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NKT cell activation by *Leishmania mexicana* LPG: Description of a novel pathway

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ABSTRACT

NKT cells have been associated with protection against *Leishmania donovani*, yet their role in infections with *Leishmania mexicana* has not been addressed, nor has the activation pathway been defined after stimulation with *Leishmania mexicana* lipophosphoglycan (LPG). We analyzed the activation of NKT cells and their cytokine production in response to *Leishmania mexicana* LPG. Additionally we compared NKT-cell numbers and cytokine profile in lymph nodes of skin lesions induced by *Leishmania mexicana* in BALB/c and C57BL/6 mice. We show that LPG activates NKT cells primarily through the indirect pathway, initiating with TLR2 stimulation of dendritic cells (DC), thereby enhancing TLR2, MHC II, and CD86 expressions and IL-12p70 production. This leads to IFN-γ production by NKT cells. C57BL/6 mice showed enhanced DC activation, which correlated with augmented IFN-γ production by NKT cells. Additionally, infected C57BL/6 mice showed elevated percentages of NKT cells with higher IFN-γ and IL-4 production in lymph nodes.

We conclude that the response of NKT cells towards *Leishmania mexicana* LPG initiates with the indirect activation, after binding of LPG to TLR2 in DC. This indirect activation pathway enables NKT cells to produce IFN-γ during the innate phase of *Leishmania* infection, the magnitude of which differs between mouse strains.

1. Introduction

NKT cells have features of both the innate and adaptive immune responses: they share surface markers with NK cells such as NK1.1 and they also have an invariant T-cell receptor (TCR) characterized by Vα14–Jα18 (Bendelac et al., 2007). NKT cells make up 0.1–0.5% of the peripheral blood leucocytes, they produce cytokines such as IFN-γ, TNF-α, IL-4, IL-10 and IL-13 and additionally are cytotoxic cells capable of destroying cells after TCR recognition, as well as independently of TCR recognition. Furthermore, NKT cells also enhance cytotoxicity of NK cells through their IL-2 secretion (Godfrey et al., 2000; Coquet et al., 2008; Metelitsa et al., 2001). NKT cells are classically activated by glycolipid antigens presented by CD1d molecules of DC (Slauenwhite and Johnston, 2015). One of the best-studied glycolipids capable of activating NKT cells through the direct activation pathway is α-Galactosylceramide (α-GalCer), which induces both IFN-γ and IL-4 production (Juno et al., 2012). However, NKT cells can also be activated through an indirect pathway, where DCs are firstly stimulated by PAMPs (pathogen associated molecular patterns) inducing their IL-12 production, which in turn activates NKT cells (Zajonc and Girardi, 2015).

NKT cells play an important role in autoimmunity and cancer and they also protect against intracellular pathogens, mainly through their IFN-γ production (Terabe et al., 2008; Tsuji, 2006; Ranson et al., 2005). Thus, IFN-γ produced by liver NKT cells was shown to control intravenous infections with *Leishmania donovani* (Amprey et al., 2004). One of the *Leishmania* molecules capable of activating NKT cells is LPG, the most abundant glycolipid covering the surface of the parasite in the promastigote stage of the life cycle (Späth et al., 2003). LPG consists of four domains: (i) a con-

Abreviations: LPG, lipophosphoglycan; DC, dendritic cells; TCR, T-cell receptor; α-GalCer, α-galactosylceramide; PAMPs, pathogen associated molecular patterns; BMDC, bone marrow-derived dendritic cells.

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served GPI anchor [1-*O*-alkyl-2- lyso-phosphatidylinositol (PI)]; (ii) a core [Gal(α1-6)Gal(α1-3)Gal$_f$(β1-3)[Glc(α1)PO4]Man(α1-3)Man(α1-4)-GlcN(α-1) heptasaccharide]; (iii) disaccharide repeats [Gal(β1-4)Man (α1)PO4 units] and (iv) a neutral oligosaccharide forming the terminal ("cap") (Ibraim et al., 2013). Even though NKT cells have been associated with protection against intravenously administered *Leishmania*, their role in skin infections with *Leishmania mexicana* has not been addressed, nor has the activation pathway after LPG stimulation been defined.

The aim of this study was to analyze the activation pathway induced by *Leishmania mexicana* LPG in NKT cells and to comparatively study the cytokine production and infiltration kinetics of NKT cells in lymph nodes draining *Leishmania mexicana*-infected lesions of BALB/c and C57BL/6 mice.

We here demonstrate that *Leishmania mexicana* LPG activates NKT cells mainly through the indirect pathway involving TLR2 stimulation in DC by *Leishmania* LPG, the magnitude of which differs between mouse strains. We furthermore show differences in kinetics and cytokine production of NKT cells in lymph nodes from C57BL/6 and BALB/c mice infected with *Leishmania mexicana*.

2. Materials and methods

2.1. Animals

Male BALB/c and C57BL/6 mice, aged 8–10 weeks, were bred and housed at the animal facilities of the Unidad de Investigación en Medicina Experimental of the Medical School, UNAM, and their handling was done following the National Ethical Guidelines for Animal Health NOM-062-ZOO-1999 and the guidelines recommended for animal care by the Ethical Committee of the Medical School of the UNAM. The animals were kept in pathogen-free micro-isolation cages and received water and food *ad libitum*.

2.2. Leishmania mexicana *infections*

L. mexicana promastigotes were grown in 199 culture medium (Invitrogen Cat. 12350039), supplemented with 10% FBS at 26 °C. After 5 days of culture, promastigotes were harvested in the stationary growth phase. Anesthetized BALB/c and C57BL/6 mice were infected subcutaneously in the earlobe dermis with 1×10^5 parasites suspended in 5 μL isotonic saline solution. On days 1, 3, 5 and 7 post-infection, mice were sacrificed by cervical dislocation.

2.3. Monitoring of lesion size

A comparative study of earlobe lesions was made between BALB/c and C57BL/6 mice infected with 1×10^5 parasites throughout 8 weeks of evolution. Lesion size was evaluated using a Vernier.

2.4. Limiting dilution analysis of parasite burden

Parasite load was determined using the quantitative Limiting Dilution Assay (LDA) described by Titus et al. [15]. Infected ears were aseptically removed from infected BALB/c and C57BL/6 mice. Homogenized tissues were diluted in RPMI-1640 medium supplemented with 10% heat inactivated FBS (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 100 U penicillin/ml and 100 mg/ml streptomycin. The samples were serially diluted into 96-well plates containing biphasic blood agar (Novy-Nicolle-McNeal) medium and incubated at 26 °C during one week. The wells showing parasite growth were registered according to specific dilutions and parasite burdens of tissues were calculated.

2.5. NKT cell count in lymph nodes

NKT cells were comparatively analyzed in the cervical lymph nodes closest to the infection sites in both mouse strains. The cervical lymph nodes were aseptically removed and placed in a Petri dish containing cold PBS. The tissue was disrupted in a 100 μm nylon cell strainer (BD Falcon) and the isolated cells were centrifuged at $800 \times g$ during 10 min at 4 °C. Cells (1×10^6) were suspended in RPMI supplemented with 10% FBS, incubated with 1 μg/ml monensin (00-4505-51 e-Bioscience) during 2 h and stained for FACs analysis. NKT-cell counts were made on days 1, 3, 5 and 7 post-infection. On the day of their peak increase, IFN-γ and IL-4 were analyzed by flow cytometry in purified NKT cells of both mouse strains.

2.6. NKT-cell enrichment from spleen cells

Spleen cells were separated by Ficoll-Hypaque gradient (Sigma) and mononuclear cells were washed twice with PBS. T cells were purified using a Pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). This method yields a cell suspension enriched in NKT cells (Lotter et al., 2009).

2.7. Lipophosphoglycan purification

LPG was purified from *L. mexicana* promastigotes grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS at 26 °C. Parasites were harvested on day 5, which corresponded to the stationary culture phase. LPG was purified from 10^{10} promastigotes as described in the literature (McConville et al., 1987) with some modifications. Briefly, the parasites were centrifuged at $3200 \times g$, the supernatant was removed and the pellet was extracted with chloroform/methanol/water (1:2:0.5; v/v) for 2 h at RT. The insoluble material was used for LPG extraction with 9% 1-butanol in water (2×50 ml) and the pooled supernatants were vacuum dried. LPG was purified from this fraction by octyl-sepharose chromatography in HPLC using a 1-propanol gradient (5–60%) in 0.1 M ammonium acetate. The preparations were negative for the presence of endotoxin using the *Limulus* sp. amebocyte lysate assay (E-Toxate Kit; Sigma). Polymyxin B (5 g/ml) was also used to confirm the absence of contaminating LPS. A sample was analyzed for protein contaminants by SDS–PAGE with silver staining. The preparation was devoid of protein contaminants.

2.8. Bone marrow-derived dendritic cells (BMDC)

Bone marrow was flushed from the femurs and tibiae of eight-week old C57BL/6 and BALB/c mice and plated in 100 mm bacteriological Petri dishes (Falcon) at a concentration of 2×10^6 leukocytes in 10 ml of medium. Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS (endotoxin tested), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg 2-mercaptoethanol (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 25 mM HEPES buffer, and 24 mM NaHCO3 (Sigma–Aldrich Co., St. Louis, MO, USA). Complete medium was supplemented with 10% GM–CSF-containing supernatant from X-63 cell line transfected with the murine GM–CSF gene, shown to be equivalent to 200 U/ml of GM–CSF (Zal et al., 1994). The X-63 cell line was kindly donated by Dr. Brigitta Stockinger (Medical Research Council, Mill Hill, London, UK). On day 3 of culture, 10 ml of GM–CSF-containing medium were added to each dish and on day 6, 10 ml of the culture medium was replaced with fresh GM–CSF-containing medium. On day 8, non-adherent cells were harvested, centrifuged and suspended in complete RPMI medium. Flow cytometry showed that cells were 85% CD11c+ pure.