain interactions with the inhibitory isoforms of CD32. Preliminary analysis of CD200R signaling suggest that upon receptor engagement by ligand or agonist Abs, CD200R is rapidly tyrosine phosphorylated and subsequently recruits secondary signal transduction mediators.

CD200R was variably expressed on in vitro differentiated mouse and human mast cells, and was shown to be prominently expressed

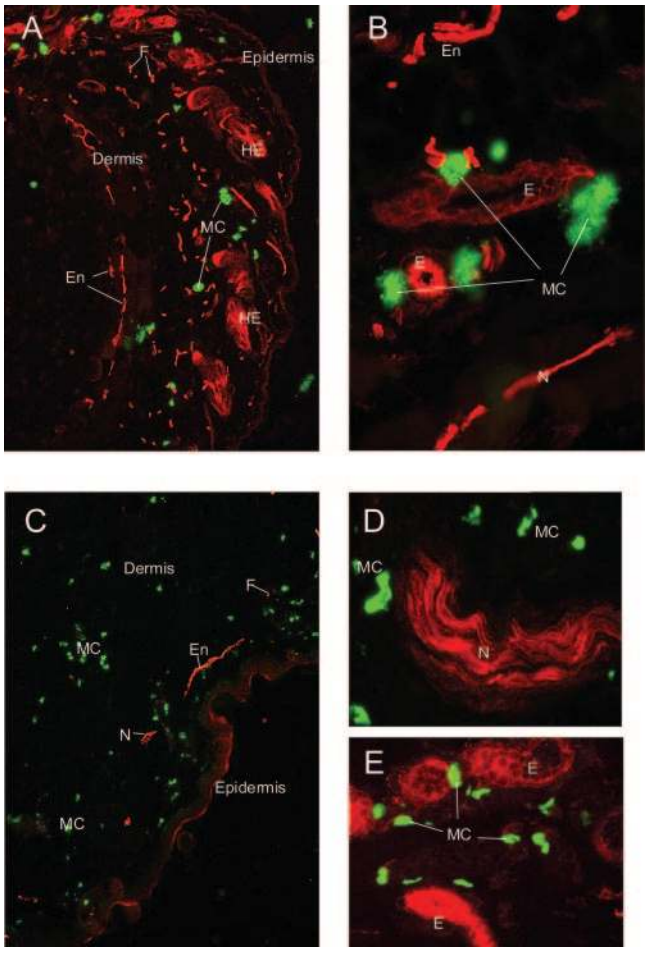

FIGURE 8. Localization of CD200 and mast cells in mouse and human skin. *A,* Frozen sections of mouse BALB/c skin (original magnification, ×10) were stained with anti-mCD200 mAb, OX2 (red), and FITC avidin (green). mCD200 was prominently expressed on endothelial cells (En), glandular epithelial cells (E), hair follicle epithelial cells (HE), nerve bundles (N), and fibroblasts (F). Mast cells (green) did not express mCD200; however, mast cells were occasionally seen in close proximity to mCD200-expressing cells (*B*, original magnification, ×40). *C,* Frozen sections of normal human abdominal skin (original magnification, ×10) were stained with anti-hCD200 mAb, DX136 (red), and FITC avidin (green). hCD200 was prominently expressed on endothelial cells (En), some glandular epithelial cells (E), a few hair follicle epithelial cells (HE) nerve bundles (N) and fibroblasts (F). Mast cells (green) did not express hCD200, however, mast cells were occasionally seen in close proximity to CD200-expressing cells (*D–E*). Sections were analyzed by standard fluorescent microscopy and arrows indicate epidermal regions of the skin.

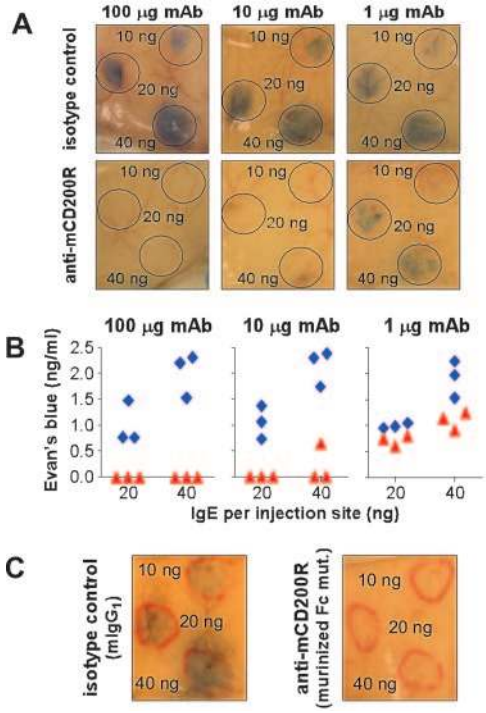

FIGURE 9. Effects of anti-mCD200R Abs on mouse skin PCA reaction. Mice were injected i.v. with 1, 10, or 100 μg of anti-mCD200R (DX109) or an isotype control mAb just before intradermal injection of IgE into local skin sites. Sixteen to 24 h later, 50 μg of DNP-HSA (Sigma-Aldrich) and 0.5% Evan's blue in PBS was injected i.v. in a final volume of 100 μl. *A,* Inverted dorsal skin samples are shown from two representative mice treated with either control rat IgG1 or anti-mCD200R mAb. The amounts of IgE injected intradermally are indicated. *B,* Evan's blue was extracted from biopsies taken from PCA reaction sites of three control IgG1-treated mice as well as three anti-CD200R mAb-treated mice, and the amount of Evan's blue was quantitated. The amounts of Evan's blue at the sites of injection of 10 ng of IgE was below the level of detection for this assay so only results for 20 and 40 ng of IgE are shown. *C,* To investigate whether FcR binding was required for anti-mCD200R-mediated inhibition of mast cell degranulation in the PCA model. Mice were injected i.v. with 10 μg of anti-mCD200RmFcmut (DX109 mAb murinized and Fc mutated) or isotype control mAb (mouse IgG1) just before injection of IgE into local skin sites. Sixteen to 24 h later, 50 μg DNP-HSA (Sigma-Aldrich) and 0.5% Evan's blue in PBS was injected i.v. in a final volume of 100 μl. Inverted dorsal skin samples are shown from two representative mice. Amounts of IgE injected intradermally are indicated.

on the majority of leukocytes localized in the dermis of both human and mouse skin by immunohistochemistry. Analysis on the distribution of CD200, the natural ligand for CD200R, clearly showed that, although it was broadly expressed on a variety of cell types in the skin, the majority of skin mast cells were not observed in direct contact with CD200-expressing cells. CD200 was also never coexpressed with CD200R on skin mast cells suggesting that most dermal mast cells are not constitutively inhibited by constant interaction of the CD200R with its natural ligand. Indeed, in vivo studies clearly demonstrated that systemic administration of an agonist anti-mCD200R Ab into mice could potently inhibit dermal mast cell IgE-dependent degranulation and that this inhibition was not dependent upon IgG FcR engagement. The ability of anti-CD200R Ab to inhibit in vivo mast cell FcϵRI-dependent responses without additional cross-linking suggests that mast cells in situ are more susceptible to CD200R triggering than in vitro cultured mast cells. Preliminary comparative phenotypic analysis indicates that freshly isolated mast cells from skin express significantly higher levels of CD200R than tissue culture bone-marrow-derived mast cells and that this differential expression may explain the enhanced efficacy of the anti-CD200R Ab in vivo. In addition, the ability of anti-CD200R Ab to readily inhibit FceRI dependent cytokine secretion would support the notion that the in vivo efficacy of the anti-CD200R Ab is also reflected in its inhibition of cytokine secretion as well (56). The ability to target CD200R and inhibit mast cell inflammatory functions opens the potential for a novel therapeutic strategy for the management of mast cell dependent pathologies.

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