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Dual Role for Nitric Oxide in Paracoccidioidomycosis: Essential for Resistance, but Overproduction Associated with Susceptibility

Flávia R. F. Nascimento, Vera L. G. Calich, Dunia Rodríguez, and Mommchilo Russo

Using a murine model of susceptibility and resistance to paracoccidioidomycosis, we have previously demonstrated that immunosuppression occurs in susceptible (B10.A), but not in resistant (A/Sn), mouse strains. Accumulating evidence shows that NO is involved in the induction of T cell immunosuppression during infection as well as in the killing of Paracoccidioides brasiliensis. In the present work, we focused on NO and other macrophage products that could be associated with resistance or susceptibility to paracoccidioidomycosis. A striking difference was related to NO and TNF production. Macrophages from B10.A mice produced high and persistent NO levels, while in A/Sn animals, TNF production predominated. In in vitro cultures, P. brasiliensis-infected macrophages from A/Sn mice also produced large amounts of TNF, while B10.A macrophages only produced NO. TNF production by B10.A macrophages appeared to be suppressed by NO, because the addition of aminoguanidine sulfate, an inducible NO synthase (NOS2) inhibitor, resulted in TNF production. These results suggested that enhanced TNF or NO production is associated with resistance and susceptibility, respectively. However, regardless of the mouse strain, NOS2-deficient or aminoguanidine sulfate-treated mice presented extensive tissue lesions with increased fungal load in lungs and liver compared with their controls. We conclude that NOS2-derived NO is essential for resistance to paracoccidioidomycosis, but overproduction is associated with susceptibility.


N
infection by the dimorphic fungus Paracoccidioides brasiliensis (Pb) can result in a systemic granulomatous disease designated paracoccidioidomycosis (Pb mycosis). Pb mycosis presents a gamut of clinical forms ranging from a localized and benign disease to a relentlessly progressive and potentially lethal systemic infection (1). The outcome of the infection depends on several factors, such as the host responses and the virulence of the infecting agent (2, 3).

We have previously characterized an isogenic murine model in which polar forms of the disease were reproduced. The B10.A mouse strain was found to be susceptible while the A/Sn strain was extremely resistant to Pb infection. Resistant mice are able to control the infection, while susceptible animals develop a progressive disseminating disease with increased recovery of fungal cells from several organs (4). Although the mechanism(s) involved in resistance to Pb infection is poorly understood, it appears that macrophages are fundamental for protection, as mice with blockade of macrophage functions by colloidal carbon are extremely susceptible to infection (5).

A key antimicrobial molecule involved in the killing of intracellular pathogens is NO, which is generated from the amino acid L-arginine by the inducible isoform of NO synthase (iNOS or NOS2). Although NO production is involved in Pb killing (6), it can also be involved in immunosuppression, because NO inhibits lymphoproliferation, TNF-α production, and MHC class II molecule expression (7, 8).

We have previously shown that in susceptible (B10.A), but not in resistant (A/Sn), mice, Pb infection is accompanied by suppression of the Ab response to unrelated Ags and an impaired delayed-type hypersensitivity reaction (9, 10). In the present work, we selected these two polar mouse strains to ascertain by comparison which of the macrophage-derived products could be associated with resistance or susceptibility to Pb infection. We quantified the production of NO and other macrophage-derived metabolites, such as hydrogen peroxide and TNF-α, in infected B10.A and A/Sn mice. We also determined the macrophage expression of MHC class II molecule (Iaβ), as these two strains are identical in the IA region. In this study, we show that the major difference in macrophage responses observed in these strains was related to NO and TNF-α production. Macrophages from susceptible mice released high and persistent levels of NO throughout the infection, while macrophages from resistant mice presented persistent TNF-α production. Incubation of resident macrophages from susceptible or resistant mice with Pb resulted in NO or TNF production, respectively. TNF-α production by B10.A macrophages appeared to be suppressed by NO, because the addition of an NOS2 inhibitor, aminoguanidine hemisulfate (AG), restored TNF-α production. These results indicate that NO might play a negative role in Pb infection. However, B6 mice with NOS2 deficiency were more susceptible to infection than NOS2-sufficient mice. Moreover, treatment with AG during the first 10 days of infection resulted in increased fungal load and more severe infection in both susceptible and resistant animals.
Therefore, we conclude that NOS2-derived NO is essential to confer resistance to paracoccidioidomycosis regardless of the animal genetic background. However, high levels and persistent NO production are associated with susceptibility, indicating that in this situation NO plays a negative role in paracoccidioidomycosis.

Materials and Methods

Mice

Mouse strains susceptible (B10.A) and resistant (A/Sn) to paracoccidioidomycosis were obtained from our Isogenic Breeding Unit (Department of Immunology, Instituto de Ciências Biomédicas, Universidade de Sao Paulo, Sao Paulo, Brazil). C57BL/6 NOS2-deficient and NOS2-sufficient mice were used in one set of experiments. Animals were age and sex matched, fed sterilized food and acidified water, and tested according to Instituto de Ciências Biomédicas, Universidade de Sao Paulo Animal Welfare guidelines.

Fungus

A highly virulent isolate of P. brasiliensis, designated Pb18, was used throughout this study. To ensure the maintenance of its virulence, the isolate was used after three serial animal passages. Pb18 yeast cells were then maintained by weekly subcultivation in semisolid Fava Netto’s culture medium at 35°C and used on the seventh day in culture (11). The yeasts were washed in PBS (pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions, determined by Janus Green B vital dye (Merck, Darmstadt, Germany) (12), was always >80%.

Macrophages

Peritoneal exudate cells were obtained from control or infected mice by washing their peritoneal cavities with 5-ml ice-cold PBS. The cells from individual mice were centrifuged (160 × g, 10 min, 4°C), resuspended in complete RPMI 1640 medium, and adjusted to 2 × 10⁶ cells/ml. Total and differential cell counts were performed on fixed and stained cell suspensions with 0.05% crystal violet dissolved in 3% acetic acid (final solution). Cell suspensions used in all experiments consisted of at least 85% macrophages. For culturing, 100-μl cell suspension was plated onto each well of 96-well flat-bottom tissue culture plates (Corning, Corning, NY), and cultures were incubated at 37°C in 5% CO₂, 95% air.

Experimental protocol

Mice were infected i.p. with 5 × 10⁶ yeast cells of P. brasiliensis. Control animals received sterile PBS. The animals were sacrificed 1, 2, 3, or 4 wk postinfection (p.i.). For in vitro infection, yeast cells were adjusted to 2 × 10⁶ cells/ml and osonopent with complement by incubation for 10 min at 37°C with 10% fresh mouse serum in RPMI medium (13). Thereafter, 10 μl of this suspension was added to 96-well macrophage cultures at a 1:10 ratio (Ph-macrophages). In preliminary experiments, we found that one yeast cell per macrophage induced cell death, while a 1:1 ratio was marginally effective in stimulating macrophages. Plates were incubated for 24, 48, and 72 h for determinations of NO and TNF levels.

AG hemisulfate treatment

To inhibit NO production, mice were given a 2.5% solution of AG (Sigma-Aldrich, St. Louis, MO) in sterilized drinking water for 10 consecutive days beginning 1 day before P. brasiliensis infection. In macrophage cultures, 1 mM AG was used (14).

Macrophage hydrogen peroxide (H₂O₂) release

H₂O₂ production was measured by the modified HRP-dependent phenol red oxidation microassay (15, 16). After peritoneal cells were harvested, as described earlier, the cell suspensions were centrifuged for 10 min at 150 × g and adjusted to 2 × 10⁵ cells/ml in freshly prepared, ice-cold Dulbecco’s PBS containing 5.5 mM dextrose, 0.56 mM phenol red (Sigma-Aldrich), and 8.5 U/ml HRP type II (Sigma-Aldrich). One hundred microliters of the cell suspension was plated onto each well of a 96-well flat-bottom tissue culture plate (Corning), and 10 μl PMA (1 μg/ml; Sigma-Aldrich) was added to each well. The plates were incubated in humidified atmosphere at 37°C for 1 h. Thereafter, 10 μl 1 N NaOH was added to stop the reaction. The absorbance at 620 nm was determined with a microplate reader. The conversion of absorbance to micromolar concentrations of H₂O₂ was deduced from a standard curve. All determinations were performed in quadruplicate and expressed as micromolar concentrations of H₂O₂.

Macrophage MHC class II molecule (IAα) expression

IAα expression by the peritoneal macrophages was determined by cell ELISA using a mouse H-39.487.7 (IgG2a) anti-IAα (17) mAb immediately after cell harvesting. Briefly, 2 × 10⁶ cells (100 μl/well) were incubated in PBS/10% FCS for 1 h; thereafter, when nonadherent cells, mainly lymphocytes, were washed off by three washes with PBS/5% FCS. The remaining adherent cells were fixed with 1% paraformaldehyde in PBS for 15 min, washed twice with PBS/0.05% Tween 20, and incubated for 30 min with 1 μg/ml biotinylated mAb anti-IAα. The bound Abs were revealed by ExtrAvidin-peroxidase (Sigma-Aldrich), followed by o-ortophe- nyldiamine (Sigma-Aldrich) in sodium citrate buffer (pH 5.0) and H₂O₂. The enzymatic reaction was stopped by the addition of 50 μl 4 N sulfuric acid. The absorbance at 492 nm was determined with a microplate reader. All determinations were performed in quadruplicate. The biotinylated anti-IAα was prepared by reacting 1 ml Ab in PBS (1 mg/ml) that was dialyzed at 4°C overnight against 0.2 M borate buffer (pH 8.5) with 100 ml N-hydroxysuccinimidobiotin in DMSO (4 mg/ml) for 4 h at room temperature, followed by overnight dialysis against PBS at 4°C (18).

Macrophage NO production

NO production was quantified by the accumulation of nitrite (as a stable end product) in the supernatants by the standard Griess reaction. Briefly, 50 μl of the supernatants were removed from 96-well plates and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthyle diamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate reader. Conversion of absorbance to micromolar concentrations of NO was deduced from a standard curve using a known concentration of NaNO₂ diluted in RPMI medium. All determinations were performed in quadruplicate and expressed as micromolar concentrations of NO.

TNF activity

TNF activity was evaluated by measuring TNF levels in supernatants of cultured macrophages or by direct macrophage cytoxicity against L929 tumor cells as follows. TNF levels were determined by the standard L929 cytotoxicity assay (19). Briefly, 100 μl of the diluted samples were pipetted into 96-well microtiter plates containing target cells (3.5 × 10⁴/100 μl) in the presence of actinomycin D (2 μg/ml). The samples were incubated with the cells for 20 h at 37°C in 5% CO₂. Thereafter, the remaining viable adherent cells were fixed and stained with 0.05% crystal violet and 3% acetic acid (final dilution) for 10 min. Excess stain was removed by rinsing with tap water, and the plate was air-dried. A volume of 100 μl absolute methanol was added to each well to dissolve the stain. The absorbance was read at 620 nm (Titer-Tek Multiskan; Helsinki, Finland). Macrophage cytotoxicity was determined by coculturing L929 cells with different concentrations (10³–10⁶) of peritoneal macrophages for 4 h. The cultures were treated with actinomycin D (2 μg/ml), and after 20 h of coculture the viable cells were fixed, stained, and processed as described earlier.

The TNF titer (units per milliliter) was defined as the reciprocal of the dilution that induced 50% L929 cell lysis. The macrophage cytolitic activity was determined as the percentage of tumor cytotoxicity, where the percent cytotoxicity = 1 – (OD of L929 cells cocultured with macrophages/OD of control L929 cells) × 100. One unit of TNF-dependent cell cytotoxicity was defined as the number of macrophages that induced 50% lysis of L929 cells. Cytotoxic activity was expressed as TNF units per 10⁵ cells. The specificity of the reaction was evaluated in both assays by the addition of 20 μg/ml polyclonal anti-TNF Abs (Endogene, Cambridge, MA).

CFU assay

The number of viable micro-organisms in the lungs, liver, and spleen was determined by counting the number of CFU. Six to eight animals from each group were sacrificed at 4 wk p.i., and the enumeration of viable organisms in the three organs was performed as previously described (20). Briefly, aliquots (100 μl) of the cellular suspension of each organ were plated on brain-heart infusion agar (Difco, Detroit, MI) supplemented with 4% (v/v) horse serum (Instituto Butantan, Sao Paulo, Brazil) and 5% Pb912 culture filtrate, the latter constituting a source of growth-promoting factor. Plates were incubated at 35°C, and colonies were counted daily until no increase in counts was observed. The number of viable P. brasiliensis colonies per gram of tissue was expressed as the mean ± SEM.

Histopathologic analysis

Livers were removed from different groups of mice and fixed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced, and 5-μm sections were stained by H&E.
for light microscopic examination. The size (area) and number of granulomatous lesions in the liver were quantified by histocytometry using an image analyzer (BioScan/OPTIMAS; Media Cybernetics, Silver Spring, MD). Values were expressed as the mean ± SEM of triplicate sections.

Statistical analysis

The data showing normal distribution were submitted to Student’s t test when comparing two groups or ANOVA for more than two groups. The fungal load data were analyzed using the Mann-Whitney U test. All analyses were made using the Instat Program (GraphPad software, San Diego, CA).

Results

Macrophages from susceptible mice do not release TNF nor present TNF-dependent macrophage cytotoxicity

As TNF is considered a key molecule in granuloma formation (21), we evaluated the capacity of macrophages from infected mice to release this cytokine. Fig. 1A shows that TNF activity was not detected in supernatants obtained from macrophage cultures of susceptible mice. Conversely, macrophages obtained from resistant mice released significant levels of TNF at all time periods examined (Fig. 1A). To further explore the TNF activity, we cocultured macrophages from infected mice with L929 tumor cells and determined the tumor cell cytotoxicity. As shown in Fig. 1B, macrophages from susceptible mice did not exhibit TNF-dependent cell cytotoxicity, while macrophages from resistant mice were able to kill L929 tumor cells at 1, 3, and 4 wk p.i. These results document that TNF production is impaired in susceptible mice.

Macrophages from susceptible mice present prominent and persistent NO production

It is known that TNF is involved in the generation of NO, whereas NO may suppress TNF production (7). Thus, we next determined NO production by macrophages from susceptible and resistant mice. Strikingly, susceptible (B10.A) macrophages presented persistent NO production, while NO production by resistant (A/Sn) macrophages was only detected at 1 wk p.i. (Fig. 2). However, at this time point, the NO levels of susceptible animals were significantly higher (~2-fold) than those of resistant mice. Moreover, except for wk 2 p.i., NO release by susceptible macrophages was prominent (>35 μM), while NO release by resistant mice was <20 μM (Fig. 2). Thus, it is apparent that persistent and exacerbated NO production is associated with susceptibility.

H2O2 production and MHC class II molecules (IAk) expression by macrophages obtained from susceptible and resistant mice

A previous study indicated that Pb infection inhibited MHC class II Ag expression (8). As susceptible and resistant animals in our experimental model share the same IAk molecule in MHC class II complex, we could compare macrophage IAk expression during Pb infection in these mouse strains. Fig. 3A shows that during Pb infection IAk expression increased in both mouse strains except at 2 wk p.i., when IAk expression of A/Sn mice returned to control levels. Surprisingly, the expression of IAk molecule by macrophages from B10.A mice was higher than that presented by A/Sn animals, except at 2 wk p.i. (Fig. 3A). These results indicate that macrophage IAk expression is not impaired in susceptible animals.

Next, we determined PMA-triggered H2O2 production. Fig. 3B shows that macrophage H2O2 production reached maximum levels at 1 wk p.i., decreased at 2 wk, and reached control values at 3 wk p.i. Although H2O2 production was significantly higher in resistant mice at 1 wk p.i., macrophages from susceptible mice produced significant amounts of H2O2, indicating that the difference in H2O2 production by these strains might not influence the outcome of infection.

Differences in NO and TNF production by susceptible and resistant macrophages after infection with P. brasiliensis in vitro

Data obtained in vivo indicated that NO and TNF production is polarized when comparing susceptible and resistant mice. To further investigate whether this polarization could also be reproduced in macrophage cultures, we established an in vitro infection model. Resident macrophages were infected with yeast cells, and TNF and NO levels in supernatants were quantified at 24, 48, and 72 h of coculture. As shown in Fig. 4A, significant NO production by susceptible (B10.A) macrophages was apparent after 24 h and persisted thereafter, whereas NO production by resistant (A/Sn) macrophages was <1 μM at all time points examined. Regarding TNF production, Pb-infected macrophages from both mouse strains released detectable levels...
of TNF at 48 and 72 h, but not at 24 h, of culture (Fig. 4B). However, the production of TNF by resistant macrophages was significantly higher (5- and 3-fold) than that of susceptible animals at 48 and 72 h, respectively (Fig. 4B). Thus, the macrophage-polarized responses were also reproduced in vitro.

AG, a NOS2 inhibitor, blocks NO production and increases TNF release by susceptible macrophages

To identify the isoenzyme responsible for NO production in susceptible macrophages and to ascertain whether NO might have a suppressive effect on TNF production (7), we added AG, a more selective NOS2 substrate inhibitor (22), to Pb-infected macrophage cultures. As shown in Fig. 5, the addition of AG blocked NO production (A) and concomitantly increased TNF release substantially (B). These results indicate that NOS2-derived NO inhibits TNF production and suggest that NO might have a deleterious effect on Pb infection.

NOS2-deficient mice are extremely susceptible to Pb infection

To evaluate the role played by NOS2 in vivo, we compared the outcome of infection of B6 NOS2-deficient with B6 control mice. Infected animals were analyzed at 4 wk p.i. In line with the results obtained with B10.A mice, macrophages from Pb-infected B6 mice released significant levels of NO, while, as expected, NO production was absent in NOS2-deficient mice (Fig. 6A). We next determined the fungal load (monitored by CFU counts) in lungs, liver, and spleen. Fig. 6B shows that NOS2-deficient mice are more susceptible to Pb infection, as CFU counts of lungs, spleen, and liver were, respectively, 2-, 3-, and 4-fold higher than those in B6 mice. The increased susceptibility of NOS2-deficient mice was also apparent when the number and size of granulomatous lesions were determined. As shown in Fig. 6, C and D, the number and size of granulomas in liver were roughly 2- and 4-fold higher in NOS2-deficient mice than in NOS2-sufficient mice. Thus, NOS2-derived NO appears to be essential for the control of fungal growth.

Transient inhibition of NOS2 activity exacerbates Pb infection of susceptible and resistant mice

Our results indicated that either persistent NOS2 activity seen in B10.A mice or persistent NOS2 deficiency appears to be associated with increased susceptibility to Pb infection. To elucidate this
apparent paradox, we asked whether transient inhibition of NOS2-derived NO would have a beneficial effect on Pb infection. To test this hypothesis, susceptible mice were treated with 2.5% AG solution in drinking water for 10 consecutive days beginning 1 day before Pb infection. In parallel experiments, resistant mice received a similar treatment. At 4 wk p.i., the fungal loads of lungs and liver were evaluated. As shown in Fig. 7A, transient inhibition of NO production in B10.A mice resulted in a greater fungal load in these organs compared with nontreated mice. In resistant mice, AG treatment also increased the fungal load compared with nontreated animals (Fig. 7B). However, the fungal load of B10.A mice was significantly higher than that of A/Sn mice when comparing nontreated or AG-treated animals (Fig. 7, A and B). Transient inhibition of NOS2 in Pb-infected B10.A animals resulted in higher NO production at 4 wk p.i. than that in nontreated animals (Fig. 7C). Interestingly, although AG treatment increased the fungal load of resistant mice, the macrophage NO production was still very low and did not differ significantly from that of nontreated animals. Finally, the effect of AG treatment on Pb infection in both mouse strains was evaluated by histology (Fig. 8, A–C) and histocytometry (Fig. 8, E–F) of liver sections. Liver sections from nontreated susceptible mice presented small granulomatous lesions (roughly two lesions per 10 mm²), while in resistant mice no tissue lesions were observed (Fig. 8, A and B, and E and F). Conversely, after AG treatment, the number and size of tissue lesions of susceptible mice increased drastically (25 granulomas/10 mm² with a mean area of 0.8 mm²) as shown in Fig. 8, C, E, and F. Although in resistant mice AG treatment resulted in fungal dissemination to the liver, both the number and the size of granulomas were significantly lower (three granulomas per 10 mm²) than in susceptible mice (Fig. 8, B, E, and F). The above results indicate that transient suppression of NOS2-derived NO production during early infection increases the severity of the disease.

Discussion

In the present work, we compared the macrophage responses to P. brasiliensis infection in resistant or susceptible mice. Macrophage responses were assessed by the production of NO, TNF, and H₂O₂ and by the expression of IAk molecules. These molecules were selected for analysis based on previous reports suggesting that they might influence the outcome of P. brasiliensis infection (7, 8, 23, 24). Minor differences in macrophage responses of susceptible or resistant mice were observed when IAk expression and H₂O₂ production were analyzed, while major differences were related to NO and TNF production. It has been reported that Pb infection impairs

FIGURE 6. NOS2 deficiency enhances fungal dissemination and increases the number of granulomatus lesions. NOS2-deficient or NOS2-sufficient mice were infected i.p. with 5 × 10⁸ yeast cells of P. brasiliensis, and at 4 wk p.i., the following parameters were determined: NO production (A); CFU of lungs, liver, and spleen (B); and area and number of granulomatus lesions (C and D). One representative experiment of two is shown. Results are expressed as the mean ± SEM (n = 5). *, Significant difference (p < 0.05) between NOS2-deficient vs NOS2-sufficient mice.

FIGURE 7. Transient inhibition of NOS2 activity increases NO production and fungal load. Mice were treated or not with 2% AG dissolved in drinking water for 10 consecutive days. On day 2 of AG treatment, mice were infected i.p. with 5 × 10⁸ yeast cells, and 4 wk later, CFU of lungs, liver, and spleen (A and B) and macrophage NO production (C and D) were determined. One representative experiment of two is shown. Results are expressed as the mean ± SEM (n = 5). *, significant difference (p < 0.05) between AG-treated vs untreated group.
MHC class II expression (8) and that macrophages from susceptible mice are less effective in Ag presentation (25). However, our results with IA<sup<k</i> expression are in contrast to those reports, as we showed that macrophage IA<sup>k</i> expression of susceptible mice was not impaired and actually was higher in susceptible than in resistant mice throughout the infection. These discrepancies may be due to the different experimental protocols used. We analyzed IA<sup<k</i> expression by freshly explanted macrophages, while Bocca et al. (8) used thioglycolate-elicited macrophages that were incubated with IFN-γ. Regarding APC function it will be of interest to ascertain whether the Ag presentation of freshly explanted macrophages from susceptible mice is different from that of resistant mice.

We have previously shown that heat-killed yeast cells were as potent as PMA in triggering macrophage H<sub>2</sub>O<sub>2</sub> release (26). This fact suggests that fungal cells might actively induce the release of reactive oxygen intermediates at inflammatory sites. Association between reactive oxygen intermediates release and resistance to infection has been observed with other parasites. For instance, H<sub>2</sub>O<sub>2</sub> release by macrophages, following in vivo challenge with BCG, was higher in resistant than in susceptible animals (27). Furthermore, macrophages from resistant mice infected with Leishmania donovani were shown to be superior H<sub>2</sub>O<sub>2</sub> producers, following IFN-γ treatment, than susceptible macrophages (28). However, in Pb infection, fungal killing seems to be independent of H<sub>2</sub>O<sub>2</sub> production (29). In the present work, we demonstrated that the resistance to Pb infection is not directly correlated with H<sub>2</sub>O<sub>2</sub> levels, because its production was almost similar in both mouse strains. Moreover, we could not detect any significant killing when yeast cells were incubated with different concentrations of H<sub>2</sub>O<sub>2</sub> and HRP (data not shown).

Accumulating evidence points to TNF and/or NO as major molecules involved in resistance to infections by bacteria (Mycobacterium tuberculosis and Salmonella spp.), protozoa (Leishmania major, Plasmodium chabaudi, Toxoplasma gondii, and Trypanosoma cruzi) and helminthes (Schistosoma mansoni) (24, 30; reviewed in Refs. 31 and 32). Regarding Pb mycosis, TNF could participate in granuloma formation that may restrict fungal dissemination (23), while NO might participate in the killing process (6) or in down-regulation of immune responses (7). The fact that we used mouse strains with polarized phenotypes to Pb infection allowed us to test these possibilities. We found that the most striking difference between susceptible and resistant mice was related to NO and TNF production. Macrophages from susceptible mice produced prominent and persistent NO levels throughout the infection, while in resistant mice, Pb infection elicited a low and transient NO production at 1 wk p.i. that was terminated at 2 wk p.i. Conversely, TNF production predominated in resistant animals, but was almost absent in susceptible animals. Strikingly, similar results were obtained when peritoneal cells from resistant or susceptible mice were infected in vitro with Pb. Thus, the differential phenotypic expression of susceptible and resistant macrophages is established during the initial contact of Pb with the host cells. We found that the addition of AG, an NOS2 inhibitor, to macrophage cultures of susceptible mice resulted in significant TNF production, indicating that NO was down-regulating this cytokine. Our results are in consonance with a previous report showing that during Pb infection, inhibition of NO production increased

**FIGURE 8.** Transient inhibition of NOS2 activity exacerbates infection of susceptible and resistant mice. Mice were treated or not with 2% AG dissolved in drinking water for 10 consecutive days. On day 2 of AG treatment, mice were infected i.p. with 5 x 10<sup6</i> yeast cells, and 4 wk later the histopathology and histocytometry of liver sections were performed. Representative liver sections stained with H&E show infected animals (A and C) and infected and AG-treated animals (B and D). Note two small granulomatous lesions in a susceptible animal (A) and the absence of lesions in a resistant animal (C). After AG treatment, the susceptible strain presents multiple and large granulomatous lesions (B), while in resistant mice the liver lesions became apparent. Histocytometry counts confirm the histological findings showing that the area (E) and the number (F) of granulomatous lesions increase after AG treatment. One representative experiment of two is shown. Results are the mean ± SEM (n = 5).
TNF production (7). Similarly, AG treatment increased TNF release of peripheral blood monocytes obtained from premenopausal women (33). We suggest that high and persistent NO production might be detrimental to the host, probably due to inhibition of TNF production. Our results clearly indicate that macrophage TNF production is associated with resistance to Pb infection. Indeed, TNF receptor-deficient mice are extremely susceptible to Pb infection (23).

To further define the role of the NOS2 enzyme in Pb infection, we examined the outcome of infection in B6 mice that are genetically NOS2 deficient. It should be noted that B6 mice are relatively more susceptible than resistant to Pb infection (4). Our results showed that NOS2-deficient mice were more susceptible to Pb infection, as revealed by extensive granulomatous lesions in lungs and in the parenchyma of liver in these animals. Thus, it is clear that NO plays a fundamental role in the effector mechanism underlying resistance.

As NOS2 deficiency permanently impairs NO production, we next examined the effect of transient inhibition of NOS2 activity in susceptible or resistant mice. For this, we treated the animals with AG starting 1 day before infection and continued the treatment for 9 consecutive days. At 4 wk p.i. it was found that in both mouse strains, the transient NOS2 inhibition exacerbated the infection, i.e., the infection disseminated extensively to lungs and liver. These data suggest that regardless of the mouse strain, NO plays an essential role in the control of fungal dissemination. Our results confirm previous reports showing that NO is able to kill fungus and hence control parasite multiplication (6, 7). In addition, we show that paradoxically, macrophages from susceptible mice produce very high levels of NO. It is likely that this high and sustained NO production may be involved in immunosuppression. Indeed, this “two-edged sword” nature of NO (protection vs immunosuppression) has been described in Salmonella, ToxoplasmA, and Trypanosoma infections as well as in tumors (32, 34, 35). We have previously shown that Pb infection in susceptible mice leads to immunosuppression of humoral and cellular responses (9). More recently, it was found that the low lymphoproliferative response of B6 mice infected with Pb was prevented by treatment with Nω-nitro-L-arginine, an inhibitor of NOS1 and NOS2, during infection (7). It is noteworthy that even after AG treatment, the differences between susceptible and resistant mice were still apparent. It will be of interest to determine whether the relative difference between susceptible and resistant mice will decrease after prolonged treatment with AG.

NO production by B10.A macrophages was higher in AG-treated animals than in nontreated animals, while TNF production was not detected. As transient AG treatment increased fungal multiplication at 4 wk of infection, it is possible that the increased fungal load stimulated macrophages to produce more NO. These results indicate that transient inhibition of NOS2 activity quantitatively altered NO production but did not qualitatively change the macrophage responses of susceptible mice. It is noteworthy that the augmented NO levels observed in AG-treated mice did not result in enhanced resistance to Pb infection. We have recently shown that NO is necessary, but not sufficient, to induce macrophage leishmanical activity, because the addition of PGE₂ to macrophages infected with Leishmania amazonensis, increased NO production, but abolished macrophage leishmanical activity (36).

Collectively, our results indicate that macrophage responses are genetically determined and that distinct phenotypic patterns of responses, mainly related to NO and TNF production, are established during the infection. Moreover, although NO derived from NOS2 activity is critical for the control of fungal multiplication in susceptible and resistant mice, high and persistent NO production is the hallmark of susceptibility, while persistent TNF production is associated with resistance to Pb infection. It seems that in susceptible animals, NO production can be viewed in two ways; it controls fungal multiplication, but at the same time inhibits TNF production, which is also essential for the control of fungal dissemination (23). This interpretation is in line with the idea that NO may exert positive and negative effects on the regulation of host defense (32, 37).

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