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CD200 increases alternatively activated macrophages through CREB - C/EBP-beta signaling

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Abstract

The concept of macrophage polarization toward different phenotypes after CNS injury has been increasingly discussed. Here, we propose that CD200 treatment may help shift pro-inflammatory macrophages to an Arg1-, TGM2- and TGF-β-positive phenotype. Rat macrophages were stimulated by IFN-γ and LPS to induce pro-inflammatory phenotypes. Treatment with human CD200-Fc upregulated expression levels of alternatively activated M2-like markers such as Arg1 and TGM2 but suppressed pro-inflammatory M1-like markers such as TLR4, IL-1β, IL-6 and GM-CSF. Concomitantly, CD200-Fc enhanced C/EBP-beta promoter activity, whereas NF-κB activity was suppressed. Treatment with CD200-Fc also upregulated potentially beneficial TGF-β expression in macrophages. When C/EBP-beta signaling was suppressed with siRNA, the effect of CD200-Fc on Arg1, TGM2 and TGF-β upregulation was canceled. Taken together, these data provide proof-of-principle that targeting CD200 signaling may be a novel therapeutic approach to shift macrophages towards M2-like polarization via modulating CREB-C/EBP-beta transcriptional activity.

Keywords

CD200-Fc; alternatively activated Mϕ; CREB; C/EBP-beta; NF-κB

Introduction

The adult mammalian brain possesses many endogenous elements for recovery after injury and disease (Moskowitz *et al.* 2010). Neuroplasticity and neurogenesis can help replace damaged areas, axonal remodeling can help restore damaged circuits, and angiogenesis can help repair neurovascular integrity. Furthermore, beyond central responses per se, dynamic crosstalk between the brain and systemic responses, such as circulating blood cells, may also play an important role (Offner *et al.* 2006). In particular, macrophages may contribute to both brain damage and remodeling after injury depending on the inflammatory circumstance (Iadecola & Anrather 2011). Macrophage phenotypes may range from deleterious cells to

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potentially beneficial cells. Thus, understanding the mechanism that governs macrophage phenotype is important to promote brain recovery.

CD200 (OX2) is widely expressed in the CNS under physiological and pathological conditions. As a component of the neuroimmunoregulatory molecule family (NIRegs), CD200 can bind to CD200 receptors, which are predominantly expressed on myeloid lineage cells, and act to suppress inflammatory responses (Griffiths *et al.* 2010). In this proof-of-concept study, we explored the hypothesis that CD200 signaling may affect macrophage polarization.

Materials and Methods

Peritoneal macrophage isolation

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health guidelines and with the United States Public Health Service's Policy on Human Care and Use of Laboratory Animals and following the Animals in Research: Reporting *In vivo* Experiments (ARRIVE) guidelines. Macrophages were isolated from the peritoneal cavity of male SD rat (12-13 weeks, Charles River Laboratories). After collecting cells from the cavity, they were seeded on non-coated six-well plates and grown in RPMI medium 1640 containing 10% FBS, 1% penicillin/ streptomycin. On the next day, attached cells were cultured in RPMI medium 1640 without serum for 3 hours and stimulated by rat interferon-γ (IFN-γ, 100 ng/ml) (BioLegend) and lipopolysaccharide (LPS, 100 ng/ml) (Sigma Aldrich) to induce pro-inflammatory macrophage for 72 hours. Human CD200-Fc (10 μg/ml) (Sino Biological Inc.) or control human IgG (10 μg/ml) (Thermo scientific) was co-incubated with pro-inflammatory macrophage.

Immunocytochemistry

Immunocytochemistry was performed as described before (Hayakawa *et al.* 2012). After staining with primary antibody, fluorescent-tagged secondary antibody, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Immunostaining was analyzed with a fluorescence microscope (Nikon ECLIPSE Ti-S) interfaced with a digital charge-coupled device camera and an image analysis system. Antibodies for CREB (1:500, Cell Signaling Technology) and C/EBP-beta (1:200, Santa Cruz Biotechnology) were used.

Western blot analysis

Western blot procedure was performed as described before (Hayakawa et al. 2012). After blocking, membranes were incubated overnight at 4°C with the following primary antibodies: anti-pSTAT1, anti-pSTAT6, anti-Arg1, anti-TGM2, anti-TLR4 and anti-C/EBP-beta (purchased from Santa Cruz Biotechnology); anti-pCREB (1:1000) (obtained from Cell Signaling Technology); IL-6 antibody (1:200) (purchased from Novus biologicals); anti-TGF-β (1:500) (purchased from Abcam). Signals were visualized by peroxidase-conjugated secondary antibodies (GE Healthcare) and chemiluminescence (Thermo Scientific).

Enzyme-linked immunosorbent assays (ELISA)

Multiple cytokine assay using macrophage conditioned media was performed by rat Multi-Analyte ELISArray kit (QIAGEN) or rat TGF-β ELISA kit (ScienCell Research Laboratories). Pro-inflammatory macrophages were induced by IFN-γ (100 ng/ml) and LPS (100 ng/ml) treatment for 72 hours. Then CD200-Fc (10 μg/ml) or control IgG (10 μg/ml) was co-incubated with pro-inflammatory macrophage for another 24 hours, and then conditioned media were collected for ELISA.

Immunoprecipitation

Immunoprecipitation assay was performed between CREB and C/EBP-beta according to the instruction of Pierce Direct IP kit (Thermo SCIENTIFIC). Total protein of 500 μg of Mϕ was incubated with anti-C/EBP-beta antibody or control IgG for 2 hours at 4°C with end-over-end mixing. Then, the bound CREB to C/EBP-beta was detected by SDS-PAGE-western blot method.

siRNA experiment in Mϕ culture

siRNA transfection was performed as described before (Hayakawa et al. 2012). Control siRNA and rat C/EBP-beta siRNA (sense: CCGUUUCGAGCAUUAAAGUTT, antisense: ACUUUAAUGCUCGAAACGGTT or sense: GGCCCUGAGUAAUCACUUATT, antisense: UAAGUGAUUACUCAGGGCCCG) were obtained from Santa Cruz Biotechnology or QIAGEN. Each siRNA was co-incubated with macrophages for 24 hours, and western blot was performed to confirm C/EBP-beta protein level.

Reporter assay

NF-κB and C/EBP reporter assays were performed using Cignal reporter (GFP) assay kit (QIAGEN). Briefly, rat peritoneal macrophages (1×10^5 cells/well) cultured in 96 wells were transfected with NF-κB or C/EBP reporter for 24 hours. After transfection, cells were treated with CD200-Fc or IFN-γ/LPS or IFN-γ/LPS/CD200-Fc for another 24 hours. GFP expression was analyzed by fluorescence microplate reader at Ex (488 nm)/Em (515 nm).

Statistical analysis

Results were expressed as mean ± SD. Multiple comparisons were evaluated by Tukey-Kramer's test after one-way ANOVA. $P<0.05$ was considered to be statistically significant.

Results

It is now well accepted that macrophages can be polarized towards a diverse range of phenotypes after CNS injury or disease (Hu *et al.* 2014, Sica & Mantovani 2012). Here, we tested the hypothesis that as a key component of neuroimmunoregulatory molecules (NIRegs), CD200-Fc can shift classically activated pro-inflammatory macrophages towards alternatively activated forms. Immunostaining confirmed that isolated rat peritoneal macrophages were highly purified (Fig. 1a). Pro-inflammatory macrophages were initially prepared using the standard approach of stimulation with IFN-γ and LPS for 72 hours. Then CD200-Fc was co-incubated with these macrophages for 24 hours, and phenotypes were

examined. CD200-Fc treatment appeared to shift pro-inflammatory macrophages into an elongated cell shape consistent with alternatively activated macrophages (McWhorter *et al.* 2013) (Fig. 1b). Western blots showed that CD200-Fc upregulated Arg1 and TGM2 but suppressed TLR4 (Fig. 1c and 1d). In addition, immunocytochemistry confirmed that CD200 treatment increased Arg1 positive macrophages in IFN-γ/LPS-stimulated macrophages (Fig. 1e). Next, we assessed macrophage soluble factors using multiple cytokine array analysis (Supplementary Fig. 1). In IFN-γ/LPS-stimulated macrophages, levels of IL-1α, IL-1β, IL-6, and GM-CSF were increased; CD200-Fc treatment appeared to suppress these elevations of IL-1β, IL-6, and GM-CSF. On the other hand, CD200-Fc significantly increased TGF-β secretion in the pro-inflammatory phenotype. CD200 treatment did not affect IL-2, IL-4, IL-10, IL-12, IL-13, TNF-α, and RANTES.

Next, we investigated the cellular signaling and transcriptional activity following CD200-Fc stimulation in macrophages. Phosphorylation of STAT1 is known to increase expression of pro-inflammatory M1-like markers in macrophages, whereas STAT6 phosphorylation may reflect M2-like macrophages (Sica & Mantovani 2012). In our model system, CD200 enhanced CREB phosphorylation but neither STAT1 nor STAT6 (Fig. 2a and 2b). Because CREB may closely link to C/EBP-beta transcriptional activation in M2-like macrophages (Ruffell *et al.* 2009), we assessed this pathway in our model system. Immunocytochemistry and immunoprecipitation analysis demonstrated an interaction between CREB and C/EBP-beta after CD200-Fc treatment (Fig. 2c-2e). Consistent with this proposed shift in macrophage polarization, NF-κB activity was significantly increased in IFN-γ/LPS-stimulated macrophages, whereas CD200-Fc enhanced C/EBP-beta transcriptional activity and suppressed NF-κB activity (Fig. 2f).

The prototype anti-inflammatory cytokine TGF-β is known to be released by alternatively activated macrophages. Treating our IFN-γ/LPS-stimulated macrophages with CD200-Fc also upregulated intracellular TGF-β (Fig. 3a and 3b). To assess causality, we used siRNA to interfere with these pathways. We confirmed that our siRNA sequence efficiently and specifically suppressed C/EBP-beta (Supplementary Fig. 2 and Supplementary Fig. 3). Compared to control scrambled siRNA, siRNA suppression of C/EBP-beta blocked CD200-Fc-induced elevations in TGF-β levels (Fig. 3a and 3b, Supplementary Fig. 3). Additionally, upregulation of Arg1 and TGM2 following CD200-Fc treatment were canceled when C/EBP-beta was suppressed by siRNA (Fig. 3c and 3d, Supplementary Fig. 3). This suggests that CD200 treatment may enhance CREB-C/EBP-beta signaling and thus polarize macrophages toward an anti-inflammatory phenotype along with TGF-β upregulation (Fig. 3e).

Discussion

In recent years, it has been well accepted that systemic responses outside the brain are important after CNS injury. Peripheral monocytes/macrophages can cross the disrupted BBB and influence the progression of brain injury. However, the role of these systemic macrophages remains complex and poorly understood. Under some circumstances, macrophages play positive roles by assisting with debris clean-up and tissue repair. But under different circumstances, macrophages can have deleterious roles by exacerbating

neuroinflammation and secondary injury. Hence, an important goal is to find ways to modulate macrophage polarization so as to shift them towards beneficial phenotypes. In the present study, we showed that (i) CD200-Fc treatment upregulated Arg1, TGM2, TGF-β, and decreased TLR4, IL-1β, IL-6, and GM-CSF in IFN-γ/LPS-stimulated macrophages, (ii) CD200-Fc enhanced CREB phosphorylation and C/EBP-beta activity and suppressed inflammatory NF-κB activity, and (iii) C/EBP-beta suppression in CD200-Fc-stimulated macrophages decreased Arg1, TGM2, and TGF-β. These findings provide proof-of-concept that CD200 signaling may play a key role in regulating macrophage polarization toward Arg1-, TGM2-, and TGF-β-positive alternatively activated forms.

Recently, it has been shown that the multifunctional enzyme transglutaminase 2 (TGM2) and arginase I (Arg1) are conserved markers which provide a consistent activation biomarker for both human and mouse M2 macrophages in prototypic Th2 pathology asthma (Martinez *et al.* 2013). In addition, emerging data indicate that upregulation of TGF-β signaling reflects alternative activation in macrophage in vivo and in vitro (Sica & Mantovani 2012, Gong *et al.* 2012). Moreover, macrophage-derived TGF-β is essential for reducing infarct bleeding and restoring neurovascular function in murine stroke model (Gliem *et al.* 2012). Hence, it is possible that TGM2, Arg1 and TGF-β may represent surrogate markers for an alternatively activated phenotype in our study. Here, our data showed that CD200 may upregulate TGM2, Arg1 and TGF-β in IFN-γ/LPS-induced pro-inflammatory macrophages. From a basic science perspective, this finding may be important because it provides a novel pathway for macrophage regulation. From a translational perspective, this finding may be relevant because it provides a potential method for shifting macrophages and promoting recovery in damaged and diseased CNS.

Traditionally, CD200-CD200 receptor interaction is thought to induce inhibitory signals through an inhibition of the Ras-MAPK pathway (Zhang *et al.* 2004). In vivo, CD200 expressing reactive astrocytes may provide immunosuppression to restrict further brain damage in multiple sclerosis (Chitnis *et al.* 2007). CD200-deficiency may promote classical activation of microglia and increase BBB permeability along with immune cells infiltration into the brain (Denieffe *et al.* 2013). More recently, we found that in vivo CD200-Fc administration boosts white matter recovery by suppressing macrophage attack and phagocytosis of susceptible oligodendrocyte precursors through TLR4 downregulation (Hayakawa *et al.* 2015). Taken together, these previous studies suggest that CD200 may promote the shifting of macrophages into potential beneficial forms. In this present study, we showed that CD200 may be linked to networks of signaling molecules and transcription factors that have been proposed to be involved in macrophage polarization. Canonical IRF/STAT1 and NF-κB signaling pathways activated by IFN-γ and LPS increase pro-inflammatory phenotype, while STAT6 and C/EBP-beta pathways induce alternatively activated M2-like phenotype (Sica & Mantovani 2012, Ruffell et al. 2009). Indeed, we found that CREB was highly phosphorylated by CD200-Fc, whereas STAT6 was not phosphorylated. It is known that phosphorylated CREB functionally regulates C/EBP-beta transcription factor (Niehof *et al.* 1997) and directly inhibits NF-κB activation (Wen *et al.* 2010). Intriguingly, when macrophage NF-κB was activated by IFN-γ and LPS stimulation, CD200 suppressed inflammatory cytokines such as IL-1β, IL-6, and GM-CSF. But CD200

treatment in control macrophages did not downregulate IL-1β and IL-6, suggesting that CD200 treatment may induce gene expression regulated by both C/EBP-beta-dependent mechanism and CREB-mediated NF-κB inhibition. The ability of CD200 to shift pro-inflammatory macrophages towards anti-inflammatory phenotype appeared to be dependent on upregulation of C/EBP-beta and downregulation of NF-κB signaling.

Nevertheless, there are several issues that warrant further consideration. First, macrophage polarization is highly complex beyond a simple good-versus-bad dichotomy (Hu et al. 2014, Sica & Mantovani 2012). Instead macrophage phenotypes comprise a large spectrum that spans from classically activated pro-inflammatory forms (M1-like) to alternatively activated forms (M2-like). M1-like macrophages may release proinflammatory cytokines and worsen brain damage, while M2-like macrophages may accelerate CNS repair through debris clean-up and producing pro-neurogenic and pro-recovery factors such as TGF-β (He *et al.* 2014). However, beyond M1-like or M2-like phenotype per se, macrophages which do not fit into artificially-defined M1 and M2 dichotomies will surely exist. For example, dermal wound repair may be promoted by a complex of macrophage phenotypes which are associated with alternative and classical activation (Daley *et al.* 2010). After ischemic stroke, CD200 positive macrophages express both M1-like and M2-like phenotype, suggesting the presence of “halfway activated macrophage” or unknown signals that induce a third phenotype in inflammatory circumstances. To determine macrophage phenotype, functional endpoints are required, not just the measurement of markers. Nevertheless, our data here may provide a potential mechanism for the potentially beneficial actions of the CD200-shifted macrophage that we previously reported (Hayakawa *et al*. 2015). Second, downregulation of NF-κB may lead to complex downstream effects. NF-κB pathways are involved in a wide spectrum of CNS responses. How these network signals modulate the good-vs-bad balance of inflammation in injured brain tissue requires further study. Third, how would CD200 treatment fit with the natural timing of macrophage evolution after stroke? Recent reports suggest that a complex spatial and temporal balance exists between M1-like and M2-like macrophages after stroke (Hu *et al.* 2012). Further studies are needed to determine how to optimize CD200 signaling in the context of this natural evolution in macrophage profiles. Fourth, our study is only focused on macrophages. However, after stroke and brain injury, resident microglia also play key roles. How CD200 may affect microglia phenotypes should be carefully assessed. Finally, our proof-of-concept study only suggests that CD200 and CREB signaling can help polarize macrophages towards a more M2-like form. But whether and how these CD200-shifted macrophages can actually protect and repair injured neurons remains to be tested.

In conclusion, this study provides proof-of-concept that CD200 signaling may switch macrophage phenotype from pro-inflammatory phenotype to anti-inflammatory phenotype. Regulating this mechanism of CD200-CD200 receptor signaling may lead to new therapeutic opportunities to increase function in potentially beneficial macrophages and promote brain repair processes after CNS injury and disease.

ARRIVE guidelines have been followed:

Yes

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=> if Yes, insert “All experiments were conducted in compliance with the ARRIVE guidelines.”

Conflicts of interest: none

=> if ‘none’, insert “The authors have no conflict of interest to declare.”

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviation

Arg1	arginase 1
TGM2	transglutaminase 2
IFN-γ	interferon γ
LPS	lipopolysaccharide
TLR4	toll-like receptor 4
TGF-β	transforming growth factor beta 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
IL-10	interleukin 10

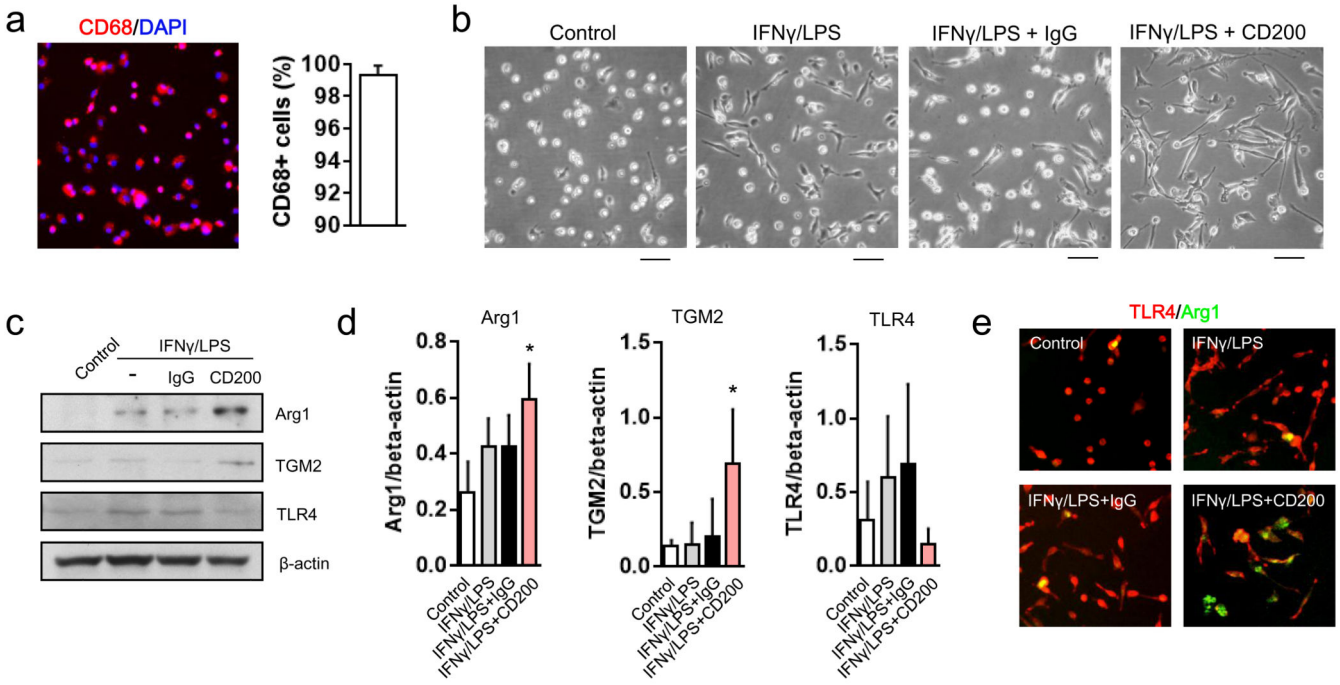

Figure 1. Upregulation of alternative activation markers in rat peritoneal macrophage (Mϕ) following human CD200-Fc treatment. (a)

Rat peritoneal macrophages were stained by a macrophage marker, CD68. Immunostaining confirmed the purified macrophage culture in this study. **(b)** Rat macrophages were initially stimulated by IFN-γ (100 ng/ml) and LPS (100 ng/ml) for 72 hours to induce classical pro-inflammatory phenotype. Treatment with CD200-Fc (10 μg/ml) in IFN-γ/LPS-stimulated macrophage changed cell shape to elongated phenotype. Scale: 50 μm. **(c and d)** Western blot confirmed upregulation of Arg1 and TGM2 following CD200-Fc (10 μg/ml) treatment. Individual signal was normalized by β-actin (n=4). **(e)** Immunocytochemistry showed that Arg1 positive macrophages were increased following CD200 treatment in IFN-γ/LPS-stimulated Mϕ. **P*<0.05, ***P*<0.01 vs control. All data are presented as mean±SD.

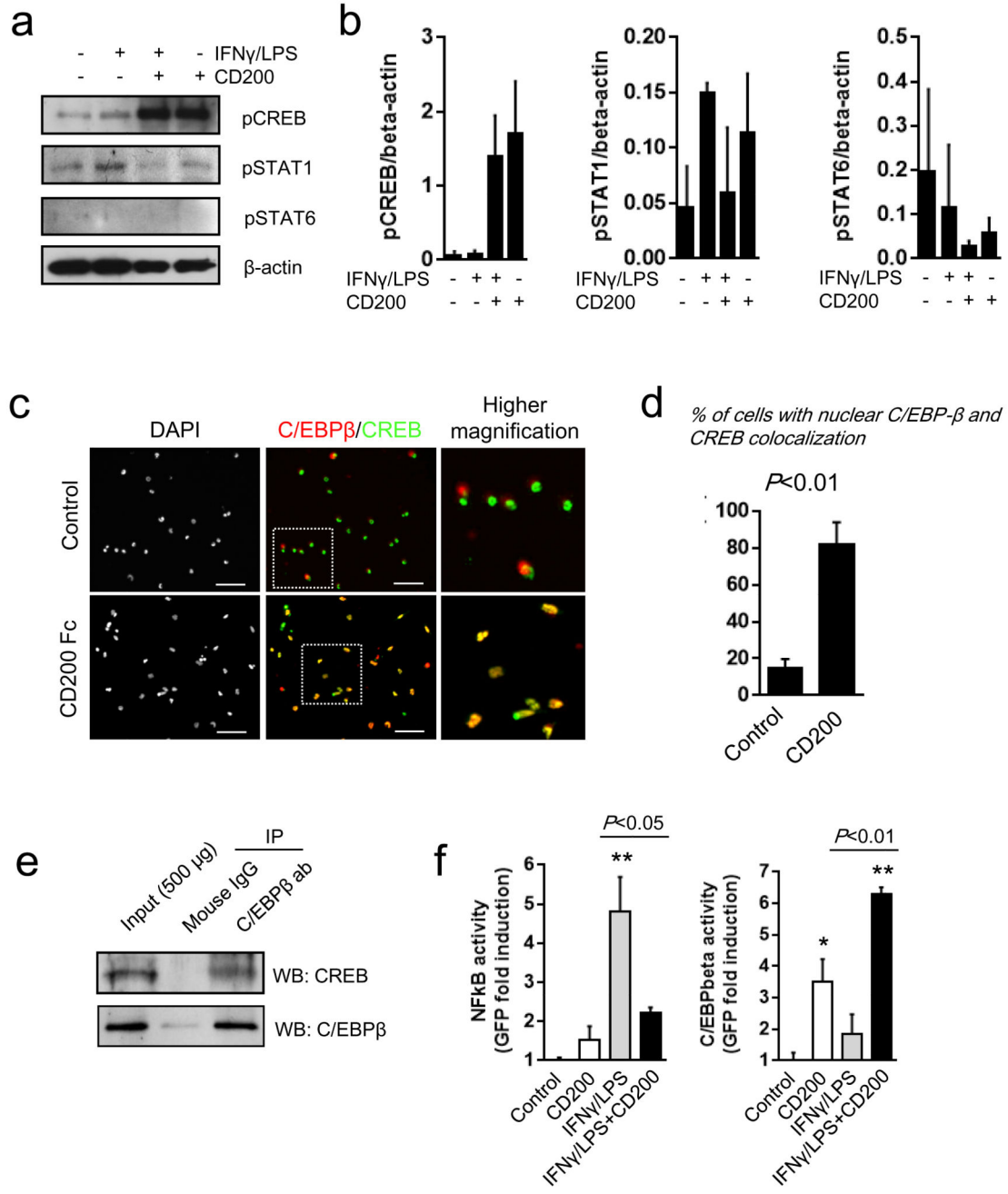

Figure 2. CD200-Fc promoted CREB phosphorylation and C/EBP-beta transcriptional activity, while NF-κB activity was suppressed by CD200 treatment in IFN-γ/LPS-stimulated Mϕ. (a and b)

Western blot analysis confirmed that CD200 (10 μg/ml) promoted CREB phosphorylation in IFN-γ/LPS-stimulated Mϕ (n=3). **(c and d)** Immunocytochemistry demonstrated that CD200-Fc (10 μg/ml) induced translocation of CREB into nucleus that was co-localized with transcription factor, C/EBP-beta. Scale: 100 μm. **(e)** Immunoprecipitation analysis confirmed that C/EBP-beta bound to CREB following CD200 (10 μg/ml) stimulation. **(f)** IFN-γ/LPS-stimulated Mϕ increased NF-κB transcriptional activity. CD200 (10 μg/ml)

clearly attenuated NF-κB activity (Control: n=6, CD200-Fc: n=6, IFN-γ/LPS: n=5, IFN-γ/LPS/CD200-Fc: n=5). Importantly, CD200-Fc (10 μg/ml) markedly increased C/EBP-beta activity (Control: n=4, CD200-Fc: n=4, IFN-γ/LPS: n=4, IFN-γ/LPS/CD200-Fc: n=4). **P*<0.05, ***P*<0.01 vs control. All data are presented as mean±SD.

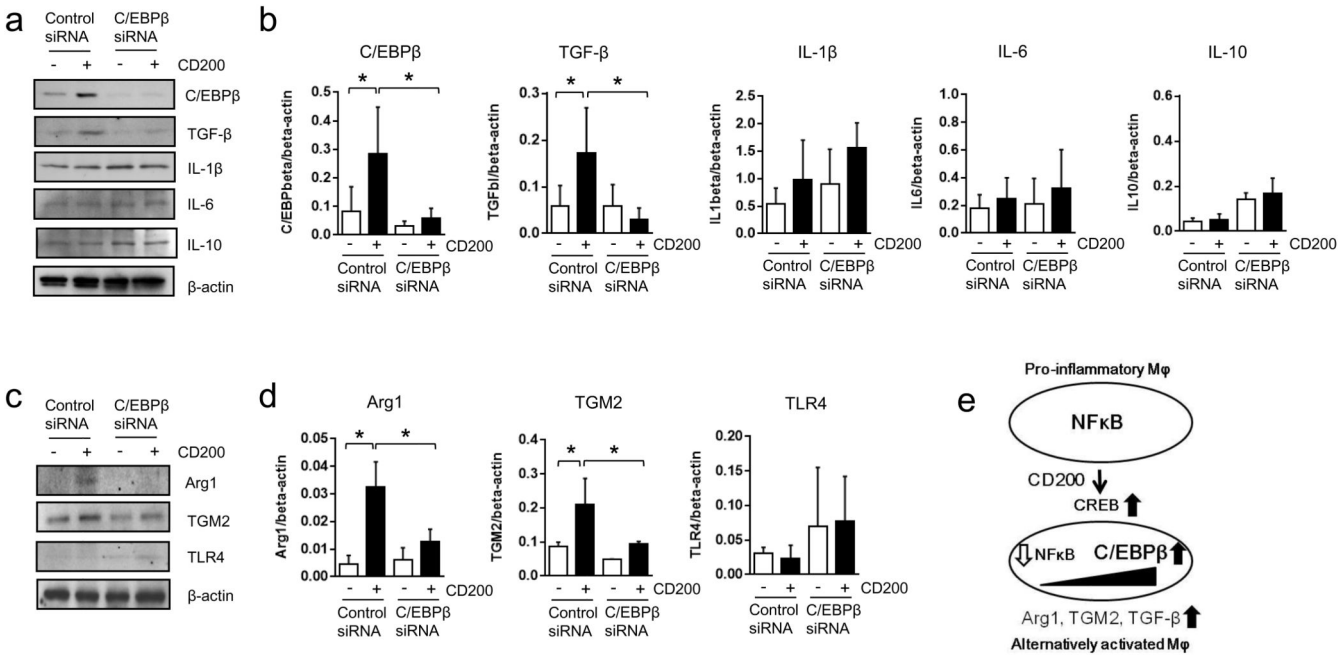

Figure 3. CD200-Fc upregulates TGF-β through a transcription factor, C/EBP-beta: (a) Representative images of western blot. **(b)** Data analysis showed that CD200-Fc (10 μg/ml) treatment increased C/EBP-beta protein, and siRNA transfection to suppress the C/EBP-beta was successfully performed (n=4). CD200-Fc (10 μg/ml) significantly increased TGF-β, and C/EBP-beta siRNA suppressed this effects (Control siRNA: n=7, Control siRNA + CD200-Fc: n=7, C/EBP-beta siRNA: n=4, C/EBP-beta siRNA + CD200: n=4). CD200 - C/EBP beta signaling did not affect IL-1β, IL-6, IL-10 expression (Control siRNA: n=11, Control siRNA + CD200-Fc: n=11, C/EBP-beta siRNA: n=4, C/EBP-beta siRNA + CD200-Fc: n=4). All data are presented as mean±SD. **(c and d)** CD200-Fc (10 μg/ml) significantly increased Arg1 and TGM2, and C/EBP-beta siRNA suppressed this effects. **(e)** Schematic for the hypothesis. CD200 may shift pro-inflammatory macrophage to alternatively activated macrophage through CREB-C/EBP-beta signaling pathway.