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Article

Leishmania Promotes Its Own Virulence by Inducing Expression of the Host Immune Inhibitory Ligand CD200

Mauro Cortez,[1,2] Chau Huynh,[1,2] Maria Cecilia Fernandes,[1] Kathleen A. Kennedy,[3] Alan Aderem,[3] and Norma W. Andrews[1,2,*]
[1]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA
[2]Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06510, USA
[3]Institute for Systems Biology, Seattle, WA 98103, USA
*Correspondence: andrewsn@umd.edu
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SUMMARY

Leishmania parasites infect macrophages, cells normally involved in innate defense against pathogens. *Leishmania amazonensis* and *Leishmania major* cause severe or mild disease, respectively, consistent with each parasite's ability to survive within activated macrophages. The mechanisms underlying increased virulence of *L. amazonensis* are mostly unknown. We show that *L. amazonensis* promotes its own survival by inducing expression of CD200, an immunoregulatory molecule that inhibits macrophage activation. *L. amazonensis* does not form typical nonhealing lesions in *CD200*$^{-/-}$ mice and cannot replicate in *CD200*$^{-/-}$ macrophages, an effect reversed by exogenous administration of soluble CD200-Fc. The less virulent *L. major* does not induce CD200 expression and forms small, self-healing lesions in both wild-type and *CD200*$^{-/-}$ mice. Notably, CD200-Fc injection transforms the course of *L. major* infection to one resembling *L. amazonensis*, with large, nonhealing lesions. CD200-dependent iNOS inhibition allows parasite growth in macrophages, identifying a mechanism for the increased virulence of *L. amazonensis*.

INTRODUCTION

The parasitic protozoan *Leishmania* infects millions of people worldwide. Leishmaniasis is a growing public health threat due to recent increases in transmission by infected sand flies. Different species of *Leishmania* cause a wide spectrum of human disease, from self-healing cutaneous lesions to lethal visceralizing infections. The parasite factors responsible for the different forms of clinical disease have not been elucidated. In this study we investigate this issue by comparing two species that cause cutaneous lesions, *L. amazonensis* and *L. major* (Afonso and Scott, 1993; Jones et al., 2000, 2002). Infections with *L. amazonensis* and *L. major* cause severe or mild disease, respectively (Barral et al., 1991; McMahon-Pratt and Alexander, 2004), and these differences in virulence have been proposed to be associated with the ability of each pathogen to survive within activated macrophages (Barral et al., 1991; Gomes et al., 2003; McMahon-Pratt and Alexander, 2004; Qi et al., 2004; Scott and Sher, 1986). However, the molecular mechanisms responsible for the resistance of *L. amazonensis* to microbicidal responses and for its enhanced virulence remain largely unknown.

Immune responses of myeloid cells can be controlled by a number of inhibitory receptors (Ravetch and Lanier, 2000). Among the best-characterized immune inhibitory ligand-receptor pairs is CD200 and its receptor CD200R. CD200 (OX2) is a cell surface glycoprotein from the immunoglobulin superfamily that is expressed in several cell types, particularly lymphoid cells, neurons, and endothelium (Barclay et al., 2002). CD200 has a very short intracellular tail that does not contain any known signaling motif. By engaging CD200R, a cell surface receptor that contains three conserved tyrosines in its cytosolic domain, CD200 generates an inhibitory signal that prevents local macrophage activation (Foster-Cuevas et al., 2004; Hoek et al., 2000; Wright et al., 2000). CD200R is widely expressed in myeloid cells, but resting macrophages express low or undetectable levels of CD200 ligand (Mukhopadhyay et al., 2010). In this study we show that *L. amazonensis*, but not *L. major*, has the ability to induce macrophages to express CD200, resulting in a dampening of macrophage microbicidal responses and an increase in parasite intracellular survival and virulence.

RESULTS

L. amazonensis-Induced Expression of CD200 in Host Macrophages Is Required for Intracellular Replication of the Parasites

Transcriptome analysis of C57BL/6 bone marrow macrophages (BMMs) infected with *L. amazonensis* detected an increase in CD200 mRNA levels (C.H., K.A.K., A.A., and N.W.A., unpublished data). Real-time PCR analysis confirmed a marked increase in CD200 mRNA 1 hr after infection of BMM with *L. amazonensis* axenic amastigotes (Figure 1A), with a concomitant increase in CD200 protein levels (Figure 1B). Interestingly, at 6 or 24 hr after infection, CD200 levels were again reduced, indicating that the signals responsible for the activation of CD200 expression are restricted to early stages of interaction of the parasites with the macrophage. The small but significant increase in CD200 expression detected at 48 hr after infection (Figure 1A) may reflect reinfection of macrophages by parasites released from neighboring cells.

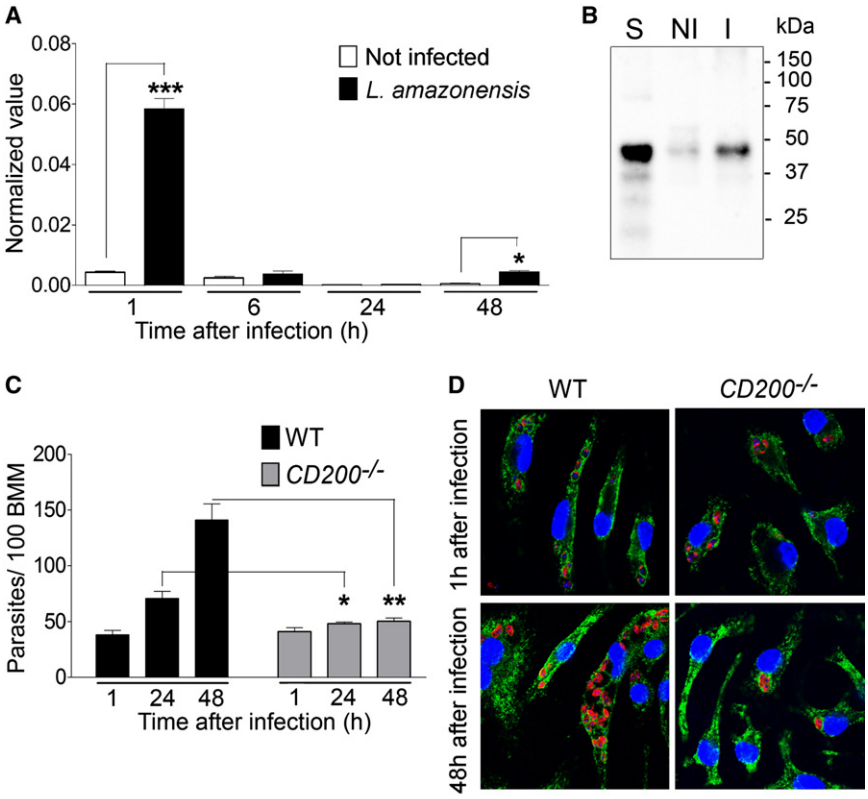

Figure 1. CD200 Expression Is Required for *L. amazonensis* Growth in Macrophages

(A) CD200 transcript levels measured by qPCR in BMMs infected for 1 hr with *L. amazonensis* axenic amastigotes and either processed immediately or after incubation for 6, 24, or 48 hr. Results correspond to mean ± SD of triplicates. ***$p < 0.0001$; **$p < 0.0075$ (Student's t test).

(B) Immunoblot of CD200 protein levels in BMMs infected (I) or not (NI) for 1 hr with axenic amastigotes. Mouse splenocytes (S) were used as a positive control.

(C) Intracellular *L. amazonensis* over time in WT and *CD200*$^{-/-}$ BMMs infected for 1 hr with axenic amastigotes and either fixed immediately or after incubation for 24 or 48 hr. Results correspond to mean ± SD of triplicates. *$p < 0.0270$; **$p < 0.0035$ (Student's t test).

(D) Images of *L. amazonensis*-infected WT or *CD200*$^{-/-}$ BMMs, 1 and 48 hr after infection. Nuclei were stained with DAPI (blue); parasitophorous vacuole membranes were stained with antibodies to the lysosomal glycoprotein Lamp1 (green); parasites were stained with anti-*Leishmania* antibodies (red).

(E) Intracellular *L. amazonensis* in WT and *CD200*$^{-/-}$ BMMs infected for 1 hr in the presence or not of CD200-Fc. Results correspond to mean ± SD of triplicates. *$p < 0.0138$, 24 hr; *$p < 0.0150$, 48 hr (Student's t test).

We found that CD200 expression was required for *L. amazonensis* replication in macrophages. The number of intracellular parasites doubled approximately every 24 hr in wild-type (WT) BMMs, while no significant growth was observed in BMMs isolated from *CD200*$^{-/-}$ mice (Figures 1C and 1D). Importantly, treatment of *CD200*$^{-/-}$ BMMs with a CD200-Fc fusion protein (Snelgrove et al., 2008) rescued their ability to support the intracellular replication of *L. amazonensis*, while not affecting parasite growth in WT BMM (Figure 1E). A control IgG1-Fc fusion protein lacking the CD200 sequence had no effect on the intracellular growth of *L. amazonensis* in BMM (Figure S1).

CD200 Expression Inhibits *L. amazonensis*-Induced iNOS and NO Production

Nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) is a major mechanism used by macrophages to kill intracellular *Leishmania* (Liew et al., 1990; Qadoumi et al., 2002; Wei et al., 1995) and to prevent reactivation of latent leishmaniasis (Stenger et al., 1996). Of particular relevance for the present studies, iNOS/NO production by macrophages also plays a prominent role during early stages of the innate immune response against *L. major* (Diefenbach et al., 1998). Since CD200 signaling was shown to suppress iNOS-mediated generation of NO (Banerjee and Dick, 2004; Cameron et al., 2005; Copland et al., 2007), we investigated if this mechanism was involved in the reduced growth of *L. amazonensis* after infection of *CD200*$^{-/-}$ BMMs. Low levels of iNOS expression were detected by western blot in WT BMM exposed to *L. amazonensis* axenic amastigotes, whereas higher levels were consistently observed in *CD200*$^{-/-}$ BMMs (Figures 2A and 2B). In agreement with an involvement of iNOS in the restriction of the intracellular growth of *L. amazonensis* in macrophages lacking CD200, the specific iNOS inhibitor L-NMMA rescued parasite replication in *CD200*$^{-/-}$ BMMs. In contrast, the inactive isomer D-NMMA had no effect (Figures 2C and 2D). A sensitive fluorometric assay revealed that NO produced by *CD200*$^{-/-}$ BMMs infected with *L. amazonensis* was elevated when compared to infected WT BMM (Figure 2E). These NO levels were markedly lower than what is observed in infected or noninfected BMMs exposed to LPS and IFN-γ (Figure S2), consistent with the observed inhibition in parasite replication instead of

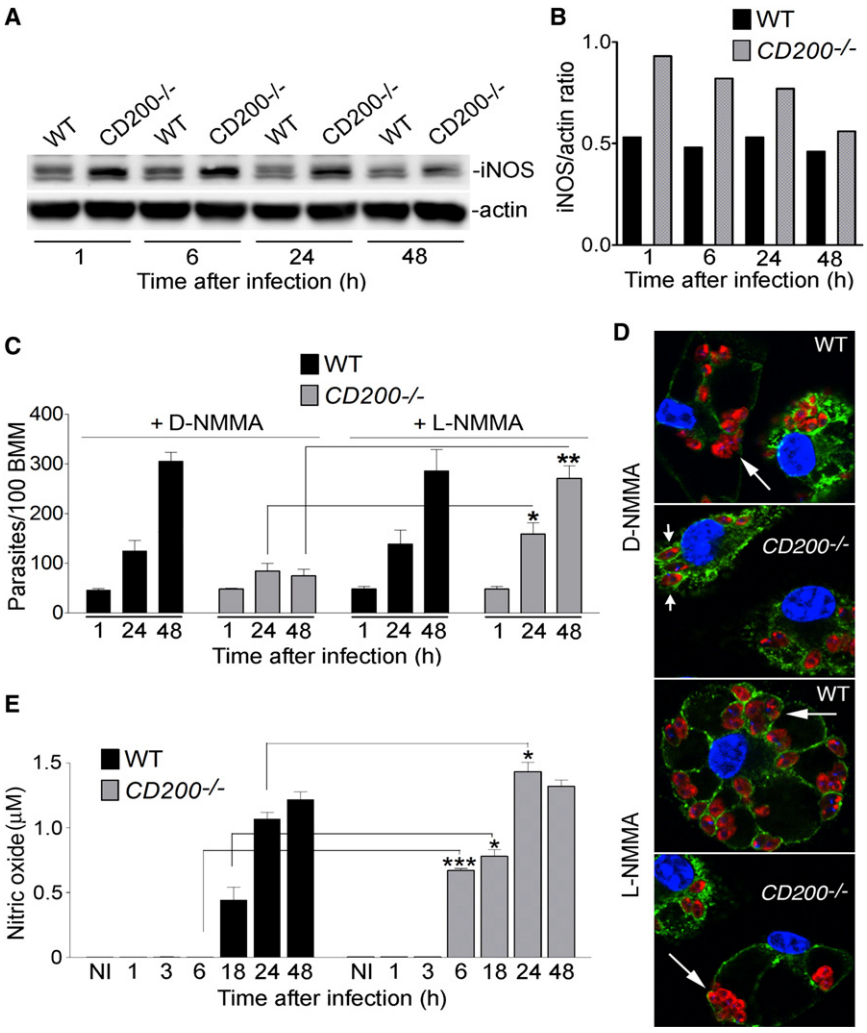

Figure 2. CD200 Promotes *L. amazonensis* Intracellular Growth by Downregulating iNOS

(A) iNOS immunoblot in WT and *CD200*$^{-/-}$ BMMs infected for 1 hr with *L. amazonensis* axenic amastigotes and incubated for the indicated time points. No iNOS band was detected in lysates of noninfected BMMs (not shown). Blots were also probed with anti-actin as a loading control.

(B) Densitometry analysis of the data in (A), expressed as iNOS/actin ratio.

(C) Intracellular *L. amazonensis* over time in WT or *CD200*$^{-/-}$ BMMs pretreated with 500 μM D- or L-NMMA, infected for 1 hr with axenic amastigotes, and either fixed immediately or after incubation for 24 or 48 hr. Results correspond to mean ± SD of triplicates. *$p < 0.0444$; **$p < 0.0023$ (Student's t test).

(D) Images of WT and *CD200*$^{-/-}$ BMMs pretreated with D- or L-NMMA, 48 hr after infection. Nuclei were stained with DAPI (blue); parasitophorous vacuole membranes were stained with anti-Lamp1 (green); parasites were stained with anti-*Leishmania* (red). Long arrows point to replicating amastigotes; short arrows point to single, nonreplicating amastigotes.

(E) NO levels in the supernatant of WT and *CD200*$^{-/-}$ BMMs measured with a fluorometric assay after 1 hr infection with axenic amastigotes. Results correspond to mean ± SD of triplicates. ***$p = 0.0001$; *$p = 0.038$ (18 hr); $p = 0.014$ (24 hr) (Student's t test).

parasite death. No differences in iNOS transcript levels were observed between *CD200*$^{+/+}$ and *CD200*$^{-/-}$ infected BMMs (results not shown), consistent with previous reports suggesting that *L. amazonensis* inhibits iNOS expression in host cells by a posttranscriptional mechanism (Balestieri et al., 2002; Wilkins-Rodríguez et al., 2010).

CD200 Expression Is Required for *Leishmania* Virulence In Vivo

Next, we examined the progression of cutaneous lesions after inoculation of *L. amazonensis* into mouse footpads. WT *CD200*$^{+/+}$ mice showed the rapidly growing, nonhealing lesions that are typical of *L. amazonensis* infections in C57BL/6 mice. In sharp contrast, *CD200*$^{-/-}$ mice infected with a similar parasite dose developed smaller lesions that healed by the 12th week following inoculation (Figure 3A). Quantification of viable *L. amazonensis* in infected footpads revealed a ~100-fold expansion in the parasite population between weeks 3 and 5 in WT mice, while no significant increase was detected during the same period in *CD200*$^{-/-}$ mice (Figures 3B and S3). Thus, CD200 expression, which promotes *L. amazonensis* growth in macrophages, appears to also lead to more severe cutaneous lesions in vivo.

The progressive, nonhealing pattern of *L. amazonensis* lesion formation in mice (Jones et al., 2002) resembles the human disease caused by this *Leishmania* species in Central and South America (Barral et al., 1991; McMahon-Pratt and Alexander, 2004). *L. major*, an agent of cutaneous leishmaniasis in the Old World, causes a more benign, self-healing form of human disease, which can be reproduced in mice of the C57BL/6 background (Belkaid et al., 2000; Jones et al., 2002). In marked contrast to *L. amazonensis*, *L. major* caused small lesions that healed within 8–9 weeks in both WT and *CD200*$^{-/-}$ mice (Figure 3C). Tissue load assays also showed that CD200 expression did not affect the numbers of viable *L. major* between 3 and 5 weeks after infection (Figures 3D and S3). These results suggested that *L. major* differs from *L. amazonensis* in its capacity to engage CD200 as a strategy for intracellular growth in macrophages.

Since *L. major* cannot be grown axenically, we used lesion-derived amastigotes from both species for our subsequent in vitro studies of the CD200 effect. We found that *L. major* infection, in contrast to *L. amazonensis*, did not induce CD200 transcription in BMMs (Figures 3E and S4), although infection levels with both parasite species were comparable (Figure 3F). We also compared iNOS induction in macrophages infected with either *L. amazonensis* or *L. major.* As observed in infections performed

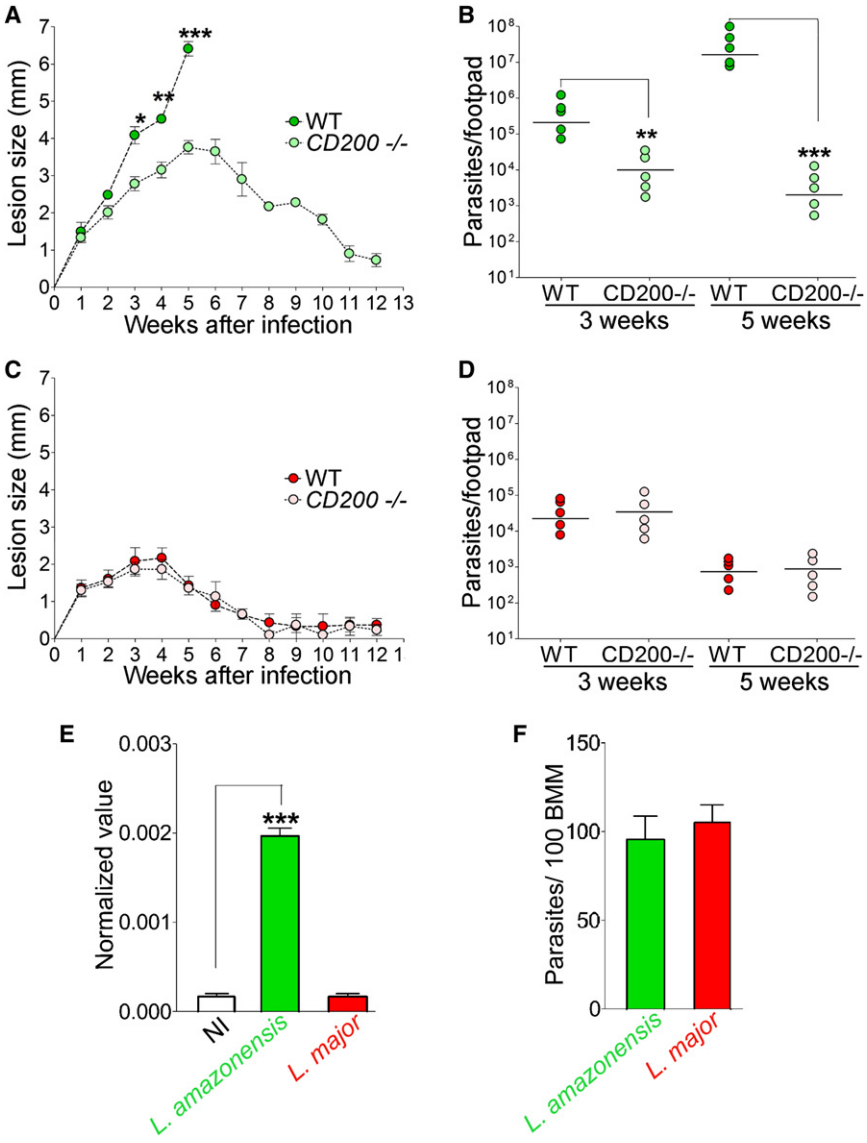

Figure 3. The Reduced Virulence of *L. major* Is Associated with Its Inability to Induce CD200 Expression

(A) Lesion size during infection of WT and *CD200*$^{-/-}$ mice with *L. amazonensis*. Results correspond to mean ± SD (n = 5). *p < 0.021; **p < 0.0037; ***p < 0.0006 (Student's t test).

(B) Parasite load in footpads from WT and *CD200*$^{-/-}$ mice infected with *L. amazonensis*. Results correspond to mean ± SD (n = 5). **p < 0.0025; ***p < 0.0001 (Student's t test).

(C) Lesion size during infection of WT and *CD200*$^{-/-}$ mice with *L. major*. Results correspond to mean ± SD (n = 5).

(D) Parasite load in footpads from WT and *CD200*$^{-/-}$ mice infected with *L. major*. Results correspond to mean ± SD (n = 5).

(E) CD200 transcript levels determined by qPCR in BMMs infected for 1 hr with *L.* amazonensis (green) or *L. major* (red) lesion-derived amastigotes. Results correspond to mean ± SD of triplicates. ***p = 0.0001 (Student's t test).

(F) Numbers of intracellular parasites in the experiment shown in (E). Results correspond to mean ± SD of triplicates.

with axenic amastigotes (Figures 2A and 2B), iNOS levels were higher in *CD200*$^{-/-}$ BMMs when compared to WT BMMs after 1 hr of infection with *L. amazonensis* lesion-derived amastigotes (Figures 4A and 4B). However, the outcome of infection with *L. major* lesion-derived amastigotes was markedly different. iNOS expression was detected after 1 hr of infection in both populations of BMMs, independent of CD200 expression (Figures 4A and 4B). These iNOS expression levels were apparently sufficient to restrict the early intracellular growth of *L. major*, since the specific iNOS inhibitor L-NMMA rescued parasite replication after 48 hr in both WT and *CD200*$^{-/-}$ BMM, whereas the inactive isomer D-NMMA had no effect (Figure 4C).

The Mild, Self-Healing Course of Infection with *L. major* Is Transformed into a Virulent Nonhealing Pattern by Administration of Soluble CD200-Fc

As observed with axenic amastigotes (Figures 1C and 1E), *L. amazonensis* lesion-derived amastigotes grew poorly in *CD200*$^{-/-}$ BMMs, and extracellular addition of CD200-Fc rescued parasite replication (Figure 5A). In contrast, *L. major* growth was limited in both WT and *CD200*$^{-/-}$ BMMs (Figure 5B), consistent with the small, self-healing lesions observed in mice (Figure 3C). Furthermore, the addition of CD200-Fc to both WT and *CD200*$^{-/-}$ BMMs promoted vigorous intracellular growth of *L. major* (Figure 5B). In vivo results further supported the hypothesis that the induction of CD200 is critical for *Leishmania* virulence. Administration of CD200-Fc to *L. amazonensis*-infected WT mice resulted in slightly larger lesions and increased tissue parasite load (Figures 5C and 5D). Remarkably, in vivo inoculation of CD200-Fc converted the mild *L. major* phenotype into a much more virulent one, resembling that of *L. amazonensis* including nonhealing lesions and a six-log increase in parasite load (Figures 5C and 5D).

DISCUSSION

Negative regulatory mechanisms within the immune system are essential to prevent pathology driven by unrestrained inflammatory responses to self and foreign antigens. The widely expressed and inducible ligand CD200 plays an important role in this process, by suppressing inflammatory responses on myeloid cells expressing CD200 receptors (CD200R) (Barclay et al., 2002). In this study, we demonstrate that the protozoan parasite *L. amazonensis* manipulates this potent immune inhibitory pathway to its advantage by inducing the expression of CD200. In the absence of CD200, the virulence of

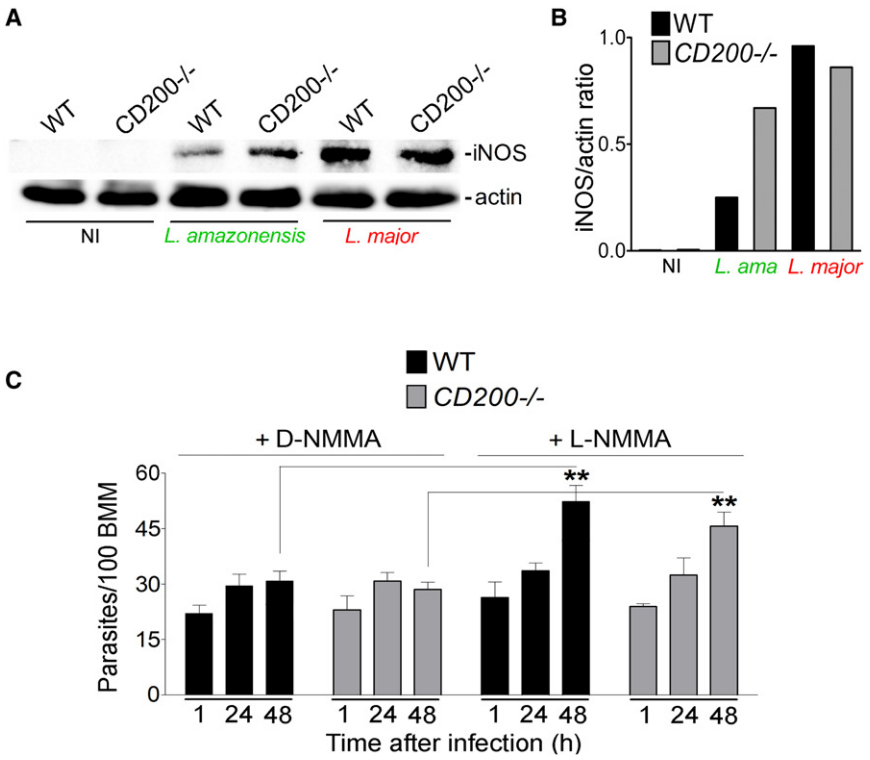

Figure 4. The Reduced Ability of *L. major* to Replicate in Macrophages Is Associated with Induction of iNOS Expression

(A) Immunoblot of iNOS in WT and *CD200*$^{-/-}$ BMMs not infected (NI) or infected for 1 hr with *L. amazonensis* or *L. major* lesion-derived amastigotes. Anti-actin was used as loading control.

(B) Densitometry analysis of the data in (A), expressed as iNOS/actin ratio.

(C) Intracellular *L. major* over time in WT or *CD200*$^{-/-}$ BMMs pretreated with 500 μM D- or L-NMMA and infected for 1 hr with lesion-derived amastigotes and either fixed immediately or after incubation for 24 or 48 hr. Results correspond to mean ± SD of triplicates. **p = 0.0079 (WT); p = 0.0027 (*CD200*$^{-/-}$) (Student's t test).

L. amazonensis is attenuated to that resembling *L. major*, a strain that does not induce CD200. Concordantly, exogenous CD200 is capable of increasing the virulence of *L. major* to levels normally observed for *L. amazonensis*.

The detailed mechanism by which CD200 expression promotes *Leishmania* virulence in vivo remains to be fully elucidated. However, we found a role for inhibition of iNOS expression and NO production, a process known to be mediated by CD200 binding to CD200R (Banerjee and Dick, 2004; Cameron et al., 2005; Copland et al., 2007). Expression of iNOS (NOS2) in macrophages is stimulated by numerous cytokines and microbial products, which often act in synergistic pairs (MacMicking et al., 1997). The effect of LPS and IFN-γ in inducing iNOS expression and NO-mediated killing of *L. major* (Liew et al., 1990) has been the subject of numerous studies, which have largely focused on the potent microbicidal effect associated with the establishment of a Th1 helper lymphocyte response (Reiner and Locksley, 1995). However, more recent studies revealed that the role of iNOS expression in *L. major* infections is not restricted to the adaptive phase of the immune response. iNOS-mediated antimicrobial activity dependent on type 1 interferons (IFN-α, IFN-β) was detected within the first day after parasite inoculation into mice and identified as part of the innate immune response to *Leishmania* (Bogdan et al., 2000; Buxbaum, 2010; Diefenbach et al., 1998). IFN-α was also shown to synergize with subactivating doses of LPS, activating macrophages to produce NO and kill intracellular *L. major* (Shankar et al., 1996). The fact that we detected iNOS expression and NO production by macrophages after short periods of infection (1–6 hr) adds significant additional information to the poorly understood innate response to *Leishmania* and indicates that parasite components may be sufficient to provide the molecular signals required for this early response. Despite the detectable levels of iNOS present in isolated WT macrophages infected with *L. amazonensis*, NO production by these cells could only be measured 18–24 hr after infection. In contrast, in infected macrophages lacking CD200, iNOS levels were elevated and resulted in detectable NO production as early as 6 hr after infection. It is important to note that the NO levels we observed in these assays, using a sensitive fluorescence-based assay, are markedly lower than the levels observed after lipopolysaccharide (LPS) and IFN-γ stimulation of macrophages (see Figure S2). This observation is consistent with our finding that CD200 deficiency in macrophages impairs parasite replication over a period of 48 hr, rather than parasite killing, which is observed in IFN-γ-activated macrophages and appears to require higher NO levels (Liew et al., 1990).

Our in vivo experiments show that the progressive cutaneous lesions typical of *L. amazonensis* infections shift to a healing pattern in CD200-deficient mice and that *L. major*-induced lesions can be transformed into a nonhealing pattern by CD200 inoculation. Taken together with our observations in isolated macrophages, these results suggest that the CD200-mediated suppression of iNOS expression seen shortly after cell entry may also play a role in the stimulation of lesion progression in-vivo. However, important aspects of the long-term in vivo immune responses that develop in the presence or absence of CD200 remain to be determined, such as whether CD200 expression affects the normal Th1 subset development that occurs in mice infected with *L. major* (Reiner and Locksley, 1995). It will also be of interest to determine if parasite-induced expression of CD200 plays a role in the functional impairment of dendritic cells infected with *L. amazonensis* (Boggiatto et al., 2009; Xin et al., 2008).

The increased susceptibility of Balb/c mice to *L. major* infection, with extensive parasite proliferation at sites of inoculation in vivo, is well documented (Nasseri and Modabber, 1979; Belosevic et al., 1989; Reiner and Locksley, 1995). In resistant mice from the C57BL/6 background, the *L. major* tissue load

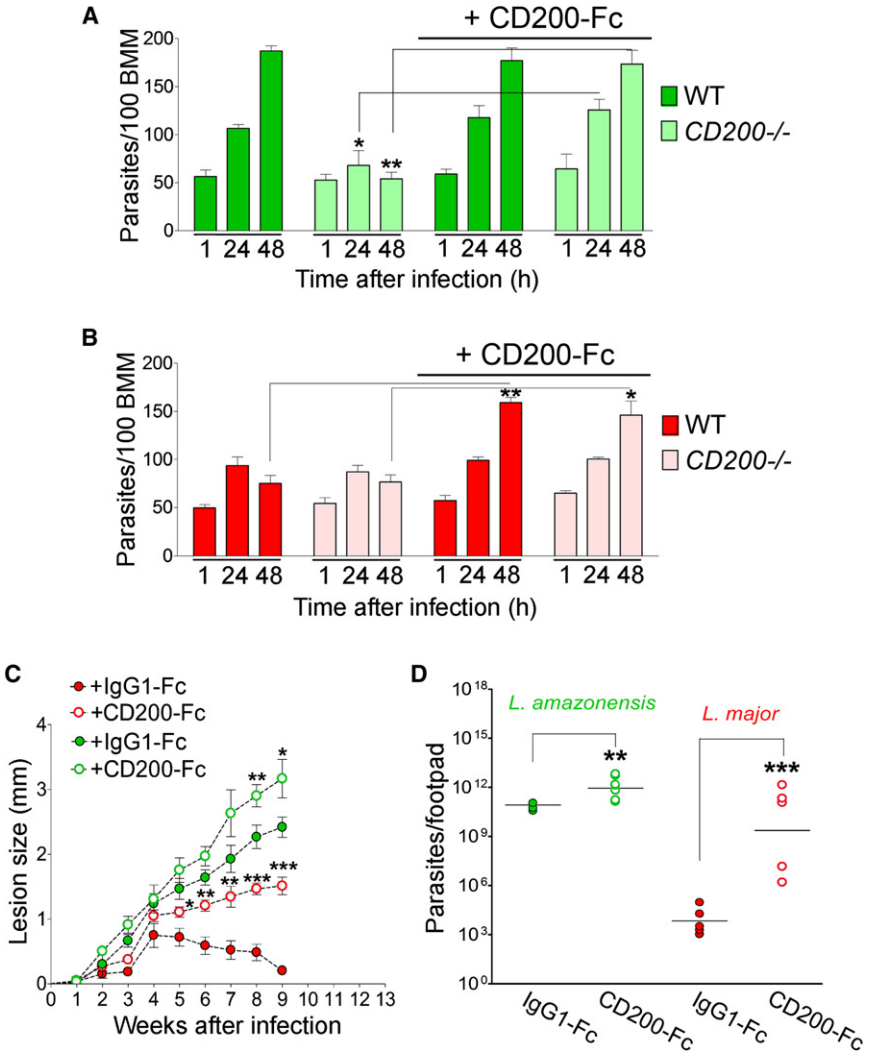

Figure 5. CD200 Transforms the Mild *L. major* Infection into a Virulent Pattern Typical of *L. amazonensis*

(A) Intracellular parasites in WT or *CD200*$^{-/-}$ BMMs infected for 1 hr with *L. amazonensis* lesion-derived amastigotes and either fixed immediately or after incubation for 24 or 48 hr. Results correspond to mean ± SD of triplicates. *p < 0.0379; **p < 0.0018.

(B) Intracellular parasites in WT or *CD200*$^{-/-}$ BMMs infected for 1 hr with *L. major* lesion-derived amastigotes, in the presence or not of CD200-Fc. Results correspond to mean ± SD of triplicates. **p < 0.0011; *p < 0.0142 (Student's t test).

(C) Lesion size during *L. amazonensis* (green) or *L. major* (red) infection in WT mice, treated with CD200-Fc (open circles) or control IgG1-Fc (closed circles). Results correspond to mean ± SD (n = 5). *L. amazonensis*: **p = 0.038; *p = 0.077; *L. major*: *p = 0.039, **p = 0.005 (6th week), p = 0.0054 (7th week), ***p = 0.0002 (8th week), p = 0.0001 (9th week) (Student's t test).

(D) Parasite load in footpads from the experiment in (C), 9 weeks following infection with *L. amazonensis* (green) or *L. major* (red). Results correspond to mean ± SD (n = 5). *L. amazonensis* *p = 0.025; *L. major* ***p = 0.0004 (Student's t test).

increases slowly in the first few weeks after infection, followed by a decline that coincides with lesion healing (Sacks and Noben-Trauth, 2002). However, very few studies have addressed in detail the rate of *L. major* replication in isolated macrophages from susceptible and resistant mouse strains. Most reports show increases in intracellular parasite numbers only after long periods of infection, such as 72 hr (Blos et al., 2003) or longer. The numbers of intracellular *L. major* were reported to remain constant in the first 48 hr of infection in Balb/c BMMs (Muleme et al., 2009), and studies performed with C57BL/6 or CH3/HeNHSD BMMs showed either a very small increase (Green et al., 1990; Mattner et al., 2000) or a reduction in parasite numbers during this period (Kuang et al., 2009). These observations are consistent with our present study, where we show that only *L. amazonensis* is able to double its numbers intracellularly every 24 hr, while the number of intracellular *L. major* stays constant during this period. Our results suggest that CD200-mediated suppression of *Leishmania* intracellular growth may also influence lesion progression in vivo, although additional factors must also come into play as the acquired immune response develops in infected animals.

Consistent with the increased susceptibility of *CD200*$^{-/-}$ animals to autoimmune and inflammatory diseases (Hoek et al., 2000), deficiency in CD200 or CD200R leads to exacerbated responses to infections with microbes such as influenza (Snelgrove et al., 2008) and *Neisseria meningitidis* (Mukhopadhyay et al., 2010). In those studies, the observed pathology was mediated by an uncurtailed systemic response against the pathogen, rather than an intrinsic increase in pathogen virulence. In sharp contrast with these observations, we found that CD200-deficient mice are more resistant to infection with virulent *L. amazonensis,* without any obvious evidence of additional pathology. The anti-inflammatory environment generated during *Leishmania* infections (Sacks and Noben-Trauth, 2002) may play a role in this process, by masking the damaging consequences of systemic CD200 deficiency.

CD200 is thought to control the activation of CD200R-expressing myeloid cells by establishing *trans*-cellular interactions (Barclay et al., 2002; Hatherley and Barclay, 2004). Thus, we envision that CD200 molecules expressed in response to *L. amazonensis* infection interact with CD200R on neighboring macrophages, dampening the overall ability of the host cell population to control intracellular parasite growth through suppression of the microbicide NO. Our experiments demonstrate that signaling mediated by CD200 can suppress NO production and promote *Leishmania* growth within a homogeneous population of cultured macrophages. In vivo, it is conceivable that additional cell types may also respond to *L. amazonensis* infection by

expressing CD200, expanding the parasites' ability to evade host microbicidal activity.

EXPERIMENTAL PROCEDURES

Leishmania Culture and Purification

L. amazonensis (IFLA/BR/67/PH8) and *L. major* (clone VI, MHOM/IL/80/Friedlin) were propagated as promastigotes at 26°C in M199 media supplemented with 5% penicillin/streptomycin, 0.1% hemin (25 mg/ml in 0.1 N NaOH), 10 mM adenine, and 10% FBS (pH 7.5). Metacyclic forms of *L. amazonensis* and *L. major* were purified by agglutination of stationary-phase promastigote cultures using the 3A.1 mAb (Pinto-da-Silva et al., 2005) and peanut agglutinin (Sigma) (Sacks and Melby, 2001), respectively. To generate *L. amazonensis* axenic amastigotes, cultures rich in metacyclic forms were incubated in M199 media supplemented with 0.25% glucose, 0.5% trypticase, 40 mM sodium succinate (pH 4.5), 20% FBS, and 5% penicillin/streptomycin at 2 × 10^6/ml at 32°C for a minimum of 6 days and then cultured axenically at 32°C. Parasites were washed three times in PBS before use in experiments. To obtain lesion amastigotes, female Balb/c mice were infected with purified metacyclic promastigotes of *L. amazonensis* or *L. major*. After 4–5 weeks, the animals were euthanized and the parasites recovered from footpad lesions, as described (Sacks and Melby, 2001). The lesion homogenate rich in amastigotes was submitted to sequential 2000× *g* centrifugation steps to separate parasites from tissue debris.

Mouse Infections and Quantification of Parasite Load

Female WT C57BL/6 mice (obtained from Jackson Laboratory) and *CD200*$^{-/-}$ mice generated in the C57BL/6 background (Hoek et al., 2000) (obtained from Schering-Plough BioPharma) were bred and used under protocols approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine. Female WT or *CD200*$^{-/-}$ 8-week-old C57BL/6 mice were injected in the left hind footpad with 10^6 purified metacyclic promastigotes of *L. amazonensis* or *L. major*, and lesion progression was followed by blinded weekly measurements with a caliper. Inoculations of CD200-Fc or IgG1-Fc were done as described (Snelgrove et al., 2008), with some modifications. Mice were injected intraperitoneally with 10 μg of recombinant CD200-Fc chimera (3355-CD, R&D Systems) or control human IgG1-Fc (110-HG, R&D Systems) in PBS on days 1, 3, and 6 after infection, followed by weekly injections throughout the experiment. The total number of parasites in the injected footpad was estimated by a limiting dilution assay as previously described (Sacks and Melby, 2001; Titus et al., 1985), with minor modifications. Footpad tissues were ground in a Medimachine (Becton Dickinson) and mechanically dissociated using a pellet pestle in 100 μl of Dulbecco's modified Eagle's medium containing 100 U/ml penicillin and 100 μg/ml streptomycin. Tissue homogenates were filtered in a 70 μm pore-size cell strainer (Falcon Products, Inc.) and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium prepared using 30 μl of NNN medium containing 20% defibrinated rabbit blood and overlaid with 100 μl of M199 medium. The number of viable parasites was calculated from the highest dilution at which promastigote growth could be observed after 10 days of incubation at 26°C.

Affymetrix Gene Chip Analysis

C57BL/6 BMMs plated at 2.5 × 10^5 in 10 cm dishes were either not infected or infected with *L. amazonensis* amastigotes at moi = 10 for 30 min at 34°C. After infection, cells were washed three times in PBS and further incubated for the appropriate periods. Total RNA was isolated using TRIzol (Invitrogen) and analyzed using an Affymetrix Gene Chip protocol, as previously described (Wilson et al., 2008). The Affymetrix protocol, used to analyze RNA extracted from BMMs infected for 48 hr with *L. amazonensis* amastigotes, was essentially as described (GeneChip Expression Analysis Technical Manual, Affymetrix). cRNA was hybridized for 16 hr to Affymetrix GeneChip Mouse Genome 430 2.0 Array, which contain 45,000 probe sets for the analysis of 39,000 transcripts and variants from over 34,000 mouse genes. Normalization was performed using G-C-corrected robust multiarray average (GC-RMA). Identification of significantly perturbed genes was done using significance analysis of microarrays. The false positive rate was 0.1%.

Quantitative Real-Time PCR

Real-time PCR was performed using a BioRad iQ icycler Detection system (BioRad Laboratories) using SYBR green fluorophore (BioRad Laboratories) according to the manufacturer's instructions. Oligonucleotide primers 5′-GAA TCA AAC AAT ACA GAA TG- 3′ and 5′-TGC CCC CCA CCA GTA ACA TGG- 3′ were used to amplify a portion of the *CD200* cDNA or 5′-TCA GTC AAC GGG GGA CAT AAA-3′ and 5′-GGG GCT GTA CTG CTT AAC CAG-3′ to amplify the control cDNA *HPRT1*. The reaction was incubated for 3 min at 95°C and then for 45 cycles of 20 s at 95°C followed by 30 s at 55°C. Fluorescence was detected at each annealing step, and the cycle threshold (C_t) was calculated by determining the point at which the fluorescence exceeded a threshold limit. All reactions were performed in triplicate, and negative controls (no template cDNA) were included in each experiment. The data were normalized by the level of HPRT1 expression in individual samples.

Quantification of Leishmania Intracellular Growth in Macrophages

Mouse BMMs were prepared as previously described (Becker et al., 2009). A total of 1 × 10^5 BMMs were plated on glass coverslips in 3 cm dishes 24 hr prior to experiments. Purified recombinant CD200-Fc (3355-CD, R&D Systems) or the control construct IgG1-Fc (110-HG, R&D Systems) was added at 2 μg/ml. The nitric oxide synthase inhibitors *N*-methyl-L-arginine (L-NMMA, active form) or N-methyl-D-arginine (D-NMMA, inactive form) were added at 500 μM (Sigma) (Iniesta et al., 2001; Koblish et al., 1998) for 30 min, followed by a PBS wash prior to infection. Axenic or lesion amastigotes were then added at a moi = 2 in RPMI 10% FBS for 1 hr at 34°C. After invasion, cells were washed three times in PBS and incubated for the indicated times at 34°C. Coverslips were then fixed in 4% paraformaldehyde (PFA), and host cell and parasite DNA were stained with 10 μg/ml DAPI for 1 hr, after permeabilization with 0.1% Triton X-100 for 10 min. The number of intracellular parasites was quantified by scoring the total number of macrophages and the total number of intracellular parasites (clearly identifiable by visualization of the parasitophorous vacuole by phase microscopy) per microscopic field (100× N.A. 1.3 oil immersion objective, Nikon E200 epifluorescence microscope). The results were expressed as intracellular parasites per 100 macrophages. At least 300 host cells, in triplicate, were analyzed for each time point. For immunofluorescence, PFA-fixed cells were washed with PBS, quenched with 15 mM NH_4Cl for 15 min, and permeabilized with saponin/PBS (0.1%) prior to staining with rat anti-mouse Lamp1 mAb (Developmental Studies Hybridoma Bank) for 1 hr, followed by 1 hr incubation with anti-rabbit IgG Alexa Fluor 488. For parasite staining, coverslips were permeabilized with 0.1% Triton X-100 for 3 min and incubated with mouse polyclonal antibodies prepared against axenic amastigotes of *L. amazonensis*, followed by anti-mouse IgG Texas red. All samples were incubated with 10 μg/ml DAPI for nuclei staining. Secondary antibodies were purchased from Molecular Probes. Images were either acquired through a 100× objective using a Zeiss Axiovert microscope equipped with a Hamamatsu Orca II cooled CCD camera controlled by Metamorph Software (Molecular Devices) or with a Leica SPX5 confocal microscope using a 63×/1.4 objective.

Western Blot and Immunoprecipitation

A total of 15 μg of protein/well was separated under reducing conditions on a 10% SDS polyacrylamide gel, blotted onto nitrocellulose membranes using a Trans-blot Transfer system (Bio-Rad Laboratories) overnight at 40V, and probed with rabbit anti-mouse iNOS (Abcam) and mouse anti-mouse actin (Sigma) followed by peroxidase-conjugated secondary antibodies. CD200 protein detection was performed using the Pierce Crosslink Immunoprecipitation Kit as described by the manufacturer (Thermo Scientific). Briefly, samples were added to columns containing goat anti-mouse CD200 antibodies (R&D Systems) crosslinked to protein G/A beads. After several washes, elution buffer (20 μl) was added, and the eluate samples were separated under reducing conditions on a 10% SDS polyacrylamide gel, blotted onto nitrocellulose membranes, and probed with goat anti-CD200 antibodies followed by peroxidase-conjugated secondary antibodies. Immunoblots were developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific) and detected using a Fuji LAS-3000 Imaging System and Image Reader LAS-3000 software.

Nitric Oxide Measurement

Nitrite concentrations in BMM culture supernatants were used as a measure of NO production and quantified using a Fluorometric Assay Kit (Biovision) or modified Griess reagent (Sigma), as described by the manufacturers. Before quantification, all samples were centrifuged at 800× g to remove cellular debris and filtered in Amicon Ultra device filters (Millipore). Absorbance was measured at 450 nm after excitation at 360 nm for the Fluorometric Assay Kit and at 540 nm for the modified Griess reagent. The concentration of NO (total nitrite concentration) in each sample was calculated from standard curve determinations as provided by the manufacturers.

Statistical Analysis

Statistical significance between means of various groups was determined using an unpaired Student's t test for independent samples (GraphPad Prism software). Values of 0.05 or less were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.chom.2011.04.014.

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