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17 β -ESTRADIOL INCREASES *LEISHMANIA MEXICANA* KILLING IN MACROPHAGES FROM DBA/2 MICE BY ENHANCING PRODUCTION OF NITRIC OXIDE BUT NOT PRO-INFLAMMATORY CYTOKINES

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Abstract

We have previously shown that female DBA/2 mice are significantly more resistant to *Leishmania mexicana* compared with males. Here, we have analyzed the effect of 17 β -estradiol (E₂) on function and cytokine production in male and female DBA/2 macrophages *in vitro*. We show that E₂ increases NO production and parasite killing in *L. mexicana*-infected male and female DBA/2 macrophages without increasing production of pro-inflammatory cytokines. These data indicate that E₂ may enhance leishmanicidal activity in macrophages by directly regulating production of NO.

INTRODUCTION

Leishmania parasites mainly infect macrophages known to produce a wide range of biologic products that include multiple enzymes, enzyme inhibitors, complement components, reactive oxygen and nitrogen intermediates, and several important cytokines.¹ It is widely accepted that testosterone and 17 β -estradiol (E₂) are critically involved in the control of sexual dimorphism of the immune system.¹ Macrophages, peripheral mononuclear cells, and T cells express surface receptors for E₂, whereas B-lymphocytes express intracellular receptors for this molecule.^{2–5}

We previously showed that gender associated differences in susceptibility to *Leishmania mexicana* in DBA/2 mice are associated with differential expression of IFN- γ and TNF- α production in males and females.⁶ Resistance in female DBA/2 mice is associated with preferential induction of Th1 response and higher production of IFN- γ , whereas susceptibility in males is associated with increased levels of TNF- α but less IFN- γ than

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females.⁶ In humans with *L. mexicana* infections, male infections are more frequent than female infections, and female resistance is associated with increased levels of serum GM-CSF.^{7,8} Despite these observations, little is known about sexual dimorphism of macrophage response in DBA/2 mice infected with *L. mexicana* because studies investigating the immunologic mechanisms mediating sex-determined resistance in female DBA/2 mice have been mainly focused on T cells. In the present study, we examined the effect of E₂ on *L. mexicana* killing and cytokine production in bone marrow derived macrophages (BMDM) derived from male and female DBA/2 mice.

MATERIALS AND METHODS

L. mexicana (MNYC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated subcutaneously into the shaven rumps of 129SvE mice. Breeding pairs of DBA/2 mice were purchased from B and K Universal (Yorkshire, UK). The mice were maintained and bred at the SPF animal facility at The Ohio State University animal facilities in accordance with institutional guidelines. In each experiment, 10 male or female DBA/2 mice were used for BMDM isolation. Bone marrow from femurs and tibias of mice were dispersed and cultured at 37°C and 5% CO₂ in RPMI-1640 culture media (Sigma, St. Louis, MO) containing 100 U/mL penicillin (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Inc., Kerrville, TX) and 20% of supernatant from cultured L-929 cells. Cells were grown in 75-cm² cell culture flasks (Corning Inc., Corning, NY) and collected after 5 days of culture with or without (sham controls) pharmacological concentrations of E₂ (10⁻⁶ M; Sigma). At this time, cells were washed to remove this hormone (E₂) with Hanks' balanced salt solution (HBSS, GIBCO BRL) and plated into 24-well plates as indicated below. More than 90% of differentiated cells were positive for CD11b, a monocyte/macrophage marker as tested by flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA).

BMDM (0.5 × 10⁶) were infected overnight with 2.5 × 10⁶ metacyclic promastigotes from stationary phase of *L. mexicana* (ratio 5:1) in 24-well tissue-culture plates (Corning, Inc.). After this time, cells were extensively washed with HBSS to eliminate non-phagocytosed parasites, and, after stimulation with LPS (1 µg) and IFN-γ (100 U), cells were incubated for 1, 2, 3, and 4 days. Supernatants from each time point were kept at -80°C, and cells stained with Giemsa stain (Sigma). Parasite growth in macrophages was measured 1, 2, 3, and 4 days after stimulation with LPS and IFN-γ, and infection level and infection rate were recorded. In each experiment, infection rates and levels were measured by counting 100 cells on each slide in triplicate at each time point in blinded fashion.

Levels of IL-6 and IL-12 in supernatants were measured using sandwich ELISA methods with recombinant cytokines as standards (BD PharMingen, San Diego, CA). Antibody clones used were as follows: IL-6 clone MP5-20F3 (capture) and clone MP5-32C11 (detection); IL-12, clone C15.6 (capture; p40/p70), clone C17.8 (detection). Nitric oxide determination was performed with the Griess reagent and read at 570 nm. Results were extrapolated from a standard curve prepared with Na NO₂ (0–400 µM).

Student's *t* test was used for statistical analysis, and linear regression analysis was used to determine correlation between NO production and infection level.

RESULTS AND DISCUSSION

After *in vitro* infection with *L. mexicana*, untreated (sham) male-derived macrophages displayed significantly higher infection rates and contained relatively more parasites as compared with female derived macrophages on day 1 post-infection, suggesting that female DBA/2 macrophages are inherently more resistant to *L. mexicana* infection (Figure 1a and 1b). However, both female- and male-derived macrophages treated with E₂ were more resistant to *in vitro* infection with *L. mexicana* as compared with sham controls. By day 4, E₂-treated macrophages from both groups displayed significantly lower infection rates and contained fewer parasites as compared with corresponding sham controls, indicating that E₂ can also enhance leishmanicidal activity in male-derived macrophages and that female-derived macrophages are inherently more resistant to *L. mexicana* infection than males (Figure 1a and 1b).

Some studies indicate that E₂ stimulation might enhance or reduce production of TNF- α production depending on the concentration used to stimulate macrophages *in vitro*.^{9,10} Other reports have indicated that, at physiologic concentrations, E₂ exerts a pro-inflammatory effect, while at pharmacological concentrations it shows an anti-inflammatory effect.¹¹ Estrogens can be converted to intracellular downstream metabolites in peripheral macrophages, and this activity is increased by IL-1 β , IL-6, and TNF- α .¹¹ Furthermore, it has also been shown that E₂ does not affect IL-12 production in murine macrophages but does inhibit production of IL-1 α and IL-6 in these cells, which is associated with decreased LPS-induced NF κ B binding.¹² In the present study, infected cells from female mice produced significantly more NO than males at all time points. Furthermore, E₂ treatment significantly increased NO production in both groups at all different time points (Figure 1c), and higher NO production correlated with lower infection level in both male- and female-derived macrophages at day 4 post-activation ($r = -0.75$ and -0.96 for males and females, respectively). Although female-derived macrophages produced significantly more TNF- α as compared with males on day 1 post-infection, no significant differences were noted in TNF- α production between the groups at all other time points (data not shown). We found that sham-treated BMDMs derived from females produced more IL-6 and IL-12 as compared with BMDMs derived from males throughout the course of the infection. Interestingly, E₂ treatment inhibited IL-6 and IL-12 production in BMDMs derived from female mice but not from male mice (Figure 1d and 1e). This is particularly interesting because IL-12 is down-regulated after treatment with E₂ and during *L. mexicana* infections in mice.^{11,13} Taken together, these findings indicate that E₂ enhances leishmanicidal activity in *L. mexicana*-infected male and female DBA/2-derived macrophages by increasing NO production via a pro-inflammatory cytokine-independent mechanism. Similarly, it has recently been shown that E₂ induces NOS expression and NO production in a dose-dependent manner and is associated with an increase in E₂ R(β) expression on rat peritoneal macrophages.¹⁴ Endogenous E₂ contribution in the two different populations of macrophages studied and intracellular signaling are issues that we are currently addressing.

It was recently reported that E₂ induced NO-associated S-nitrosylation of the E₂ receptor (ER), resulting in selective inhibition of DNA-binding at a specific estrogen-responsive element (ERE). This cross-communication between NO and ER may favor rapid (nongenomic) signaling pathways and subsequent modulation of downstream genomic activity.¹⁵ It will be interesting, for future investigations, to find out if ER in DBA/2 macrophages is S-nitrosylated and whether this down-regulates production of pro-inflammatory cytokines but enhances intracellular *L. mexicana* killing.

In conclusion, our study shows that pharmacological concentrations of E₂ can induce a reduction of parasites counts after *in vitro* infection with *L. mexicana*, which correlates with an increase NO production and which is not due to an increase in production of pro-inflammatory cytokines from macrophages.

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Figure 1. Effect of E₂ on parasite survival (**A, B**) nitric oxide (NO) (**C**) and IL-12 and IL-6 (**D, E**) production in BMDMs from female or male DBA/2 mice. In each experiment, infection rates and levels were measured by counting 100 cells on each slide, in triplicate, at each time point in blinded fashion. At these time points, NO levels in supernatants from individual well were analyzed using Griess reagent, and IL-6 and IL-12 levels were measured by ELISA. Level of significance was determined by Student's *t* test. *P < 0.05. Data (*N* = 9) are expressed as mean ± SEM and are representative of three separate identical experiments.