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Research paper

Myeloid cells do not contribute to gender-dependent differences in disease outcome in murine cutaneous leishmaniasis

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ABSTRACT

Gender-associated differences in the outcome of infections are well known. Apart from behavior-released differences in their incidence, immunological factors also contribute to disease outcome. The underlying mechanisms are often unknown. Here, we show that in murine experimental leishmaniasis, female mice develop larger skin lesions that harbor significantly more parasites, exhibit increased parasite dissemination to visceral organs associated with a shift towards T helper (Th) 2 immunity with increased levels of IL-4. Antigen presenting cells (APC) responsible for T cell priming, such as macrophages or dendritic cells, were not involved in the process. Additionally, in adoptive transfer experiments, we show that differences in the lymphoid lineage are also not critical for mediating these gender-dependent effects. In summary, neither myeloid nor lymphoid cells contribute to disease outcome against this important human pathogen, but stromal cells influenced by e.g. hormonal effects in addition to other parts of the immune system might play a role.

1. Introduction

The protozoan parasite *Leishmania* threatening millions of people worldwide manifests in disease forms ranging from skin lesions to fatal systemic infections [1,2]. The disease is endemic in (sub-) tropical countries. Disease outcome is dependent on the causing subspecies of *Leishmania*, but also on the immune status of the patient. As such, co-infections with e.g. HIV lead to more severe courses of disease, to reactivation of healed leishmaniasis and to treatment resistance [3,4].

The parasite is transmitted to the skin by the bite of an infected sand fly. Within the skin, they are rapidly ingested by skin-resident macrophages (MΦ) and neutrophils. Within MΦ, they transform into the obligate intracellular life form, the amastigotes. After silent replication, parasites are released into the tissue to infect adjacent cells, such as dendritic cells (DC). In contrast to MΦ, which are silently invaded by the parasite, infected DC are activated, process parasite antigen, migrate to draining lymph node cells and release proinflammatory cytokines. Antigen presentation to T cells ultimately leads to lesion resolution in that the induction of IFNγ-producing Th1/Tc1 cells aids MΦ activation. Activated MΦ, in turn, are rendered capable to produce NO to efficiently kill the parasite. Thus, various previous studies have shown that cells of the adaptive immune system are ultimately responsible for parasite elimination and life-long protective immunity [5].

Infections with the parasite are known to show gender-dependent courses of disease in humans and mice. Male and female patients differ in their ability to cope with a variety of diseases. In addition to differences in the likelihood of exposure to parasites due to behavioral and social factors, females typically show increased immunologic responses [6,7]. This elevated immunity, on the other hand, also increases the prevalence of autoimmune reactions. However, it is thought to be beneficial in case of parasitic diseases, since males show increased parasitism in many cases [6–8].

Various studies using mice as model organism for analyzing gender-dependent differences in the outcome of *Leishmania* infections showed contradictive results. Subcutaneous infection of female DBA/2 mice with 5×10^4–10^5 amastigotes revealed that they were more resistant compared to males, but the latter healed their lesions, while corresponding females did not [9]. In contrast, intradermal promastigote inoculation into male B10.129 mice rendered them more resistant compared to female littermates, which developed non-healing ulcers and finally succumbed to

Abbreviations: DC, dendritic cell; MΦ, macrophages; Th, T helper cells; *L. major*, *Leishmania major*; ACP, antigen presenting cell.

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infection [10]. Finally, i.v. infections with 10^7 amastigotes into male BALB/c or DBA/2 mice resulted in worsened disease compared to females [11]. Previously, we demonstrated that transgenic female C57BL/6 mice overexpressing IL-12p40 show dramatically increased lesion development compared to male littermates, when infected with 2×10^5 *L. major* parasites. This was shown to be due to increased levels of the inhibitory IL-12p40 homodimer, IL-12p80, produced by female dendritic cells (DC) [12].

In the present study we now investigated the gender-dependent difference in disease outcome after intradermal application of physiological low-dose [13] infections with 1000 metacyclic *L. major* promastigotes in detail. We utilized C57BL/6 mice, since *L. major* infection of resistant C57BL/6 mice closely mimics human cutaneous leishmaniasis [5]. Interestingly, our study reveals that the more susceptible phenotype of female C57BL/6 mice is likely driven by differences in the behavior of stromal cells, hormones or other factors, but not by cells of myeloid lineage.

2. Material and methods

2.1. Animals

6–8-Week-old C57BL/6 mice were purchased from Janvier and housed under specific pathogen free (SPF) conditions in the Translational Animal Research Center (TARC) of the Johannes Gutenberg-University, Mainz. All animals were housed in accordance with institutional and federal guidelines. All experiments were undertaken with approved license from the Animal Care and Use Committee of the Region Rhineland-Palatinate.

2.2. Parasites and infections

Metacyclic promastigotes or amastigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously [14]. Amastigotes were prepared from infected ears of BALB/c mice as described before [15]. Isolated parasites were opsonized with 5% normal mouse serum for 10 min (37 °C) and washed before *in vitro* or *in vivo* infections. For production of soluble *Leishmania* antigen (SLA), infectious-stage metacyclic promastigotes were isolated from stationary cultures of *L. major* by positive selection using a biphasic Ficoll gradient (10%/20%) as described [15]. Parasites were dissociated by freeze-thaw cycles followed by vigorous vortexing.

2.3. Cells

Inflammatory skin-derived MΦ were elicited by subcutaneous injection of polyacrylamide beads and enriched to homogeneity as described [14,16]. Bone marrow-derived DC were generated in GM-CSF- and IL-4-containing media and harvested on day 6 of cell culture [14].

2.4. Phagocytosis and cytokine release of antigen presenting cells

Isolated cells were sub-cultured in medium (RPMI 1640/5% FCS) at 2×10^5/ml and parasites were added at an MOI of 5 [14]. After 18 h, cells were harvested and cytospins were prepared. Diff Quick-stained cells were analyzed for the presence of intra-and extracellular parasites. At least 200 cells were counted per sample.

Supernatants from parasite/cell co-cultures were collected and assayed for the presence of various proinflammatory cytokines by ELISA (R&D Systems; BD).

2.5. In vivo infections

Male and Female C57BL/6 mice were infected with 10^3 metacyclic *L. major* promastigotes in a volume of 10 µl by intradermal injection into ear skin applied by 0.3 mm diameter needles. Lesion volumes were measured weekly in three dimensions and are reported as ellipsoids: $[(a/2 \times b/2 \times c/2) \times 4/3 \times \pi]$. Parasites present in lesional and spleen tissue were enumerated using a limiting dilution assay as previously described [14]. For measurement of antigen-specific cytokine production in lymph node (LN) cells, single cell suspensions were plated at 1×10^6 LN cells/200 µl complete RPMI 1640 (BioWhittaker) in 96-well plates in the presence of 25 µg/ml SLA. Supernatants were harvested after 48 h of stimulation and assayed using ELISAs specific for IL-12p40 and IFNγ (R&D Systems), as well as IL-4 and IL-10 (BD) [17].

2.6. Bone marrow chimera

Female and male donor bone marrow was used to reconstitute lethally irradiated (9 Gy) recipient female C57BL/6 mice. The resulting chimeric mice were allowed to reconstitute their hematopoietic compartment for at least 6 weeks before analysis. Subsequently, mice were infected with physiologically relevant low dose inocula of *L. major* and lesion development was assessed weekly.

2.7. Statistical analysis

Statistical analysis was performed using StatView software and unpaired students *t*-test.

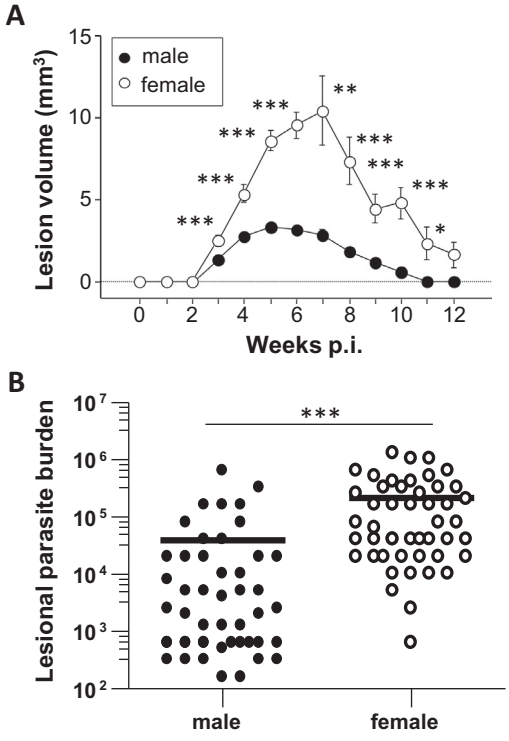

Fig. 1. Gender-dependent course of disease. Female and male C57BL/6 mice were infected intradermally with metacyclic *L. major* promastigotes into ears. A, Lesion development at inoculation sites were monitored and calculated as ellipsoids (mm^3). B, Parasite numbers were assessed by limited dilution assay 6 weeks post infection (dots represent single mice, bars indicate means). A + B, Significant differences between males and females are shown as $^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.005$, and $^{***} = p \leqslant 0.002$ (unpaired students *t*-test, n = 5 independent experiments with ⩾ 24 mice/group).