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Essential roles for Dok2 and RasGAP in CD200 receptor-mediated regulation of human myeloid cells[1]

Robin Mihrshahi, **A. Neil Barclay**, and **Marion H. Brown**[2]
Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom

Abstract

The CD200 receptor (CD200R) acts as a negative regulator of myeloid cells by interacting with its widely expressed ligand CD200. Using mutants expressed in U937 cells, we show that inhibition is mediated by the PTB domain binding motif (NPLY) in the receptor's cytoplasmic region. The adaptor protein downstream of tyrosine kinase 2 (Dok2) bound directly to the phosphorylated NPLY motif with a tenfold higher affinity (K_D ~ 1 μM at 37°C) than the closely related Dok1. Both of these proteins have been suggested to play a role in CD200R signaling in murine cells. Dok2 was phosphorylated in response to CD200R engagement and recruited RAS p21 protein activator 1 (RasGAP). Knockdown of Dok2 and RasGAP by RNA interference revealed that these proteins are required for CD200R signaling, while knockdown of Dok1 and the inositol 5-phosphatase SHIP did not affect CD200R mediated inhibition. We conclude that CD200R inhibits the activation of human myeloid cells through direct recruitment of Dok2 and subsequent activation of RasGAP, which distinguishes this receptor from the majority of inhibitory receptors that utilize immunoreceptor tyrosine-based inhibitory motifs (ITIM) and recruit phosphatases.

Keywords

Human; Monocyte/Macrophage; Signal Transduction

Introduction

The CD200 receptor (CD200R) is an immunoglobulin (Ig) superfamily transmembrane glycoprotein present on most leukocytes, but with relatively higher expression levels on cells of the myeloid lineage (1, 2). CD200R interacts with its structurally related but more widely expressed ligand CD200 through its N-terminal Ig V-type domain (3). The cytoplasmic tail of CD200R contains three conserved tyrosine residues, while CD200 has only a very short intracellular domain without any known signaling motifs (1, 2). The functional consequences of ligand engagement of CD200R were revealed *in vivo* in genetically manipulated mice lacking CD200. These mice exhibited a hyperactivated and hyperproliferative myeloid compartment and were more susceptible to induction of auto-immune disorders (4). The phenotype of mice lacking CD200R subsequently confirmed that the effects of CD200 deficiency were, indeed, due to loss of ligand induced inhibitory signalling through the receptor (5). *In vitro* studies showed that engagement of CD200R caused inhibition of cellular activation in human and mouse mast cells (5), macrophages (6, 7), mixed lymphocyte reactions (8, 9) and basophils (10).

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[2]Address correspondence and reprint requests to Dr Marion H. Brown, phone: +44 1865 275595, fax: +44 1865 275591, marion.brown@path.ox.ac.uk.

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The significance of the CD200/CD200R pathway for control of leukocyte activation is illustrated through its subversion by viruses which inhibit anti-viral host responses by expressing CD200-like proteins that mimic host-derived CD200 (7, 10-13). CD200 is also a marker for various human cancer or cancer stem cells, where it enhances evasion of immune recognition by inhibiting the activation of CD200R bearing leukocytes (9, 14-17).

CD200R is unusual amongst inhibitory receptors, as it does not contain any immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITIMS are present in a large number of inhibitory receptors and mediate inhibition through the recruitment of protein tyrosine phosphatases such as Src homology 2 domain-containing phosphatase (SHP) 1, SHP2, or the inositol phosphatase SHIP upon phosphorylation (18). The cytoplasmic region of CD200R contains three tyrosine residues of which the membrane distal one is located within a phosphotyrosine-binding (PTB) domain recognition motif (NPxY) (19). Interestingly, the chicken CD200R does contain an ITIM (N**V**I**Y**NS**V**) instead of the PTB domain motif (**NP**L**Y**DTV) found in human, mouse, rat and cow (1, 2, 20) suggesting that the mammalian receptor may possibly have evolved from an ITIM bearing precursor, which has been retained in chicken. The NPxY motif of murine CD200R has been suggested to bind the PTB domain-containing adaptor proteins downstream of tyrosine kinase 1 (Dok1) and Dok2 upon tyrosine phosphorylation, resulting in the recruitment of SHIP and RAS p21 protein activator 1 (RasGAP) (21, 22).

In this study, we investigated the molecular mechanisms of CD200R signaling in human myeloid cells. We show that Dok2 can directly interact with the NPxY motif of human CD200R and that Dok2 and RasGAP, but not Dok1 and SHIP are required for CD200R mediated cellular inhibition.

Materials and Methods

Antibodies

Polyclonal goat (sc-8130) and rabbit anti-human Dok2 (sc-13952), monoclonal mouse anti-human RasGAP (sc-63) and monoclonal mouse anti-human SHIP (sc-8425) antibodies were from Santa Cruz Biotechnology. A polyclonal rabbit anti-human Dok1 antibody (23) was a kind gift from Dominique Davidson and André Veillette. The monoclonal mouse anti-human CD200R antibody OX108 has been described previously (2). Biotinylated mouse monoclonal anti-phosphotyrosine antibody (B1531) and peroxidase conjugated polyclonal anti-mouse, anti-rabbit and anti-goat antibodies and ExtrAvidin® were from Sigma-Aldrich Ltd. Phycoerythrin-conjugated donkey anti-mouse IgG F(ab')$_2$ fragment (715–116–151) was from Jackson ImmunoReasearch Laboratories Inc.

CD200-COMP

Pentameric human CD200 (CD200-COMP) consisting of the extracellular region of human CD200 (2) linked to domains 3 and 4 of rat CD4 followed by an 11-amino-acid linker sequence (NSGGGSGGGTG) and the rat COMP (cartilage oligomeric matrix protein) oligomerization domain was generated as previously described (24). 293T cells were transiently transfected with pEF-BOS vector containing the CD200-COMP construct, and tissue culture supernatant was collected, concentrated and dialyzed into PBS. Protein activity was tested by surface plasmon resonance (SPR) on a BIAcore™ 3000, which showed strong binding to recombinant human CD200R with binding characteristics similar to those of OX108 mAb (24). Titrations of the concentrate were used in IL-8 assays with CD200R transduced U937 cells as described below to determine optimal working dilutions.

Generation of CD200R mutant cell lines

Mutants of human CD200R were generated by overlap extension PCR mutagenesis. Fragments were amplified from the wild-type gene using terminal and internal primers, with the N-terminal primer introducing a Bgl II restriction site and the internal primers overlapping and containing a single base change (A to T) to change a tyrosine to a phenylalanine codon of each of Y291, Y294 and Y302, referred to hereafter as Y1, Y2 and Y3. To generate a truncated mutant of the receptor, a stop codon followed by a Sal I restriction site was inserted, resulting in the removal of the last 40 residues (a.a. 286–325) of the cytoplasmic tail of human CD200R. The resulting PCR products were digested with Bgl II and Sal I and cloned into a BamH I and Xho I cut bicistronic, emerald (Em) enhanced green fluorescent protein (eGFP) expressing pHR-SIN-BX-IRES-Em lentiviral expression vector. The constructs were transfected into 293T cells at 60% confluence in 175 cm^2 culture flasks. 15 μg expression vector, 10 μg pCMV-ΔR8.91 packaging vector and 10 μg pMD2G envelope plasmid that had been pre-incubated with polyethylenimine (PEI) at a ratio of 1:7 (w/w) for 15 min at room temperature was added to cell cultures in 20ml X-Vivo media. After overnight incubation, the media was replaced with 20 ml RPMI 1640 containing 5% FCS, penicillin and streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Supernatant containing viral particles was then collected and replaced with fresh media after 24, 48 and 72 h. Cells of the human monocyte-like histiocytic lymphoma line U937 were transduced by incubating for 24–48 h in neat supernatant and then purified based on CD200R expression by magnetic bead isolation using OX108 mAb on an autoMACS™ separator (Miltenyi Biotech). For some experiments, cells were sorted into high and low CD200R expressing populations by fluorescent-activated cell sorting (FACS) based on eGFP expression. Transduced cells were maintained at densities below 5×10^5 per ml prior to experimental use.

Preparation of IL-4 activated human macrophages

Monocytes (>90% pure) were isolated from healthy donor buffy coats (Bristol Blood Donor Services) by two-step centrifugation over Ficoll and 46% Percoll gradients (25) and differentiated into macrophages for 7 days in X-Vivo 10 supplemented with 1% autologous serum. Macrophages were alternatively activated by culturing for an additional 2 days in the presence of 20 ng/ml recombinant human IL-4 (Peprotech).

Cytokine assays

For IL-8 assays using CD200R transduced U937 cells, OX108 mAb diluted at the indicated concentrations in phosphate buffered saline (PBS) was immobilized overnight at 4°C to wells of a 96-well tissue culture plate. The plate was then blocked with PBS, 5% FCS at room temperature for at least 2 h, followed by addition of 5×10^4 U937 cells per well. For experiments using soluble OX108 or pentameric human CD200 (CD200-COMP), these reagents were added at the indicated concentrations to wells containing 5×10^4 U937 cells. In experiments using Maja cells, a CD200 positive human B cell line, to engage CD200R on the surface of U937 cells, 4×10^5 Maja cells that had been irradiated at 30 Gy were added to 10^5 U937 cells in the wells of a 96-well tissue culture plate. After 30 min incubation on ice, lipopolysaccharide (LPS), ethanol-killed *Neisseria meningitides* cells (26) or IFN-γ were added at the indicated concentrations to stimulate IL-8 production. The total volume of media was 200 μl per well. After overnight incubation at 37°C, supernatants were collected and assayed for the presence of IL-8 by ELISA (BD Pharmingen).

RNA interference

SiRNA duplexes containing 2 thymidine 3′ overhangs were purchased from Sigma-Aldrich Ltd. (siDok1, siDok2, siSHIP and siScr) and NBS Biologicals (siRasGAP and siNeg). The

sequences were: 5′-GAAUGCUGCACCCGCUACA -3′ for siDok2, 5′-GGUCAUGUUCUCUUUCGAG -3′ for siDok1, 5′-GCUAAGUGCUUUACGAACA -3′ for siSHIP, 5′-ACGCAUGUACACACUCGCG -3′ for a randomly Dok-2 scrambled sequence (siScr) (27) and 5′-AUAAUGGAAAGCAAGCAGUCUUGUGAG-3′ for siRasGAP (28). 10^6 U937 cells sorted for high expression of wild-type or truncated human CD200R were transfected with 1.5–4 μg siRNA using kit C of the Amaxa nucleoporation system according to the manufacturer's instructions. In order to maximize transfection efficiency, cells were re-transfected after 36 h. Cells were used in IL-8 cytokine assays with 2.5 μg/ml soluble OX108 and 50 ng/ml LPS as described above, 24–48 h after the second transfection. The remaining cells where washed in PBS and lysed at 2.5×10^7 cells/ml in NP-40 lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.02% (w/v) NaN3, 1% (v/v) NP-40, 1 mM sodium pervanadate, 1mM NaF and 10% protease inhibitor cocktail for mammalian cells (Sigma)) for 20 min at 4°C. Lysates were cleared by centrifugation at 16000 g for 10 min at 4°C and equal amounts of protein (as determined by Bradford assay) were resolved under non-reducing conditions on NuPAGE® Bis-Tris gradient (4 to 12%) gels (Invitrogen). Proteins were transferred to nitrocellulose membranes in a Novex XCell II ™ Blot Module and western blotted using the SNAP i.d. ™ Protein Detection System (Millipore) to determine the effect of RNA interference on protein expression.

Immunoprecipitations

U937 cells expressing wild-type or truncated CD200R were pre-incubated on ice for 30 min at ~1.5×10^7 cells/ml in the presence of CD200-COMP concentrate diluted 1:100 in RPMI. Cells were then warmed to 37°C for 5 min, washed in ice-cold PBS containing 1mM sodium pervanadate and lysed for 10 min at 4°C. Lysates were cleared by centrifugation at 16,000 g for 10 min at 4°C and incubated for 30 min at 4°C in the presence of 1 μg of antibody per 10^7 cells. Protein G Sepharose beads (Amersham) were washed 3 times in PBS and re-suspended as a 50% slurry in lysis buffer of which ~10 μl was added per 10^7 cells. After incubating at 4°C for 30 min, beads were washed 3 times in PBS 1mM pervanadate. Proteins were eluted by boiling the beads at 95°C for 10 min in non-reducing SDS sample buffer, resolved on gradient gels and transferred and blotted with the indicated antibodies as described. For immunoprecipitations from primary human cells, 2×10^7 IL-4 activated human macrophages were cultured in T175 tissue culture flasks at 37 °C for 30 min in RPMI without supplements followed by addition of OX108 or OX21 mAb at a final concentration of 10 μg/ml. After 7 min at 37 °C, flasks were placed on ice and cultures washed once with ice-cold PBS, 1mM pervanadate. Cells were lysed at 10^7 cells/ml and immunoprecipitations were performed as described above.

BIAcore analysis

Recombinant human Dok1 PTB domain (amino acids 140–268), Dok2 PTB domain (amino acids 133–259) were provided by Louise Bird (Oxford Module Consortium [www.omc.ox.ac.uk]). Surface plasmon resonance analyses using a BIAcore™ 3000 were carried out essentially as described previously (29). In brief, ~4000 response units (RU) of streptavidin were immobilized at 25°C to CM5 chips by amine coupling followed by immobilization of 50–150 RU of biotinylated peptides (Peptide Protein Research Ltd. and Sigma). Flow cells with non-phosphorylated peptides or with streptavidin only were used as controls. Increasing concentrations of monomeric, recombinant, soluble protein were then passed over the chip at 37°C to determine equilibrium binding. Response units from empty control flow cells were subtracted from those of experimental flow cells and the resulting data points plotted and fitted to hyperbolas. Sequences of peptides from human proteins used in BIAcore experiments are shown in Table I.

Results

Generation of cell lines expressing mutant and wild-type human CD200R

To generate a cellular system in which to dissect the signaling pathway of human CD200R, we used lentiviral transduction to create stable lines of U937 cells expressing wild-type or mutant (cytoplasmic domain truncated or with each of its three tyrosines mutated to phenylalanines) human CD200R. No endogenous CD200R was detected on untransduced U937 cells (data not shown). After transduction, cells expressing high levels of CD200R were purified by magnetic (MACS) bead isolation, which resulted in stable cell lines expressing similar levels of wild-type or mutant receptor (Fig. 1).

CD200R engagement causes inhibition of IL-8 secretion from activated U937 cells

One previous study showed that ligation of CD200R on transfected U937 cells inhibits the secretion of various pro-inflammatory mediators including the neutrophil-chemoattractant IL-8 (CXCL8) (6). We confirmed this finding using U937 cells expressing wild-type and truncated human CD200R (Fig. 2). Generation of cells expressing a signaling deficient form of CD200R allows for the use of the same extracellular conditions in both samples and controls. This eliminates potential artefacts caused by different degrees of cell-cell contact or differences in Fc-receptor stimulation by antibodies. The inhibitory effect we observed did not depend on the method of receptor ligation; plate-bound or soluble mAb with or without secondary crosslinking antibody, soluble multimeric CD200 or CD200 expressing cells (Fig. 2A) or the type of activation stimulus; LPS, IFN-γ or ethanol-killed *N. meningitides* (Fig. 2B). U937 cells express no or minimal levels of CD200 and cells were maintained at densities which minimized cell contact. The inhibition caused by soluble reagents (CD200-COMP and OX108), which can block the CD200-CD200R interaction (data not shown and M. Foster-Cuevas and A. Neil Barclay, unpublished) is, therefore, not due to blocking the engagement of CD200R by endogenously expressed CD200, but rather represents an agonistic effect. Crosslinking with OX108 mAb thus mimics the effect of natural ligand engagement in our assays.

The effect of CD200R ligation on other cytokines was also investigated. LPS stimulation of CD200R transduced U937 cells did not elicit TNF and IL-1β secretion. IL-10 secretion was detectable and, similar to IL-8, was inhibited by soluble or plate-bound OX108 or CD200-COMP (data not shown). However, the effect on IL-10 was more variable and less pronounced than that on IL-8 secretion.

CD200R signaling is dependent on its third intracellular tyrosine residue

To determine the relative contribution of each of the three cytoplasmic tyrosine residues of human CD200R to signaling, we incubated U937 cells expressing wild-type or mutant (truncated, Y1F, Y2F and Y3F) CD200R in the presence of plate-bound OX108 mAb and 20 ng/ml LPS. IL-8 secretion was strongly inhibited in the presence of wild-type CD200R, while the Y3F mutation completely abrogated this effect. The Y1F mutation relieved inhibition only slightly, while the Y2F mutation had no functional effect (Fig. 3). Together, these results show that CD200R mediates active inhibitory signaling which is dependent on its third intracellular tyrosine residue. The first tyrosine plays a minor role and the second one is dispensable in this assay.

The third intracellular tyrosine residue of CD200R binds Dok2 with higher affinity than Dok1

Previous studies suggest that the cytoplasmic domain of murine CD200R, when phosphorylated, can recruit the adaptor molecules Dok1 and Dok2 (21, 22). We used SPR to determine whether these interactions occur in human cells and whether they are direct or

indirect. We measured direct binding of both Dok proteins to phosphorylated peptides corresponding to parts of the human CD200R cytoplasmic domain (Fig. 4). Dok2 and Dok1 both bound to two phosphopeptides (pY3 and pY1,3), but Dok2 bound with a tenfold higher affinity (K_D ~ 1 μM at 37°C; Fig. 4B) than Dok1 (Fig. 4C). The difference in binding affinity between Dok1 and Dok2 was not due to differences in protein activity, as Dok1 bound more strongly than Dok2 to several other peptides (Table II and data not shown). The interaction between CD200R and Dok2 was dependent on phosphorylation of the functionally most important third tyrosine residue. Neither Dok1 nor Dok2 bound strongly to the first or second tyrosine when these were phosphorylated alone or in combination (Table II), although co-phosphorylation of the first tyrosine caused a slight increase in affinity compared to phosphorylation of the third tyrosine alone (Fig. 2).

Hierarchy of interactions suggests indirect recruitment of Dok1 and SHIP

Dok1 and Dok2 have been reported to form phosphorylation dependent homo- and heterodimers resulting from an interaction between their PTB domains and Tyr^{146} of Dok1 or Tyr^{139} of Dok2 (30, 31). To investigate the possibility of indirect recruitment of Dok1 to the CD200R signaling complex, we measured interactions between both Dok proteins and peptides corresponding to sequences surrounding these tyrosines. Dok2 did not bind to either of the two peptides. The PTB domain of Dok1, on the other hand, was found to interact with both peptides, but with a slight preference for the Dok2 derived sequence (Table II). This suggests that Dok1 may be recruited to CD200R indirectly via Dok2.

Dok1 has been shown to interact directly with the inositol phosphatase SHIP, and this interaction was dependent on both, the PTB domain of Dok1 and the SH2 domain of SHIP (32). The C-terminal part of SHIP contains two NPxY motifs that are likely to be involved in the formation of this complex. We measured interactions between Dok proteins and phosphopeptides corresponding to sequences surrounding these NPxY motifs. Dok2 did not bind to the sequence surrounding Tyr^{1022} and only very weakly (K_D > 30μM) to the sequence surrounding Tyr^{917} of SHIP. The PTB domain of Dok1 bound both of the SHIP NPxY motifs, although this interaction was not particularly strong (Table II). Sequence analysis of Dok1 and Dok2 in the context of a published SHIP SH2 domain consensus sequence (33) suggests that SHIP is more likely to bind to Dok1 (especially Tyr^{377} and Tyr^{398}) than Dok2.

Engagement of CD200R on U937 cells causes phosphorylation of Dok2 and recruitment of RasGAP

As recruitment of downstream effector molecules via Dok2 depends on phosphorylation of its C-terminal tail (34), we tested whether CD200R engagement results in phosphorylation of Dok2 in human myeloid cells. Wild-type or truncated CD200R was engaged on the surface of U937 cells using soluble CD200-COMP, followed by lysis and immunoprecipitation of Dok2. Analysis of precipitates by western blot showed that engagement of wild-type but not truncated CD200R caused phosphorylation of Dok2 (Fig. 5A).

Concomitant with phosphorylation of Dok2, engagement of CD200R resulted in recruitment of RasGAP (Fig. 5A). Phosphorylation of Dok2 and recruitment of RasGAP were dependent on signalling by the intact cytoplasmic region of CD200R. The levels of SHIP co-precipitated with Dok2 from lysates of cells expressing wild type or truncated CD200R were the same (Fig. 5A), suggesting that SHIP co-precipitation was non-specific. Lack of a specific effect of CD200R on SHIP activity was confirmed by immunoprecipitation of SHIP (Fig. 5B), which showed no difference in SHIP phosphorylation or its interaction with other phosphoproteins in response to CD200R engagement (Fig. 5B).

To determine whether Dok2 and RasGAP are also involved in CD200R signaling in primary human cells, Dok2 was immunoprecipitated from IL-4 activated macrophages that had been treated with CD200R mAb (OX108) or isotype control (OX21). Treatment of macrophages with OX108 resulted in phosphorylation of Dok2 and recruitment and phosphorylation of RasGAP. RasGAP also co-precipitated with Dok2 in lysates of OX21 treated cells, but phosphorylation of Dok2 and RasGAP was substantially stronger with OX108 treatment (Fig. 5 C). No phosphoproteins corresponding to the molecular weight of Dok1 or SHIP were detectable (data not shown).

Dok2 and RasGAP are essential for CD200R signaling

Our functional analysis of mutant receptors and SPR binding data indicate that Dok2 binding to phosphorylated Y3 and recruitment of RasGAP is essential for signalling by CD200R, while Dok1 and SHIP are less important. To test this, we used RNA interference to knock down expression of Dok1, Dok2, SHIP and RasGAP in CD200R transduced U937 cells and assess the effects on CD200R signalling. We compared the effects of the RNAi on CD200R mAb (OX108) induced CD200R signalling in cells expressing wild type and truncated receptor (Fig. 6). Knockdown of Dok2 completely abrogated CD200R mediated inhibition of IL-8 secretion from LPS stimulated U937 cells (Fig. 6A). In contrast, knockdown of Dok1 did not affect CD200R signalling in this assay (Fig. 6B).

Knockdown of RasGAP also abrogated CD200R signalling consistent with this being an effector enzyme recruited by CD200R via Dok2. Knockdown of SHIP, on the other hand, had no specific effect on CD200R mediated inhibition (Fig. 6C), although it increased overall IL-8 production by about 30–50% (data not shown). Thus our results suggest essential roles for Dok2 and RasGAP, but not Dok1 and SHIP in human CD200R signalling.

Discussion

The CD200R mediates inhibitory signals but is distinguished from the large number of other leukocyte inhibitory receptors that function by recruiting phosphatases through ITIM motifs (18). We show that the membrane distal tyrosine in CD200R is essential for CD200R mediated inhibition of IL-8 secretion in U937 cells and that a phosphotyrosine peptide from this region binds the adaptor Dok2 through its PTB domain. The affinity of Dok2 for this peptide (K_D ~ 1 μM at 37°C) is within the range of other functional interactions between phosphotyrosine peptides derived from cell surface receptors and cytoplasmic adaptor or signaling proteins that have been measured at physiological temperature (29, 35). It is about tenfold weaker than that reported for the PTB domain of Src homology 2 domain containing transforming protein (Shc), but these measurements were made at lower temperatures (36), and Shc does not bind strongly to CD200R ((19) and our unpublished observations). The direct interaction between CD200R and Dok2 is thus likely to be crucial in initiating signaling, and its involvement is confirmed by our RNA interference experiments (Fig. 6).

Studies on murine CD200R suggested that Dok1 and Dok2 are involved in CD200R signaling as both were precipitated from mouse mast cells using phosphopeptides corresponding to sequences in the cytoplasmic domain of murine CD200R and were phosphorylated and co-precipitated CD200R in response to receptor engagement (21). We also observed phosphorylation of Dok2 (Fig. 5A) and Dok1 (unpublished) in response to CD200R engagement in human cells. However we found that Dok1 bound much more weakly to CD200R peptides than Dok2, and RNA interference of Dok1 expression had no effect on CD200R signaling in our assays (Fig. 6). Dok1 and Dok2 form phosphorylation dependent homo- and heterodimers (30, 31), and our BIAcore experiments show that Dok1 has a twofold higher affinity for phosphorylated Dok2 than for CD200R. Dok1 is thus more likely to be recruited to CD200R via Dok2 than by direct association with CD200R.

The first two intracellular tyrosine residues of CD200R are also conserved between human and mouse. An inhibitory role for the phosphorylated membrane proximal tyrosine demonstrated in mouse CD200R signalling (22) was substantiated in our human model. The contribution of Y1 to CD200R signalling was greater in mouse cells (22) than we observed in our human system. This may either be due to species differences or variations in assay sensitivity. Neither Dok1 nor Dok2 bound strongly to CD200R peptides phosphorylated at the first and/or second tyrosine residues although co-phosphorylation of Y1 resulted in a slightly higher affinity of both Dok proteins for Y3. Phosphorylation of the first tyrosine may thus stabilize interactions with the third. In neither mouse (22) nor human *in vitro* models, was an effect of the second tyrosine detected.

Inhibitory signalling of CD200R was functionally dependent on Dok2, as knockdown of Dok2 completely abrogated CD200R mediated inhibition of IL-8 secretion. Knockdown of Dok1 had virtually no effect. Thus the functional data are consistent with the hierarchy of interactions established through quantitative biochemistry. Dok1 and 2 play a role in various inhibitory signaling pathways, downstream of tyrosine kinases and growth factor receptors (summarized in (37)) and are involved in the negative regulation of B-cell receptor (38) and T cell-receptor signaling (27, 39). The primary signalling mechanisms of these receptors are mediated by activating tyrosine kinases, while the Dok proteins only play secondary, inhibitory roles in down-regulating or terminating these pathways. Thus CD200R appears to be unusual in that it uses a Dok protein as the initiators of its primary signaling pathway.

Upon phosphorylation of their C-terminal tyrosine residues, Dok1 and Dok2 can recruit SH2 domain containing proteins, most notably RasGAP (40, 41). RasGAP recruitment is essential for the ability of Dok1/2 to inhibit Ras-ERK signaling (30, 34). This interaction involves five tyrosines in Dok1 (42) and at least two tyrosines in Dok2 (34) indicating that RasGAP recruitment is the primary function of these adaptor proteins. RasGAP is one of several Ras GTPase activating proteins and an important negative regulator of the Ras-ERK and PI3K signaling pathways (summarized in (43)). The functional significance of these pathways can be inferred from the observation that mutations or mis-expression of Ras are found in approximately 30% of all human cancers (44). Our immunoprecipitation and RNA interference experiments suggest that recruitment of RasGAP is the primary mechanism by which Dok2 affects cellular activation in response to CD200R engagement in human myeloid cells.

The inositol phosphatase SHIP has been shown to be phosphorylated and bind Dok1 in response to CD200R signaling in mouse mast cells (21). We did not observe any effect of CD200R engagement on SHIP phosphorylation or its interaction with other phosphoproteins in our human system. Moreover, knockdown of SHIP had no effect on CD200R signaling in our RNA interference experiments. In agreement with studies in mouse cells (21), BIAcore experiments (Table II) and the SHIP SH2 domain consensus sequence (33) suggest that SHIP is more likely to interact with Dok1 than Dok2. Neither Dok1 nor SHIP, however, was found to play an important role in CD200R mediated inhibition of IL-8 secretion in our assays. The interaction between Dok1 and SHIP has been shown to be dependent on both, the PTB domain of Dok1 and the SH2 domain of SHIP (32). Quantitative binding analyses show that the affinity of the PTB domain of Dok1 for two phosphorylated NPxY motifs in SHIP is rather weak (K_D ~ 13 μM at 37°C; Table II), but binding of the SHIP SH2 domain to phosphorylated tyrosine residues in Dok1 may stabilize this complex. SHIP is thus unlikely to bind to Dok1 if the latter's PTB domain is already occupied, but may form complexes with phosphorylated free Dok1.

Our results show that CD200R signals through a pathway involving recruitment and activation of RasGAP by the adaptor molecule Dok2. This pathway is novel in so far as it

causes cellular inhibition independent of phosphatases and because it uses Dok2 as a primary signal transducer rather than a modulator of other pathways.

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Abbreviations used in this paper

CD200R	CD200 receptor
Dok	downstream of tyrosine kinase
PTB	phosphotyrosine binding
RasGAP	RAS p21 protein activator 1
RU	response units
SH2	SRC homology 2
Shc	Src homology 2 domain-containing transforming protein
SHP	SRC homology 2 domain-containing phosphatase
siRNA	small interfering RNA
SPR	surface plasmon resonance

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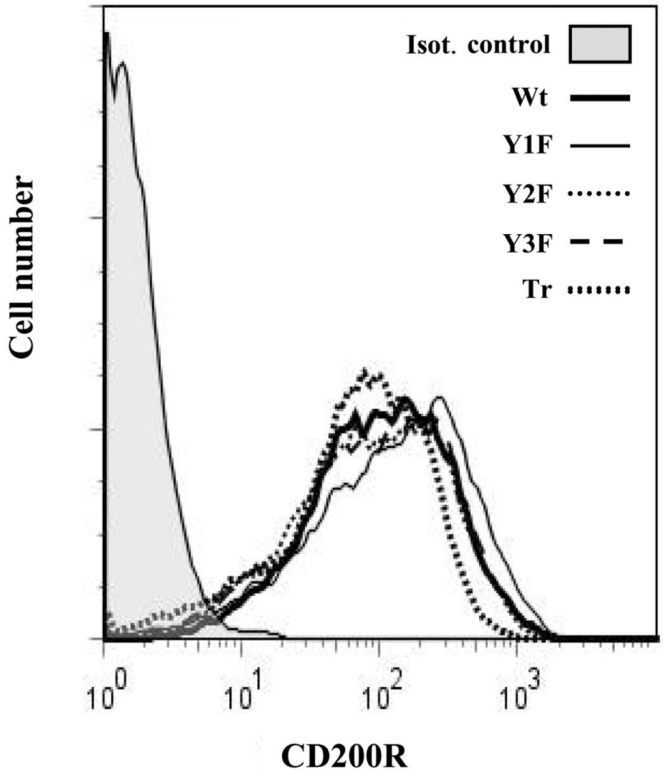

Fig. 1.

CD200R mutants are expressed on transduced U937 cells. U937 cells were lentivirally transduced to express wild-type (Wt) or signaling deficient (cytoplasmic domain truncated [Tr] or with each of its three tyrosines mutated to phenylalanines [Y1F, Y2F, Y3F]) human CD200R. Expression of wild-type and mutant CD200R is shown after magnetic (MACS) beads separation using OX108 mAb. Cells were stained with OX108 followed by phycoerythrin-conjugated anti-mouse IgG $F(ab')_2$.

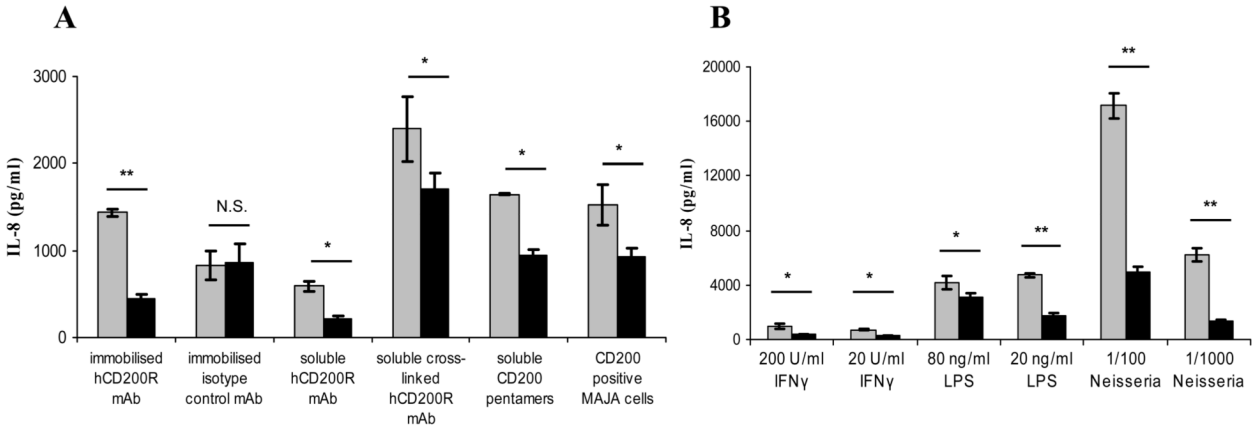

Fig. 2.

CD200R engagement inhibits activation of U937 cells. (A) Wild-type (black) and truncated (grey) CD200R were engaged on U937 cells using different reagents as indicated. Plate-bound OX108 or isotype control were immobilized at 40 μg/ml, soluble OX108 was used at 10 μg/ml and crosslinking anti-mouse IgG at 20 μg/ml. Cells were then stimulated overnight with 20 ng/ml LPS and culture supernatants assayed for IL-8 by ELISA. (B) Wild-type or truncated CD200R was engaged on U937 using 100 μg/ml plate-bound OX108 mAb and cells were stimulated overnight using different reagents as indicated. Neisseria = ethanol-killed *Neisseria meningitides* diluted in culture medium. * = $p < 0.05$ according to two-tailed student's t-test, ** = $p < 0.005$, N.S. = non-significant. Results are expressed as means of duplicate or triplicate wells ± standard deviation and are representative of two or more independent experiments.

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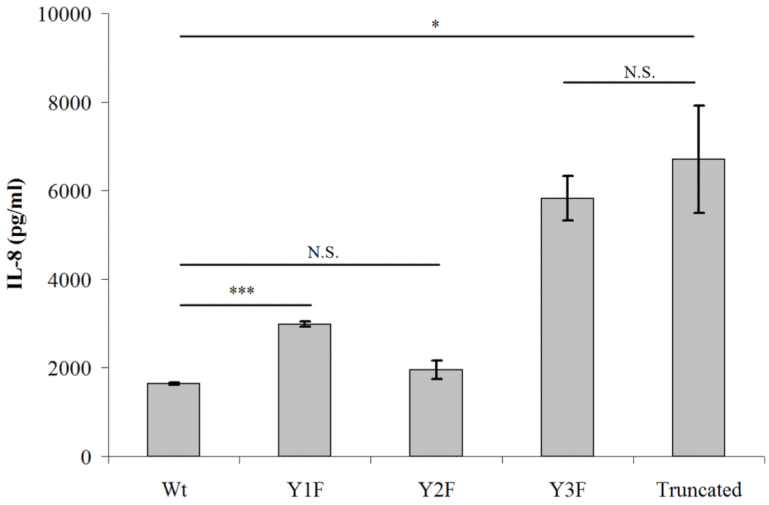

Fig. 3.

The innhibitory effect of CD200R is mainly mediated by its third intracellular tyrosine. CD200R (wild-type and mutants) was engaged on U937 cells using 100 μg/ml plate-bound OX108 mAb and stimulated and assayed as in Fig. 2. * = $p < 0.05$, *** = $p < 0.0005$, N.S. = non-significant. Results are expressed as means of triplicate wells ± standard deviation and are representative of six experiments. No significant effects of tyrosine mutations were observed in the absence of OX108 mAb or in the presence of an isotype control mAb (data not shown).

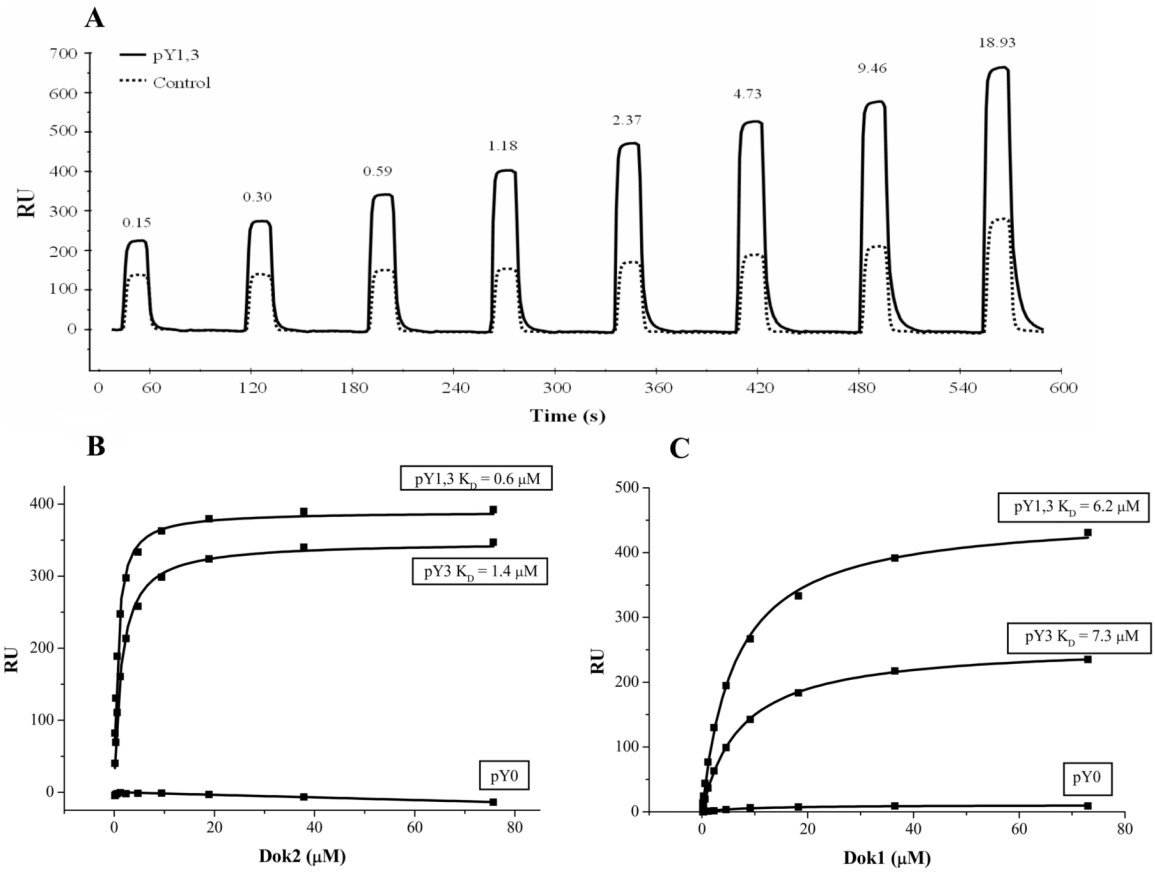

Fig. 4.

Dok2 binds the cytoplasmic tail of CD200R with higher affinity than Dok1. Singly (pY3) or doubly (pY1,3) phosphorylated peptides corresponding to parts of the cytoplasmic tail of human CD200R were immobilized on a BIAcore chip. (A) Binding of various concentrations (μM) of recombinant PTB domain of human Dok2 passed over flow cells containing immobilized unphosphorylated (pY0) control or pY1,3 phosphopeptide. (B) and (C), Equilibrium binding values at each concentration for Dok2 (B) and Dok1 (C) binding to pY3 and pY1,3 phosphopeptides. The hyperbolas represent best fits used for affinity calculations. Results are representative of two independent experiments.

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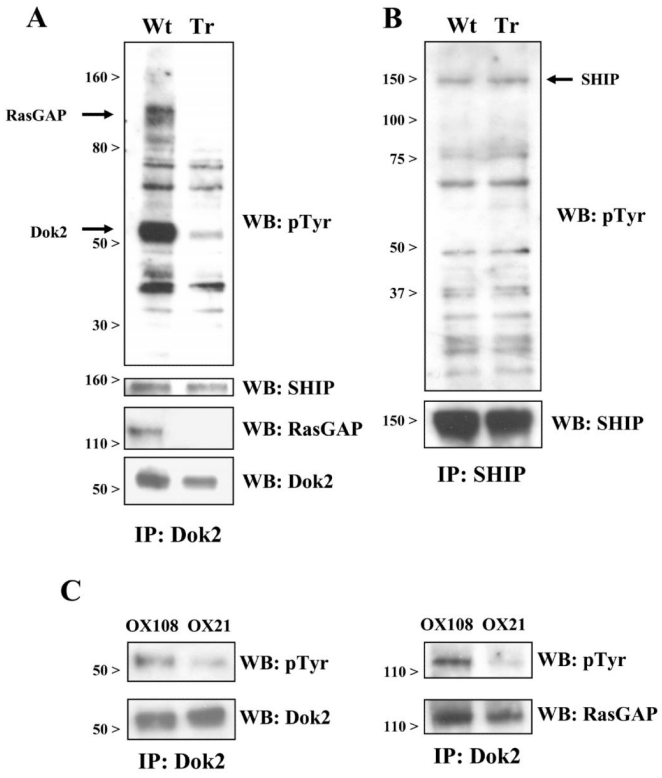

Fig. 5.

Phosphorylation of Dok2 and recruitment of RasGAP following engagement of CD200R. U937 cells expressing wild-type or truncated CD200R were incubated for 5 min at 37°C in the presence of CD200-COMP. Cells were then lysed and Dok2 (A) or SHIP (B) was immunoprecipitated from lysates. Immunoprecipitates were blotted with biotinylated anti-phosphotyrosine mAb. Membranes were then stripped and re-blotted with specific antibodies against the indicated proteins. Results are representative of at least three independent experiments. (C) IL-4 activated human macrophages were treated with CD200R mAb (OX108) or isotype control (OX21) for 7 min, followed by Dok2 immunoprecipitation and Western blotting with the indicated antibodies. Results are representative of two independent experiments conducted with macrophages from three different donors.

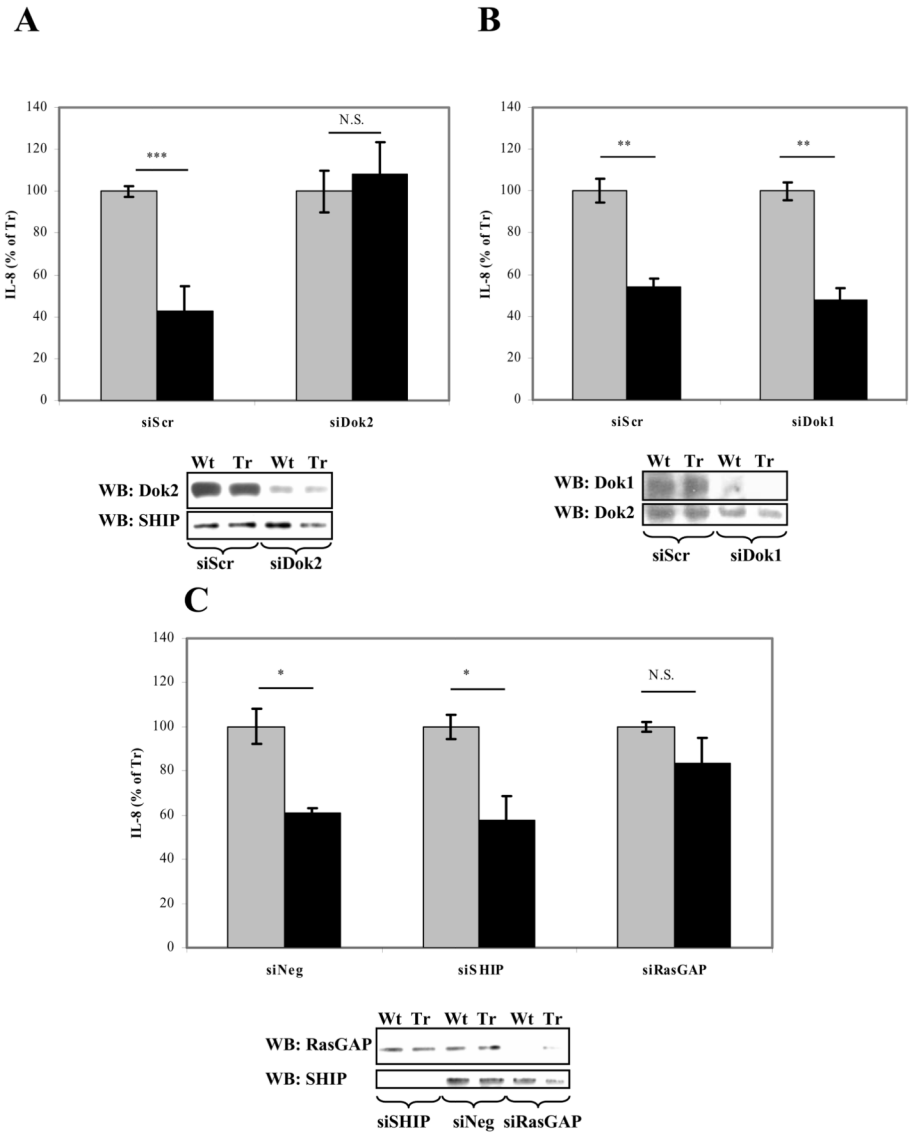

Fig. 6.

Knockdown of Dok2 and RasGAP abrogates CD200R signaling in U937 cells. U937 cells expressing wild-type (black) or truncated (grey) CD200R were transfected twice with siRNA against Dok2 (A), Dok1 (B), SHIP or RasGAP (C). 24–48 h after the second transfection, cells were incubated overnight in the presence of 2.5 μg/ml soluble OX108 mAb and 50 ng/ml LPS and the supernatants assayed for IL-8. The remaining cells were lysed and lysates were western blotted to assess the degree and specificity of protein knockdown. A scrambled (siScr) Dok2 siRNA sequence or a non-targeting control siRNA sequence (siNeg) was used as negative control. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, N.S. = non-significant. Results are expressed as means of triplicate wells normalized to the amount of IL-8 produced by cells expressing the truncated CD200R ± standard deviation. Results are representative of at least three independent experiments.

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Table I

Peptides used in BIAcore experiments[1]

Peptide	Sequence
CD200R pY0	Biotin-DEMQPYASYTEKNNPLYDTTN
CD200R pY1	Biotin-DEMQP(pY)ASYTEKNNPLYDTTN
CD200R pY3	Biotin-DEMQPYASYTEKNNPL(pY)DTTN
CD200R pY1,2	Biotin-DEMQP(pY)AS(pY)TEKNNPLYDTTN
CD200R pY1, 3	Biotin-DEMQP(pY)ASYTEKNNPL(pY)DTTN
Dok1 pY146	Biotin-EMLENSL(pY)SPTW
Dok2 pY139	Biotin-CMEENEL(pY)SSAV
SHIP pY917	Biotin-TEIINPN(pY)MGVGP
SHIP pY1022	Biotin-EMFENPL(pY)GSLSS

[1] pY = phosphotyrosine.

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Table II

Dissociation constants of interactions between Dok PTB domains and biotinylated peptides[2]

	Dok1 PTB	Dok2 PTB
CD200R pY0	N.B.	N.B.
CD200R pY1	14 μM	N.B.
CD200R pY1,2	8.4 μM	N.B.
CD200R pY3	7.3 μM	1.4 μM
CD200R pY1,3	6.2 μM	0.6 μM
Dok1 pY146	6.9 μM	N.B.
Dok2 pY139	3.3 μM	N.B.
SHIP pY917	13 μM	> 30 μM*
SHIP pY1022	13 μM	N.B.

[2] N.B. = no binding (K_D > 50 μM or no specific interaction detectable).

* Interaction was dependent on high peptide density and was not observed under standard conditions. Results are representative of at least two experiments.