# A Comparison of the Biological Properties of Small Molecular Weight Agonists and Antagonists of CD200:CD200R Interactions

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**Abstract:** Our laboratory and others have documented in some detail the immunological consequences which follow from interaction of the ubiquitously expressed molecule CD200 with its receptor(s) CD200R (expressed predominantly on cells of myeloid and lymphoid origin). In particular, there is evidence that these interactions lead to immunosuppressive signals which modulate graft rejection responses; decrease the manifestations of arthritis in rodent models; diminish mast cell mediator release in models of allergic disease; and favour the growth of tumors in both mice and humans. The development of small molecular weight agonists (and/or antagonists) of these interactions would thus likely have significant clinical importance. The data reported herein characterizes several such molecules in a number of rodent models.

**Key Words:** Tolerance, immunosuppression, inflammation, immunoglobulin superfamily.

## INTRODUCTION

"Fine-tuning" of immune cell activation uses receptor molecules encoded by gene families whose members express opposing functions, namely inhibitory and activating pairs, with the former expressing functional cytoplasmic immunoreceptor tyrosine-based inhibitory (ITIM) motifs and the latter signaling *via* adapter molecules, DAP10/DAP12, bound to motifs in their transmembrane domains) [1,2]. Inhibitory signals delivered by many of these receptors are implicated in natural tolerance of self [1,3]. Alternate signals from pattern-recognition receptors (PRRs) can often over-ride these inhibitory signals leading to activation [4-6]. For the TREM family (triggering receptors expressed by myeloid cells), the *functions* regulated by inhibitory and activating isoforms are also not the same with the one regulating inflammation the other dendritic cell (DC) development [7].

CD200R is a member of the TREM family, with 5 members, R1-5, existing in mice, and two (CD200R1/2) in man. The molecules share structural homology with the immunoglobulin and lectin-like superfamilies, which includes MHC Class I molecules [8], sialic acids [9], CD47 [3] and, the CD200R ligand, CD200 [10]. CD200 and the CD200Rs are transmembrane proteins of the Ig superfamily, and all are conserved during evolution [11]. While CD200 is ubiquitously expressed [10,12], expression of different isoforms of CD200R for both mouse and human is tissue-restricted [13,14], and this may help explain the heterogeneity of function in the CD200R family [14,15]. The cytoplasmic domain of CD200 lacks signaling motifs, and the transmembrane domain lacks motifs which might bind other signaling molecules and thus immunoregulation mediated by CD200 is thought to reflect binding to signaling receptors, CD200Rs [13,14]. CD200 ligation of CD200R1 leads to phosphorylation of ITIM-like motifs in the cytoplasmic tail and association with *Dok* [16-19], while triggering of CD200R2/3 results in signaling *via* DAP-10/-12 signaling molecules [13,16,18,20]. CD200R1 is expressed on macrophages/DCs and a subpopulation of T cells. Its activation regulates inflammation and T cell function directly [7,21] while in contrast, alternate CD200Rs are expressed primarily on cells of the myeloid lineage and like other TREM family members, their triggering alters myeloid cell differentiation [7,22].

We reported a correlation between CD200 over-expression and decreased skin/renal allograft rejection [23] which was abolished by anti-CD200 antibody and restored by a fusion protein linking the extracellular domain of CD200 to the Fc region of IgG2a, CD200Fc [24]. CD200 expression was also shown to suppress spontaneous fetal loss [25]; collagen-induced arthritis (CIA) in DBA/1 mice [26]; and tumor immunity [27,28]. A viral homolog of CD200 has also been shown to modulate both macrophage and mast cell activation following interaction with CD200R1, as well as viral immunity to human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) [20,29].

Molecular modeling of CD200 (and CD200R), revealed domain structures (CDR1, CDR2 and CDR3) typical of all immunoglobulin family members [13,30-32]. Structural considerations suggest that the N-terminal region of both CD200 and CD200R is important in their mutual interaction [30]. Using a consecutive series of 15-mer peptides for both mouse and human CD200 to study inhibition of full-length CD200 activity in binding assays *in vitro*, and inhibition of the immunosuppression induced in cell populations *in vitro* following CD200:CD200R1 interactions [33], we also concluded that $NH_2$-terminal peptides conforming to regions of the CDR1, CDR2 and CDR3 regions of the CD200 V-region domain were important in CD200:CD200R recognition. Given the evidence that the functional activity induced by triggering alternate CD200Rs is not the same as that following CD200:CD200R1 interaction, we have explored the ability of CD200-derived peptides to function as agonists for CD200Rs, or as antagonists of CD200:CD200R interactions,

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using responder cells from wild-type mice or newly created mice selectively lacking expression of CD200R1 [34]. In this case we define an agonist (of soluble CD200Fc) as a peptide which produces the equivalent biological function as the full-length CD200Fc molecule, while a (competitive) antagonist is a peptide which can block the observed biological function of full-length CD200Fc used alone.

## MATERIALS AND METHODS

### Mice

Female C3H/HEJ, C57BL/6 and BALB/c mice were purchased from the Jackson laboratories, Bar Harbour, Maine. A CD200R1 KO mouse (BL/6 background), in which the genetic region encoding the extracellular domains of CD200R1 have been deleted, was generated and characterized as described elsewhere [34]. All mice were housed 5/cage and allowed food and water ad libitum. All mice were used at 8-12 weeks of age.

### Purification of CD200Fc from Transfected CHO Cells

Mouse CD200Fc fusion protein was cloned into CHO cells using a PIRES neo vector (Clonetech, Palo Alto, CA, USA). The Fc construct used was a mouse IgG2a, obtained from Dr. T.Strom [24]. Preparation of purified fusion proteins was as described in a previous report [33]. The molecular mass of the recombinant product was within the range of that anticipated for the naturally (cell-surface) expressed glycosylated molecules, i.e. ~66kDa for CD200Fc.

Rat monoclonal antibodies to mouse CD200Fc were prepared following immunization of rats with purified fusion proteins, with hybridomas selected for specificity in ELISA and FACS, using Hek293 cells transduced with PIRES vectors encoding CD200 after pre-screening to remove anti-Fc reactive hybridomas.

### Peptide Synthesis

15-mer peptides corresponding to regions encompassing the CDR1-3 regions of mouse CD200 were synthesized by the Hospital for Sick Children's protein sequence facility (University of Toronto, ON). All peptides were stored at a concentration of 10mg/ml. Fig. **1** and Table **1** shows a schematic illustrating the sequence and location of the CD200 peptides used. A peptide was characterized as an agonist if it produced functional (suppressive) effects analogous to full-

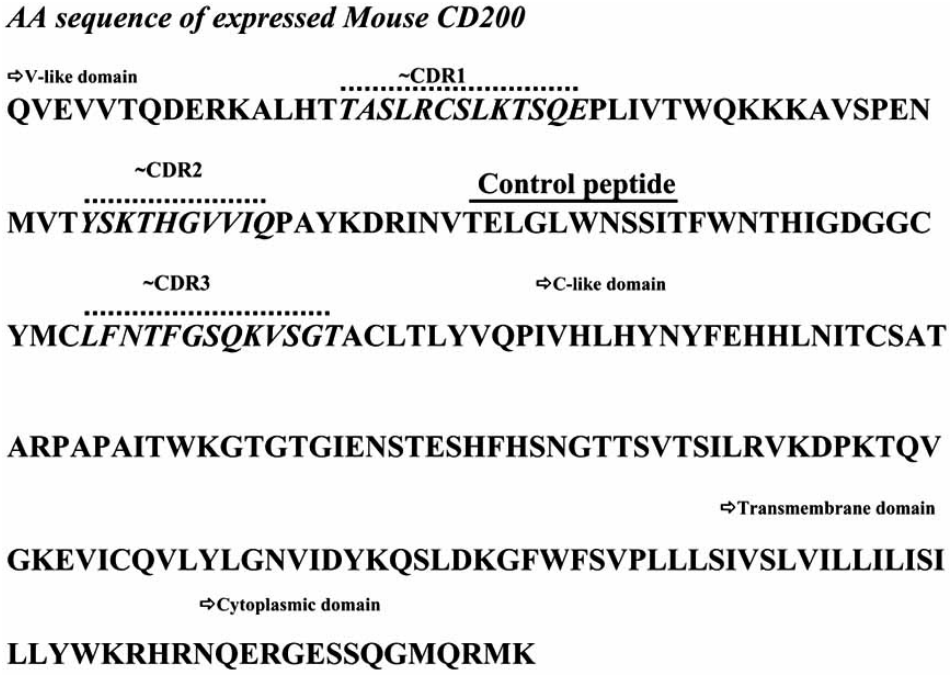


**Fig. (1).** Amino acid sequence for murine CD200, with the location of the important structural domains, including CDR regions and the important "faces" of the 3-dimensional CD200 molecule which interact with CD200R1 [32].

**Table 1. Primary and Secondary Sequence Identification of CD200 Peptides**

| Peptide Sequence | Peptide Sequence | Secondary Sequence Region |
|---|---|---|
| TASLRCSLKTSQE | #4004 | CDR1⇨FR2 |
| SPENMVTYSKT | #4005 | FR2⇨CDR2 |
| TYSKTHGVVTQ | #4012 | CDR2⇨FR3 |
| TELGLWNSSIT | #8000 | Control peptide |
| LFNTFGSQKVSGT | #4013 | CDR3 |

length CD200Fc, but as an antagonist if it blocked CD200Fc function.

### Preparation of Cells, Cytotoxicity and Cytokine Assays

Single cell suspensions from different tissues of C57BL/6 or CD200R1KO mice were prepared aseptically by incubation of teased tissue in collagenase for 30 minutes at 37°C, and, for bone marrow, by expression of cells from the femurs. After centrifugation, cells were resuspended in α-Minimal Essential Medium supplemented with 2-mercaptoethanol and 10% fetal calf serum (αF10).

Bone marrow derived dendritic cells (DCs) were generated in 8-day cultures of marrow cells incubated with IL-4+GM-CSF [22]. In some cultures various CD200-derived peptides were added [35]. In allogeneic mixed leukocyte cultures (MLC) used to assess generation of CTL, responder spleen cells were stimulated with equal numbers of mitomycin-C treated (45 min at 37°C) stimulator cells in triplicate in αF10. CD200Fc (and/or putative agonist or antagonist peptides) were added into the MLC cultures at the concentrations indicated. Where LPS-induced TNFα was measured, cells were incubated with LPs (1μg/ml) with/without CD200Fc and/or peptides, and supernatants pooled at 40hr from replicate wells and assayed in triplicate in ELISA assays.

Biotinylated detection anti-mouse TNFα(G281-2626) mAb for ELISA was obtained from Pharmingen (San Diego, CA). Capture goat heteroantibody to TNFα, along with Streptavidin-horse radish peroxidase, was purchased from Cedarlane Laboratories (Hornby, ON, Canada). Recombinant TNFα for standardization of ELISA assays was purchased from Pharmingen. Varying volumes of supernatant were bound in triplicate at 4°C to plates pre-coated with 100ng/ml mAb, washed x3, and biotinylated detection antibody added. After washing, plates were incubated with strepavidin-horse radish peroxidase (Cedarlane Labs), developed with appropriate substrate and $OD_{405}$ determined using an ELISA plate reader. The assay sensitivity was ~100pg/ml.

Where cytotoxicity was assayed, cells were harvested from MLC cultures at 6 days and titrated at different effector:target ratios for killing (6 hrs at 37°C) of $^{51}$Cr-labeled 72-hr ConA activated spleen target cells.

## RESULTS

### Evidence for Agonist/Antagonist Activity of Defined CD200 Peptides in Spleen Cells Stimulated with LPS or Allogeneic Cells

Previous studies using point mutational analysis of CD200R, and biochemical/structural analysis of CD200, have shown the most important interaction domains between CD200 and CD200R1 map to the GFCC' face of the $NH_2$-terminal domain of CD200R1 (Fig. **1**) [30,32,36]. We provided data consistent with this interpretation from studies examining suppression of CD200Fc binding to CD200R1 in FACS/ELISA by 15-mer CD200 peptides [33]. Given the heterogeneity of structure in the CD200R family, it has to date proven difficult to investigate the possibility that small 15-mer (or less) CD200 peptides might show subtle differences in binding to the different CD200R isoforms, and in turn produce functionally unique effects. WE have made us of a newly generated CD200R1 KO mouse in order to address this issue.

We have reported that in this CD200R1KO mouse, CD200 interactions with N-terminal regions of CD200R1 were eliminated [34]. Accordingly, we have explored the possibility that direct suppression of MLC reactivity *in vitro* by individual peptides (agonist activity) or suppression of protection by CD200Fc (antagonist activity) might differ using either wt or CD200R1KO cells as responder cells. The data in Figs. **2/3** show results for one of three studies assaying MLC responses using cells from wild-type *vs* KO mice as responders, in the presence of CD200Fc (± peptides). Similar data in Figs. **4/5** assess LPS-induced TNFα production from wild-type *vs* KO cells under the same circumstances (addition of CD200Fc± peptides).

It is clear from comparison of panels a and b in Figs. **2** and **4** (MLCs or LPS-induced TNFα production, using cells from control BL/6 mice) that several peptides defining the CDR2 region of CD200 (#s 4005 and #4012) all showed agonist activity, defined as an ability to suppress CTL or TNFα induction in a similar fashion to that shown by full-length CD200 (far left of Figures). In contrast while peptides defining the CDR1 (#4004) or CDR3 (# 4013) regions showed no direct agonist activity, these peptides had marked antagonist activity for CD200R1, defined by an ability to abolish the suppression mediated by full-length CD200 (panel b). A control peptide (#8000) showed no activity in either assay (panels a/b).

When responder cells were derived from CD200R1 KO mice (Figs. **3** and **5**) quite different data were obtained, which we interpret to reflect the effects of CD200 and peptides derived thereof on the alternate CD200Rs [33]. Thus, as shown in panel a of Figs. **3** and **5**, full-length CD200 itself actually augmented MLC responses and TNFα induction over 5d (or 40hr respectively) *in vitro*, as indeed did peptides mapping in the CDR1 (#4004) and CDR3 region (#4013). This effect we presume to represent agonist responses for alternate receptor function. In this context, abolition of this enhancement (mediated by full-length CD200) in panel b in each Figure, as shown by peptide #4012 we presume reflects an antagonist function of CD200 binding with reference to alternate receptors.

### Peptides Defining Interacting Regions of CD200: CD200R1 Modulate Dendritic Cell Development from Bone Marrow Cultures In Vitro

In previous studies we have shown that antibodies directed at alternate CD200Rs expressed on myeloid cells alter development of dendritic cells (DCs) *in vitro* from bone marrow precursors in the presence of GMCSF and IL-4 [22]. In particular, in the presence of antibodies to CD200R2 we have shown conclusively that "tolerogenic" DCs develop, able to induce populations of $CD4^+CD25^+Foxp3^+$Treg from allogeneic $CD4^+$ T cells [35]. Once again these studies were complicated by the coexistence of CD200R1 on the cells of interest. By making use of cells from CD200R1 KO mice, we were in a position to iso0late the activity of the CD200-derived peptides defined in MLC assays in Figs. **2-5** to

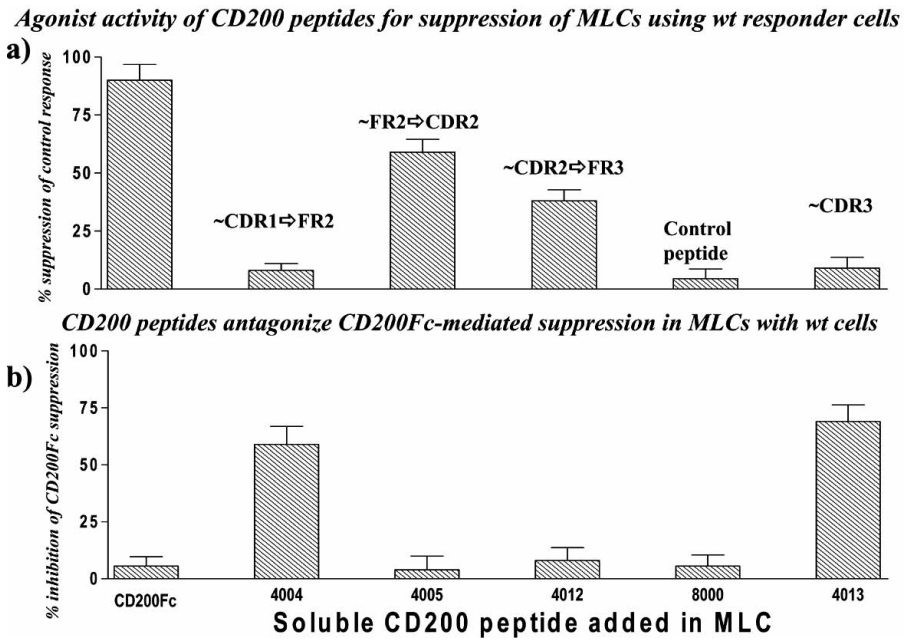


**Fig. (2).** Agonist (panel a) and anatagonist (panel b) activity of defined CD200-derived peptides (see Table **1**) in MLCs using mitomycin-c treated BALB/c stimulator cells and control BL/6 spleen responder cells, as defined by CTL analysis at day 5. In panel a, control cultures with no CD200Fc gave 28±2.5% specific lysis (30:1, E:T ratio) respectively for $10^4$ targets. All data are shown as a percent inhibition of this control response, with CD200Fc used at 4μg/ml, and all peptides at 60μg/ml. In panel b data are expressed as a percent inhibition of the suppressed (by CD200Fc) response seen in panel a.

modulate the interaction of CD200 with alternate CD200Rs, as described in the following study.

Bone marrow cultures from control or CD200R1 KO BL/6 mice were initiated with GMCSF and IL-4, with/without addition of CD200Fc (4μg/ml), CD200 peptides (60μg/ml), or a combination of CD200Fc with different peptides. After 8d cells in all cultures were washed, stimulated with LPS (1μg/ml) for 8hr and, after exhaustive washing, used to stimulate C3H splenocytes in MLCs for a further 5d. $^{51}$Cr assays were performed using EL4 tumor target cells, with typical data from one of three studies shown in Figs. **6** and **7**.

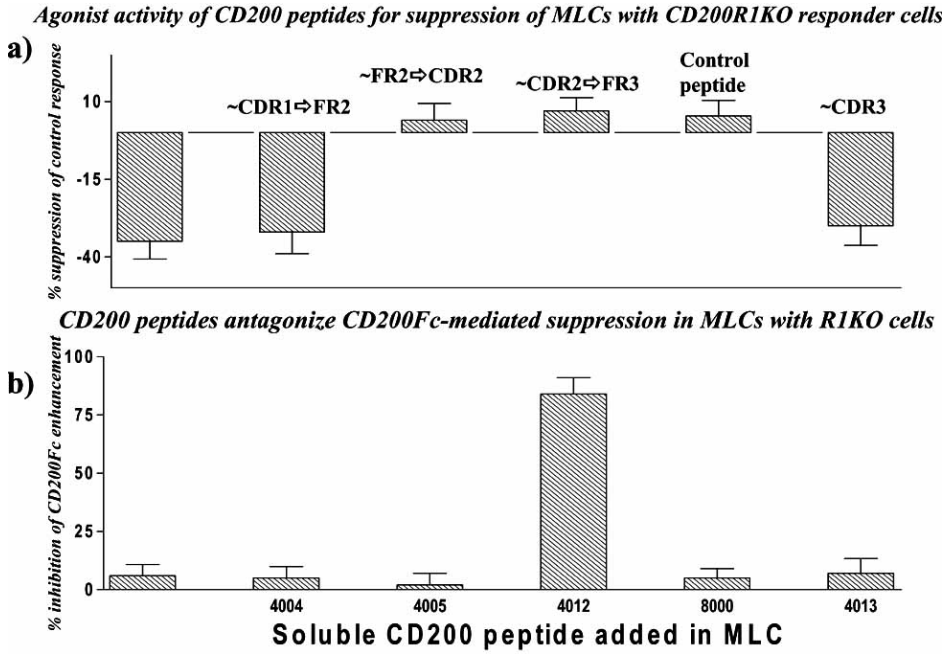


**Fig. (3).** Agonist (panel a) and anatagonist (panel b) activity of defined CD200-derived peptides (see Table **1**) in MLCs using mitomycin-c treated BALB/c stimulator cells and CD200R1KO BL/6 spleen responder cells, as defined by CTL analysis at day 5. In panel a, control cultures with no CD200Fc gave 34±2.1% specific lysis (30:1, E:T ratio) respectively for $10^4$ targets. All data are shown as a percent inhibition/enhancement of this control response, with CD200Fc used at 4μg/ml, and all peptides at 60μg/ml. In panel b data are expressed as a percent inhibition of the enhanced response (over control) induced by CD200Fc in panel a.

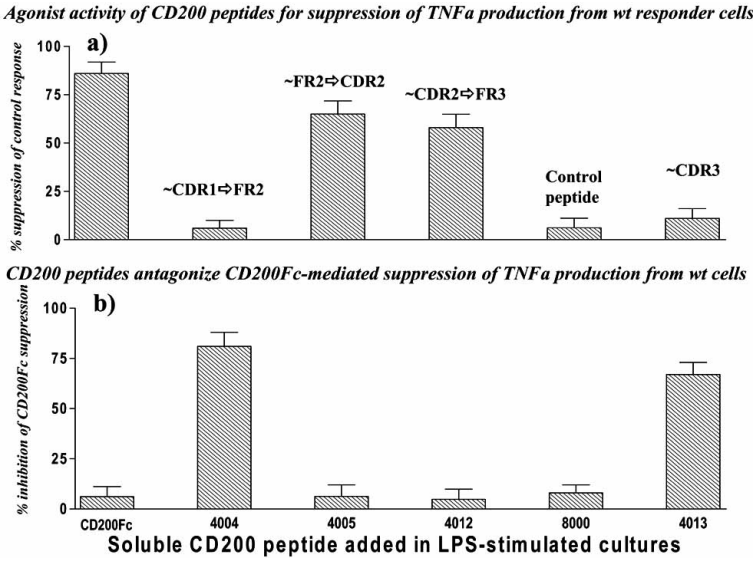


**Fig. (4).** As for Figure **2**, except ELISA assays measured TNFα production at 40hr from LPS-stimulated spleen cells of control mice, again subsequently expressing data as agonist function (suppression of TNFα production-panel a), or antagonist function in panel b (inhibition of suppression mediated by full-length CD200Fc, shown to left in panel a). Control cultures (no peptides or CD200Fc added) produced 935±125pg/ml TNFα.

Comparison of these data with those in previous Figures reveal several important features. Using bone marrow precursors from wt mice (Fig. **6**), peptide 4004 was most active (agonist) in inducing tolerogenic DCs (as assayed by the suppression of the control response seen with DCs incubated in the absence of peptides or CD200Fc), while peptide 4012 showed the greatest ability to prevent the effect seen using CD200Fc as agonist to induce tolerogenic DCs (panel b of Fig. **6**). Intriguingly, these are the same relative activities seen in Figs. **3** and **5**, measuring MLC responses in CD200 R1KO mice, and indeed we reported that induction of 0tolerogenic DCs *in vitro* was primarily a function of non-CD200R1 receptor engagement [35]. When similar studies were performed with marrow cells from CD200R1 KO mice (Fig. **7**) the data obtained paralleled that seen in Fig. **6**. Only peptide 4012 was a competitive antagonist of the activity induced by CD200Fc, while #4004, and to a lesser degree peptides 405 and 4013 showed agonist activity, in this case clearly for alternate (non-CD200R1) peptides.

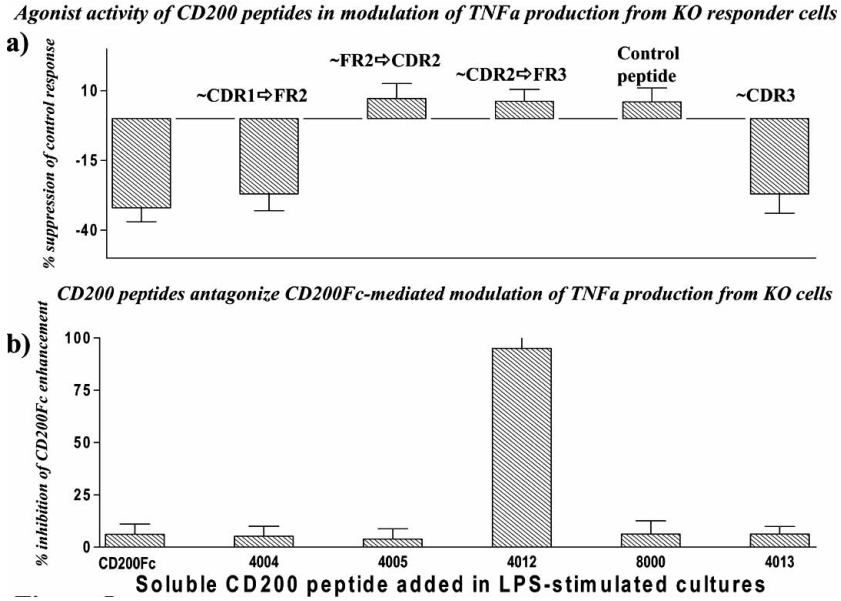


**Fig. (5).** As for Figure **4**, except ELISA assays measured TNFα production at 40hr from LPS-stimulated spleen cells of CD200R1 KO mice, again subsequently expressing data as agonist function (enhancement of TNFα production-panel a), or antagonist function in panel b (inhibition of enhancement mediated by full-length CD200Fc, shown to left in panel a). Control cultures (no peptides or CD200Fc added) produced 1275±135pg/ml TNFα.

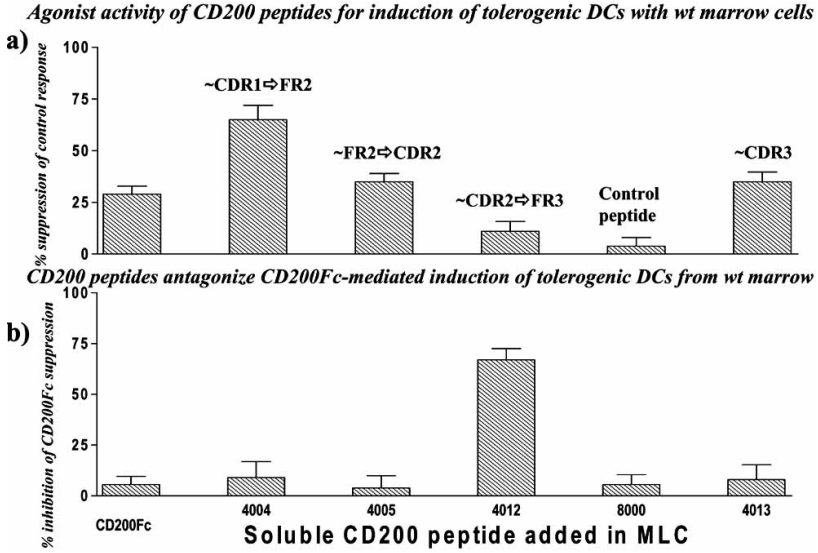


**Fig. (6).** Agonist (panel a) and antagonist (panel b) activity of defined CD200-derived peptides (see Table **1**) assayed for their ability to induce tolerogenic DCs *in vitro* from duplicate cultures of $5x10^6$ C57BL/6 wt bone marrow cells cultured for 8d with GMCSF and IL-4. As in previous Figures CD200Fc or peptides were added into marrow cultures at 4μg/ml or 60μg/ml respectively.

Cells were harvested from 8-day marrow cultures 8hr after further LPS treatment (1μg/ml), washed, and $2.5x10^5$ mitomycin-c treated cells used in MLCs to stimulate $1x10^6$ C3H responder cells in triplicate *in vitro*. CTL for $^{51}$Cr-labeled EL4 targets were assayed 5days later. Control groups (of bone marrow cells) received no peptides or CD200Fc. % lysis from MLCs stimulated with control DCs at 30:1 effector:target was 29±3.2. Agonist activity was expressed as inhibition of this control response, while antagonist activity was expressed as a percent inhibition of the relative suppression (~28%) of the MLC response seen (in panel a) using DCs from bone marrow cells cultured in the presence of CD200Fc alone.

### Use of CD200/CD200R1 Peptides In Vivo to Block Suppression of Skin Allograft Rejection by CD200Fc

In a final study we asked specifically whether the agonist/antagonist peptides identified in the assays above showed similar activity *in vivo* in skin graft rejection assays, using as control the suppression of rejection previously reported for CD200Fc, a CD200R1-mediated function [24,33]. For these studies only wild-type mice were used in skin graft rejection assays. C57BL/6 wt mice received C3H skin grafts along with CD200Fc or mouse IgG2a alone (20μg/mouse iv at 60 hr intervals), peptide alone (500μg/mouse iv at 60hr intervals), or both CD200Fc and peptide. Data from pooled from two studies, each using 6 mice/group, are shown in Fig. **8**. Graft rejection was recorded daily by an observer blinded to the respective groups, beginning on day 9 post transplantation.

Consistent with data in a previous publication, peptide #4013, an antagonist of CD200Fc mediated suppression *in vitro* (Figs. **2** and **4**), also inhibited CD200Fc suppression of graft rejection (Fig. **8b**). In contrast, #4005, an effective agonist *in vitro*, functioned also as an agonist equivalent to CD200Fc itself, *in vivo* (Fig. **8a**).

## DISCUSSION

Previous studies from our laboratory have documented a role for increased expression of the molecule CD200 in immunosuppression [14,24,26]. A solubilized form of CD200, CD200Fc, also produced suppression, measured in a graft rejection model by a polarization in cytokine production away from type-1 cytokines and towards type-2 cytokines, and decreased induction of graft specific CTL. These effects are mediated following engagement of an "immunosuppressive-signaling" receptor (CD200R1) expressed on macrophages/activated T cells [13,14,37].

A number of membrane proteins implicated in either innate and or acquired immune responses are now known to be members of an extended Ig supergene family, commonly expressing a simple structure containing two major domains, like CD200 and CD200R1 [38]. Characteristically IgSF molecules interact through their N-terminal domains [39], and abundant data has been accumulated from both our own group and others to suggest this is true for CD200:CD200R1 interactions [13,30,32,33]. Our data supporting this hypothesis was based on functional studies using a series of synthetic peptides, encompassing the complete V- and C-region domains of human CD200, as well as a number of peptides overlapping the CC'C" and FG faces of both CD200 and CD200R1, to explore perturbation both of the molecular interactions of CD200R1 with CD200, as well as the functional effects of those interactions.

The data in the current manuscript have begun to address the issue of whether CD200-derived peptides active as agonists or antagonists of CD200:CD200R1 interactions might also function to modulate interactions of CD200 with alternate CD200Rs. Several groups, including our own, have described evidence for the existence of a number of other members of the CD200R family (including in mouse, CD200R2, R3 and R4, and in man CD200R2) [13,14]. There remains some controversy as to whether CD200 is a ligand for all CD200Rs, or whether other ligands for alternate receptors also exist [40], although it is clear that the signaling pathways activated following CD200 engagement of CD200R1

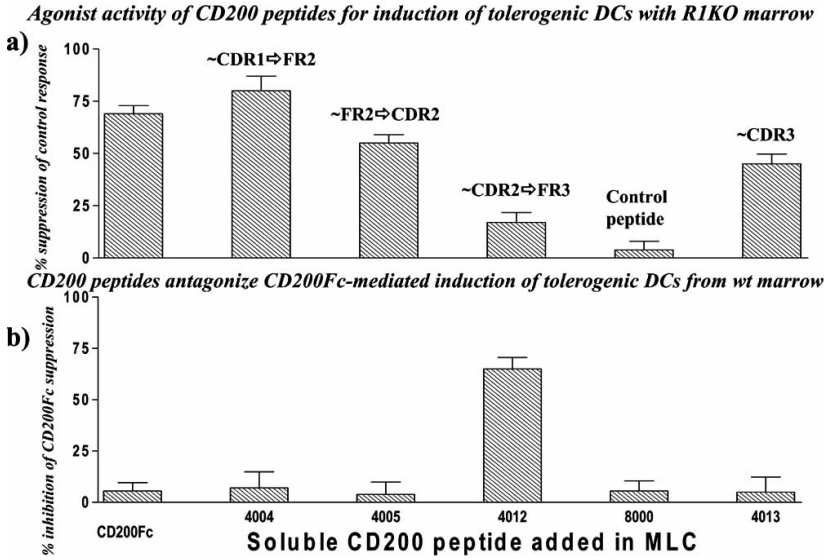


**Fig. (7).** As for Figure **6**, defining agonist (panel a) and antagonist (panel b) activity of CD200-derived peptides as measured by their ability to induce tolerogenic DCs *in vitro* from duplicate cultures of $5x10^6$ C57BL/6 CD200R1KO bone marrow cells cultured for 8d with GMCSF and IL-4. Once again control groups received no peptides or CD200Fc. In this case the response stimulated by DCs from control cultures (no peptide; no CD200Fc) showed 35±3.6% lysis at a 30:1 effector:target. Agonist activity was expressed as inhibition of this control response, while antagonist activity was expressed as a percent inhibition of the relative suppression (~70%) of the MLC response seen (in panel a) using DCs from bone marrow cells cultured in the presence of CD200Fc alone.

or alternate CD200Rs is not the same [16,18,41]. We have also suggested that the functional consequences of CD200 interaction with CD200R1 are clearly different from those observed following interaction with other isoforms [22]. Making use of a newly described CD200R1 KO mouse, in which the only CD200R genes remaining encode for alternate receptors [34], we have also explored the outcome of adding various CD200-derived peptides to development of alloreactivity in MLCs, and to development of tolerogenic DCs *in vitro*, described as being a characteristic function of engagement of alternate receptors [22]. These data consolidate the studies in defined assays with cells from wt mice (see generation of tolerogenic DCs, a function of alternate receptors), and show clearly that unique CD200 peptides can act as agonists (and antagonists) for CD200R1 and alternate receptors. Moreover, in limited *in vivo* studies, the functional activity (agonist/antagonist) of peptides defined *in vitro* was maintained *in vivo* (Fig. **8**).

The possibility that unique CD200-derived peptides can inhibit selectively the interaction of CD200 with distinct CD200R isoforms, and thus selectively interrupt modulation afforded by unique isoforms, is one of the most intriguing aspects of the described studies. Future work is expanding on these preliminary observations, and investigating the activity of the peptides documented in other biological systems in

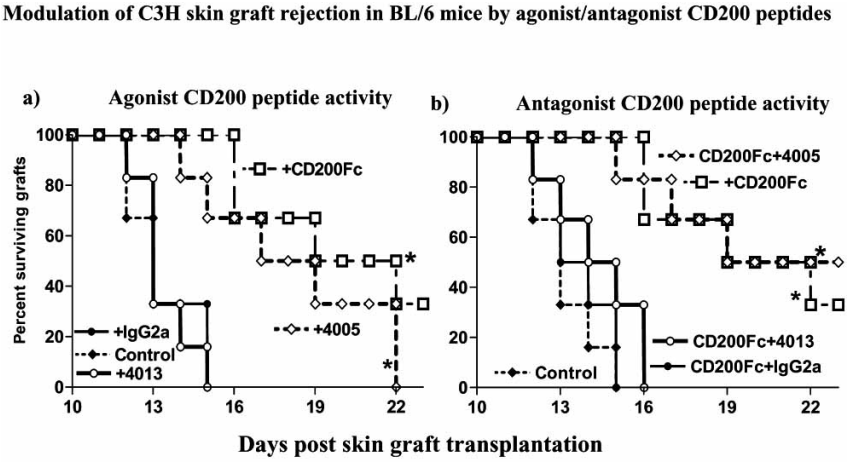


**Fig. (8). a**). CDR2 peptide for mouse CD200 (#4005) suppresses graft rejection *in vivo* in equivalent fashion to that mediated by CD200Fc *in vivo*. See text for more details. Data show graft survival/group summed over 2 independent studies (6 mice/group in each experiment). Control C57BL/6 groups received C3H skin grafts only, or grafts with mouse IgG2a.

**b**). CDR3 peptide for mouse CD200 (#4013) blocks suppression of graft rejection mediated by CD200Fc. All groups received C3H skin grafts along with CD200Fc and either mouse IgG2a or peptides as shown.

***** indicates different from control group (Mann-Whitney U-test, $p<0.05$).

which CD200 is know to play an important modulatory role (collagen-induced arthritis; fetal loss syndrome; suppression of tumor growth)[25-27].

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

DC = Dendritic cells

MLC = Mixed leukocyte culture

Mph = Macrophages

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