CD200 Is a Ligand for All Members of the CD200R Family of Immunoregulatory Molecules

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CD200Fc, a chimeric molecule including the extracellular domain of CD200 and a murine IgG2a Fc region, regulates immune responses following engagement of a cell surface receptor, CD200R, expressed on cells of the myeloid and T cell lineage. A recent report focused attention on a family of CD200Rs, but concluded that only one member used CD200 as its ligand. We have also cloned and sequenced a family of CD200Rs, but identify an amino terminus to two of the three isoforms not recognized by previous researchers. We show by FACS, using FITC-labeled CD200Fc, that COS7 cells transfected with all CD200R isoforms bind CD200 as ligand, although the functional consequences of this binding likely differs between the different isoforms. mAbs directed against the CD200 R1/R4 isoforms altered IL-2/IL-4 cytokine production and suppressed CTL responses in a fashion comparable to CD200Fc, with a significantly lesser effect seen following addition of anti-CD200 R2/R3.


Material and Methods

Animals

Eight-week-old C3H/HeJ and C57BL/6 mice (H2k/k and H2b/b, respectively) were obtained from The Jackson Laboratory (Bar Harbor, ME) and kept five per cage with food and water ad libitum. Mice were entered into experiments at 9 wk of age. All animal experiments in the study were conducted according to the guidelines of the Canadian Council on Animal Care and approved by the University of Toronto and University Health Network Committees on Animal Experimentation.

Cloning of a family of murine CD200 receptors

Using expressed sequence tag analyses, 5’-RACE, cDNA, and genomic DNA clone analysis, we have identified a family of CD200R molecules in the mouse genome. cDNA clones representing all of these CD200R isoforms (hereafter CD200R1–4) have been prepared. As documented elsewhere, the 5’ sequences of CD200R2 and R3 we identified differ significantly from the homologous sequences reported by Wright et al. (9) (CD200Rlc and CD200RLb in their terminology). In other general aspects, including sequence of CD200R1 and R4, chromosomal location, and tissue distribution (by Southern blots; real-time PCR), our data on the expression of CD200R isoforms is in accord with that of Wright et al. (9).

Evidence for binding of CD200Fc to CD200R isoforms

To assess binding of different CD200R isoforms with CD200, we developed transiently and stably transfected COS7 cell lines expressing murine CD200R1–4 using a dominant selectable marker. Cells were transfected with linearized empty pBK vector (control cells) or pBK vectors containing the full-length open reading frames for the various receptors. Pools of transfectants were selected in G418 (400 μg/ml) using the pBK neomycin resistance gene. FITC-conjugated CD200Fc, a chimeric molecule representing the extracellular domain of CD200 fused to the Fc regions of murine IgG2a (2), was used in FACS analysis (with or without excess unlabeled CD200Fc) to assess binding to COS7 transfected pools, using approaches described elsewhere (1).

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Anti-CD200R Abs

Using predicted amino acid sequence comparisons, Kyte-Doolittle hydrophobicity plots, and three-dimensional modeling of the CD200R isoforms, the following peptides were synthesized: 313015, STPDHSPELQISAVTLPQHELQGTYTC; 313417, CEAMAGKPAAQISWTPDGD; 313418, KPRGQPSCIMAYKVETKET; 313016, CSVKGREEIPPDDSFPFSDDN; and 313017, LQQISKKICTERGTTRVPAHHQSS.

Peptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits (American Peptide, Sunnyvale, CA) or rats (Immuno-Precise Antibodies, Victoria, British Columbia) for production of heterologous rabbit or rat mAbs to mouse CD200Rs, following fusion with rat hybridoma parent cells established and maintained as described previously (3). Abs were prescreened in ELISA using plates coated with the relevant peptides (100 ng/ml). The specificity of all sera and mAbs was assessed by FACS analyses of COS7 cells expressing the different isoforms of the CD200Rs or empty vector. FITC-labeled anti-rabbit Ig and anti-rat Ig Abs were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). In control experiments, cells were stained with FITC-labeled Abs alone.

FACS analysis and immunohistochemistry

Bone marrow and PBMC were obtained by centrifugation over murine lymphopaque (Cedarlane Laboratories) and represent pools derived from five C57BL/6 mice per preparation. Activated splenocytes and thymocytes were obtained using standard approaches (48-h LPS (10 μg/ml) or Con A (5 μg/ml), respectively), again from C57BL/6 mice. Five × 10^6 cells were the various anti-CD200R Abs at 4°C for 45 min, washed three times with cold PBS, and signal detected with FITC-labeled anti-rabbit or anti-rat Ig.

Ag stimulation in vitro and cytokine assays

Mouse spleen cells were cultured in triplicate in microtiter plates (1 × 10^6 cells/well of each responder and stimulator) in 300 μl of anti-MEM with 10% FCS (anti-F10) in the presence or absence of various concentrations of anti-CD200R Abs. Cytokines in culture supernatants (data for IL-2 and IL-4 only shown in Fig. 3a) were assayed by ELISA using commercial cytokine-specific mAbs and biotin-labeled secondary Abs as described earlier (3). In all cases, streptavidin-coupled alkaline phosphatase with appropriate substrate was used to develop the assay. Recombinant mouse cytokines (Endogen, San Diego, CA) were used to quantitate assays. CTL activity in stimulated cultures was assessed at 5 days in 51 Cr release assays using labeled EL4 tumor target cells as described previously (2). Control cultures contained only unstimulated responder cells. All assays were performed in triplicate and repeated at least three times.

Statistical analyses

All data are reported as the mean value ± SEM, with sample sizes (n) stated throughout. Values of p < 0.05 were considered to be significant.

Results

Evidence for a family of CD200 receptors in the murine genome

In addition to the recently described murine CD200R (GenBank accession number AF231393), named CD200R1 hereafter, we have isolated cDNA and genomic clones for CD200R2, R3, and R4. The sequences for murine CD200R2–R4 were submitted to
GenBank (GenBank accession numbers AY230198, AY230199, and AY230200, respectively). Data shown in Fig. 1 represent an amino acid comparison of the four CD200Rs, indicating that CD200R2–4 are highly related to, but not identical with, murine CD200R1. Comparison with sequence data from Wright et al. (9) clearly indicates significant NH2-terminal differences for CD200R2 and R3 (RLc and RLb in Wright et al.’s terminology), with our reported sequences (Fig. 1) containing previously unrecognized additional sequence. The predicted transmembrane domains of CD200R2–4 were highly related to each other, each sharing a highly charged lysine residue within transmembrane-spanning regions. These residues are known to be important “docking sites” for transmembrane adaptor proteins. Many of these adaptors, such as the CD3ζ, DAP-12/KARAP, and the γ-chain of FcRs (FcγR), contain immunoreceptor tyrosine-based activating motifs implicated in the recruitment of protein tyrosine kinases, such as Syk and/or ZAP-70, among others (11). As reported (9) and in contrast to CD200R2-R4, CD200 and CD200R1 do not have a charged residue in transmembrane-spanning regions. CD200R2, R3, and R4 have short 15-aa cytoplasmic tails that are highly related in sequence but not identical. In contrast with CD200 and CD200R2–R4, murine and rat CD200R1 have long 67-aa cytoplasmic tails, with three conserved tyrosine residues.

One of these residues is contained within an NPXY sequence motif, which is known to represent a target for signaling adapter molecules that recognize phosphotyrosine-binding domains (12). Based upon the phenotype of the CD200-deficient mouse and the known structural features of the CD200 molecule, it is reasonable to infer that CD200/CD200R1 engagement elicits an inhibitory signal in myeloid cells of the innate immune system that express CD200R1. As such, CD200R1 activation may be mechanistically related to receptors of the innate immune system that contain related cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs)4 (13). Genes encoding ITIM-bearing receptor families often contain related molecules in the genome that, although highly related in the sequences of exofacial domains, lack ITIM motifs and have contrasting signaling properties (13).

Interaction between CD200R isoforms and CD200

In a recent report, Wright et al. (9) concluded that isoforms of CD200R1 did not bind CD200 as ligand. Our sequence analysis indicates that, at least for CD200R2 and R3, significant NH2-terminal sequence is absent from the clones identified by the Wright

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4 Abbreviation used in this paper: ITIM, immunoreceptor tyrosine-based inhibitory motif.
Accordingly, we have investigated whether these newly identified full-length cDNA sequences for the different CD200Rs encode molecules that bind CD200. FACS analysis confirmed data from Wright et al. (8) that CD200R1 could indeed serve as a binding molecule for CD200. FITC-labeled CD200Fc bound to pools of COS7 cells transiently transfected with CD200R1 but not to cells transfected with empty expression vector. Importantly and in marked contrast to the recent studies of Wright et al. (9), FITC-labeled CD200Fc also bound to pools of COS7 cells transfected with CD200R2, R3, or R4. This binding, as anticipated, was blocked by inclusion of a 20-fold excess of non-FITC-labeled CD200Fc in the binding reaction. Data for representative FACS studies are shown in Fig. 2.

Functional properties of anti-CD200R Abs

CD200R peptides were used to generate rabbit polyclonal and rat mAbs. The predicted ability of the various peptides to discriminate between the murine CD200R isoforms is indicated in Table I. Anti-313015 would be predicted to be relatively specific for R1/R4; anti-313418 to be relatively specific for R2; and anti-313016 and anti-313017 to be relatively specific for R3. All of the anti-peptide Abs detected the expression of the murine CD200R isoforms heterologously expressed in COS7 cells (see Fig. 3 for mAb data: polyclonal Ab data not shown). Variations in the level of staining seen may reflect minor variation in the relative steady-state protein expression of the varied CD200R isoforms in COS cells as well as differences in the affinities of the Abs. We have not been able to use our Abs to discriminate between CD200R1 and CD200R4. Data in Fig. 4 shows FACS analyses of the different anti-peptide Abs (both polyclonal Abs and rat mAbs) using single-cell suspensions prepared from different murine tissues.

We reported earlier that CD200Fc blocked CTL responses in allostimulated MLCs (2). We have compared and contrasted the effects of CD200Fc vs the rat mAbs described above on cytokine and CTL responses generated in allostimulated MLCs. C3H cells were stimulated with C57BL/6 spleen stimulator cells in the presence or absence of CD200Fc and anti-CD200R Abs. We stress again that there were no important differences in the ability of the anti-CD200 R1/R4, anti-CD200R2, and anti-CD200R3 Abs to bind their cognate cell surface targets and that expression of CD200R2 and CD200R3 mRNA is readily detected in splenic tissue.

The addition of anti-CD200 R1/R4 modulated IL-2:IL-4 cytokine production (Fig. 5a) and inhibited CTL responses (>75%, Fig. 5b) in a manner analogous to the effects of CD200Fc addition (2). The addition of anti-CD200R2 or anti-CD200R3 caused significantly less perturbation of cytokine production and inhibition of CTL responses (<35%) when compared with the addition of anti-CD200 R1/R4 or CD200Fc. It is important to note that in an alternate assay reported elsewhere, in which anti-CD200Rs were used to modulate differentiation of bone marrow-derived dendritic cells in vitro, it was in contrast to anti-CD200R2/R3 which showed the greatest effect relative to anti-CD200R1/R4 (10).

Table I. Amino acid homology amongst CD200R isoforms for peptides used for anti-CD200R immunization

<table>
<thead>
<tr>
<th>Peptide</th>
<th>CD200R1</th>
<th>CD200R2</th>
<th>CD200R3</th>
<th>CD200R4</th>
</tr>
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<tr>
<td>313015, 24 aa</td>
<td>24/24</td>
<td>20/24</td>
<td>8/24</td>
<td>23/24</td>
</tr>
<tr>
<td>313417, 19 aa</td>
<td>18/19</td>
<td>19/19</td>
<td>14/19</td>
<td>19/19</td>
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<tr>
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<td>12/19</td>
<td>19/19</td>
<td>7/19</td>
<td>8/19</td>
</tr>
<tr>
<td>313016, 21 aa</td>
<td>4/21</td>
<td>0/21</td>
<td>21/21</td>
<td>4/21</td>
</tr>
<tr>
<td>313017, 24 aa</td>
<td>4/24</td>
<td>3/24</td>
<td>24/24</td>
<td>4/24</td>
</tr>
</tbody>
</table>

*Based on comparisons shown, anti-313015 and anti-313016, anti-313017 would be most likely to discriminate well between CD200R1/CD200R4 and CD200R3; anti-313016, anti-313017 discriminate CD200R2 and CD200R3; anti-313418 would be most likely to discriminate between CD200R2 and all other CD200Rs.
bodies and a minimum of SD of three independent assays for two each of the rabbit/rat heteroantis. Results are pooled from a mean

ded both morphologically and functionally. Recent evidence has implicated an important role for members of the Ig

cyte differentiation and activation, relatively less is known about much has been learned concerning the mechanism(s) of lympho-

eloid lineage which deliver secondary cellular signaling events. Engagement of cell surface receptors of the innate immune system,

molecules in the mouse genome and deduced development of bone marrow cells in vitro (10). These results are consistent with previously described reports on the

moiety and the phenotype of a CD200-decient mouse (4). Heterologous COS7 cells that stably expressed unique CD200R isoforms were developed, with all such CD200R-

transfected cells subsequently shown to bind the ligand CD200 (Fig. 2), at least when expressed as the soluble molecule CD200Fc (2). Our data thus provide evidence for a family of CD200R mo-

lecules in the mouse genome and define the existence of previously unrecognized diversity in the CD200:CD200R immunomodulatory gene member family. Although our data document a CD200 binding

function for all CD200Rs, it is nevertheless possible that only CD200R1, with a cytoplasmic tail containing a PTB-signaling motif, is a functional member of this family. CD200R2–R4 might alternatively serve as decoy receptors for CD200. We do not favor this model and instead suggest that CD200R2–4 serve an alternative function (10).

We hypothesize that the family of CD200-binding receptors (CD200Rs) provides a further example of activating and inhibitory isoforms in the myeloid cells of the immune system (13). Formal proof of this model awaits future detailed studies of the biochemical properties of the different receptors. Based upon the phenotype of the CD200-deficient mouse (4) and the known structural features of the CD200 molecule, we infer that CD200:CD200R1 engagement elicits an inhibitory signal in myeloid cells of the innate immune system expressing CD200R1. Thus, CD200R1 activation may be mechanistically related to receptors of the innate immune system that contain related cytoplasmic (ITIMs) (11–13). Although we show that CD200Fc interacts physically with each of the CD200Rs when they were expressed on the surface of a heterologous cell type (Chinese hamster ovary, see Fig. 2), CD200R2, R3, and R4 are distinct from CD200R1 in that their short cytoplasmic tails lack putative ITIMs, but instead have charged residues within their membrane-spanning domains. Future studies will need to define whether our documented CD200R2–R4 are associated with immunoreceptor tyrosine-based activation motif-bearing proteins, although data from Wright et al. (9), using the incomplete CD200R2/R3 sequences, support this idea.

FACS analyses indicated that the cell surface expression of the CD200Rs defined by mAbs (Fig. 3) exhibited unique patterns of cell- and tissue-specific expression (see Fig. 4). For example, thymus and PBMC exhibited the greatest expression of CD200R1/R4, with some expression by splenic cells. Bone marrow expressed both CD200R2 and CD200R3. Thus, the family of CD200Rs has broad but unique tissue and cellular distribution and expression. Some other ITIM-bearing molecules and their activating partners are highly restricted in expression to hemopoietic tissues.

Evidence that the newly described CD200R2–R4 isoforms have functional properties comes from independent series of studies, reported in greater detail elsewhere (10). Inclusion of anti-peptide Abs in allogeneic MLCs in vitro led to both altered cytokine production and inhibition of CTL development (Fig. 5), as well as to modified development of bone marrow cells in vitro (10). These results are consistent with previously described reports on the functional effects of a soluble ligand for CD200R (CD200Fc) (2, 6) and the phenotypic properties of a CD200-deficient mouse (4).

**FIGURE 5.** Inhibition of generation of cytokines (a) or CTL (b) in spleen cell MLCs using Abs to different peptides (see earlier). Data are shown for splenic MLCs using C3H responder cells and C57BL/6 stimulator cells. Cytokines were assayed by ELISA at 40 h, and CTL were assayed at day 5 in 51Cr release assays with EL4 target cells. Lysis by CTL is shown for a single (50:1) E:T ratio. Results are pooled from a mean ± SD of three independent assays for two each of the rabbit/rat heteroantibodies and a minimum of five mAbs of each specificity.

**Discussion**

Regulation of the acquired immune system is mediated by physical interactions between lymphocytes and APC derived from the myeloid lineage which deliver secondary cellular signaling events. Engagement of cell surface receptors of the innate immune system, in the form of ligand and counterligand pairs, is a key determinant of the acquired immune response that develops (14). Although much has been learned concerning the mechanism(s) of lymphocyte differentiation and activation, relatively less is known about factors governing regulation of myeloid cells and their descendants. It is known that there is considerable heterogeneity in such cells, as defined both morphologically and functionally. Recent evidence has implicated an important role for members of the Ig superf gene family in controlling the differentiation and functional properties of cells of myeloid origin, such as CD200 (1, 3, 4), CD47 (15), and sialic acids (16) among others.

Use of a CD200-deficient mouse model provided evidence for a role for CD200-CD200R interactions in regulation of myeloid differentiation and/or activation (4). Other data suggest a key role for CD200-CD200R interactions in regulating acquired immunity: transplant rejection (3), autoimmunity (4, 6, 7), and fetal loss (17). An intracellular signaling role for CD200R in this interaction was predicted from structural analysis of the CD200 molecule, showing that it lacked signaling domains in the cytoplasmic tail or “docking motifs” within membrane-spanning regions, and was confirmed in several independent structural and functional studies (3, 4, 8). Understanding the biology of CD200R is a key to understanding “cross-talk” that exists between the innate and acquired immune system. The proposal by Wright et al. (9) that newly identified members of the CD200R family did not use CD200 as ligand was surprising to us, and, as we report above, in marked contradistinction to our own data.

Using 5'-RACE, cDNA, and genomic DNA clone analysis, we have defined a family of CD200Rs in mouse, which we have named CD200R1, R2, R3, and R4. CD200R1 had been previously reported (3, 4). Heterologous COS7 cells that stably expressed unique CD200R isoforms were developed, with all such CD200R-transfected cells subsequently shown to bind the ligand CD200 (Fig. 2), at least when expressed as the soluble molecule CD200Fc (2). Our data thus provide evidence for a family of CD200R molecules in the mouse genome and define the existence of previously unrecognized diversity in the CD200:CD200R immunomodulatory gene member family. Although our data document a CD200 binding function for all CD200Rs, it is nevertheless possible that only CD200R1, with a cytoplasmic tail containing a PTB-signaling motif, is a functional member of this family. CD200R2–R4 might alternatively serve as decoy receptors for CD200. We do not favor this model and instead suggest that CD200R2–4 serve an alternative function (10).

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Evidence that the newly described CD200R2–R4 isoforms have functional properties comes from independent series of studies, reported in greater detail elsewhere (10). Inclusion of anti-peptide Abs in allogeneic MLCs in vitro led to both altered cytokine production and inhibition of CTL development (Fig. 5), as well as to modified development of bone marrow cells in vitro (10). These results are consistent with previously described reports on the functional effects of a soluble ligand for CD200R (CD200Fc) (2, 6) and the phenotypic properties of a CD200-deficient mouse (4).
In each of these assays, the addition of anti-CD200 R1/R4 elicited immunomodulatory responses that were comparable to findings with CD200Fc and qualitatively and/or quantitatively different from those observed following addition of anti-CD200 R2/R3 (see Fig. 5). Whether these findings reflect the dichotomy in the signaling responses discussed above remains to be defined.

A recent paper by Wright et al. (9) reached conclusions similar to those above in certain instances, while differing in other important regards. Both studies document a CD200R1 with equivalent gene localization and a DNA/protein sequence that binds CD200Fc and contains intracytoplasmic signaling motifs. Our data extend these studies to show that engagement of this CD200R1 results in delivery of an immunosuppressive signal in vitro (Fig. 5). Wright’s group provided evidence that the variant CD200Rs can interact with DAP12 adapter protein. This finding is consistent with our proposed model that suggests an alternative signaling modality from that used by CD200R1 and might be implicated in the important differences we report between CD200R1 and other CD200R family members (10). Wright’s group in contrast reported that CD200R2 and R3 (CD200Rc and CD200Rlb in their terminology) differ in sequence at the NH2-terminal from the structures reported by us and showed no evidence for binding of CD200Fc. Using a combination of approaches, especially 5’-RACE, to define the full coding sequences for these variants, our data indicate that the NH2-terminal domains reported by Wright et al. for CD200R2 and CD200R3 are incomplete. Thus, comparison of the NH2 terminus of CD200RLb with CD200R3 indicates they have failed to define the complete NH2-terminal coding exon of this gene and lack important regions of the signal peptide. A full open reading frame was also not defined for CD200R2. This may, in part, explain their failure to define a CD200-binding function for these variants. The explanation for their failure to define a CD200Fc-binding function for the CD200R4 variant however remains elusive, although these data are not shown in their article.

The explanation for their failure to define a CD200-binding function for CD200R4 variant may, in part, explain their failure to define an alternative signaling pathway for CD200Rs. This gene localization and a DNA/protein sequence that binds CD200Fc and contains intracytoplasmic signaling motifs. Our data extend these studies to show that engagement of this CD200R1 results in delivery of an immunosuppressive signal in vitro (Fig. 5). Wright’s group provided evidence that the variant CD200Rs can interact with DAP12 adapter protein. This finding is consistent with our proposed model that suggests an alternative signaling modality from that used by CD200R1 and might be implicated in the important differences we report between CD200R1 and other CD200R family members (10). Wright’s group in contrast reported that CD200R2 and R3 (CD200Rc and CD200Rlb in their terminology) differ in sequence at the NH2-terminal from the structures reported by us and showed no evidence for binding of CD200Fc. Using a combination of approaches, especially 5’-RACE, to define the full coding sequences for these variants, our data indicate that the NH2-terminal domains reported by Wright et al. for CD200R2 and CD200R3 are incomplete. Thus, comparison of the NH2 terminus of CD200RLb with CD200R3 indicates they have failed to define the complete NH2-terminal coding exon of this gene and lack important regions of the signal peptide. A full open reading frame was also not defined for CD200R2. This may, in part, explain their failure to define a CD200-binding function for these variants. The explanation for their failure to define a CD200Fc-binding function for the CD200R4 variant however remains elusive, although these data are not shown in their article.

An additional and/or contributing explanation for the discrepancies in sequence reported by our two groups is that there is allelic variation in CD200Rs, although our limited data to date (unpublished) would not support this interpretation. The choice of cell type and experimental design used to investigate binding, and the possibility of variable levels of heterologous protein expression may be other important variables.

We have provided in this report the first evidence that all members of the CD200R family can bind CD200. Our other (unpublished) studies indicate that CD200R diversity exists within the human CD200R family, which seems to contain only two family members (homologues of murine CD200R1 and CD200R2). Sequence inspection again reveals features consistent with the hypothesis of independent signaling functions for CD200R1 vs CD200R2 following CD200 binding, although it remains to be determined whether, as in the mouse (see above), human CD200R2 is functionally expressed.

There are limitations to the interpretation of our data, some of which have already been addressed. Potential structural heterogeneity in the functional form of the CD200R expressed at the cell surface has not been investigated. We have not yet examined the possibility that multiple CD200Rs are coexpressed on individual cells, constitutively or following immune induction, and the potential functional consequences this might have. Thus, the potential for CD200R homodimerization and/or heterodimerization is unknown. Indeed, the Ab reagents described in this report do not yet allow for analysis of this question. As but one example, none of the mAbs or heteroantibodies described unequivocally discriminate between CD200R1 and R4. Cytoplasmic-tagged CD200R constructs are in preparation to investigate this issue. In addition, the avidity of any one CD200R for its ligand, CD200, has not been investigated, although preliminary studies are examining the ability of titrated concentrations of CD200Fc to block binding of FITC-CD200Fc to COS7-transfected cells (see assay of Fig. 2). Taken together, our data provide evidence for a family of CD200-binding CD200R molecules in the mouse genome and defines the existence of previously unrecognized diversity in the CD200:CD200R immunomodulatory gene member family. This adds important information to our understanding of the role and importance of cell surface interactions occurring on cells of the myeloid lineage in regulation of immunity. Evidence for molecular heterogeneity in the CD200Rs and unique patterns of tissue and cell expression provides an example of the plasticity that exists in lymphocyte and myeloid cell interactions and may be of practical importance for the clinical care of patients.

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