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CD200R expression and regulation on macrophages

Expression of the inhibitory CD200R is associated with alternative macrophage activation

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Submitted

Abstract

The mechanisms by which type II cytokine-associated M2 macrophages maintain their functions are not fully understood. However, signaling pathways known to be important in type I cytokine-associated classically activated M1 macrophages, such as MAPK p38, ERK, and JNK, can be inhibited by signaling through the inhibitory receptor CD200R. Here, we show that CD200R expression was induced on human in vitro-polarized macrophages of the alternatively activated M2a subtype, generated by incubation with IL-4. CD200R induction was restricted to M2-inducing conditions, in contrast to the mannose receptor, which is currently viewed as the most common M2a marker in human. Experimental parasite infections in mice evoke highly polarized type 2 immune responses correlating with an M2 macrophage phenotype. Peritoneal M2a macrophages, elicited during infection with Taenia crassiceps or Trypanosoma b. brucei expressed increased CD200R levels compared to those derived from uninfected mice. However, in vitro stimulation of mouse peritoneal macrophages and T. crassiceps infection in IL-4$^{-/-}$ and IL4R$^{-/-}$ mice showed that, in contrast to human, induction of CD200R in mice was not IL-4 dependent. Our data identify CD200R as a suitable marker for alternatively activated macrophages in human and corroborate observations of distinct mechanisms regulating macrophage polarization in mouse and man. Since the effects of CD200R triggering involve the inhibition of pathways of classical M1 activation, CD200R expression on M2 cells might form a mechanism to keep M2 cells in a polarized state.

Introduction

Macrophages are versatile players in the immune system. They effectively neutralize pathogens and harmful endogenous products via phagocytosis, but also contribute to efficient immunity by production and secretion of multiple mediators like cytokines and chemokines. Next to pro-inflammatory activities, macrophages possess important regulatory activities during the resolution phase of an immune response and at sites where antigenic material is ignored (lung and colon) or tolerated (eye, brain, ovary, and testis). Opposing macrophage activities result from the initiation of distinct gene expression programs.[23,172-174] IFN-γ and LPS induce classic activation of macrophages. These macrophages, also known as M1 cells, secrete high amounts of pro-inflammatory cytokines and reactive oxygen species and efficiently clear pathogens either directly or by facilitating Th1-mediated responses. In contrast, stimulation with IL-4, IL-10, IL-13, or glucocorticoids triggers pathways that program macrophages to exert anti-inflammatory functions. These type 2 cytokine-associated M2 cells can be further classified into M2a, M2b, and M2c cells that each emphasize specific functions.[23] For example, IL-4 generates M2a cells that are considered to be involved in Th2-mediated responses and are most commonly characterized by mannose receptor (MR, CD206) expression.[172] IL-10 and glucocorticoids lead to M2c-polarized cells that mediate tissue remodeling and matrix deposition and are characterized by high levels of the scavenger receptor CD163.[175]

The way M2 cells maintain their immune suppressive properties has not been completely elucidated. However, immune inhibitory receptors such as the membrane glycoprotein CD200R may be involved for several reasons. CD200R, mainly expressed on myeloid cells and on a subset of T and B cells,[121,122,125] inhibits inflammatory macrophage activation as illustrated by studies showing that absence of its ligand CD200 aggravates, accelerates, or increases the susceptibility of inflammatory models such as experimental autoimmune encephalomyelitis, uveoretinitis, and collagen-induced arthritis.[125,126] Indeed, CD200R signaling profoundly inhibits the MAP kinases p38, ERK, and JNK, the common signaling pathways involved in classical activation of macrophages.[123] As M2 cells can mediate a type II immune response, by producing and secreting anti-inflammatory mediators, and reducing inflammatory cytokines like TNF, it is interesting that some of these effects have also been reported for CD200R.[124] However, the expression of CD200R or other immune inhibitory molecules on M2 cells has never been studied thoroughly.

Methods

Cell culture

Monocytes were purified from human peripheral blood mononuclear cells and either matured into macrophages for 4 days and then polarized for 3 days with IFN-γ (50 ng/ml), IL-1β (40 ng/ml), TNF (50 ng/ml), GM-CSF (50 ng/ml), M-CSF (25 ng/ml), IL-4 (40 ng/ml), IL-13 (50 ng/ml), IL-10 (50 ng/ml) (all Peprotech, London, UK), or dexamethasone (2 μM, Sigma Aldrich, Zwijndrecht, The Netherlands), or were directly matured and polarized for 3 days in the presence of these stimuli, as described previously.[176] Mouse peritoneal macrophages were isolated as described previously[177] and were stimulated with 50 ng/ml mIL-4 (R&D Systems, Minneapolis, MN) or 50 ng/ml mIL-10 (Peprotech) for 3 days.

Quantitative PCR

Total RNA (10 ng per reaction) was transcribed into first-strand cDNA and analyzed by quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI Prism 7300 Sequence Detection System (Applied Biosystems) as described elsewhere.[111] Target genes were human CD200R (forward primer 5′-GAGCAATGGCACAGTGACTGTT-3′, reverse primer 5′-GTGGCAGGTCACGGTAGACA-3′), MR (forward primer 5′-TGCAGAAGCAAACCAAACCTGTAA-3′, reverse primer 5′-CAGGCCTTAAGCCAACGAAACT-3′), CD163 (forward primer 5′-ACATAGATCATGCATCTGTCATTTG-3′, reverse primer 5′-ATTCTCCTTGGAATCTCACTTCTA-3′), TREM2 (forward primer 5′-CCACCCACTTCCATCCTTCT-3′, reverse primer 5′-GTCCCTGGCTTCTGTCCAT-3′), and SIRPα (forward primer 5′-GTCTGGAGCAGGCACTGA-3′, reverse primer 5′-GGACTCGCAGGTGAAGCT-3′) and mouse CD200R (forward primer 5′-TGCCATCCTGCACAATAGCA-3′, reverse primer 5′GGAGGCCCAGGTGATGTT-3′). Targets were normalized to 18S ribosomal RNA (forward primer 5′-TAGTCGCCGTGCCTACCAT-3′, reverse primer 5′-CCTGCTGCCTTCCTTGGA-3′).

Flow cytometry

Cells were incubated with anti-human Alexa 674-labeled CD200R (Serotec, Oxford, UK), APC-labeled MR (BD Biosciences, San Jose, CA), PE-labeled CD163 (BD Biosiences), and biotinylated TREM2 (Peprotech) antibodies. Appropriate Ig isotypes were used as negative control. Staining was measured on a FACSCalibur and analyzed with FlowJo software version 8.7.1 (Treestar, Inc. Ashland, OR).

Parasite infections

Inoculation of C57BL/6 and Balb/c mice with *Taenia crassiceps* and of C57BL/6 x BALB/c (F1) mice with PLC$^{-/-}$ *Trypanosoma b. brucei* has been described previously.[177]

Statistical analysis

Data were analyzed by one-way ANOVA and post-hoc Dunnett's Multiple Comparison Test using GraphPad Prism 5.01 (GraphPad Software Inc. La Jolla, CA, USA). A p value < 0.05 was considered significant.

Results and Discussion

To determine the expression of the immune inhibitory receptor CD200R on polarized macrophages, we subjected human monocytes, purified from peripheral blood mononuclear cells (PBMCs), to a panel of pro- and anti-inflammatory stimuli for 3 days, as described previously,[176] to initiate either M1 activation (INF-γ, IL-1β, TNF, GM-CSF) or M2 activation (IL-4, IL-13, IL-10, M-CSF or dexamethasone) (Fig. 1).[178] Analysis of CD200R mRNA expression in these cells, performed by quantitative real-time PCR (qPCR), revealed a significant induction after stimulation with IL-4 or IL-13 (Fig. 1). On the protein level, CD200R expression was robustly increased by IL-4 stimulation and slightly but not significantly following IL-13, IL-10, and the glucocorticoid dexamethasone (Fig. 1). These results indicate that CD200R is solely expressed on alternatively activated M2a macrophages, which are at present most commonly identified by expression of the MR.[172] We therefore compared the expression pattern of CD200R with that of MR mRNA by qPCR and protein by flow cytometry in the same series of experiments. MR mRNA was indeed induced by IL-4 and IL-13, but also by the M1 activator GM-CSF, confirming previously reported data,[179] and to some extent by IL-1β, although the latter was not significant (Fig. 1). Different from M2a cells are M2c cells that are induced by IL-10 and glucocorticoids and are characterized by CD163 expression.[23] As expected, CD163 mRNA and protein was induced only by IL-10 and the glucocorticoid dexamethasone and not by IL-4 or IL-13 (Fig. 1), confirming the M2c specificity of this marker. Thus, whereas CD163 is an excellent marker for M2c cells, CD200R is expressed on M2a cells with higher specificity than MR and therefore appears to be a novel reliable marker for this type of macrophage *in vitro*.

We next tested whether two other inhibitory molecules expressed on myeloid cells, TREM2 and SIRPα,[113,137,180] were also primarily expressed by alternatively acti-

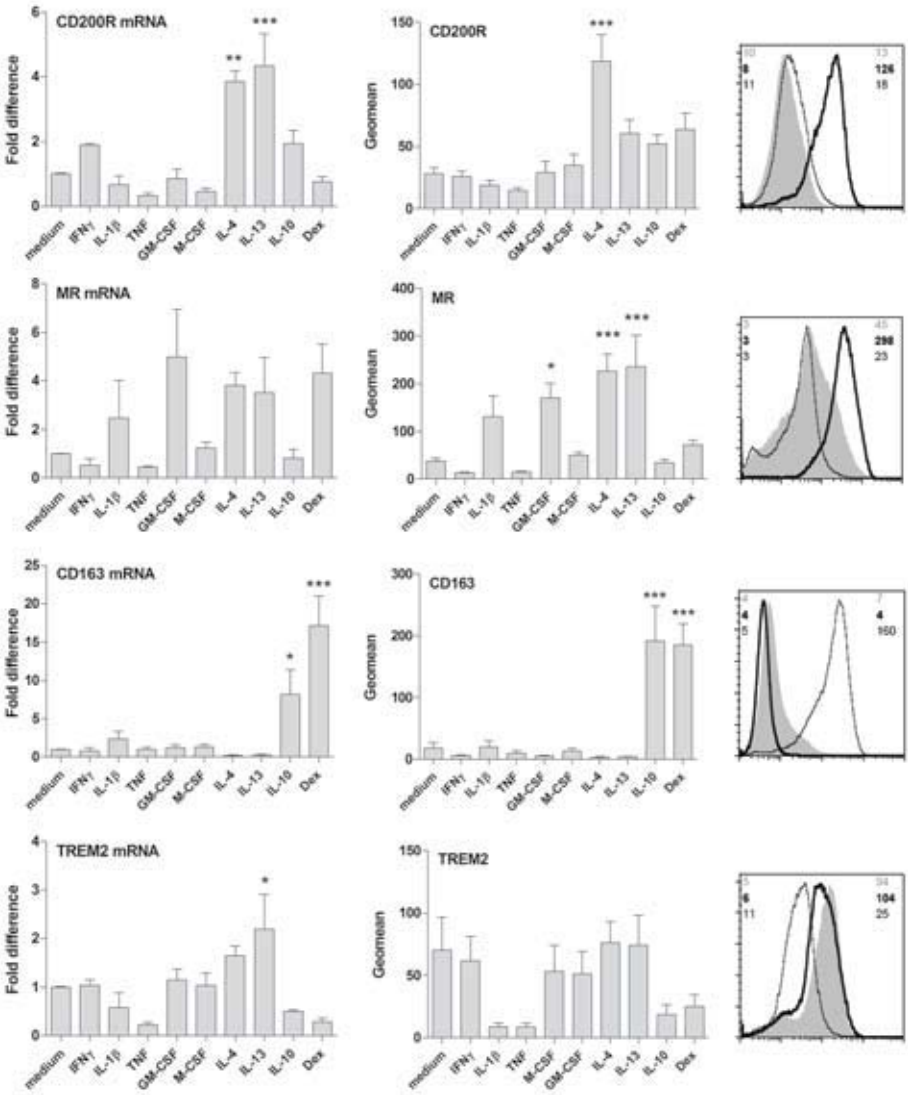

Fig. 1 Expression of CD200R by human macrophages maturated under polarizing conditions. Human primary monocytes were isolated from PBMCs and differentiated into M1 macrophages by stimulation with IFN-γ, IL-1β, TNF, or GM-CSF or into M2 macrophages by stimulation with M-CSF, IL-4, IL-13, IL-10, or dexamethasone. After 72 hours, mRNA and protein expression of CD200R, MR, CD163, and TREM2 was determined. Transcript data (left panels) are depicted as mean-fold induction ± SEM compared to macrophages cultured in medium only (n = 3). Protein data (middle panels) are depicted as mean fluorescence intensity (geomean) ± SEM (CD200R, n = 12; MR, n = 5; CD163, n = 6; TREM2, n = 3). Representative histograms (right panels) show protein expression levels of macrophages stimulated with IL-4 (bold line), IL-10 (thin line), or unstimulated (medium, grey) with geometric means indicated in the numbers with corresponding colors (isotype controls left and antibodies right). Single asterisk indicates $0.01 < p \leq 0.05$; double asterisks indicate $0.001 < p \leq 0.01$; triple asterisks indicate $p < 0.001$.

vated macrophages. A significant induction of TREM2 was found after IL-13 stimulation on mRNA level, but not on protein level (Fig. 1). IL-4 did not induce TREM2 expression. Expression of SIRPα, determined at the mRNA level, was not induced by any of the investigated stimuli (data not shown), indicating that induction of immune inhibitory molecules is not a common phenotype of experimentally polarized human M2 macrophages, and seems restricted to CD200R.

To further analyze the induction of CD200R expression on M2 cells, freshly isolated monocytes from PMBCs were first matured for 4 days and then stimulated with IL-4 or IL-13 for another 3 days. Figure 2 shows that this procedure induced a dramatic increase in CD200R mRNA expression (Fig. 2) compared to cells that were stimulated directly after isolation (Fig. 1). Similar to CD200R, also the increase in MR expression was much stronger when cells were matured prior to stimulation (Fig. 2). As expected, expression of CD163 was unaffected by IL-4 or IL-13 (Fig. 2).

Experimental infection of mice with parasites is known to provoke strong type 2 immune responses correlating with an M2 signature of the elicited macrophages[177] To test whether CD200R is expressed on M2 cells *in vivo*, we analyzed peritoneal macrophages isolated from animals infected with either the helminth *Taenia crassiceps* or the protozoan *Trypanosoma b. brucei*, inoculated as described previously.[177] Indeed, macrophages from T. crassiceps-infected mice (C57BL/6 and BALB/c) showed increased CD200R mRNA expression compared to those derived from uninfected mice (Fig. 3A). Mice infected with phospholipase C-deficient ($PLC^{-/-}$) *T. b. brucei* display a switch from a type 1 cytokine milieu during the early stage of the

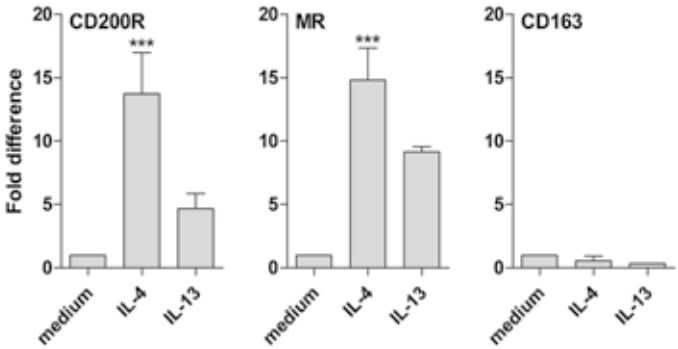

Fig. 2 Expression of CD200R by maturated human macrophages that subsequently were polarized into M2 cells. Human primary monocytes were isolated from PBMCs and adhered to the culture dish for 4 days to obtain matured macrophages, and were then differentiated with IL-4 or IL-13. After 3 days, mRNA expression of CD200R, MR and CD163 was determined by qPCR. The mRNA data are depicted as mean-fold induction ± SEM as compared to macrophages cultured in medium only (n = 4-6). Triple asterisks indicate $p < 0.001$.

infection to a type 2 environment in the chronic phase of the disease.[177] CD200R expression was unaltered or even slightly decreased on peritoneal macrophages derived from the acute phase, but robustly up-regulated on macrophages derived from the chronic phase (Fig. 3A). In both parasite models, regulation of CD200R expression correlated well with the established mouse M2 markers arginase-1 and 'found in inflammatory zone 1' (Fizz1)[177] (data not shown). These results indicate that CD200R is also induced on mouse M2 macrophages *in vivo*.

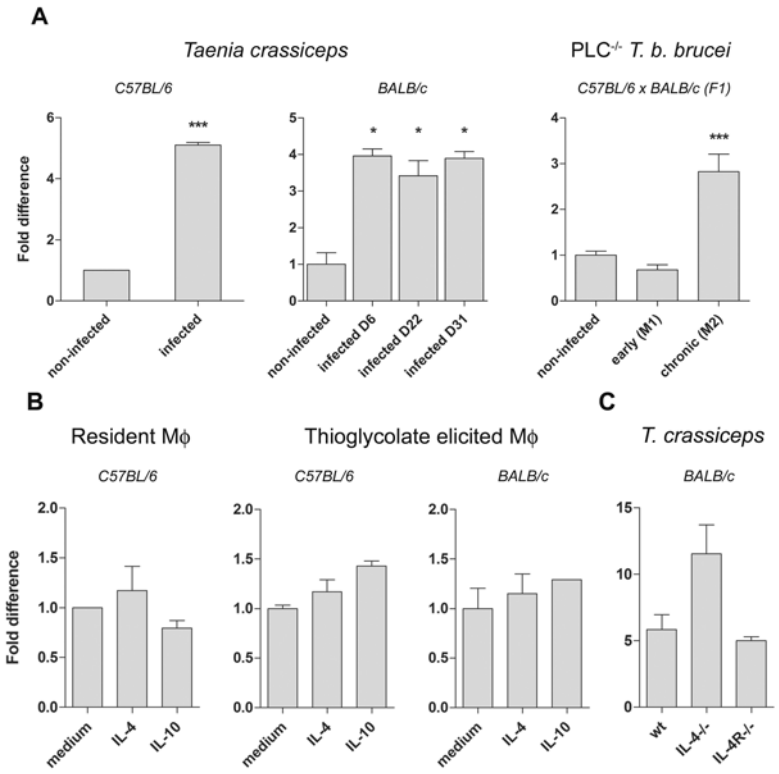

Fig. 3 CD200R expression in mouse alternatively activated macrophages *in vivo* and *in vitro*. (A) Transcription of CD200R was determined in peritoneal macrophages isolated from C57BL/6 mice at 4 weeks after infection with *Taenia crassiceps* (left panel), and from BALB/c mice at 6, 22, and 31 days after infection (central panel). CD200R expression was also determined in peritoneal macrophages isolated from C57BL/6 x BALB/c (F1) mice in the early (2 weeks) and chronic (3-4 months) stage of infection with PLC$^{-/-}$ *Trypanosoma b. brucei* (right panel). (B) Induction in CD200R mRNA in resident peritoneal macrophages (Mφ) isolated from C57BL/6 mice and thioglycolate-elicited macrophages isolated from C57BL/6 and BALB/c mice, stimulated with IL-4 or IL-10. (C) Induction of CD200R mRNA on peritoneal macrophages isolated from BALB/c wild type, IL-4$^{-/-}$ and IL-4R$^{-/-}$ mice infected with *T. crassiceps*. Data are depicted as mean-fold induction ± SEM compared to macrophages from non-infected mice (A), untreated cultures (B), and non-infected wild-type mice (C) (n = 2-6). Single asterisk indicates $0.01 < p \leq 0.05$; triple asterisk indicates $p < 0.001$.

We next determined whether CD200R expression on M2 macrophages in mice was IL-4 dependent, as we showed in human macrophages. Surprisingly, mIL-4 or mIL-10 failed to induce CD200R mRNA expression in resident peritoneal macrophages from C57BL/6 mice, and in thioglycolate-elicited peritoneal macrophages from either C57BL/6 (Th1-prone) or BALB/c (Th2-prone) mice (Fig. 3B). *In vivo* we confirmed that the elevated CD200R expression in M2 cells was indeed IL-4 independent as no abrogation of CD200R induction was observed in IL-4$^{-/-}$ or IL-4R$^{-/-}$ mice infected with *T. crassiceps* (Fig. 3C).

In summary, we here demonstrate that the immune inhibitory molecule CD200R is induced on *in vitro*-polarized human macrophages following IL-4 stimulation and can be used as a novel cell surface marker for M2a cells in human, with even higher specificity than MR. Furthermore, CD200R is also expressed on mouse M2 cells, but in contrast to human, is not induced by IL-4. In fact, it was recently shown that IFN-γ was able to induce CD200R expression in mouse peritoneal macrophages.[181] Since we show that IFN-γ does not affect CD200R expression in human macrophages, these data corroborate previous findings implying differences in human versus mouse macrophage biology.[177,182] The current classification in macrophage polarization represents extremes of a continuum, indicating that CD200R may also be expressed on macrophages with phenotypes lying in between these extremes. However, the present data imply that rather than merely cellular silencing, it is now more likely that CD200R may be actively involved in maintaining M2 cells in their polarized state, which enables them to create a milieu that favors a type 2 immune response. Lack of this regulatory pathway, for instance in CD200$^{-/-}$ mice, could then lead to exacerbation of (auto)immune diseases as reported previously.[125,126,181]

Acknowledgments

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