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Article

Immune Inhibitory Ligand CD200 Induction by TLRs and NLRs Limits Macrophage Activation to Protect the Host from Meningococcal Septicemia

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SUMMARY

Macrophage activation is essential for protection against bacterial pathogens but needs to be regulated to prevent damage to the host. We show a key role for the immune inhibitory receptor CD200R and its ligand CD200 in the context of infection with the Gram-negative human pathogen *Neisseria meningitidis*. *N. meningitidis* induced CD200 but downregulated CD200R on macrophages in a manner dependant on *Neisserial* lipopolysaccharide, Toll-like receptor-4 (TLR-4), and the MyD88 pathway but independent of a known *Neisserial* receptor, scavenger receptor A (SR-A). Agonists of the pattern-recognition receptors nucleotide oligomerization domain 2 (NOD2) and NACHT-LRR protein 3 (NALP3) also induced CD200. The NF-κB member c-Rel was essential for TLR-, NOD2-, and NALP3-mediated induction of CD200. $CD200^{-/-}$ animals showed higher lethality in response to experimental meningococcal septicemia, induced higher levels of proinflammatory cytokines, and recruited increased numbers of activated leukocytes, despite comparable bacterial clearance. Thus CD200 is induced by TLR-, NOD2-, and NALP3-mediated pathways, limiting their function and protecting the host from excessive inflammation.

INTRODUCTION

Activated macrophages (Mϕ) initiate phagocytosis and inflammation in response to infection and injury, promoting killing and repair. However, uncontrolled host responses often lead to inflammatory pathologies. Pathogen-sensing pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs, including NOD1/2 and NALPs) induce "innate immune activation" of Mϕ after recognition of microbial or endogenous "danger" molecules (Creagh and O'Neill, 2006). Phagocytic PRRs such as scavenger receptors (SRs) and C-type lectin receptors (CLRs) contribute to innate activation either by collaboration with TLRs and NLRs or by directly modulating adhesion and migration of Mϕ (Mukhopadhyay et al., 2004; Underhill, 2003). In addition to innate activation, T helper 1 (Th1) and Th2 lymphocyte-derived cytokines interferon-γ (IFN-γ) and interleukin-4 (IL-4) can induce distinct "classical" (Mosser, 2003) and "alternative" (Gordon, 2003; Martinez et al., 2009) Mϕ activation, respectively, and further regulate inflammatory responses of innately activated Mϕ. Functional dysregulation of PRRs and of IFN-γ and IL-4 pathways has been implicated in a variety of inflammatory disorders (Farrar and Schreiber, 1993; Karin et al., 2006; Wynn, 2003). Therefore, endogenous regulatory pathways must exist to protect the host by limiting excessive inflammation. Indeed, a number of negative regulators of inflammation have been identified, but their therapeutic potential remains to be determined (Han and Ulevitch, 2005; Nathan, 2002).

Numerous inhibitory receptors have been described on myeloid cells and are likely to be involved in the control of immune responses (Ravetch and Lanier, 2000; Yamada and McVicar, 2008). CD200 and its receptor CD200R is one of the best-characterized immune inhibitory ligand-receptor pairs (Barclay et al., 2002; Nathan and Muller, 2001). Manipulation of this pathway has been shown to have therapeutic promise in experimental models of chronic inflammation (Gorczynski, 2005). CD200 and CD200R are both cell-surface glycoproteins that contain two immunoglobulin superfamily domains. CD200 is broadly expressed on many cell types, whereas CD200R expression is present on most leukocytes but at higher levels on myeloid cells. Interaction between CD200 and CD200R through their membrane-distal Ig domains transmits an inhibitory signal to the myeloid cells through CD200R, which contains three conserved tyrosine residues. By contrast, CD200 is thought to be signal incompetent, because of its short intracellular tail without any known signaling motifs. $CD200^{-/-}$ animals show increased susceptibility to a range of autoimmune and inflammatory diseases (Hoek et al., 2000). This is due to loss of ligand-induced inhibitory signaling through CD200R. A similar phenotype has been seen in wild-type (WT) animals after

perturbation of CD200-CD200R interaction by blocking antibodies (Wright et al., 2000) and confirmed by the phenotype of mice lacking CD200R (Boudakov et al., 2007). By contrast, infusion of CD200-Ig fusion proteins and agonistic antibody to CD200R ameliorated even established pathologies (Copland et al., 2007; Snelgrove et al., 2008). These findings confirmed a nonredundant myeloid-specific inhibitory function for CD200-CD200R interaction. Most of the studies utilized chronic autoimmune models of disease, usually driven by antigen-specific lymphocytes, yet no evidence was found for primary lymphocyte hypersensitivity, and hyperactivated Mϕ populations almost always dominated inflammation (Hoek et al., 2000; Wright et al., 2000). The essential requirement for adjuvants in the induction of disease further points to a role for innate immune activation of Mϕ (Nathan and Muller, 2001).

We investigated how CD200 and CD200R are regulated during innate and cytokine-mediated Mϕ activation and defined the role of CD200 in modulating various innate receptor pathways to limit inflammation. We report that the pathogen-sensing PRRs TLRs, nucleotide oligomerization domain 2 (NOD2), and NACHT-LRR protein 3 (NALP3), but not phagocytic scavenger receptor A (SR-A), differentially upregulate CD200 and downregulate CD200R expression on innately activated Mϕ through the NF-κB family transcription factor c-Rel. In turn, CD200-CD200R interaction specifically inhibits TLR-, NOD2-, and NALP3-mediated innate inflammatory responses, both ex vivo and in vivo, and is essential to protect the host from experimentally induced lethal meningococcal septicemia.

RESULTS

Neisseria meningitidis Induces CD200 and Downregulates CD200R Expression on Various Mϕ Populations

We compared CD200 and CD200R expression on defined Mϕ populations such as biogel-elicited peritoneal Mϕ (Bg-PM), thioglycollate-elicited peritoneal Mϕ (TPM), and bone marrow culture-derived Mϕ (BMDM) after innate (*Neisseria meningitidis* [NM] stimulated), classical (IFN-γ-treated), and alternative (IL-4-treated) activation. Analysis showed no CD200 surface expression in unstimulated BMDM and Bg-PM from Balb/c mouse strain (Figure 1A, and low level basal expression in Bg-PM from C57BL/6J strain (Figure 1B). However, irrespective of genetic background, CD200 expression was significantly induced in these Mϕ populations after NM treatment (Figure 1A and data not shown). In contrast, CD200 was constitutively expressed at high levels on Mϕ elicited with thioglycollate broth (TPM), and NM treatment failed to further increase CD200 expression in this population (Figure 1A and data not shown). Interestingly, IFN-γ or IL-4 treatment did not induce CD200 in any of these Mϕ populations (Figure S1, available online), indicating that CD200 is an inducible and selective marker for Mϕ innate activation. In contrast to CD200, all three Mϕ populations (Bg-PM, BMDM, and TPM) constitutively expressed high levels of CD200R, which showed a marked downregulation after NM treatment in Bg-PM and TPM and to a lesser extent in BMDM (Figure 1A). We have confirmed that downregulation in CD200R expression was not due to enhanced cell death following stimulation (data not shown).

Neisseria meningitidis Differentially Regulates CD200 and CD200R, Independent of SR-A, but Does Require the LPS-TLR-4-MyD88 Signaling Axis

TLR-4 and SR-A are two major PRRs on Mϕ that recognize NM through lipopolysaccharide (LPS)- and -independent mechanisms, respectively (Peiser et al., 2002). To determine the mechanism by which NM differentially regulates CD200 and CD200R, we tested the potential contributions of SR-A, TLR-4 pathways, and *Neisserial* LPS. No difference in CD200 induction or CD200R downmodulation was observed between NM-treated WT and SR-A$^{-/-}$ Bg-PM (Figure 1B), indicating that SR-A was not essential for NM-mediated regulation of these two molecules.

To test potential involvement of *Neisserial* LPS on CD200 and CD200R regulation, we stimulated Bg-PM from WT mice with either an LPS-sufficient NM strain *H44/76* or its LPS-deficient isogenic mutant strain *H44/76lpxA* and analyzed CD200 and CD200R expression. The *H44/76lpxA* strain failed to upregulate CD200 or downregulate CD200R expression on Mϕ (Figure 1C), indicating that *Neisserial* LPS is essential for differential regulation of these two surface proteins.

The role of TLR-4 in the regulation of CD200 and CD200R was investigated by comparing CD200 and CD200R expression on Bg-PM between C3H/HeJ (lacking functional TLR-4) and C3H/HeN (corresponding WT control) mouse strains after stimulation with the LPS-sufficient NM strain MC58. NM failed to induce CD200 and downregulate CD200R on TLR-4-deficient Mϕ, confirming the essential requirement of TLR-4 (Figure 1D). TLR-4 utilizes both MyD88- and TRIF-dependent signaling pathways. NM failed to induce CD200 or downmodulated CD200R expression in MyD88$^{-/-}$ Mϕ (Figure 1E), confirming that the MyD88-dependent TLR-4 signaling pathway is responsible for differential regulation of CD200 and CD200R. We further confirmed that CD200R downregulation is independent of CD200, since NM-mediated CD200R downregulation occurs efficiently in CD200$^{-/-}$ Mϕ (data not shown).

The NF-κB Family Member c-Rel Is Essential for TLR-Mediated Transcription of CD200 mRNA

To determine the mechanism controlling induction of CD200, we investigated whether LPS-mediated induction of CD200 is transcriptionally regulated and the role of c-Rel, a key member of the NF-κB transcription factor family, downstream of MyD88. LPS stimulation induced CD200 mRNA in WT BMDM. However, CD200 transcription was profoundly deficient in BMDM from c-Rel$^{-/-}$mice (Figure 2). This suggests that c-Rel controls the transcriptional activation of the CD200 gene downstream of MyD88. A range of other TLR agonists such as PGN (TLR-2/6), Poly I:C (TLR-3), and CpG DNA (TLR-9) also induced CD200 mRNA in a c-Rel-dependent manner. Thus, TLR signaling can induce CD200 transcription, and that depends on c-Rel. In contrast to their effects on CD200 expression, various TLR agonists showed only modest downregulation of CD200R mRNA synthesis in both WT and c-Rel$^{-/-}$ Mϕ (data not shown). These data suggest that although CD200 upregulation depends on c-Rel-mediated new mRNA synthesis, downregulation of CD200R is not due to reduced transcription of the *cd200R* gene and is possibly regulated by other cellular mechanisms such as altered receptor recycling, degradation, or mRNA stability.

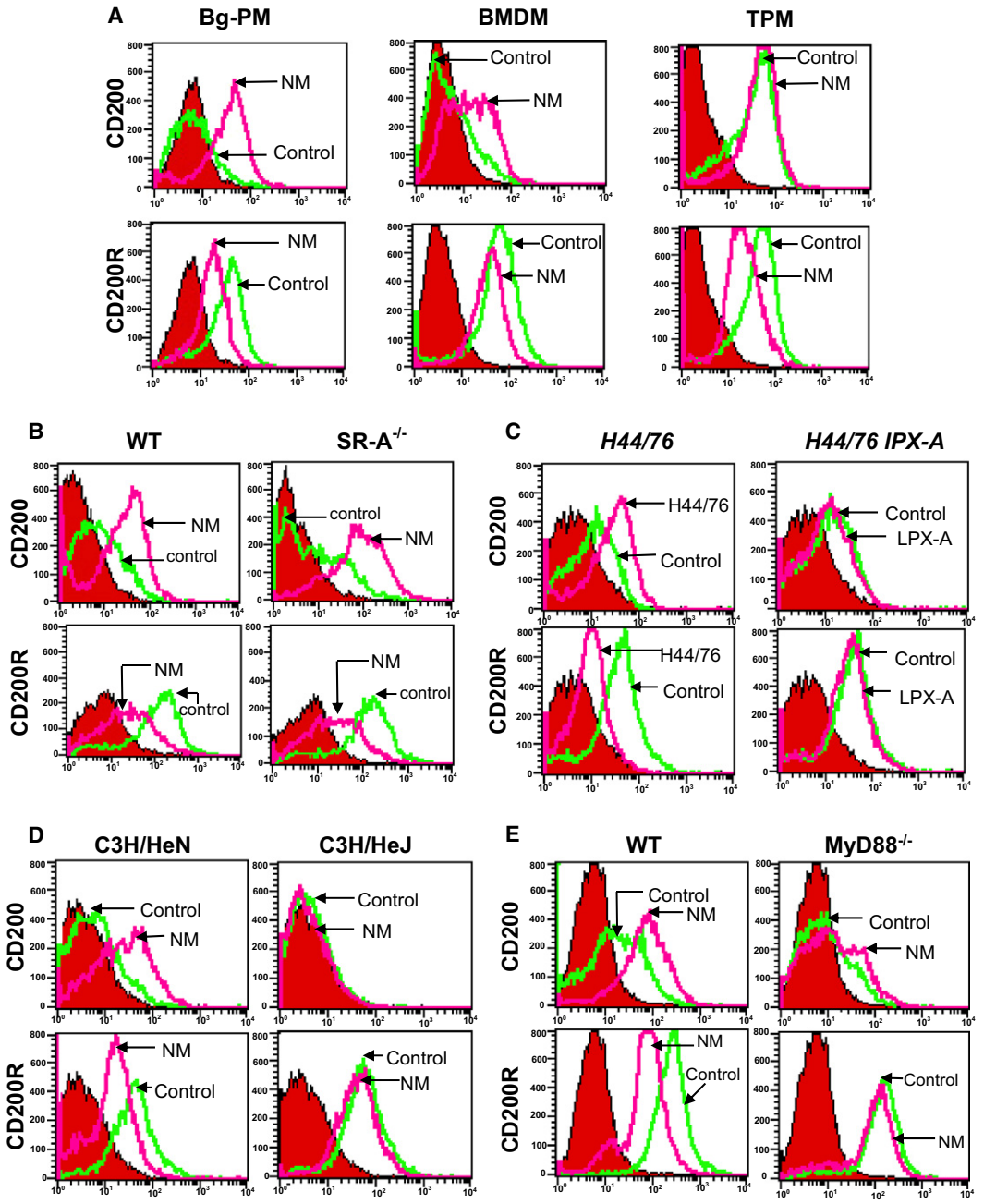

Figure 1. NM Treatment Differentially Regulates Surface Expression of CD200 and CD200R on Various Elicited Mϕ Populations

The pink lines on histograms show surface expression after NM treatment. Green lines and filled histograms represent expression in untreated control cells and after staining with isotype-matched control mAb, respectively. Each treatment condition was analyzed in triplicates and data are representative of three independent experiments.

(A) Flow cytometry showing surface expression of CD200 and CD200R on Bg-PM, BMDM, and TPM isolated from Balb/c mice after NM treatment (20 NM/Mϕ).

(B) Flow cytometry showing that the differential regulation of CD200 and CD200R surface expression is the same on both WT and SR-A$^{-/-}$ Bg-PM after NM treatment (20 NM/Mϕ); therefore, regulation is independent of SR-A.

(C) Flow cytometry showing CD200 and CD200R expression on Bg-PM after stimulation with a WT (*H44/76*) or a LPS mutant (*H44/76lpxA*) NM strain indicates that *Neisserial* LPS is essential for differential regulation of CD200 and CD200R.

(D) Flow-cytometry analysis showing CD200 and CD200R expression on Bg-PM from TLR-4 mutant C3H/HeJ and WT C3H/HeN mice, after NM treatment. Histogram shows that TLR-4 is essential for differential regulation of CD200 and CD200R expression.

(E) Flow-cytometry analysis of CD200 and CD200R expression on Bg-PM from WT and MyD-88$^{-/-}$ mice after NM treatment shows that MyD-88 is essential for differential regulation of CD200 and CD200R.

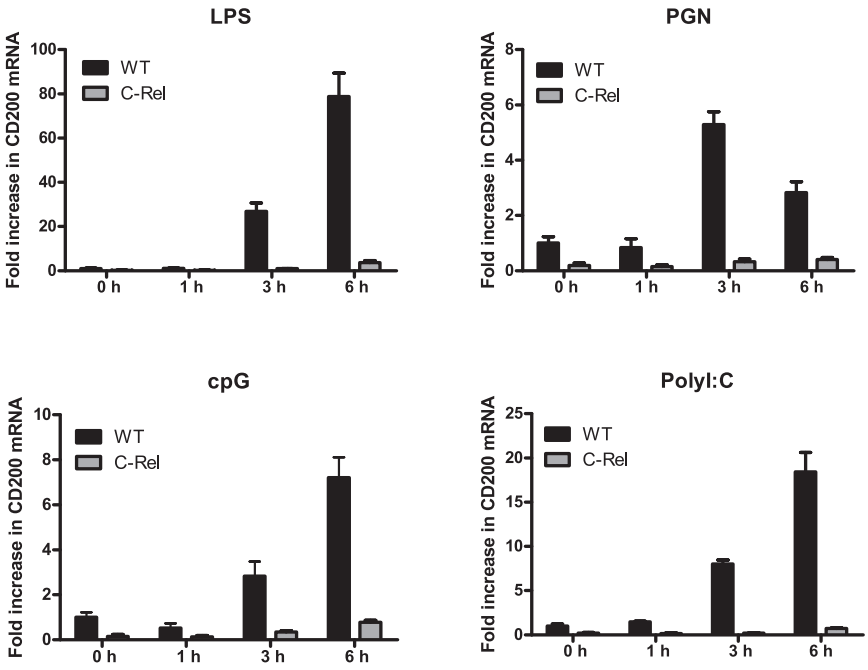

Figure 2. NF-κB Family Member c-Rel Is Essential for CD200 Induction by Specific TLR Agonists

Fold changes in CD200 mRNA expression are shown after stimulating BMDM from WT and c-Rel$^{-/-}$ mice with agonists of selected TLRs, LPS, CpG, peptidoglycan, and poly I:C for indicated times. Data are shown as mean ± SD, with 3 mice per group. Each treatment condition was analyzed in triplicate and data are representative of three independent experiments.

CD200$^{-/-}$ Mice Are More Susceptible to Experimental Meningococcal Septicemia

We studied the in vivo role of CD200 in acute inflammation induced by *N. meningitidis*, by using a well-characterized murine model of meningococcal septicemia with the serogroup B strain of *N. meningitidis* MC58. Both WT and CD200$^{-/-}$ strains showed signs of infection but at 40 hr after injection, the health of the CD200$^{-/-}$ animals declined dramatically and almost all the animals in this group had to be culled (Figure 3A). In contrast, 38.6% of the WT animals recovered and remained healthy until the experiment was terminated at 120 hr (Figure 3B). No significant differences were observed in the levels of bacteremia in blood and in the spleen of CD200$^{-/-}$ animals compared to WT animals (Figure 3C). However, levels of IL-6, a marker for systemic inflammation, were significantly higher in CD200$^{-/-}$ mice than in WT mice (Figure 3D). Taken together, these data suggest that the enhanced susceptibility of CD200$^{-/-}$ mice is associated with an exacerbated systemic inflammatory response rather than higher levels of bacteremia.

CD200$^{-/-}$ Mice Show an Enhanced Inflammatory Response during Sterile Peritonitis Induced by Inactivated NM

To define the early events of NM-mediated inflammatory response in vivo, we adopted a sterile peritonitis model utilizing inactivated NM and analyzed inflammatory cell recruitment and cytokine responses 2 hr after injection. phosphate-buffered saline (PBS)-injected CD200$^{-/-}$ mice showed significantly higher numbers of F4/80^{hi}CD11b^{hi}GR-1^{-ve} resident peritoneal Mϕ (these cells were also negative for CD11c and CD19) in their peritoneal cavity compared to WT mice. The number of resident Mϕ decreased in both strains after NM injection, possibly because of their emigration into lymphatics (Figure 4A). Resident Mϕ from PBS-injected CD200$^{-/-}$ animals expressed higher levels of major histocompatibility complex class II (MHC-II) constitutively. After NM injection, MHC-II was enhanced on this population in both strains, but induction was significantly higher in CD200$^{-/-}$ animals (Figure 4A, inset). In contrast to resident Mϕ, inflammatory monocyte (F4/80^{low}CD11b^{+ve}GR-1^{med}) and neutrophil (CD11b^{hi}GR-1^{hi}F4/80^{-ve}) populations were recruited in both mouse strains, but induced recruitment was significantly higher in CD200$^{-/-}$ mice compared to the WT strain (Figure 4A). Higher levels of TNF-α and IL-6 were present in the lavage fluid from NM-injected CD200$^{-/-}$ mice compared to WT mice (Figure 4B). No TNF-α or IL-6 was detected in the PBS-injected groups of either strain. To exclude the possibility that phagocytes from CD200$^{-/-}$ mice were more efficient in engulfing NM and therefore produced more cytokines, we compared uptake of NM in vivo between the two strains. Mice were injected intraperitoneally with fluorescently labeled inactivated NM, peritoneal exudate cells were isolated and fixed, and total NM uptake was measured by flow cytometry. No difference in uptake was observed between the two strains (Figure 4B). To evaluate the inflammatory response of CD200$^{-/-}$ Mϕ in isolation, we stimulated Bg-PM from WT and CD200$^{-/-}$ mice with different doses of inactivated NM for 16 hr and measured TNF-α and IL-6 levels in culture supernatants. CD200$^{-/-}$ Mϕ consistently produced significantly higher levels of TNF-α and IL-6, compared to WT Mϕ (Figure 4C). Similar to the in vivo experiments, WT and CD200$^{-/-}$ Bg-PM showed no significant difference in NM uptake in vitro (Figure 4C). Thus CD200$^{-/-}$ Mϕ show an exaggerated inflammatory response to NM, independent of uptake.

CD200$^{-/-}$ Mϕ Are Hyperresponsive to Purified TLR Agonists

To test whether the hyperinflammatory response of CD200$^{-/-}$ Mϕ extended to defined TLR agonists, we stimulated Bg-PM from WT and CD200$^{-/-}$ mice with different concentrations of selected TLR agonists, such as LPS (TLR-4), PGN (TLR-2/6), and poly I:C (TLR-3), and measured the levels of TNF-α and IL-6 in culture supernatants. Results confirmed that CD200$^{-/-}$ Bg-PM produced significantly higher levels of TNF-α and IL-6 in response to all three TLR agonists (Figure 5). Since CD200$^{-/-}$ Mϕ showed a heightened cytokine response to both

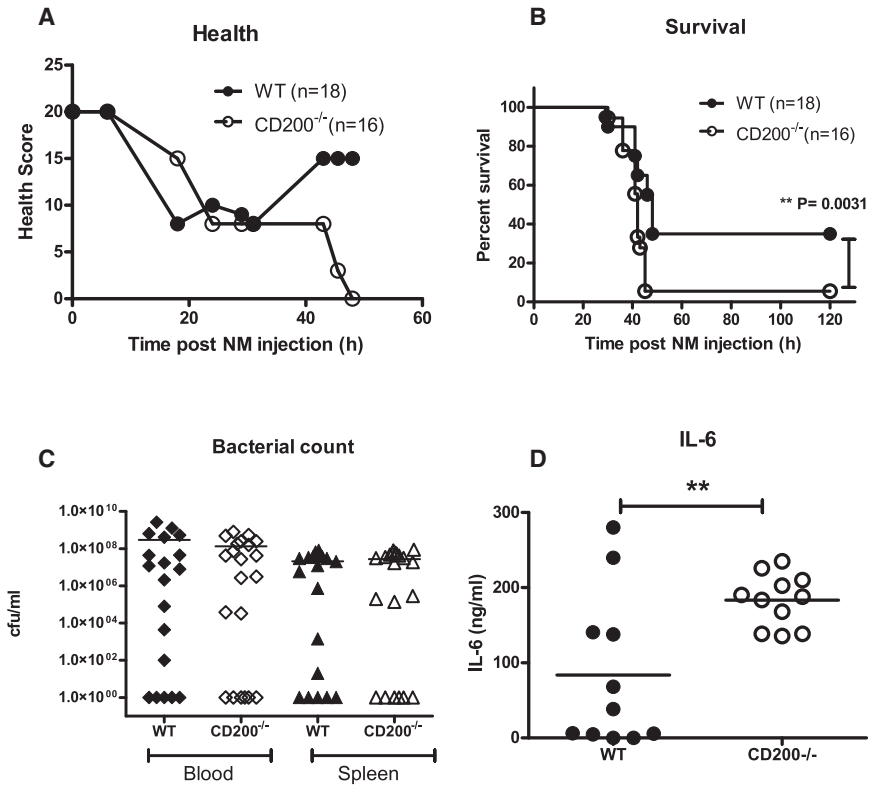

Figure 3. CD200$^{-/-}$ Mice Are More Susceptible to Meningococcal Septicemia

Cohorts of WT and CD200$^{-/-}$ mice were i.p. inoculated with live NM.

(A) The health of the animals was monitored regularly and scored by a semiquantitative method. Collated scores for each time point are plotted.

(B) The survival of each group was plotted, and the statistical comparison was performed with the log-rank (Mantel Cox) test ($p = 0.0031$).

(C) Blood was collected by cardiac puncture at termination, and serial dilutions were plated on bacterial growth medium. Spleen segments were also collected, homogenized, and plated. Plates were incubated at 37°C, 5% CO_2 overnight, and bacterial colonies counted. Results were analyzed with a two-tailed, unpaired t test. No significant difference in bacterial count was observed between two mouse strains.

(D) The concentration of IL-6 in plasma from infected animals was measured at termination with an ELISA. Results were analyzed with a two-tailed, unpaired t test to determine statistical significance. **, $p < 0.01$.

MyD88-dependent and -independent TLR stimuli, we conclude that CD200-CD200R interaction negatively regulates both arms of the TLR signaling response.

CD200 Is Induced by NOD2 Ligand and Limits NOD2-Mediated Inflammation In Vivo and In Vitro

Unlike TLRs, NOD2 is a cytosolic sensor for bacterial cell wall components such as muramyl dipeptide (MDP), but also activates NF-κB signaling through the specific adaptor RIP2 (Tattoli et al., 2007). We showed that NOD2-mediated c-Rel activation was able to induce CD200. Bg-PM from WT and c-Rel$^{-/-}$ mice were first primed with recombinant IFN-γ and then stimulated with 100 μg/ml muramyl dipeptide (MDP) to activate NOD2. Induction of CD200 mRNA was detected by quantitative PCR (qPCR) after different times. MDP stimulation significantly induced CD200 mRNA in WT Mϕ but completely failed to induce CD200 in c-Rel$^{-/-}$ Mϕ (Figure 6A), indicating that NOD2-mediated c-Rel activation can also induce CD200. To investigate any potential influence of CD200 on a NOD2-mediated inflammatory response, we primed Bg-PM from WT and CD200$^{-/-}$ mice with IFN-γ and then stimulated them with different concentrations of MDP. CD200$^{-/-}$ Mϕ produced significantly higher levels of TNF-α and IL-6 in response to MDP (Figure 6B). The levels of TNF-α and IL-6 were consistently higher in peritoneal lavage fluid from CD200$^{-/-}$ mice after intraperitoneal MDP challenge, indicating that the CD200/CD200R axis also negatively regulates NOD2-mediated inflammatory signaling (Figure 6C). To exclude the possibility that our MDP preparation was contaminated with TLR agonists, we measured TNF-α and IL-6 secretion in MyD88$^{-/-}$ mice after similar intraperitoneal MDP challenge. MyD88$^{-/-}$ mice produced similar levels of TNF-α and even higher levels of IL-6 compared to WT mice, indicating that our MDP preparation was not contaminated with TLR agonists (Figure 6C). Similar to Fig-4, PBS-injected groups did not induce any cytokine in either mouse strain (data not shown).

CD200 Is Induced by Agonists of NALP3 Inflammasomes and Limits Caspase-1 Activation and IL-1β Secretion

NALP3 is another cytosolic pathogen sensor. Unlike TLRs and NOD2, NALP3 stimulation does not activate NF-κB, but rather activates caspase-1, which in turn cleaves pro-IL-1β to mature IL-1β. However, transcriptional induction of pro-IL-1β depends on TLR stimulation. Therefore, NALP3 contributes to inflammation by regulating secretion of inflammatory mediators but, unlike TLRs and NOD2, does not regulate gene expression. Since TLRs and NOD2 induced CD200, we investigated whether direct activation of NALP3 itself can activate CD200 expression in the absence of MyD88 and c-Rel. BMDM from WT, MyD88$^{-/-}$, and c-Rel$^{-/-}$ mice were stimulated with LPS for 6 hr and briefly pulsed with 1 mM adenosine triphosphate (ATP) to activate the NALP3 inflammasome (LPS and ATP individually do not activate NALP3 but together are a potent agonist of NALP3). Induction of CD200 mRNA was analyzed by qPCR after different times following the ATP pulse. Consistent with results in Figure 2, LPS treatment alone induced high levels of CD200 in WT cells, but not in MyD88$^{-/-}$ and c-Rel$^{-/-}$ Mϕ. Interestingly, a short pulse of ATP after LPS treatment induced CD200 mRA in MyD88$^{-/-}$ Mϕ but much lower levels in c-Rel$^{-/-}$ Mϕ, suggesting that direct stimulation by the known NALP3 agonists LPS+ATP may induce CD200 independent of the MyD88-mediated pathway but still require c-Rel (Figure 7A). Although c-Rel$^{-/-}$ Mϕ show significant

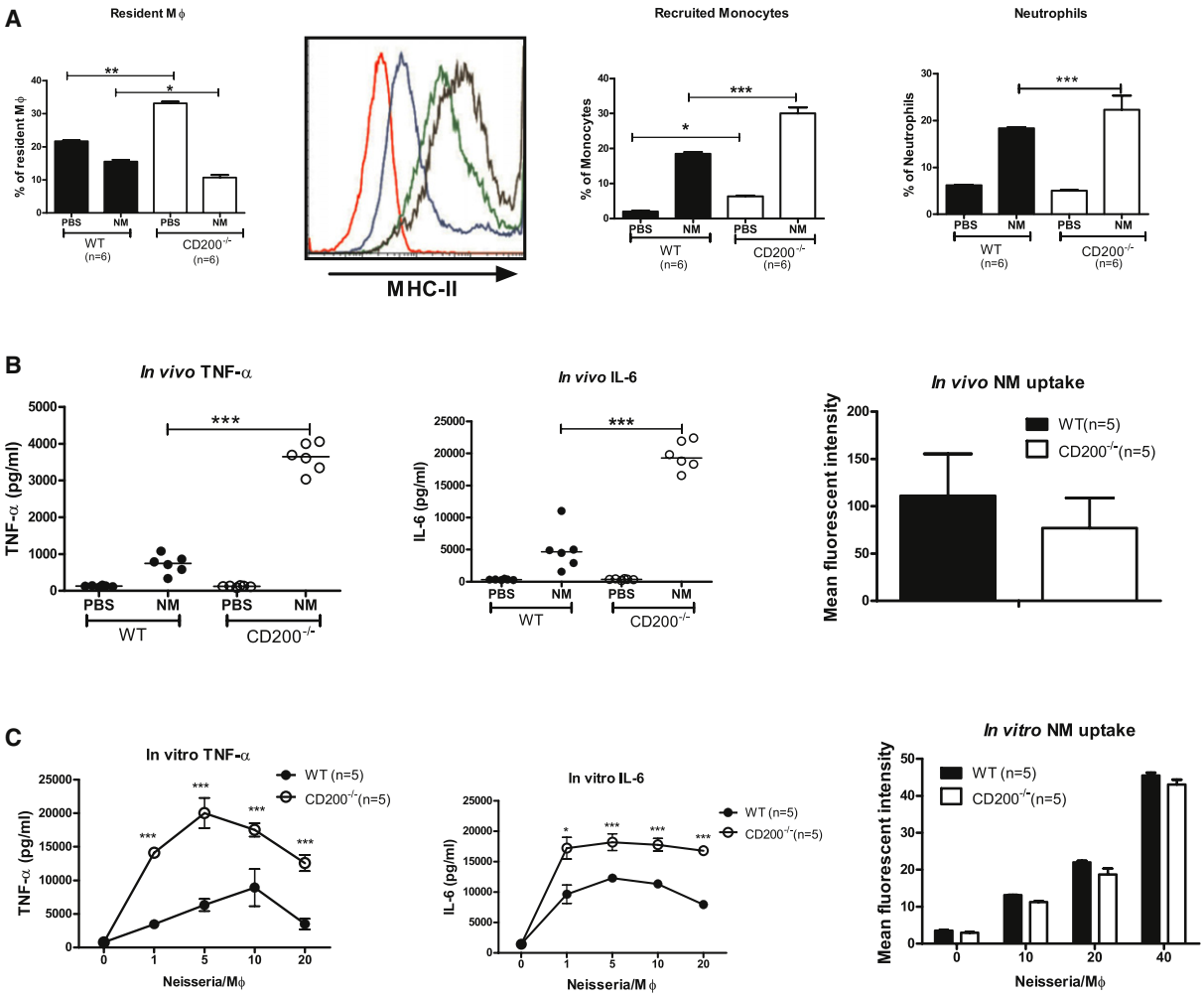

Figure 4. CD200$^{-/-}$ Mϕ Show Increased Cellular and Secretory Inflammatory Responses to Inactivated *Neisseria* In Vivo and In Vitro

(A) The distribution of leukocyte populations was determined by flow cytometry 2 hr after injection (i.p) of WT and CD200$^{-/-}$ mice with 10^5 cfu of inactivated *Neisseria* or equal volumes of control PBS. Resident Mϕ were identified as F4/80^{hi}CD11b^{hi}GR-1^{-ve}CD11c^{-ve} and CD19^{-ve}. Data are shown as mean ± SD, with 6 mice per group, and are representative of three independent experiments. The two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. Inset: histograms comparing MHC-II expression on Mϕ populations between PBS-treated WT (red line) and CD200$^{-/-}$ (blue line) mice, as well as NM-treated WT (green line) and CD200$^{-/-}$ (brown line) mice. All comparisons between two genotypes as well as comparisons between two treatment conditions within the same genotype are statistically significant, as above (not shown).

(B) Enhanced cytokine production in CD200$^{-/-}$ mice after injection of inactivated NM compared to PBS control. Secretion of TNF-α (left panel) and IL-6 (middle panel) was measured 2 hr after injection in peritoneal lavage fluid by ELISA. Two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. **, $p < 0.01$.

RdGNX-labeled NM (10^6 cfu) were injected into the peritoneal cavity of WT and CD200$^{-/-}$ mice. After 90 min, peritoneal exudate cells were harvested by lavage and bacterial uptake was analyzed by flow cytometry (right panel). No significant difference in bacterial uptake was observed between the two mouse strains. Data are shown as mean ± SD.

(C) Enhanced cytokine production in Bg-PM from CD200$^{-/-}$ mice after NM stimulation. TNF-α (left panel) and IL-6 (middle panel) concentrations were measured in supernatants after 16 hr stimulation of WT and CD200 Bg-PM with indicated doses of inactivated NM. Data are presented after normalizing the total number of cells in each well (mean ± SD). Two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. **, $p < 0.01$

Bg-PM from WT and CD200$^{-/-}$ animals show comparable levels of NM uptake. Bg-PM were incubated with indicated doses of Rd-GNX-labeled fluorescent *Neisseria* for 90 min. The mean fluorescent intensity (MFI) of each strain is presented as a bar diagram (right panel). No significant difference in NM uptake was observed between the two strains. All data shown are representative of at least three independent experiments.

attenuation of CD200 induction after stimulation, a low-level induction of CD200 mRNA was observed at a later time point. Therefore, c-Rel$^{-/-}$ Mϕ show strikingly slower kinetics of CD200 induction rather than complete inhibition.

To investigate whether CD200 also modulates IL-1β secretion, we stimulated WT and CD200$^{-/-}$ Mϕ with LPS for 6 hr to induce pro-IL-β, followed by a short pulse of ATP or monosodium urate crystal (MSU, another activator of NALP3 mediated pro-IL-1β secretion), and after further 6 hr of incubation, we harvested supernatants and measured secretion of IL-1β by ELISA. The CD200$^{-/-}$ Mϕ produced higher levels of IL-1β compared to WT Mϕ, indicating that CD200 also negatively regulated IL-1β

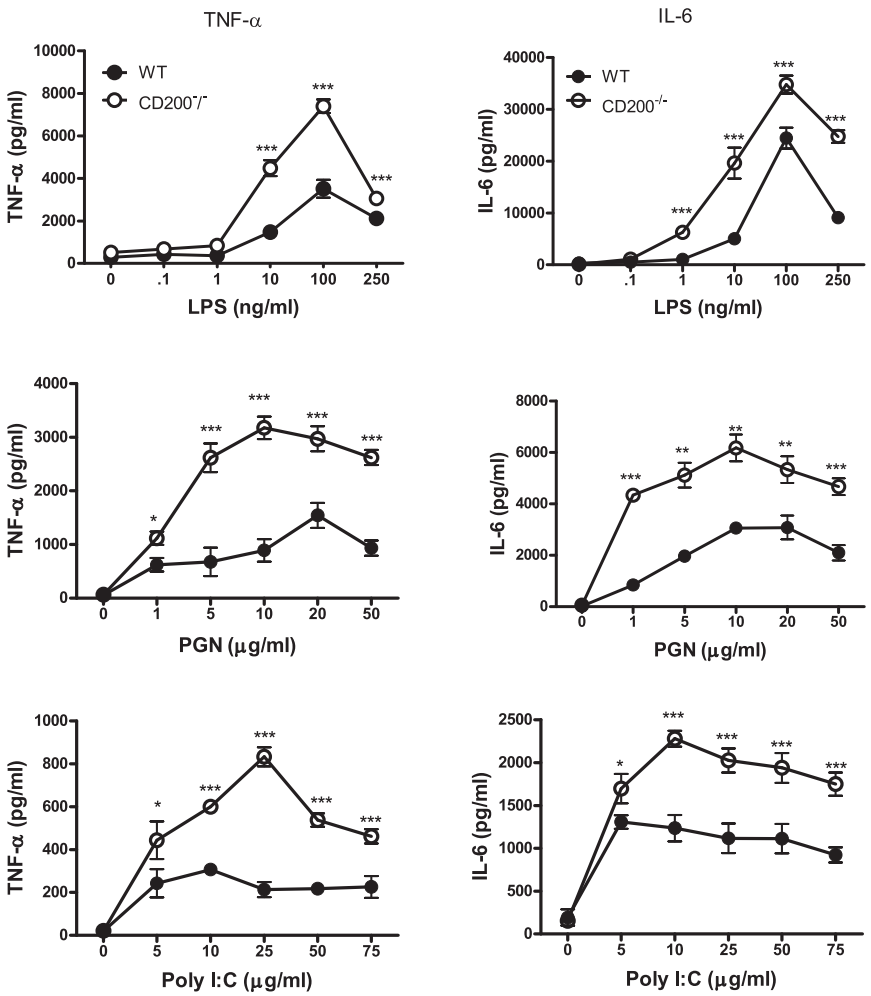

Figure 5. CD200$^{-/-}$ Mϕ Produce Enhanced Levels of TNF-α and IL-6 In Vitro, in Response to MyD88-Dependent and -Independent TLR Agonists

WT and CD200$^{-/-}$ Bg-PM were cultured on 96-well bacteriologic plastic dishes in serum-free conditions. Quadruplicate wells were stimulated with increasing concentrations of purified TLR-4 (LPS), TLR-2/6 (PGN), and TLR-3 (poly I:C) agonists. After 16 hr incubation, supernatants were harvested and TNF-α and IL-6 concentrations were measured by ELISA. Data (mean ± SD, with at least 3 mice per group) are presented after normalizing for cell numbers in each condition and are representative of at least three independent experiments. Two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. ***, $p < 0.001$.

secretion (Figure 7B). In the above assay, once the LPS priming step was replaced by NM stimulation, CD200$^{-/-}$ Mϕ still produced significantly higher levels of IL-1β (data not shown). We further validated the role of CD200 in modulating IL-1β secretion in vivo. CD200$^{-/-}$ mice produced significantly higher levels of IL-1β, confirming that CD200 also negatively regulates IL-1β release in vivo (Figure 7C). As expected, vehicle-injected groups did not induce any detectable cytokine in either mouse strain (data not shown).

We investigated whether CD200 limits IL-1β secretion just by limiting its TLR-mediated transcriptional induction or whether CD200 can also regulate caspase-1 activation, thereby limiting conversion of pro-IL-1β to active IL-1β. LPS-primed WT and CD200$^{-/-}$ Mϕ were stimulated with 5 mM ATP to activate NALP3 inflammasome. Caspase-1 activation was measured by a quantitative flow-cytometry-based method after staining the cells with a cell permeable fluorescent probe (FLICA) that specifically recognizes active caspase-1. Results show that after LPS +ATP stimulation, CD200$^{-/-}$ Mϕ expressed markedly enhanced active caspase-1 compared to WT cells (Figure 7D). LPS stimulation alone did not show any caspase-1 activation, but ATP alone induced low-level activation in both strains. These data indicate that CD200 can inhibit inflammasome activation and may regulate IL-1β secretion not just by modulating TLR pathways, but also by limiting caspase-1 activation.

DISCUSSION

We show that CD200 and CD200R are differentially regulated on innately activated Mϕ after stimulation with agonists of TLRs, NOD2 and NALP3 inflammasome, but not by the ligation of the phagocytic pattern recognition receptor SR-A. Induction of CD200 depended on the transcription factor c-Rel and negatively regulated TLR and NOD2 and inflammasome-mediated responses. Lack of CD200 also increased the accumulation of different inflammatory leukocyte populations at the site of induced inflammation. Therefore, TLR and NLR activation induces CD200 and initiates a negative regulatory loop, which in turn regulates TLR- and NLR-mediated cellular and secretory inflammatory responses. The importance of such negative regulation in host defense was evident from the finding that CD200$^{-/-}$ mice succumbed more readily to meningococcal septicemia, showing clear signs of exacerbated systemic inflammation.

TLR- and NOD2-mediated induction of CD200 was transcriptionally regulated by the NF-κB family member c-Rel. Induction of CD200 by combined LPS and ATP stimulation in the absence of MyD88 is highly suggestive of involvement of NALP3 and caspase-1 in the regulation of CD200 transcription. One unexpected finding was that such MyD88-independent CD200 induction still required c-Rel, indicating a possible transcriptional regulatory loop between caspase-1 and c-Rel. It is interesting to note that caspase-1$^{-/-}$ Mϕ show reduced NF-κB activation (Sarkar et al., 2006). Similarly, caspase-8$^{-/-}$ cells displayed diminished c-Rel activation and nuclear translocation (Falk et al., 2004). Pharmacological inhibition of caspases by the pan-caspase inhibitor ZVAD-FMK has been shown to inhibit CD200 induction (Rosenblum et al., 2004). Our finding that downregulation of CD200R is not

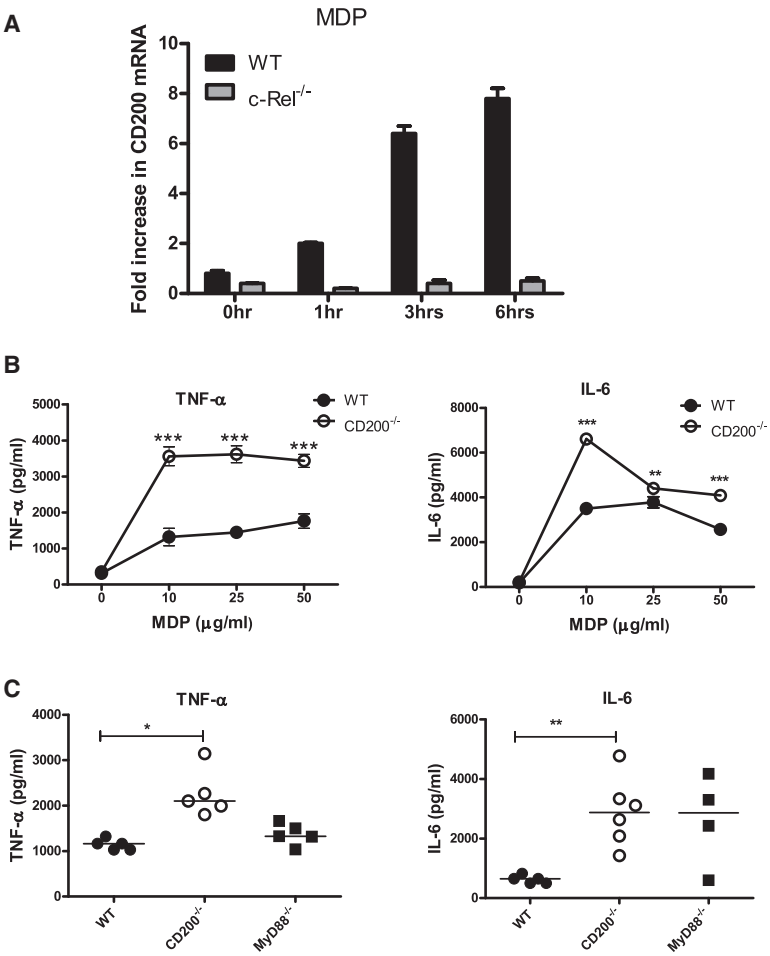

Figure 6. CD200 Is Induced by NOD2 Stimulation and Negatively Regulates Its Function

(A) CD200 mRNA was induced in Bg-PM from WT but not c-Rel$^{-/-}$ mice that had been primed overnight with 20 ng/ml of recombinant IFN-γ and then stimulated with 100 μg/ml MDP for indicated times. CD200 mRNA was determined by real-time PCR, and fold induction is presented as a bar diagram. Data are shown as mean ± SD, with 3 mice per group.

(B) Increased levels of TNF-α and IL-6 were produced in CD200$^{-/-}$ Mϕ primed overnight with IFN-γ and then challenged with indicated concentrations of MDP for 16 hr. Levels of TNF-α and IL-6 were measured by ELISA. Data are shown as mean ± SD, with at least 3 mice per group. Two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. ***, $p < 0.001$.

(C) Increased levels of TNF-α and IL-6 were produced in the peritoneal cavity of CD200$^{-/-}$ injected (i.p.) with 100 μg MDP or an equal volume of PBS. After 2 hr, animals were killed and peritoneal lavage was performed with 1 ml PBS. Levels of TNF-α and IL-6 were measured by ELISA. Two-tailed t test was performed to determine statistical significance. *, $p < 0.01$; **, indicates $p < 0.001$.

Each condition was analyzed in triplicate and representative data from at least three independent experiments are shown.

transcriptionally regulated suggests that TLRs differentially regulate expression of CD200 and CD200R by distinct mechanisms.

The inhibitory role of CD200-CD200R engagement is well characterized during chronic autoimmune inflammatory models, where adaptive immunity plays an important role in disease pathogenesis. Their role in regulating innate responses to bacterial challenge was investigated in an established experimental model of acute meningococcal septicemia. Live organisms confirmed that the enhanced susceptibility of CD200$^{-/-}$ mice to meningococcal challenge was associated with accumulation of activated leukocytes and increased secretion of inflammatory mediators, rather than bacterial overgrowth. Additional challenge with inactivated bacteria and selective agonists not only defined the specificity of each PRR system but also ruled out potential immune modulation by live organisms and the exogenous iron supplement, required for meningococcal growth in mice.

A range of negative regulators control excessive activation of various adaptive and innate immune pathways, including TLR and NLR pathways (Liew et al., 2005). Compared to other inhibitory molecules, the CD200-CD200R pathway stands out for its several distinct features.

Unlike most inhibitory receptors that signal through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and phosphatase recruitment, CD200R recruits the adaptor protein downstream of tyrosine kinase 2 (Dok2), which in turn recruits its target RAS p21protein activator 1 (RasGAP), leading to inhibition of ERK, JNK, and p38 MAPK activation (Mihrshahi et al., 2009; Zhang et al., 2004). These interactions may regulate TLR and NLR function by limiting nuclear translocation of NF-κB (Cui et al., 2007). Indeed, Dok2 has been shown to be a potent regulator of TLRs. (Shinohara et al., 2005).

CD200 is the only regulatory pathway to our knowledge that has been shown to limit functions of all three innate pathogen sensors—namely, TLR, NOD2 response, and inflammasome-mediated caspase-1 activation as well as IL-1β secretion. We show that CD200-CD200R interaction limits IL-1β secretion not just by inhibiting TLR-mediated pro-IL-β induction but also by limiting inflammasome-mediated caspase-1 activation. Since CD200-CD200R axis limits overall secretion of bioactive mature IL-1β, our data further demonstrate the central importance of this inhibitory pathway in controlling inflammation. However, CD200 is not an indiscriminate immunosuppressant, since it did not block phagocytic uptake or bacterial killing. Furthermore, CD200-CD200R interaction enhances IFN-α/β production by plasmacytoid dendritic cells after viral stimulation (Fallarino et al., 2004). However, CD200 clearly plays an important role in limiting bacterial sepsis as shown here, as well as excessive inflammation induced by viruses (Snelgrove et al., 2008) and autoimmune pathologies (Copland et al., 2007; Hoek et al., 2000).

Many negative regulators of TLRs function through *cis*-cellular mechanisms acting on the same cells, but CD200 induces its inhibitory effects through a *trans*-cellular mechanism by cell-cell contact (Hatherley and Barclay, 2004). There are not many examples showing the importance of cell-cell regulatory pathways in infection; our data clearly showed the distinct kinetics of the early inflammatory response allowing pathogen killing,

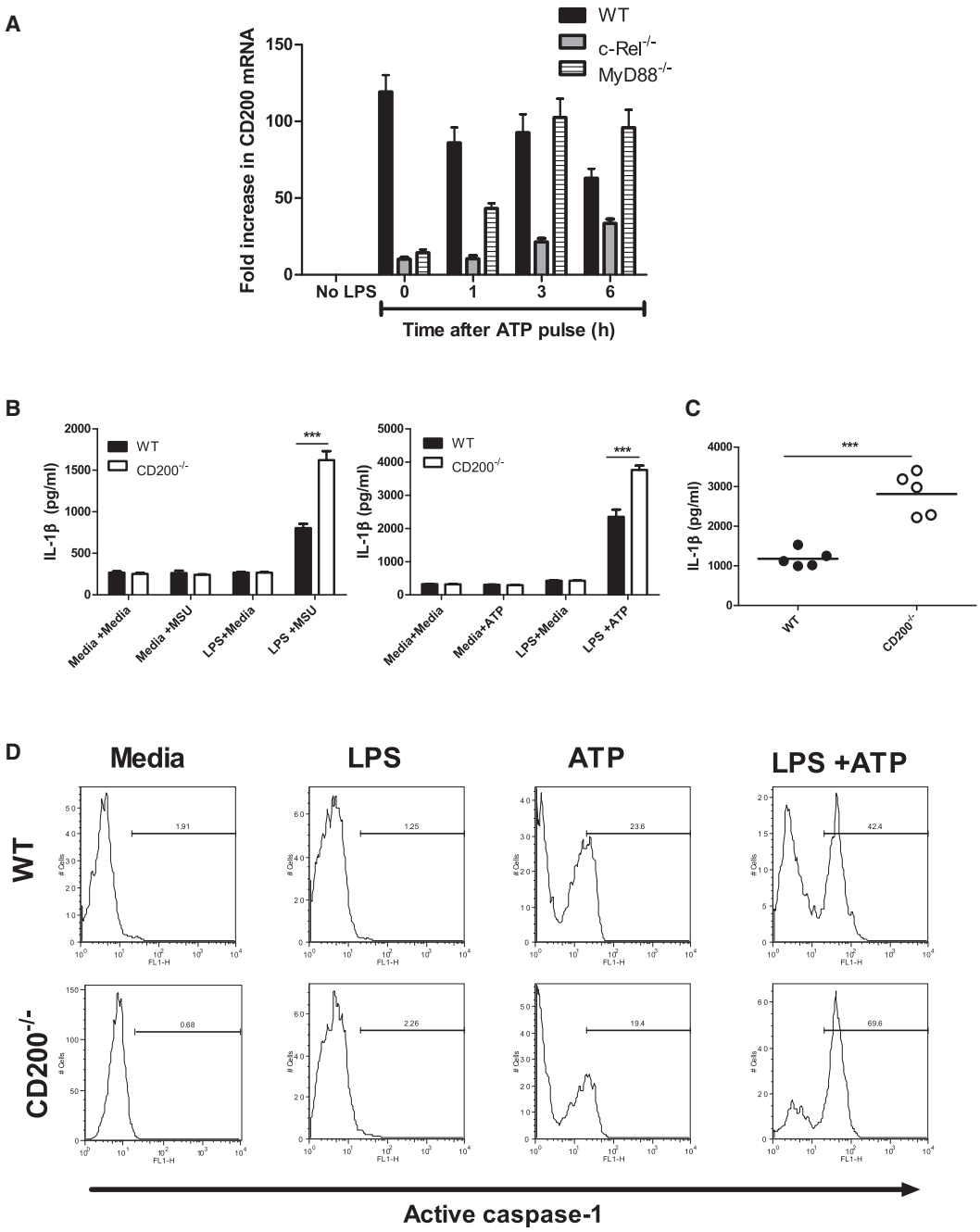

Figure 7. CD200 Is Induced by NALP3 Activation and Limits IL-1β Secretion

(A) WT, MyD88$^{-/-}$, and c-Rel$^{-/-}$ Mϕ were stimulated with 100 ng/ml of LPS for 6 hr and pulsed for 5 min with 5 mM ATP. RNA was collected for the indicated times after the pulse and induction of CD200 mRNA was determined by real-time PCR. Fold induction is presented as a bar diagram. Data are shown as mean ± SD, with 3 mice per group.

(B) WT and CD200$^{-/-}$ Mϕ were stimulated for 6 hr with indicated concentrations of LPS and either pulsed for 5 min with 5 mM ATP or stimulated with 5 μg/ml MSU. After 6 hr, supernatants were harvested and the levels of IL-1β were measured by ELISA. Data are shown as mean ± SD, with at least 3 mice per group. Two-way ANOVA with Bonferroni multiple comparison test was used to assess statistical significance. ***, $p < 0.001$.

(C) CD200$^{-/-}$ mice produce more IL-1β in peritoneal lavage fluid after i.p. injection with inactivated NM followed by ATP. After 2 hr, mice were killed and levels of IL-1β in peritoneal lavage fluid were measured by ELISA. Two-tailed t test was performed to determine statistical significance. ***, $p < 0.001$.

Each condition was analyzed in triplicate, and representative data from at least three independent experiments are shown.

(D) WT and CD200$^{-/-}$ Mϕ were stimulated with LPS (100 ng/ml), ATP (5 mM) and LPS+ATP. Induction of active caspase-1in the two strains was compared by staining with a specific fluorescent probe. Each condition was analyzed at least in duplicate and representative data from three independent experiments are shown.

followed by CD200-mediated control of the host inflammatory response. It is worth noting that CD200$^{-/-}$ mice showed enhanced disease severity only after a lag of 30–40 hr after infection. Thus direct induction of CD200 by microbial stimuli not only distinguishes cytokine versus innate activation of Mϕ but also functionally defines the latter population. Taken together, we conclude that CD200 is selectively induced by TLR- and NLR-mediated inflammatory pathways and, interacting through CD200R, limits excessive inflammation without compromising pathogen killing, therefore providing an attractive therapeutic target.

Most negative regulators are part of negative feedback loops induced by TLR signaling itself and then inhibiting TLR function. Induction of CD200 by innate stimuli is in agreement with such a paradigm, but simultaneous reduction in signal-competent CD200R may seem counterintuitive. However, similar examples are not uncommon among paired receptors. A close relative of CD200-CD200R is the paired inhibitory receptor signal regulatory protein alpha (SIRPα) and its ligand CD47. A recent report showed that SIRPα also protects the host from LPS-induced septic shock and, similar to CD200R, itself is downregulated on Mϕ after LPS stimulation (Kong et al., 2007). Downregulation of inhibitory receptors such as SIRPα and CD200R are possibly required to allow host protective physiological inflammation, but may prolong pathological inflammation. Induction of ligands such as CD200 possibly balances this risk by maximizing the chance of engagement of available receptors. We propose that distinct regulation patterns of CD200 and CD200R by inflammatory stimuli is an integral part of their inhibitory function, which allows efficient host protection but limits inflammatory pathologies. Our findings may provide a common paradigm for how these paired receptors regulate excessive inflammation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Unless stated otherwise, all chemicals were from Sigma (Poole, United Kingdom). Rhodamine green X (RdGnX) was obtained from Molecular Probes (Eugene, OR, USA). The TMB substrate reagent set was purchased from BD PharMingen (San Diego, CA). All cell-culture media were from GIBCO (Paisley, United Kingdom). All TLR and NLR agonists were obtained from Invivogen (Wiltshire, UK). The rat monoclonal antibodies against F4/80 (Austyn and Gordon, 1981) and CD11b (5C6) (Rosen and Gordon, 1987) were generated in the Gordon laboratory. The rat monoclonal antibodies against murine CD200 (OX-90) and CD200R (OX-110) were generated in the Barclay laboratory. M5114, the rat monoclonal antibody recognizing murine MHC-II, was obtained from R&D Systems (Abingdon, UK).

Bacterial Culture and Fluorescent Labeling

The encapsulated serogroup B strains of pathogenic *Neisseria meningitidis* MC58, H44/76, and its isogenic lipid A mutant strain H44/76lpxA (van der Ley et al., 1996) were cultured in an atmosphere of 5% CO_2 overnight at 37°C on brain-heart infusion (BHI) medium (Oxoid, Basingstoke, UK), supplemented with Levinthal's reagent (10% vol/vol) and solidified with agar (1% wt/vol). For fluorescent labeling, NM were harvested and resuspended in 70% ethanol overnight at 4°C and labeled with RdGnX (RdGnX-NM) (Peiser et al., 2006).

Mouse Strains

SR-A$^{-/-}$ (Suzuki et al., 1997), MyD88$^{-/-}$ (Adachi et al., 1998), CD200$^{-/-}$ (Hoek et al., 2000), and c-Rel$^{-/-}$ mouse strains were on a C57BL/6J background and kindly provided by original investigators. Balb/c, C57BL/6J, a mutant for TLR-4, and C3H/HeJ and its corresponding WT control, C3H/HeN, were from Harlan, UK. All animals were housed in specific pathogen-free conditions and procedures were conducted according to the requirements of the United Kingdom Home Office Animals Scientific Procedures Act, 1986.

Isolation and Culture of Primary Mϕ

Bg-PM and TPM were prepared by intraperitoneal (i.p.) injection of 1 ml polyacrylamide gel P-100 (Bio-Rad) beads (2% w/v suspension in endotoxin-free PBS) and thioglycollate broth (2% solution), respectively . Four days after injection, cells were harvested by peritoneal lavage with ice-cold PBS. Similarly, resident peritoneal Mϕ (RPM) were isolated from uninjected animals by lavage with PBS. Bg-PM, TPM, and RPM were plated on bacteriological plastic dishes in a defined serum-free medium, OPTIMEM-1, supplemented with 500 IU/ml penicillin-streptomycin and 2 mM-glutamine. After 3–4 hr, plates were washed three times to remove nonadherent cells and bio-gel beads. After washing, purity of Mϕ in the adherent monolayer was > 98%. Bone marrow was isolated aseptically from femur and tibia and differentiated into mature bone marrow-derived Mϕ by culturing in RPMI supplemented with 100 IU/ml penicillin-streptomycin, 2 mM-glutamine, 10% fetal calf serum (FCS), and 10% L cell-conditioned medium (LCM).

In Vitro Stimulation of Mϕ to Activate TLR and NLR Pathways

For activation of TLR pathways, Mϕ were stimulated with either indicated concentrations of inactivated NM or isolated TLR agonists. For activation of NOD2, cells were first primed with 20 ng/ml recombinant murine IFN-γ and then stimulated with muramyl dipeptide (MDP). NALP3 inflammasomes were activated by stimulating Mϕ with LPS or inactivated NM, followed by a short pulse of ATP or MSU crystals. We assessed cell viability by trypan blue exclusion method in both genotypes before and after stimulation and observed no significant cell death in any condition. After Mϕ stimulation, cytokine secretion and surface-molecule expression were analyzed.

Caspase-1 Activation Assay

WT and CD200$^{-/-}$ Mϕ were primed with 100 ng/ml LPS for 5 hr and stimulated with 5 mM ATP for 30 min. ATP-containing media were replaced with fresh media and incubated for another 30 min to allow active caspase-1 accumulation. Cells were washed and stained with an active caspase-1-specific fluorescent probe (FLICA kit, Immunochemistry Technologies, MN, USA) according to the manufacturer's instructions and analyzed by flow cytometry.

Mouse Infection Studies

This method is adapted from Gorringe et al. (2001) and Plüddemann et al. (2009). In brief, groups of 6- to 8-week-old CD200$^{-/-}$ mice and corresponding age- and sex-matched C57BL/6J were injected i.p. with 1 × 10^5 CFU of serogroup B *Neisseria* strain MC58 together with human holo-transferrin (10 mg) in a total volume of 500 μl. At 18 hr after initial injection, mice were injected i.p. with a second dose of human holo-transferrin (10 mg). The health of the animals was monitored and scored at various time points according to the symptoms presented as follows: healthy = 5, ruffled fur = 4, sticky eyes = 3, ruffled fur and sticky eyes = 2, immobile = 1. As soon as immobile mice were detected, they were killed humanely. Scores were collated and averaged for each group at the various time points. Five microliter blood samples were taken at 20 hr and 24 hr after infection, and serial dilutions were plated to determine bacteremia. At termination, blood was collected by cardiac puncture and spleens recovered. Tissue was homogenized, and serial dilutions of spleen and blood were plated to determine bacteremia. The remaining blood was centrifuged and the plasma collected and frozen at −80°C for later use.

Sterile Peritonitis Model

For the induction of sterile peritonitis, mice were injected i.p. with 2 × 10^6 cfu of ethanol-inactivated NM or 100 μg purified MDP suspended in 200 μl PBS. Mice in the control group were injected with the same volume of PBS. Two hours after injection, mice were killed and inflammatory cells were collected by peritoneal lavage with 1 ml ice-cold PBS, to avoid dilution of cytokines. Care was taken to avoid erythrocyte contamination. Cells were separated by centrifugation, and leukocyte populations were analyzed by multicolor flow cytometry by staining with well-characterized cell-type-specific markers. Cytokine levels in lavage fluid were measured by ELISA. For the assessment of NALP3-mediated IL-1β secretion, 200 μl of ATP (1 mM, pH 7.4) were injected 2 hr after the first

NM injection, and peritoneal lavage was performed after another 2 hr, modified from Griffiths et al. (1995).

Real-Time PCR

Mϕ were stimulated with 100 ng/ml LPS, 100 nM CpG, 2 μg/ml peptidoglycans, 1.8 μg/ml poly I:C, 25–100 μg/ml MDP, and/or 20 ng/ml recombinant mouse IFN-γ (BD Bioscience, San Diego). RNA was extracted with TRI-reagent, treated with Rnase-free Dnase I, and purified with an Rneasy kit (QIAGEN, USA). Two micrograms of RNA were reverse transcribed with the Omniscript RT kit (QIAGEN, USA) with random hexamer primer. Resulting cDNA was analyzed by real-time PCR. Each reaction was prepared with 0.25× SYBR Green I nucleic acid stain (Invitrogen), 10 nM FITC (Bio-Rad, USA), 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 100 μg/ml BSA, and 4 μM dNTPs (Denville) with 1.25 U "Choice" Taq (Denville, USA). Real-time PCR amplification conditions were 95°C (3 min) and 45 cycles of 95°C (15 s), 60°C (30 s), and 72°C (30 s). The following primers were used: Cd200, 5′-ATCCTGAGCACGCTGATTTT-3′ and 5′-GCCAGGGAAATGTCCTCATA-3′; GAPDH, 5′-TGGTGAAGGTCGGTGTGAAC-3′ and 5′-CCATGTAGTTGAGGTCAATGAAGG-3′.

Cytokine Analysis

Interleukin-6 and tumor necrosis factor-α (TNF-α) concentration was determined with OptEIA Mouse IL-6 and TNF-α ELISA set (BD Biosciences, San Diego), and IL-1β concentration was determined with an ELISA kit from eBioscience, UK. All analyses were performed according to the manufacturer's instructions.

Flow Cytometry to Assay Surface Antigen Expression and Association of Bacteria

Single or multicolor flow cytometry was performed on isolated primary Mϕ or a mixed population of peritoneal exudate cells to analyze expression of various surface antigens. Antibody staining was performed according to conventional protocols at 4°C in the presence of 2 mM NaN_3. Cells were blocked for 2 hr with 5% heat-inactivated rabbit serum, 0.5% BSA, 5 mM EDTA, and 10 μg/ml 2.4G2 (anti-FcγRII and III) prior to addition of primary antibodies. Directly conjugated primary antibodies were added at concentrations recommended by the supplier and incubated for another 2 hr. Biotinylated antibodies were detected with streptavidin-phycoerythrin (BD PharMingen). Cells were fixed with 1% formaldehyde in PBS for 2 hr on ice; between steps, cells were washed thoroughly with FACS wash buffer. Fixed cells were analyzed on a BD FACScalibur, with either Cell Quest or Flo-Jo software. Association of fluorescently labeled NM to various Mϕ populations was determined by flow cytometry, as reported previously (Peiser et al., 2006).

Statistical Analysis

Data were analyzed with commercial software (GraphPad Software). One-way ANOVA with Bonferroni posttests was used when multiple groups were analyzed and the two-tailed Student's t test was used for analysis of two groups. Survival data were analyzed with the log-rank test. Results were considered statistically significant with p values of less than 0.05. Unless stated otherwise, all experiments were repeated at least three times and representative data are presented.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.chom.2010.08.005.

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