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*J Immunol* 2006; 176:191-199; doi: 10.4049/jimmunol.176.1.191

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Regulation of Myeloid Cell Function through the CD200 Receptor

Maria C. Jenmalm,1 Holly Cherwinski, Edward P. Bowman,3 Joseph H. Phillips, and Jonathon D. Sedgwick3

Myeloid cells play pivotal roles in chronic inflammatory diseases through their broad proinflammatory, destructive, and remodeling capacities. CD200 is widely expressed on a variety of cell types, while the recently identified CD200R is expressed on myeloid cells and T cells. CD200 deletion in vivo results in myeloid cell dysregulation and enhanced susceptibility to autoimmune inflammation, suggesting that the CD200-CD200R interaction is involved in immune suppression. We demonstrate in this study that CD200R antagonists suppress mouse and human myeloid cell function in vitro, and also define a dose relationship between receptor expression and cellular inhibition. IFN-γ- and IL-17-stimulated cytokine secretion from mouse peritoneal macrophages was inhibited by CD200R engagement. Inhibitory effects were not universal, as LPS-stimulated responses were unaffected. Inhibition of U937 cell cytokine production correlated with CD200R expression levels, and inhibition was only observed in low CD200R expressing cells, if the CD200R agonists were further cross-linked. Tetanus toxoid-induced human PBMC IL-5 and IL-13 secretion was inhibited by CD200R agonists. This inhibition was dependent upon cross-linking the CD200R on monocytes, but not on cross-linking the CD200R on CD4+ T cells. In all, we provide direct evidence that the CD200-CD200R interaction controls monocyte/macrophage function in both murine and human systems, further supporting the potential clinical application of CD200R antagonists for the treatment of chronic inflammatory diseases. The Journal of Immunology, 2006, 176: 191–199.

Myeloid cells (i.e., macrophages, dendritic cells (DC), neutrophils, mast cells, and eosinophils) play important roles in maintaining chronic inflammation (1–5). They can be regulated through cell-cell interactions that trigger matched sets of activating and inhibitory receptors, in addition to being regulated by secreted factors (6). The regulation of myeloid cell activity by direct cell-cell contact allows a more localized control than that mediated by cytokines. The CD200-CD200R interaction also provides a cell-cell contact regulatory interaction for myeloid cells. The widely expressed glycoprotein CD200 is closely related structurally to the T cell costimulatory molecules CD80 and CD86 and is genetically linked to them on human chromosome 3 and mouse chromosome 16 (7, 8). Structurally, CD200 contains two Ig superfamily (IgSF) domains in a typical V/C2 arrangement (9).

Deletion of CD200 resulted in myeloid cell dysregulation and enhanced susceptibility to autoimmune inflammation such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) (10), suggesting that CD200 normally induces immune suppression through CD200R. Consistent with this was the demonstration that a soluble form of CD200 (mouse (m) CD200g) administered to mice blocks autoimmune inflammation in vivo (11). In terms of mechanism of action, endogenous CD200 (and soluble CD200g) could send a signal that deviates the immune response away from a damaging effector pathway, as proposed (12), and/or it could directly inhibit effector cell function (10). As in vivo studies cannot discriminate between these possibilities, a cost cell model was established to test whether CD200R directly mediates cellular inhibition. IgE-mediated activation of mast cells was inhibited by mCD200g or an agonist rat-anti-mCD200R (DX109) when the cells were transfected with mCD200R, and by human (h) CD200g or agonist rat-anti-hCD200R (DX136 and DX153) when the cells were transfected with hCD200R (13). These agonists directly blocked mast cell degranulation as well as TNF and IL-13 secretion, with a typical IC50 of 0.2–1 nM. Thus, CD200 directly triggered cellular inhibition through CD200R engagement.

The CD200R is expressed at the surface of human and mouse myeloid cells, such as macrophages, DCs, neutrophils, and mast cells, and also on T cells (14, 15). CD200R is closely structurally related to CD200, located on the same chromosome, the genes probably evolved by gene duplication (15). However, the receptor is distinct from CD200 by virtue of a longer cytoplasmic tail containing three conserved tyrosine residues, one of which is contained within an NPXY motif (14, 15). Phosphorylated NPXY motifs bind phosphotyrosine-binding domains that are present in signaling adaptor molecules such as Shc (16), suggesting that the CD200R can signal after ligation by CD200. CD200R lacks an ITIM motif present in almost all immune inhibitory receptors (17). Using the mast cell system, we have shown that the NPXY motif is critical for receptor activity, and that signaling and cellular inhibition are linked to Dok1 and Dok2 phosphorylation and subsequent Ras/MAPK pathway inhibition (18).
The aim of the present study was to further characterize the regulation of myeloid cell function through the CD200R in vitro, focusing especially on the effects of the CD200R on macrophage and DC function in human and mouse.

Materials and Methods

CD200R-binding reagents
Rat mAbs that were agonistic for mouse and human CD200R were generated in rats as previously described (15). Abs used were DX109 (ratIgG1-anti-mCD200R), DX136 (ratIgG2a-anti-hCD200R), and DX153 (ratIgG1-anti-hCD200R). DX109 does not bind the activating DNA-activating protein 12 (DAP-12)-paired mCD200RLa and mCD200RLb (15). hCD200RLa is not expressed and is probably nonfunctional (15).

Ligand (CD200)-Ig fusion proteins were generated as described (13) by fusion of the extracellular domain of human or mouse CD200 to the Fe domain of mlgG1 mutuated in the CH2 domain (D265A) to inhibit binding to FcRs (19). mCD200Ig does not bind the activating DAP-12-paired mCD200RLa and mCD200RLb (15, 20).

All mAb and protein preparations used contained <0.1 ng of endotoxin/mg protein, as determined by the Limulus Amebocyte Lysate Pyrogen Testing kit, QCL-1000 (BioWhittaker).

Mouse and cell culture

Mouse peritoneal cells. Resident peritoneal cells were obtained by peritoneal lavage of 8- to 12-wk-old C57Bl/6 mice (The Jackson Laboratory) with 5 ml of DMEM (Mediatech). A total of 1 × 10^6 cells were cultured in 1 ml of DMEM with 10% heat-inactivated FCS (HyClone) and 1% FCS and 1% penicillin and streptomycin in Falcon 24-well plates at 37°C in a humidified incubator containing 5% CO2. After a 30-min incubation, and cell-bound hCD200IgG or rat-anti-hCD200R were cross-linked with 5 μg/ml endotoxin-depleted Fab(α)2, goat anti-mouse/rat IgG, and incubated for another 30 min before addition of TT. Supernatants were collected and analyzed for IL-1β, IL-6, IL-10, IL-12p70, MCP-1/CCL2, IFN-γ, and TNF secretion by ELISA or cytometric bead array (CBA) as described below.

Human peripheral blood cells. PBMC were isolated from heparinized blood of recently tetanized toxoid (TT)-vaccinated healthy donors, using Ficoll-Paque density gradient (Amersham Biosciences). A total of 1 × 10^6 PBMC were cultured in 1 ml of RPMI 1640 with 10% heat-inactivated FCS and 1% penicillin and streptomycin in Falcon 24-well plates at 37°C in a humidified incubator containing 5% CO2. After 30-min incubation with hCD200IgG, control Ig, rat-anti-mCD200R (DX109), or rat IgG1 isotype control (BD Pharmingen), cells were stimulated with recombinant murine IFN-γ (R&D Systems), recombinant murine IL-17 (DNAz), or recombinant murine IFN-γ and TNF for 18 h. Cell supernatants were collected and analyzed for IL-6, IL-10, IL-12p70, MCP-1/CCL2, IFN-γ, and TNF secretion by ELISA or cytometric bead array (CBA) as described below.

Human peripheral blood cells. PBMC were isolated from heparinized blood of recently tetanized toxoid (TT)-vaccinated healthy donors, using Ficoll-Paque density gradient (Amersham Biosciences). A total of 1 × 10^6 PBMC were cultured in 1 ml of RPMI 1640 with 10% heat-inactivated FCS and 1% penicillin and streptomycin in Falcon 24-well plates at 37°C in a humidified incubator containing 5% CO2. After 30-min incubation with hCD200IgG, control Ig, rat-anti-hCD200R (DX153), or rat IgG1 isotype control (BD Pharmingen), hCD200R or vector control-transfected U937 cells were stimulated with 100 U/ml recombinant human IFN-γ (R&D Systems) for 18 h. In some experiments, cells were washed after the 30-min incubation and the cell-bound hCD200IgG or rat anti-hCD200R were cross-linked with 5 μg/ml endotoxin-depleted Fab(α)2, goat anti-mouse/rat IgG, and incubated for another 30 min before addition of TNF. Supernatants were collected and analyzed for TNF-α, IL-6, IL-8, CXCL8, IL-10, TNF, IL-12, CD200, and corresponding mouse IgG1, mouse IgG2a, mouse IgG2b, rat IgG1, and CD200 RECEPTOR REGULATION OF MYELOID CELLS

Flow cytometry

Cells were stained and washed in PBS containing 0.1% Na2HPO4, (Sigma-Aldrich) and either 1% heat-inactivated FCS for mouse cells or 1% heat-inactivated normal human serum (Jackson ImmunoResearch Laboratories) for human cells. To block FcRs, 1 × 10^6 cells/tube were incubated with 0.1% Fc block (anti-human FcRn, clone 2G7; Biolegend) and 10 μg/ml mouse IgG2b or 10 μg/ml heat-inactivated normal human serum for 10 min on ice. FcRn binds mouse cells or 10% heat-inactivated normal human serum for human cells. The cells were then stained with FITC-, PE-, PerCP-, allophycocyanin-, or biotin-conjugated Abs (BD Pharmingen, except as indicated) for 30 min 4°C in the dark. Biotinylated Abs were secondarily labeled with streptavidin-allophycocyanin (BD Pharmingen). Cells were stained with Abs to mouse CD11b (clone M1/70), mouse CD200R (DX109; DNAz), mouse F4/80 (clone CLA3-1; Biolegend), mouse Gr-1 (Ly6G, Ly6C; clone RB6-8C5), human CD1A (clone HI149), human CD3 (clone H57/59A), human CD4 (clone RPA-T4), human CD8 (clone HIT8a), human CD11c (clone B-Ly6), human CD14 (clone M5E2), human CD19 (clone HIB1B), human CD20 (clone L27), human CD32 (clone FLB2.6), human CD80 (clone L304.1), human CD83 (clone HB15e), human CD86 (clone FUN-1), human CD1a (clone H1L3), human CD192 (clone L243), lin 1 (mixture of CD3 (clone SK7), CD14 (clone M49P), CD16 (clone 3G8), CD19 (clone S125C1), CD20 (clone L27), and CD56 (clone NCAM16.2) and corresponding mouse IgG1, mouse IgG2a, mouse IgG2b, rat IgG1, and
Cytokine and chemokine assays

The levels of mouse TNF and IL-6, and human IFN-γ, IL-5, IL-8/CXCL8, IL-13, and TNF were determined by ELISA, as described in (22), except that Ultra-3,3',5,5'-tetramethylbenzidine substrate (Pierce) was used as substrate. The following coating Abs were used: polyclonal goat anti-mouse IL-6 (clone MP5-20F3; BD Pharmingen), mouse-anti-human IFN-γ (clone NIB42; BD Pharmingen), mouse anti-human IL-5 (clone TRFK 5; BD Pharmingen), mouse anti-human IL-8 (clone 3IL8-H110; Endogen), mouse anti-human IL-13 (clone JES10-5A2; BD Pharmingen), and mouse anti-human TNF (clone 28401; R&D Systems). Recombinant mouse TNF and IL-6 and human IL-5 (BD Pharmingen), recombinant human IFN-γ, IL-13, and TNF (R&D Systems), and recombinant human IL-10 (Endogen) were used for standards. Biotinylated polyclonal goat anti-mouse TNF (R&D Systems), rat anti-mouse IL-6 (clone MP5-32C11; BD Pharmingen), mouse anti-human IFN-γ (clone 4S.B3; BD Pharmingen), mouse anti-human IFN-γ (clone JES1-5A10; BD Pharmingen), mouse anti-human IL-8 (clone 18-S2; Endogen), mouse anti-human IL-13 (clone B69-2; BD Pharmingen), and polyclonal goat anti-human TNF (R&D Systems) were used for detection. The sensitivity limits for quantitative determinations were 2 pg/ml for human TNF, 3 pg/ml for mouse IL-6, and human IL-5, IL-8/CXCL8 and IL-13, 6 pg/ml for human IFN-γ, and 10 pg/ml for mouse TNF.

The CBA system (BD Pharmingen) was also used for simultaneous detection of cytokines and chemokines, according to the manufacturer’s instructions. The mouse inflammation (IL-6, IL-10, MCP-1/CCL2, IFN-γ, TNF, and IL-12p70), human inflammation (IL-8/CXCL8, IL-1β, IL-6, IL-10, TNF, and IL-12p70), human chemokine (IL-8/CXCL8, RANTES/CCL5, MIG/CXCL9, MCP-1/CCL2, and IP-10/CXCL10) and human Th1/Th2 (IL-2, IL-4, IL-5, IL-10, TNF, and IFN-γ) kits were used. The samples were acquired on a FACS Calibur (BD Biosciences), and the data was analyzed using the BD Biosciences CBA software.

Results

The CD200R regulates mouse peritoneal macrophage cytokine production

Resident murine peritoneal macrophages express the CD200R (Fig. 1a) and agonistic mCD200Ig or anti-mCD200R Ab (DX109) treatment inhibited IFN-γ-induced TNF secretion by these cells (Fig. 1b). Similar effects were observed when macrophages were enriched by removing nonadherent cells. In contrast, comparable LPS-stimulated TNF secretion was not affected by either agonist (Fig. 1b). Elevated TNF responses induced by higher LPS doses were also not inhibited by CD200R agonists (data not shown).

FIGURE 1. mCD200R agonists inhibit IFN-γ- and IL-17-induced peritoneal cell cytokine secretion. a, Expression of mCD200R on resident peritoneal F4/80+ macrophages. Resident peritoneal cells were stained with PE-conjugated anti-F4/80 and biotinylated anti-mCD200R (DX109, solid line) or biotinylated isotype control Ab (dotted line), followed by allophycocyanin-conjugated streptavidin. Similar results were seen when macrophages were phenotyped as CD11b+ Gr-1+ cells (data not shown). b, mCD200R agonists inhibit IFN-γ, but not LPS-induced, peritoneal cell TNF secretion (p < 0.01 for both agonists, unpaired t test). After a 30-min incubation with 3 μg/ml control Ig (gray striped bars), mCD200Ig (black striped bars), rat IgG1 isotype control (gray bars), rat-anti-mouse CD200R (black bars), or no Ab (white bars), 1 × 106 peritoneal cells were stimulated with 0.5 ng/ml recombinant murine IFN-γ or 0.02 ng/ml Salmonella typhimurium LPS for 18 h. TNF secretion in supernatants was determined by ELISA. Results are presented as mean ± SEM of triplicate cultures and are representative of six independent experiments. c, Below limit of detection (10 pg/ml). d, mCD200R agonists inhibit IFN-γ- and IL-17-induced peritoneal cell IL-6 secretion (p < 0.01 for both stimuli and agonists, unpaired t test). Cells were cultured as in b and IL-6 secretion determined by CBA. Results are presented as mean ± SEM of triplicate cultures and are representative of two independent experiments. d, mCD200R agonists do not inhibit IFN-γ-induced peritoneal cell MCP-1 secretion. Cells were cultured as in b and MCP-1/CCL2 secretion determined by CBA. Results are presented as mean ± SEM of triplicate cultures and are representative of two independent experiments. c, d, Below limit of detection (20 pg/ml).

Human cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12p70, IL-13, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, TNF, and IFN-γ) were also analyzed using the LINCOplex Simultaneous Multianalyte Detection System (Linc Research), according to the manufacturer’s instructions. Cytokine levels were quantitated using the Luminex 100 system. Values were expressed as picograms per milliliter deduced from the mean fluorescence intensity of the standard curves after subtracting the blanks, using a 5-parameter logistic algorithm with MasterPlex QT Quantitation Software version 2.0 (MiraiBio).

Gene expression analysis using TaqMan real-time quantitative PCR

Methods were essentially as described in Ref. 23.

Statistical methods

Comparisons between groups were assessed with the two-tailed unpaired t test. A p value of <0.05 was considered as statistically significant. Calculations were performed with a statistical package, StatView 5.0 for Macintosh (Acubas Concepts).
IFN-γ- and IL-17-induced IL-6 secretion was also inhibited by mCD200Ig and anti-mCD200R Ab (Fig. 1c). CD200R inhibition of IFN-γ-induced activation was not universal, as MCP-1/CCL2 production was not affected (Fig. 1d). IL-17 did not induce detectable TNF and MCP-1/CCL2 production.

**CD200R expression on human peripheral blood leukocytes**

We then explored the role of CD200R in the human system. CD200R expression was first investigated on human blood cells. Fresh ex vivo CD14+/lin− DCs expressed the highest CD200R levels, followed by CD14+/ monocytes, CD4+ T cells, and CD8+ T cells (Fig. 2). B cells did not express CD200R (15). Monocyte-derived DCs also expressed high CD200R levels (Fig. 2), and slightly down-regulated CD200R expression after further maturation with CD40L or TNF and IL-1β (data not shown).

**Inhibition of TT-induced cytokine secretion from PBMC by CD200R triggering**

We next investigated whether Ag-induced cytokine responses, requiring cooperation between Ag-presenting monocytes and T cells, were modulated by CD200R agonists. Thus, PBMC from recently TT-vaccinated donors were pretreated with CD200R agonists before stimulation with TT. TT dose-dependently induced IL-5, IL-13, IFN-γ, and TNF (data not shown), but not IL-2, IL-4, IL-10, and IL-12p70 secretion (Fig. 3). CD200R agonists inhibited production of IL-5 and IL-13, but only when the agonists were further cross-linked by F(ab′)2 goat anti-mouse/rat IgG (Fig. 3 and Table I). The effects on IFN-γ (Fig. 3c) and TNF (data not shown) were lesser and more variable. No effect of CD200R stimulation was observed at suboptimal TT doses. Data from four independent donors are summarized in Table I.

The inhibitory activity of CD200R agonists could be due to a direct or indirect effect on T cell activation, since both PBMC CD4+ T cells and monocytes express CD200R. Each cell type was isolated and separately treated with CD200R agonists to investigate whether the inhibition of TT-induced cytokine secretion required triggering of the hCD200R on monocytes, on CD4+ T cells, or on both cell types. Each cell type was incubated with an anti-hCD200R Ab and a cross-linking Ab and then mixed before stimulation with TT. Inhibition of IL-5 and IL-13 secretion was only observed when hCD200R was cross-linked on monocytes, but not when the hCD200R was cross-linked on CD4+ T cells (Fig. 4). As controls, IL-5 and IL-13 secretion was also inhibited when the CD200R was cross-linked on both monocytes and T cells, and in PBMC cultures performed in parallel (Fig. 4). In this experiment, IFN-γ secretion was not inhibited after CD200R ligation.

**Effects of hCD200R agonists on DC activation**

CD200R was expected to be functionally important on monocyte-derived DCs, since they express high CD200R levels. Thus, we investigated the effects of CD200R agonists on these cells with and without further cross-linking. DCs were activated with LPS, IFN-γ, CD40L and IFN-γ, or TNF and IL-1β. We analyzed expression of the cell surface markers CD1a, CD32, CD80, CD83, CD86, and HLA-DR, as well as secretion of IL-1α, IL-1β, IL-6, IL-8/CXCL8, IL-10, IL-12p70, IP-10/CXCL10, MCP-1/CCL2, MIG/CXCL9, MIP-1α/CCL3, RANTES/CCL5, and TNF. We did not observe any effect of CD200R agonists on these parameters (data not shown). CD200R agonists also had no effect on allodendritic DC-induced CD4+ T cell proliferation or CD4+ T cell cytokine production using TT-loaded DC (data not shown). It is likely that human DC will also be modulated through CD200R given the recent data showing effects of CD200Ig on mouse DC function (24). However, to date, we have not identified the relevant in vitro conditions needed to observe CD200R-mediated modulation of human DC function.

**Inhibition of hCD200R-transfected U937 cell cytokine production by CD200R agonists**

Human monocytic U937 cells were transfected with hCD200R to further explore CD200R regulation of human myeloid cell activation. U937 cells did not express the receptor endogenously, but when infected with a retrovirus encoding hCD200R, they express CD200R at high levels (Fig. 5a). IFN-γ stimulation resulted in increased IL-8/CXCL8, IP-10/CXCL10, and MIG/CXCL9 secretion, whereas TNF, IL-1β, IL-6, IL-10, and IL-12p70 production was not detectable (data not shown). CD200R agonists dose-dependently inhibited IFN-γ-induced IL-8/CXCL8 (Fig. 5b), MIG/CXCL9, and IP-10/CXCL10 (data not shown) secretion from the
FIGURE 3. hCD200R agonists inhibit TT-induced PBMC cell IL-5 and IL-13 secretion. After a 30-min incubation with 10 µg/ml control Ig (gray striped bars), hCD200Ig (black striped bars), rat IgG1 isotype control (gray bars), or rat-anti-human CD200R (black bars), 1 × 10^6 PBMC were washed and the hCD200Ig or rat-anti-human CD200R were cross-linked with 5 µg/ml endotoxin-depleted F(ab')2 goat anti-mouse/rat IgG, and incubated for another 30 min before addition of 1, 10, or 100 ng/ml TT. Supernatants were harvested after 6 days. IL-5, IL-13, IFN-γ secretion was determined by ELISA. Results are presented as mean ± SEM of triplicate cultures and are representative of three to four independent experiments. Statistically significant inhibition of IL-5 and IL-13 secretion by hCD200R agonists was observed (p < 0.01 for both agonists and cytokines, unpaired t test).

Table I. hCD200R agonists inhibit tetanus toxoid-induced PBMC IL-5 and IL-13 secretion

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A total of 1 × 10^6 PBMCs were cultured in 1 ml of RPMI 1640 with 10% heat-inactivated FCS, 1% penicillin and streptomycin at 37°C in a humidified incubator containing 5% CO2. After a 30-min incubation with control-Ig, hCD200Ig, rat IgG1 isotype control, or rat-anti-human CD200R, cells were washed and the hCD200Ig or rat-anti-human CD200R were cross-linked with 5 µg/ml endotoxin-cleaned F(ab')2 goat anti-mouse/rat IgG, and incubated for another 30 min before addition of tetanus toxoid. Supernatants were harvested after 6 days. IL-5, IL-13, IFN-γ, or TNF secretion was determined by ELISA, CBA, or Luminex. Data from the optimal tetanus toxoid dose (10 ng/ml for donor 1 and 100 ng/ml for donors 2, 3, and 4) are presented as mean values picograms per milliliter of triplicate cultures.

Discussion

The present study shows that triggering of CD200R suppresses myeloid cell function in vitro. Thus, after stimulation with the myeloid cell-activating cytokine IFN-γ, cytokine secretion from mouse peritoneal macrophages and human myeloid U937 cells transfected with the hCD200R was inhibited by triggering of the CD200R. IL-17-induced IL-6 production from mouse peritoneal macrophages was also inhibited by CD200R triggering. By contrast, responses after LPS stimulation were not affected. CD200R-mediated inhibition correlated with levels of CD200R on U937 cells. In cells expressing low CD200R levels, inhibition was only observed if the CD200R agonists were further cross-linked. TT-induced PBMC IL-5 and IL-13 secretion was also inhibited by cross-linked CD200R agonists. This inhibition required cross-linking the CD200R on monocytes, but not cross-linking the CD200R on CD4+ T cells. In all, our in vitro data agree well with the in vivo observations showing that targeted deletion of CD200 resulted in dysregulation of myeloid cells and enhanced susceptibility to autoimmune inflammation such as EAE and CIA (10).
As macrophages play pivotal roles in chronic inflammatory diseases through their broad proinflammatory, destructive, and remodeling capacities (1–3, 5), regulation of their function is critical for treatment of these disorders. One way of controlling inflammation is through blocking proinflammatory cytokines produced by macrophages, such as via anti-TNF therapy for rheumatoid arthritis, Crohn’s disease, and psoriasis (25). Triggering inhibitory receptors represents an alternative way of regulating myeloid cells (6), potentially superior to focused anti-cytokine therapies given the ability to affect a number of effector pathways via cellular

**FIGURE 4.** TT-induced IL-5 and IL-13 secretion is inhibited when the hCD200R is cross-linked on monocytes, but not when the hCD200R is cross-linked on CD4+ T cells. Monocytes and CD4+ T cells were isolated by MACS to >95% purity. After separate incubation of 250,000 monocytes/well and 750,000 CD4+ T cells/well with 10 μg/ml rat IgG1 isotype control (gray bars) or rat-anti-human CD200R (black bars), cells were washed and the rat-anti-human CD200R was cross-linked with 5 μg/ml endotoxin-cleaned F(ab’)_2 goat anti-mouse/rat IgG, and incubated for another 30 min before addition of 100 ng/ml TT. IL-5 (a) and IL-13 (b), mean ± SEM of triplicate cultures, was determined by the LINCplex Simultaneous Multianalyte Detection System using a Luminex. <, Below limit of detection (3 pg/ml). Inhibition was only observed when hCD200R was cross-linked on monocytes, or on both monocytes and T cells or in PBMC (p < 0.01 for both agonists and cytokines in all three cultures, unpaired t test).

**FIGURE 5.** hCD200R agonists dose-dependently inhibit IFN-γ-induced hCD200R-transfected U937 cell IL-8/CXCL8 secretion. a. Expression of hCD200R on hCD200R- and vector control-transfected U937 cells. The transfected U937 cells were stained with biotinylated anti-hCD200R (DX136, solid line) or biotinylated isotype control Ab (dotted line), followed by allophycocyanin-conjugated streptavidin. b. hCD200R agonists inhibit IFN-γ-induced hCD200R-transfected U937 cell IL-8/CXCL8 secretion. After a 30-min incubation with control Ig (circles with no connecting line), hCD200lg (circles with connecting lines), rat IgG1 isotype control (squares with no connecting line), rat-anti-human CD200R (squares with connecting lines), rat IgG1 isotype control (squares with no connecting line), rat-anti-human CD200R (squares with connecting lines), rat IgG1 isotype control (squares with no connecting line), rat-anti-human CD200R (squares with connecting lines), rat IgG1 isotype control (squares with no connecting line), rat-anti-human CD200R (squares with connecting lines), rat anti-human CD200R (squares with connecting lines), 1 × 10^6 hCD200R-transfected U937 cells were stimulated with 100 U/ml recombinant human IFN-γ (filled symbols with solid connecting lines) or cultured in medium alone (open symbols with dashed connecting lines) for 18 h. IL-8/CXCL8 secretion in supernatants was determined by ELISA and is presented as mean ± SEM of triplicate cultures. Statistically significant inhibition (unpaired t test) was observed from 0.3 μg/ml hCD200lg and 0.1 μg/ml rat-anti-hCD200R.
One potential mechanism through which CD200 may control inflammation in vivo is via inhibition of TNF and IL-6 production following stimulation of mouse macrophages with IFN-γ, a very important activator of macrophage function (28–30). TNF plays a central role in autoimmune disease (25). TNF has several proinflammatory activities such as up-regulation of adhesion molecule expression, induction of cytokines and chemokines, angiogenesis and bone resorption (1, 25). IL-6 is the most strikingly elevated cytokine in human rheumatoid arthritis (1, 31), and it is required for experimentally induced autoimmune diseases, including CIA (32) and EAE (31, 33). Clinical trials with a humanized anti-IL-6R-antibody for treatment of rheumatoid arthritis and Crohn’s disease are ongoing (34). Proinflammatory effects of IL-6 include enhancing autoimmune production, bone absorption and triggering of acute phase responses (31), and blocking regulatory T cells suppression (35).

The observation that IL-17-induced IL-6 production was suppressed by CD200R agonists is also relevant to the in vivo mechanism of CD200R. This is the first report that IL-17 activates mouse macrophages, although enhancement of IL-6 production has been observed in human rheumatoid arthritis synovial cultures and human macrophages after IL-17 stimulation (36, 37). IL-17 is a T cell-derived cytokine that exhibits pleitropic biological activities on various types of cells, such as fibroblasts, endothelial...
cells, and epithelial cells, mediating a wide range of responses, mostly proinflammatory and hemopoietic (38). Increased levels of IL-17 have been found in several human inflammatory disorders, including multiple sclerosis (39), rheumatoid arthritis (40), psoriasis (41), and asthma (42). Neutralizing IL-17 reduces the severity of CIA (43) and EAE (44), and its production is triggered by IL-23, an essential cytokine in the development of autoimmune inflammation (44).

CD200R triggering did not result in universal macrophage inhibition, since LPS-induced TNF responses were not affected. This could be a safety advantage in a therapeutic setting, especially if this holds true for other pathogen-derived TLR ligands. The differential effects of CD200R-mediated inhibition on IFN-γ, IL-17-, and LPS-induced cytokine responses is not surprising, as different signaling pathways are induced by these factors (30, 38, 45).

We also explored the role of CD200R in human systems in vitro after showing that mouse macrophages were functionally inhibited by CD200R triggering. As blood APCs and CD4+ T cells express the CD200R, we first investigated whether Ag-induced cytokine responses, requiring cooperation between Ag-presenting monocytic and T cells, could be modulated by CD200R agonists. We found that TT-induced PBMC IL-5 and IL-13 secretion was inhibited by CD200R agonists after further cross-linking. Effects on IFN-γ and TNF secretion were more minor and variable. Cytokine responses were not affected by CD200R agonists in the absence of further cross-linking. The requirement for cross-linking is not surprising, as CD200R is normally present as a cell surface protein. Engagement of CD200 and CD200R as a cell-cell interaction is likely to lead to multimerization or aggregation of receptor, with different signaling pathways are induced by these factors (30, 38, 45).

We also explored the role of CD200R in human APC function, we investigated the effects of CD200R triggering on monocyte-derived DCs, as these cells express high CD200R levels. We have not yet been able to observe any effects of CD200R agonists on a broad range of cell surface markers or cytokine/chemokine secretion, however, after stimulation of the cells with LPS, IFN-γ, CD40L and IFN-γ, or TNF and IL-1β. CD200R agonists also had no effects on allogeneic DC-induced CD4+ T cell proliferation or CD4+ T cell cytokine production when DCs were used to present TT. It is possible that other aspects of DC activation may be affected (e.g., via indoleamine 2,3-dioxygenase-based effects (24)), or that the in vitro conditions do not permit effects of CD200R triggering (e.g., contact with other cells and extracellular matrix proteins present in the in vivo environment are absent).

Human hCD200R-expressing monocyte U937 cells were used to further explore the CD200R regulation of human myeloid cell activation. CD200R engagement inhibited IFN-γ-stimulated secretion of IL-8/CXCL8, IP-10/CXCL10, and MIG/CXCL9. Inhibition of cytokine production positively correlated with CD200R expression levels. Inhibition of low CD200R-expressing cells was only observed if the CD200R agonists were further cross-linked. This may explain why inhibition of TT-induced secretion required cross-linking of the CD200R. Inhibition of secretion of the chemotactic factors IL-8, IP-10, and MIG would reduce recruitment of neutrophils and Th1 cells to sites of inflammation (46).

In conclusion, we provide direct evidence in vitro that the CD200-CD200R interaction controls monocyte/macrophage function in both mice and humans and that the efficiency of CD200R-mediated inhibition of cellular functions is proportional to the receptor density at the cell surface. Our data solidify the concept that CD200R agonism may provide a novel approach to the treatment of chronic inflammatory diseases.

Acknowledgments
We thank Janet Wagner and Sandra Zurawski for production of fusion proteins and mAbs.

Disclosures
The authors have no financial conflict of interest.

References