ORIGINAL ARTICLE

Hypoxia in *Leishmania major* Skin Lesions Impairs the NO-Dependent Leishmanicidal Activity of Macrophages

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Cure of infections with *Leishmania major* is critically dependent on the ability of macrophages to induce the type 2 nitic oxide (NO) synthase (NOS2) that produces high levels of NO in the presence of ample oxygen. Therefore, we analyzed the oxygen levels found in leishmanial skin lesions and their effect on the NOS2-dependent leishmanicidal activity of macrophages (MΦ). When *L. major* skin lesions of self-healing C57BL/6 mice reached their maximum size, the infected tissue displayed low oxygen levels ($pO_2 \sim 21$ Torr). MΦ activated under these oxygen tensions failed to produce sufficient amounts of NO to clear *L. major*. *Nos2*-deficient and hypoxic wild-type macrophages displayed a similar phenotype. Killing was restored when MΦ were reoxygenated or exposed to a NO donor. The resolution of the lesion in C57BL/6 mice was paralleled by an increase of lesional pO_2. When mice were kept under normobaric hypoxia, this caused a persistent suppression of the lesional pO_2 and a concurrent increase of the parasite load. In *Nos2*-deficient mice, there was no effect of atmospheric hypoxia. Low oxygen levels found at leishmanial skin lesions impaired the NOS2-dependent leishmanicidal activity of MΦ. Hence, tissue oxygenation represents an underestimated local milieu factor that participates in the persistence of *Leishmania*.

INTRODUCTION

Leishmania are protozoan pathogens that in their flagellated, promastigote form are transmitted by sand flies and can cause chronic cutaneous, mucocutaneous, or visceral disease in humans and animals, depending on the parasite species and strain, the inoculated parasite dose, and the type of the ensuing host immune response. The parasites are endocytosed by a diverse set of host cells such as macrophages, dendritic cells, and fibroblasts, in which they reside as aflagellated amastigotes. In mouse cutaneous leishmaniasis due to an infection with *L. major*, self-healing resistant C57BL/6 mice developed an initially progressive skin lesion that resolved

after weeks following the establishment of an anti-leishmanial immune response (reviewed in Sacks and Noben-Trauth, 2002; Mougneau *et al.*, 2011; Bogdan, 2012). In resistant mice, type 2 nitic oxide (NO) synthase (NOS2)-derived NO proved to be essential for the killing of intracellular parasites in macrophages, skin lesions, and draining lymph nodes and for the clinical cure of the disease (Liew *et al.*, 1990; Diefenbach *et al.*, 1998).

In infected wounds, low oxygen tensions (pO_2) (i.e., hypoxic conditions) prevail (Niinikoski *et al.*, 1972; Raju *et al.*, 1976), whereas oxygen supply promotes wound healing and helps to control infections (Remensnyder and Majno, 1968; Hunt *et al.*, 1975). Hypoxia can have multiple and diverse effects on host-pathogen interactions. Many infectious pathogens successfully adapt to hypoxic conditions and exhibit a state of enhanced replication or at least persistence (Peyssonnaux *et al.*, 2005; Dietz *et al.*, 2012; Nickel *et al.*, 2012; Wiese *et al.*, 2012; Cunningham-Bussel *et al.*, 2013; Szaszak *et al.*, 2013). Regarding the role of oxygen for the expression and function of NOS2 (Kwon *et al.*, 1990), hypoxia enhanced the cytokine and Toll-like receptor ligand-induced mRNA and protein expression of NOS2 in macrophage cell cultures but inhibited its NO production (Albina *et al.*, 1995; Melillo *et al.*, 1996; Wiese *et al.*, 2012) and caused a prolonged inactivation of the NOS2 protein by disrupting its interaction with the cytoskeleton (Daniliuc *et al.*, 2003).

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Based on these previous findings, we hypothesized that tissue oxygen levels in leishmanial skin lesions may be low and impair the NO-dependent leishmanicidal activity of macrophages. In order to test for these hypotheses, we resorted to the infection of C57BL/6 mice with *L. major*, because these mice display a transient disease whose healing is dependent on the ability of macrophages to produce high levels of leishmanicidal NO (Liew *et al.*, 1990; Diefenbach *et al.*, 1998; Horta *et al.*, 2012). For determination of the pO_2 in the *L. major*-infected skin, we used fluorescence-based oxygen sensors that allow transcutaneous quantitative determination of tissue oxygen levels in a non-invasive manner (Hofmann *et al.*, 2013). Next, we explored the relationship between anti-leishmanial activity and pO_2 in macrophage cultures. Finally, we exposed *L. major*-infected C57BL/6 mice to normobaric hypoxia in order to test whether constant reduction of tissue oxygen affects the control of the infection in otherwise self-healing mice.

RESULTS

Inverse relationship between the size of *L. major* lesions and tissue oxygenation

We used luminescence-2D-*in vivo* oxygen imaging using planar sensor films to determine skin oxygenation transcutaneously. Compared with the uninfected contralateral footpad (transcutaneous skin oxygen tension $(p_{tc}O_2) = 39.8$ (± 10.8) Torr; mean $\pm$ SD), the pO_2 of the infected footpad dropped to values of 20.9 (± 6.10; mean $\pm$ SD) Torr (corresponding to approximately 2.8% O_2) at day 14, when the *L. major* skin lesion reached its maximum size (Figure 1). Thereafter, tissue oxygenation returned back to normal levels (Figure 1b). This normalization of the *L. major*-infected tissue oxygen levels correlated with the clinical healing of the lesions (Figure 1a and b). Based on these observations, we hypothesized that oxygen availability may be an important parameter of *L. major*-infected tissue which affects the antimicrobial response of the host.

Oxygen tension governs the leishmanicidal activity of macrophages

In order to test this hypothesis, we analyzed whether the oxygen levels found in leishmanial lesions are sufficient to allow macrophages to control *Leishmania* parasites. Therefore, we tested whether activated macrophages cultured at pO_2 values found in infected tissues were capable of killing intracellular *L. major* parasites. When bone marrow–derived macrophages (BM-MΦ) were infected with *L. major*, costimulated with lipopolysaccharide (LPS)/IFN-γ for the induction of NOS2 and exposed to atmospheres with different pO_2, we observed that the leishmanicidal activity and NO production of the macrophages were strongly impaired below a pO_2 of ~30 Torr (4% O_2, 54 μM O_2) (Figure 2).

In order to exclude that hypoxia acted directly on the parasite and promoted its replication, we analyzed the growth of extracellular *L. major* promastigotes under hypoxic conditions (using a pO_2 of ~15 Torr or of ~4 Torr). These
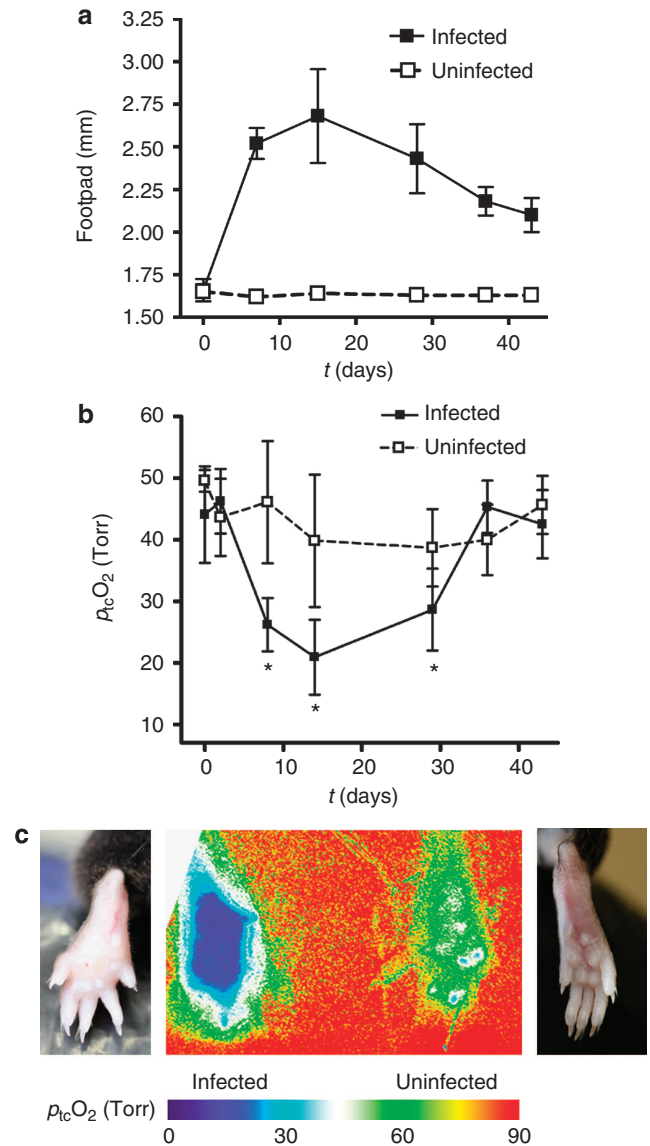

Figure 1. Inverse relationship between the size of *L. major* lesions and tissue oxygenation. (**a**) Clinical course of cutaneous *L. major* infection in C57BL/6 mice was monitored by measuring the infected and the uninfected (contralateral) footpad of five mice (means ($\pm$ SD)). Data are representative of two similar experiments. (**b**) In parallel, using ratiometric luminescence imaging and luminescence lifetime imaging (LLI), the transcutaneous skin oxygen tension ($p_{tc}O_2$) of the infected and the uninfected footpad were determined (means ($\pm$ SD); $n = 5$). Data are representative of two similar experiments. *P (vs. uninfected control) < 0.01. (**c**) Representative pseudocolour image of tissue oxygen distribution using LLI of an infected and uninfected footpad 14 days after *L. major* infection.

experiments revealed that hypoxia did not enhance the proliferation of *L. major* promastigotes (Supplementary Figure S1 online). Together, these data demonstrated that tissue oxygen levels below ~30 Torr (4% O_2) are insufficient for cytokine and Toll-like receptor-activated macrophages to eliminate *L. major* parasites and thus must be considered hypoxic.
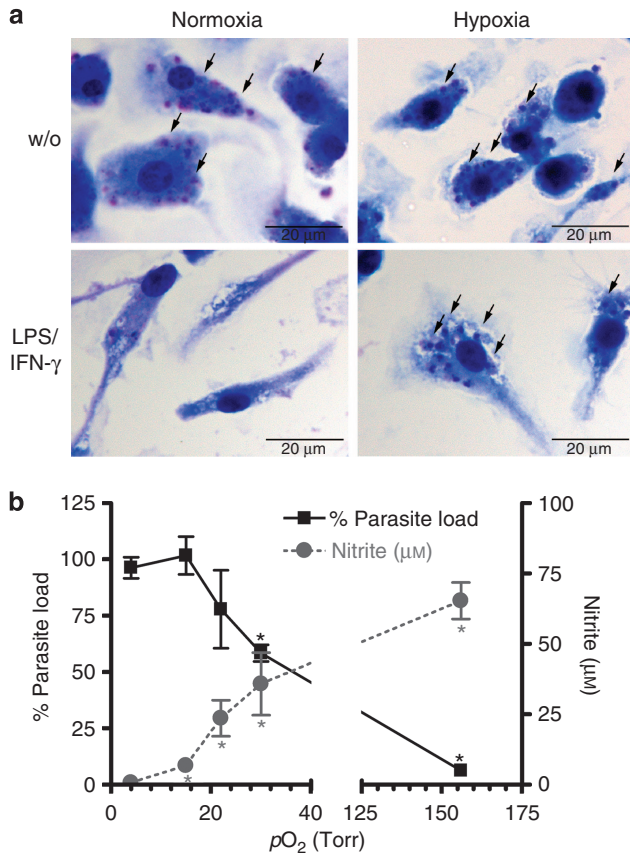
Figure 2. Activated macrophages are impaired to kill *L. major* parasites at oxygen tensions below ~30 Torr (4% O₂). Bone marrow–derived macrophages (BM-MΦ) infected with *L. major* were cultured for 72 hours under various pO_2 with or without (w/o) lipopolysaccharide (LPS)/IFN-γ. (**a**) Representative Diff-Quik staining of infected BM-MΦ cultured under normoxia and hypoxia (pO_2 ~15 Torr (2% O₂)). Representative intracellular parasites are highlighted with black arrows. Bar = 20 μm. (**b**) *L. major* load of BM-MΦ at various pO_2 was determined (black squares). In parallel, in collected supernatants nitrite was measured (grey circles). Means (±SEM) of at least three independent experiments are given. *P (vs. pO_2 4 Torr) < 0.001.

Macrophage inactivation by hypoxia does not result from the modulation of arginase 1 (*Arg1*) or *Nos2* and is reversible

The missing production of NO by NOS2 in the absence of molecular oxygen (anoxia) certainly reflects the lack of cosubstrate (Albina *et al.*, 1995). Whether this also holds true under hypoxic conditions or whether hypoxia might indirectly affect the production of NO by NOS2 needed to be tested in our system. Therefore, we analyzed the *Nos2* mRNA and NOS2 protein expression in *L. major*-infected macrophages after activation with LPS/IFN-γ under normoxic and hypoxic conditions (Figure 3a and b). These experiments revealed that hypoxia did not block the induction of *Nos2* mRNA but partly impaired translation of NOS2 protein (Figure 3a and Supplementary Figure S2 online).

Hypoxia is known to promote *Arg1* expression in macrophages (Albina *et al.*, 1995; Louis *et al.*, 1999; Takeda *et al.*, 2010), which may result in the depletion of the NOS2 substrate L-arginine (Rutschman *et al.*, 2001; El-Gayar *et al.*,

2003). Therefore, we analyzed the *Arg1* mRNA and ARG1 protein expression in *L. major*-infected macrophages. Hypoxia alone or in concert with LPS/IFN-γ stimulation very weakly induced *Arg1* mRNA, which did not lead to an ARG1 protein expression above background levels (Figure 3b). In contrast, stimulation with the known *Arg1*-inducing cytokines IL-4 and IL-10 (Modolell *et al.*, 1995) resulted in an induction of *Arg1* mRNA and ARG1 protein that was further increased by hypoxia (Figure 3b). These results indicated that hypoxia-mediated modulation of NOS2 and ARG1 abundance played only a minor or no role, respectively, for the impaired leishmanicidal activity of LPS/IFN-γ-activated macrophages under low-oxygen conditions.

Reoxygenation of infected and activated macrophages should restore their ability to kill the intracellular parasites, if the blockade of their leishmanicidal activity under hypoxic conditions is primarily or solely due to a shortage of the co-substrate oxygen. Reoxygenation did not result in the recovery of the hypoxia-induced partial impairment of NOS2 protein levels in LPS/IFN-γ-costimulated macrophages (Supplementary Figure S3 online). Nevertheless, cultures of activated and reoxygenated macrophages still produced sufficient levels of NO (Figure 3c) that allowed for the subsequent clearance of intracellular *L. major* amastigotes (Figure 3d). Thus the absent NOS2 enzyme activity rather than the reduced expression of NOS2 protein accounts for the impaired leishmanicidal activity observed under hypoxic conditions. Furthermore, hypoxia did not affect the viability of the infected macrophages, because the reduction of their anti-leishmanial activity was fully reversible as soon as a normoxic pO_2 was reached.

Comparable phenotype of hypoxic wild-type and *Nos2*-deficient macrophages

Assuming that the reduction of NO release is the primary mechanism for the defective leishmanicidal activity of hypoxic macrophages, the phenotype of hypoxic wild-type macrophages should be mimicked by normoxic or hypoxic *Nos2*-deficient cells. In line with previous observations (Diefenbach *et al.*, 1998), *Nos2*⁻/⁻ macrophages were entirely devoid of inducible NO production and were unable to reduce the number of intracellular *L. major* in response to LPS/IFN-γ stimulation (Figure 4a and b). Furthermore, the parasite load of activated *Nos2*⁻/⁻ macrophages kept under normoxic conditions reached the level of activated *Nos2*⁺/⁺ wild-type macrophages cultured under hypoxic conditions. Most notably, hypoxia did not lead to a further increase of the *L. major* load in *Nos2*⁻/⁻ macrophages (Figure 4b). The indistinguishable phenotype of normoxic and hypoxic *Nos2*⁻/⁻ macrophages and of hypoxic *Nos2*⁺/⁺ macrophages demonstrated that the *Nos2*-mediated NO production is the dominant oxygen-dependent anti-leishmanial effector mechanism that operates in macrophages and fails under hypoxic conditions.

To further corroborate this notion, we tested whether the NO donor 1-[N-(2-aminoethyl)-N-(2-aminoethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) alone can rescue the defective anti-leishmanial activity of hypoxic wild-type or of normoxic or hypoxic *Nos2*⁻/⁻ macrophages. Addition of
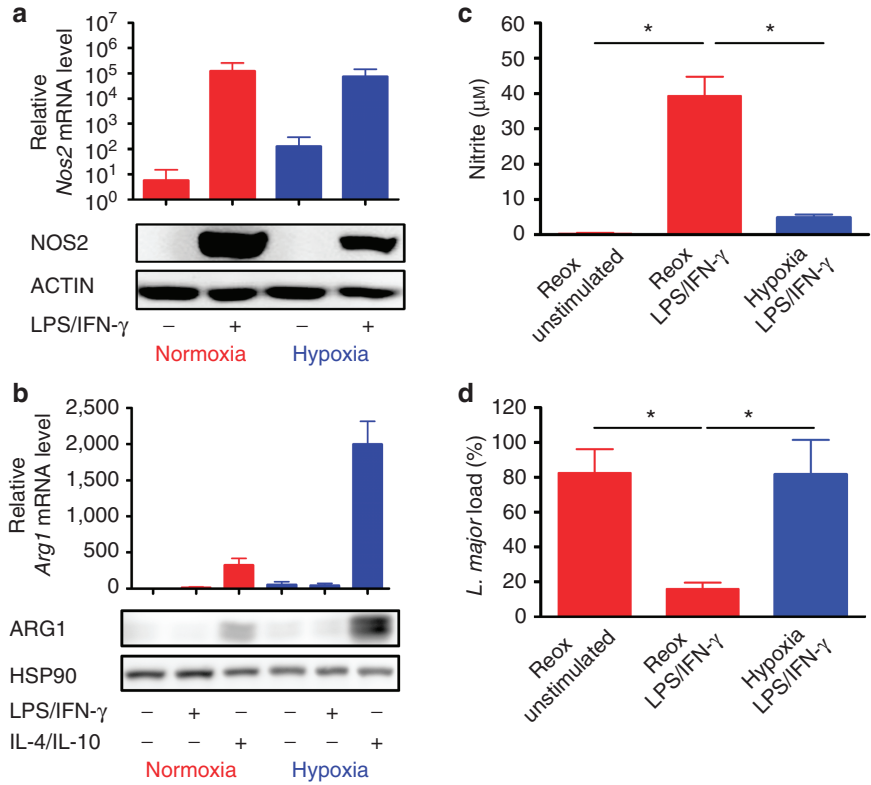
Figure 3. Hypoxia weakly modulates the expression of type 2 nitic oxide (NO) synthase (NOS2) and arginase 1 (ARG1) and does not cause irreversible inactivation of lipopolysaccharide (LPS)/IFN-γ-costimulated macrophages. Bone marrow–derived macrophages (BM-MΦ) infected with *L. major* were cultured with or without LPS/IFN-γ under normoxic or hypoxic conditions. After 24 hours, (**a**) *Nos2* and (**b**) *Arg1* relative mRNA expression were determined (means (+ SD) of at least four similar independent experiments). In addition, NOS2 and ACTIN (**a**), ARG1 and heat-shock protein 90 (HSP90) (**b**) levels were analyzed by immunoblotting. (**c**, **d**) Infected cells were incubated for 24 hours under hypoxia followed by 72 hours under normoxia (reoxygenation, Reox) or for 96 hours under hypoxia. Nitrite content of supernatants (**c**) and *L. major* load of BM-MΦ (**d**) were determined (means (+ SEM) of at least three independent experiments). *$P<0.05$.
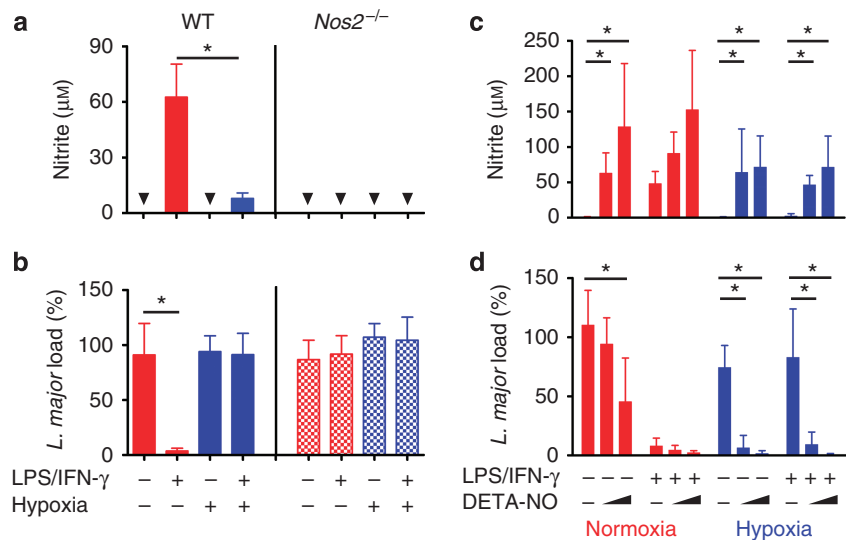

Figure 4. The defective anti-leishmanial activity of activated macrophages under hypoxic conditions results from an absent type 2 nitic oxide (NO) synthase (NOS2) activity and can be rescued by the addition of a NO donor. (**a**, **b**) Wild-type (WT) and *Nos2*$^{-/-}$ bone marrow–derived macrophages (BM-MΦ) infected with *L. major* were cultured with or without lipopolysaccharide (LPS)/IFN-γ under normoxia or hypoxia (2% O$_2$). Nitrite content of supernatants (**a**) and *L. major* load of BM-MΦ (**b**) were determined (means (+ SD) of at least two independent experiments. (**c**, **d**) WT BM-MΦ infected with *L. major* were cultured with or without LPS/IFN-γ under normoxia or hypoxia. Where indicated, DETA-NO (1-[N-(2-aminoethyl)-N-(2-aminoethyl)amino]diazen-1-ium-1,2-diolate; 50 and 150 μM) was added. Nitrite content of the supernatants (**c**) and *L. major* load of BM-MΦ (**d**) were determined. Means (+ SD) of at least three independent experiments are given. Black triangles, not detectable. *$P<0.01$.

DETA-NO increased the concentration of nitrite in the supernatants of hypoxic cells (Figure 4c) and mediated parasite killing in hypoxic macrophages to the same extent as seen in LPS/IFN-γ-costimulated normoxic cells (Figure 4d). Likewise, treatment of $Nos2^{-/-}$ macrophages with DETA-NO restored their leishmanicidal activity (Supplementary Figure S4 online).

Together, these *in vitro* data strongly support the concept that tissue hypoxia in *L. major* skin lesions may negatively affect the parasite control primarily via impeding the production of NO by NOS2.

Increased parasite burden in mice kept in a normobaric, hypoxic atmosphere

Based on the absolute requirement of oxygen for the antileishmanial activity of macrophages *in vitro*, we postulated that experimentally induced inhibition of normalization of tissue oxygenation at the site of infection will delay the clearance of *L. major* in resistant C57BL/6 mice. To test this hypothesis, *L. major*-infected C57BL/6 mice were kept in a normobaric, but hypoxic atmosphere, in which the fraction of inhaled oxygen was reduced from ∼20% to ∼9%. Exposure of infected mice to atmospheric hypoxia not only resulted in a persistently reduced $p_{tc}O_2$ of infected footpads (Figure 5a) but also in an increase of the parasite load and delayed clearance of *L. major* in the lesions (Figure 5b).

As systemic hypoxia can have pleiotropic effects on a whole organism, we set out to demonstrate that the increased parasite numbers under normobaric hypoxia are indeed due to a blunted NOS2 activity rather than to a blocked NOS2

protein induction in *L. major* skin lesions, an impaired T helper type 1 response, or the failing of another oxygen-dependent leishmanicidal mechanism. For that purpose, we performed three types of experiments. First, we analyzed the NOS2 protein abundance in infected footpads. These experiments revealed that NOS2 protein expression was not blocked in infected skin lesions under hypoxic conditions (Figure 5c). In a second approach, we assessed the *Leishmania*-specific T-cell response by analyzing the production of IFN-γ by draining lymph node cells from wild-type C57BL/6 mice after restimulation with soluble *L. major* antigen (SLA). The release of SLA-specific IFN-γ by lymph node cells from infected mice kept under hypoxic conditions was in the same order of magnitude than in the case of the normoxic controls (Supplementary Figure S5 online). In a third approach, both $Nos2^{+/+}$ and $Nos2^{-/-}$ C57BL/6 mice were infected with *L. major* and kept continuously under hypoxic conditions as described above. As seen before, hypoxia caused an approximately 100-fold increase of the parasite load in the infected footpads of wild-type mice. In contrast, there was no such effect of atmospheric hypoxia in $Nos2^{-/-}$ mice (Figure 5d). These results underline that the expression and activity of NOS2 is the dominant oxygen-dependent effector mechanism that accounts for the control of *L. major* at the infection site.

DISCUSSION

To our knowledge quantitative monitoring of skin pO_2 in *L. major*-induced lesions was previously unreported. Earlier studies, which aimed to monitor tissue oxygenation in mouse

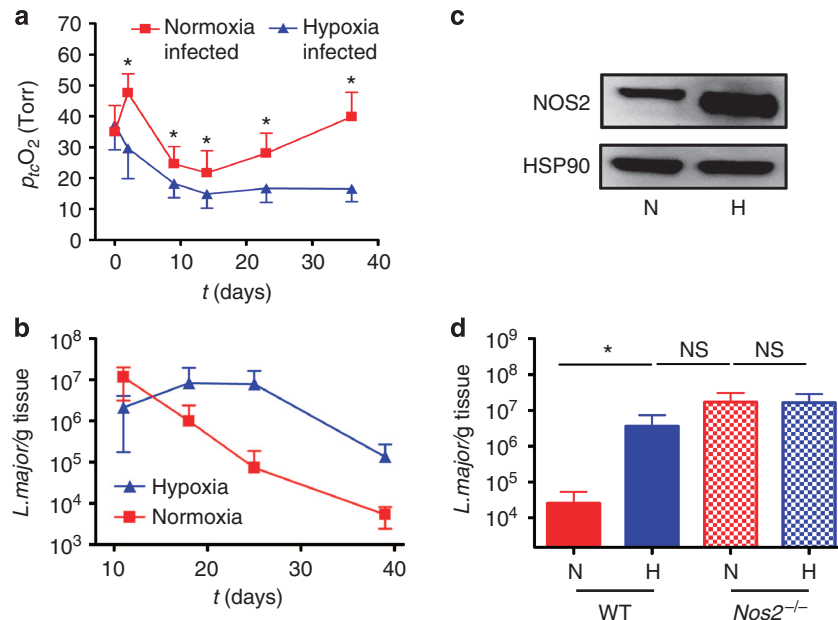**Figure 5. Systemic hypoxia lowers tissue oxygenation of the leishmanial skin lesion and impairs anti-leishmanial activity in a type 2 nitic oxide (NO) synthase (*Nos2*)-dependent manner.** Mice were infected with *L. major* in their hind footpads and subjected to hypoxic (H, ∼9% O_2) or normoxic conditions (N, ∼20% O_2). (**a**) Transcutaneous skin oxygen tension ($p_{tc}O_2$) of the *L. major* skin lesion of normoxic or hypoxic wild-type (WT) mice (means (±SD), $n \geqslant 5$ from two independent experiments). (**b**) Parasite burden in the skin lesions (means (±SD), $n \geqslant 2$ from two independent experiments). (**c**) NOS2 and heat-shock protein 90 (HSP90) levels in infected footpads (day 25, $n = 2$). (**d**) Parasite load of the skin lesions of WT and $Nos2^{-/-}$ mice was determined (day 25, means (+SD), $n \geqslant 12$ from two independent experiments). *$P < 0.05$. NS, not significant.

models of other infections, used electrochemical oxygen sensors (Melican *et al.*, 2008) or applied indirect methods, notably radiolabeled tracers (Harper *et al.*, 2012) or the injection of 2-nitroimidazole derivatives (Heng *et al.*, 2011; Araujo *et al.*, 2012). These indirect methods yielded semi-quantitative data. We resorted to the use of a quantitative method, i.e., transcutaneous luminescence-2D-*in vivo* oxygen imaging using planar sensor films. This method proved to be as reliable but less tedious than the invasive, one-dimensional, polarographic electrode technique (Hofmann *et al.*, 2013). Next, our oxygen sensors do not suffer from the disadvantages of classical electrochemical oxygen sensors (eg, Clark-type polarographic oxygen sensors). They do not consume oxygen, which could compromise precision at low pO_2, and they allow the imaging of relatively large areas within milliseconds (Wolfbeis, 2005; Schreml *et al.*, 2011). In the future, this technology may be used to determine skin tissue oxygen levels in various other skin diseases, such as deep-skin infections, skin cancer, or autoimmune diseases of the skin. In general, this may lead to a better understanding of the impact of skin pO_2 on the pathogenesis of various skin diseases.

Using this technology, we observed that tissue oxygenation dropped transiently to values around 20 Torr in the center of *L. major* lesions when maximum size of the leishmanial lesion was reached. Application of these oxygen levels to activated macrophages blocked their NOS2-dependent anti-leishmanial activity. These data are in line with findings that the ability of macrophages to produce NO correlates inversely with the parasite load and that the production of NO parallels the availability of oxygen (Albina *et al.*, 1995; Melillo *et al.*, 1996; Jantsch *et al.*, 2011; Wiese *et al.*, 2012).

In contrast to our findings, hypoxia promoted the anti-leishmanial control in *Leishmania amazonensis*-infected mouse and human macrophages (Colhone *et al.*, 2004; Degrossoli and Giorgio, 2007; Degrossoli *et al.*, 2007, 2011). This may be due to the degree of the oxygen dependency of *L. amazonensis* itself and/or due to the hypoxia-induced triggering of antimicrobial effector pathways that are specifically directed against the species *L. amazonensis*.

Resolution of *L. major* lesions in resistant mice was closely linked with the return to normal skin oxygenation levels. Subjecting *L. major*-infected macrophages *in vitro* to oxygen levels found in cured skin tissue established a substantial leishmanicidal activity and robust NO production. Our observation that normal skin displayed mild hypoxia is in perfect accordance with several publications that assessed skin oxygenation (Evans and Naylor, 1966; Sheffield, 1988; Intaglietta *et al.*, 1996; Buerk *et al.*, 1998; Evans *et al.*, 2006; Peyssonnaux *et al.*, 2008; Hofmann *et al.*, 2013).

Higher oxygen levels in the outer and/or perivascular regions are likely when the infected skin lesions reach maximum size. Therefore, after initiation of a robust *Leishmania*-specific IFN-γ response we assume that activated macrophages residing in that zone of sufficient oxygenation may start to produce leishmanicidal NO. This possibly explains why there is a gradual clearance of *Leishmania* even when transcutaneously obtained skin oxygen levels in infected tissue are low (Figure 5b). Nevertheless, preventing the reoxygenation of *L. major*-infected tissue in resistant mice by subjecting them to normobaric hypoxia delayed the clearance of *L. major* in a NOS2-dependent manner. This indicates that oxygenation is an important factor of the tissue microenvironment that provides perfectly armed immune cells with their ammunition.

The extracellular concentration of the other NOS2 substrate, L-arginine, regulates the protein expression and enzymatic activity of NOS2 in macrophage cultures (Rutschman *et al.*, 2001; El-Gayar *et al.*, 2003). There is, however, no compelling evidence for a relevant lack of L-arginine at the site of infection in self-healing mice (Modolell *et al.*, 2009).

To date, the exact regulatory mechanisms that govern local tissue oxygenation in *L.*-infected tissues are unknown. In *L. major*-infected tissues of resistant C57BL/6 mice, transient infection-induced metabolic oxygen demands (Campbell *et al.*, 2014) and/or transient impairment of local tissue perfusion (Abbot *et al.*, 1994; Melican *et al.*, 2008; Massberg *et al.*, 2010) might contribute to the low oxygen values. It is tempting to speculate that in mice displaying a non-healing phenotype (such as BALB/c mice) the NO-dependent anti-leishmanial activity of lesional macrophages may be continuously extinguished, because pO_2 on the site of infection might not return to normal levels (as in C57BL/6 healer mice).

In conclusion, we demonstrate that low tissue oxygen levels found at the leishmanial skin lesion impaired the NOS2-dependent leishmanicidal activity of macrophages. A better understanding of tissue oxygenation in infected tissues may pave the way for the development of refined antimicrobial treatment strategies that aim to enhance local tissue oxygenation.

MATERIALS AND METHODS
Reagents
LPS (*E. coli* O111:B4) was purchased from Sigma-Aldrich (Deisenheim, Germany). Recombinant murine IFN-γ IL-10, and IL-4 were purchased from eBiosciences (Frankfurt a.M., Germany) or R&D Systems (Wiesbaden, Germany). The NO-donor DETA-NO was provided by Larry K. Keefer (Center for Cancer Research, National Cancer Institute, Frederick, MD).

Parasites
Promastigotes of the *L. major* strain MHOM/IL/81/FE/BNI (Stenger *et al.*, 1996) were derived from skin lesions of BALB/c mice and propagated *in vitro* in RPMI 1640 (10% FCS) on Novy-Nicolle-MacNeal blood agar slants for a maximum of five passages.

Macrophages
BM-MΦ were generated from C57BL/6 mice (Charles River Breeding Laboratories, Sulzfeld, Germany) and from *Nos2*-deficient mice (B6;129P2-*Nos2tm1Lau*/J41 mice (The Jackson Laboratory, Bar Harbor, ME) in hydrophobic Teflon bags (FT FEP 100 C (Dupont), American Durafilm, Holliston, MA) as described earlier (Wiese *et al.*, 2010).

Macrophage infection studies
For infection, BM-MΦ were co-cultured with *L. major* promastigotes at ratios of 1:30 for 4 hours under normoxic conditions. Thereafter,

extracellular *Leishmania* were washed off, and the macrophages were further cultured in the absence or presence LPS/IFN-γ costimulation (20 ng ml^{-1} each) for a total of 72 hours under normoxic or hypoxic conditions. The infected cells were cultured under normoxic conditions in a regular humidified incubator (37 °C, 5% CO_2, ~20% O_2) or under hypoxic conditions (37 °C, 5% CO_2, 0.5% O_2, unless otherwise indicated in the figure legends) using an adjustable hypoxic humidified workbench suitable for cell culture experiments (invivo300; Ruskinn Technology, West Yorkshire, UK). To examine the influence of reoxygenation, the cells were first incubated under hypoxic conditions and then kept under normoxic conditions for the rest of the experiment (i.e., 72 hours).

The number of parasites per infected cell was determined microscopically after Diff-Quik staining (Medion Diagnostics AG, Düdingen, Switzerland) and used to calculate the number of parasites per macrophages in culture (based on the evaluation of approximately 100 macrophages in at least four different high-power fields). The *L. major* load was determined by dividing the ratio *L. major* in 100 BM-MΦ after 72 hours/*L. major* in 100 BM-MΦ after 4 hours.

RNA isolation, reverse transcription, real-time PCR, and relative quantification

Total RNA was extracted, reverse transcribed, and analyzed by real-time quantitative RT-PCR as described earlier (Jantsch *et al.*, 2011). The following assays were used (Applied Biosystems, Darmstadt, Germany): *Arg1* (Mm00475988_m1), *Hprt1* (Mm00446968_m1); and *Nos2* (Mm00440485_m1). Data were analyzed using the $\Delta\Delta C_T$ method. In unstimulated BM-MΦ, the normalized ratio of target *Nos2* or *Arg1* to the internal control *Hprt1* was set to 1.

Immunoblotting

Preparation of cell lysates and immunoblotting was performed as described earlier (Jantsch *et al.*, 2011). The following antibodies were used: rabbit-anti-ACTIN (Sigma-Aldrich); rabbit-anti-NOS2 (Biomol, Hamburg, Germany); rabbit-anti-heat-shock protein 90 (Santa Cruz Biotechnology, Dallas, TX); goat-anti-ARG1 (Santa Cruz Biotechnology); polyclonal swine anti-rabbit immunglobulins/horseradish peroxidase (HRP; Dako, Hamburg, Germany); donkey anti-goat secondary immunoglobulin G-HRP (Santa Cruz Biotechnology). Densitometry was performed with the ImageJ (1.47v, National Institutes of Health, Bethesda, MD).

Nitrite production

Nitrite accumulation in the supernatant as an indicator of NO production was determined by the Griess reaction.

Infection experiments and determination of parasite burden

All animal experiments were carried out according to protocols approved by the Animal Welfare Committee of the local government authorities (Regierung von Mittelfranken, Ansbach, Germany). We infected C57BL/6 wild-type mice and *Nos2*-deficient mice in their hind footpads with 3×10^6 of stationary-phase *L. major* promastigotes of a low *in vitro* passage (≤5) in 50 μl phosphate-buffered saline. The number of parasites in the tissue was determined by limiting dilution analysis using serial threefold dilution of tissue suspensions and 12 wells per dilution step and applying Poisson statistics and the χ^2 minimization method (L-Calc. software, Stemcell Technologies, Vancouver, Canada).

Oxygen sensing using luminescence imaging

Sensor foils for ratiometric luminescence (VisiSens, Presens, Regensburg, Germany) or luminescence lifetime imaging (LLI) contain both an oxygen-dependent probe that generates an oxygen-dependent signal by collisional quenching of luminescence. Transcutaneous pO_2 measurements of hind footpads of restrained mice using ratiometric luminescence- or luminescence lifetime imaging-based optical readouts were performed in a darkened environment as described previously (Hofmann *et al.*, 2013).

Infection experiments with animals kept under conditions of chronic hypoxia

For studies under conditions of chronic hypoxia, infected mice were exposed to ~9% O_2 in an animal hypoxia chamber (Biospherix, Lacona, NY). Normoxic control mice were kept under ambient air conditions.

In vitro restimulation with soluble *Leishmania* antigen

Single-cell suspensions from popliteal lymph nodes were prepared and restimulated with whole SLA. SLA was prepared by three freeze (−70 °C) and thaw cycles and a final sonication step for 30 seconds (level 5; Branson Sonifier, Fa. Heinemann, Schwäbisch Gmünd, Germany). After 3 days, supernatants were collected, and IFN-γ was measured by enzyme-linked immunosorbent assay (BD Biosciences, Heidelberg, Germany).

Statistical analysis

Statistical significance was calculated with the Prism v4.0 GraphPad software (GraphPad Software, San Diego, CA). For normally distributed data, a Student's *t*-test or an analysis of variance with an appropriate *post-hoc* test was used to compare groups. For non-normally distributed data, the nonparametric Wilcoxon rank-sum test or Kruskal-Wallis test with Dunn's multiple comparison tests was used.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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