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Immature Human Dendritic Cells Infected with *Leishmania infantum* Are Resistant to NK-Mediated Cytolysis but Are Efficiently Recognized by NKT Cells

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Dendritic cells (DCs) play an important role in innate and adaptive immunity, interacting with T cells, NK, and NKT cells. A critical step in the interaction of the parasitic protozoa *Leishmania* with their host is the evasion of both innate and adaptive immunity, producing a long-lasting chronic infection. There is growing evidence that these parasites can modify the Ag-presenting and immunoregulatory functions of DCs. The cells and mechanisms involved in innate immune response against *Leishmania* are still poorly understood. In this study, we investigated how *Leishmania infantum* infection affects DC interactions with NK and invariant NKT (iNKTs) cells in humans. We found that infected immature DCs (iDCs) do not up-regulate HLA class I molecules. Despite this, iDCs become resistant to killing mediated by autologous NK cells due to the up-regulation of HLA-E expression, which protects target cells from NK-mediated lysis through interaction with the inhibitory receptor CD94/NKG2A. Furthermore, iDCs infected with *L. infantum* up-regulate CD1d cell surface expression and consequently can be efficiently recognized and killed by iNKT cells that produce IFN-γ. These data suggest that *L. infantum* could be able to evade NK recognition; in contrast, iNKTs may play an important role in the immune response against *Leishmania*. *The Journal of Immunology*, 2006, 176: 6172–6179.

Dendritic cells (DCs)3 play a crucial role in the innate and adaptive immune responses to infectious organisms, acting as potent activators of T cells (1). In the last few years, a number of reciprocal interactions between DC and NK cells have been described, showing that NK cells can be activated by DC through cell contact as well as through soluble factors (2). It has also been shown that immature DCs (iDCs) can be efficiently lysed by autologous NK cells whereas mature DCs (mDCs) become resistant to the killing (3–5).

DCs also interact with a special type of T lymphocytes called NKT cells. NKT lymphocytes are CD1d-restricted T cells that express some NK markers (6). Upon activation, the cells are able to produce Th1 as well as Th2 cytokines (7) and also display cytotoxic activity (8).

The CD1 system is a family of nonpolymorphic Ag-presenting molecules structurally and functionally related to MHC class I that present lipids or glycolipids to T cells. CD1 molecules can be divided in two groups according to sequence homology and tissue distribution (9). Human CD1a, b, and c molecules are members of group I whereas group II is formed by CD1d. CD1a, b, and c molecules are expressed in professional APCs and are involved in defense against microbial infections like mycobacteria. CD1d is expressed on professional APCs and also on epithelial cells. CD1d is expressed on monocytes and this expression decreases during the differentiation process upon culture with GM-CSF and IL-4 (10). CD1d present endogenous as well as exogenous lipidic Ags to NK cells. The most potent activator of NKT cells is α-galactosylceramide (α-GalCer), a compound initially isolated from a marine sponge, which is presented by CD1d (11). The endogenous glycosphingolipid (GSL) isoglobotrihexosylceramide (iGb3) has been recently identified as a major self-Ag presented by CD1d to NKT cells (12). Microbial lipidic Ags structurally related to iGb3 and α-GalCer such as GSL isolated from LPS-negative Gram-negative bacteria (13–15) or phosphatidylinositolmannosides expressed by some mycobacteria have also been described to bind to CD1d and stimulate NKT cells (16).

NKT cells are involved in a number of immune responses including autoimmunity, tumor immunology (17), and immunity against viral, bacterial, fungal, and parasitic pathogens (10).

The protozoan parasites of the genus *Leishmania* cause different forms of diseases in humans, referred to as leishmaniasis. Inside the host, promastigotes infect phagocytic cells where they rapidly transform into the nonmotile amastigote form that replicates, rupturing the host cell and spreading the infection.

Interaction between DC and T cells is essential for the initiation of immune responses against *Leishmania* parasites. Several studies have demonstrated that DC can be infected by *Leishmania* parasites. Some species enhance the surface expression of costimulatory molecules and CD40 ligand-induced IL-12 production in DC; in contrast, other species down-regulate costimulatory molecules and inhibit IL-12 production by DCs. The intrinsic differences among *Leishmania* species with regard to their capacity to subvert
FIGURE 1. *L. infantum* infection of iDCs. a. Flow cytometry analysis of iDCs infected with CFSE-labeled *L. infantum* promastigote or amastigote. To differentiate internalized and noninternalized parasites, infected iDCs were washed with PBS-5 mM EGTA before FACS analysis. Black histogram corresponds to noninfected iDCs. Maturation markers in *Leishmania*-infected iDC. Expression of the indicated markers was analyzed by FACS analysis 48 h after parasite infection. Uninfected cells were used as a negative control. The positive control was LPS-treated DCs. Histograms from a representative experiment are shown. Numbers summarize the percentage of positive cells ± SD for each marker, or, when indicated, the mean fluorescence intensity (MFI) ± SD in independent experiments. Isotype control Ab fluorescence is indicated by a vertical line. ANOVA analysis showed statistical differences between iDCs and LPS-treated DCs, HLA class I expression (MFI) (p = 0.0015, n = 13 different individuals), HLA-DR (MFI) (p = 0.0008, n = 7 different individuals) and percent of CD86-positive cells (p = 0.0001, n = 5 different individuals). The expression of these markers in *Leishmania*-infected iDCs was not different from that in uninfected cells.

![Flow cytometry analysis of iDCs infected with CFSE-labeled *L. infantum* promastigote or amastigote](image)

DC function and maturation may contribute to the healing and nonhealing forms of the disease (18). *L. infantum*, *Leishmania chagasi*, and *Leishmania donovani* are the causative species of visceral leishmaniasis, a life-threatening condition if left untreated.

It has been shown that NKT cells play a crucial role in an early stages of protective immunity against infection with *Leishmania major* in mice (19). It has also been recently described that a subset of positive cells experiment are shown. Numbers summarize the percentage of positive cells ± SD for each marker, or, when indicated, the mean fluorescence intensity (MFI) ± SD in independent experiments. Isotype control Ab fluorescence is indicated by a vertical line. ANOVA analysis showed statistical differences between iDCs and LPS-treated DCs, HLA class I expression (MFI) (p = 0.0015, n = 13 different individuals), HLA-DR (MFI) (p = 0.0008, n = 7 different individuals) and percent of CD86-positive cells (p = 0.0001, n = 5 different individuals). The expression of these markers in *Leishmania*-infected iDCs was not different from that in uninfected cells.

**Materials and Methods**

**Parasites**

*L. infantum* (MCAN/ES/89/IPZ229/1/89) axenic amastigotes were grown as described previously (22) at 37°C. *L. infantum* promastigotes were grown at 27°C in RPMI 1640 (Invitrogen Life Technologies) containing 10% FCS (Harlan Sera-Lab). Metacyclic promastigotes were resuspended at 3.5 x 10^6 cells/ml in complete medium (RPMI 1640, 1% antibiotic/antimycotic solution (Invitrogen Life Technologies) and 10% FCS (Harlan Sera-Lab). Nonadherent cells were removed and the remaining adherent cells were cultured in complete medium, supplemented with 600 U/ml recombinant human (rh) IL-4 and 800 U/ml rhGM-CSF (Immunotools), for 5 days.

To induce maturation, cultures of monocyte-derived DC were supplemented with 10 µg/ml LPS (Sigma-Aldrich) for 48 h.

*Leishmania* parasite infection was performed by adding promastigotes or amastigotes at a parasite-DC ratio of 5:1 and coincubating for 48 h. Lipophosphoglycan (LPG; 1 µg/ml) was purified from *L. donovani* as described (24) and added to iDC cultures 48 h before use. To differentiate internalized and noninternalized parasites, DCs were incubated with *L. infantum*, amastigote and promastigote forms, labeled with CFSE (10 µg/ml) and the cells were washed with PBS-5 mM EGTA before FACS analysis. Phagocytosis was also measured by the cellular uptake of 4-µm latex beads (Red Fluorescent Microspheres; Molecular Probes) or Saccharomyces cerevisiae. DCs were cultured with latex beads for 48 h and washed twice, and the uptake was quantified by FACS. In other experiments, DCs were also cultured with *S. cerevisiae* at a yeast-DC ratio of 2:1.

**Isolation and culture of NK cells**

PBMC were allowed to adhere to plastic, and nonadherent lymphocytes were incubated with 800 IU/ml rhIL-2. After 48–72 h, cells were collected and a highly purified negatively selected population of NK cells was isolated using the NK Cell Isolation kit II Human (Miltenyi Biotec). The percentage of NK cells in that population was confirmed by flow cytometry using FITC-conjugated anti-CD3 and PE-conjugated anti-CD56 mAbs. Prior to cytotoxicity assays, rhIL-2 (50 IU/ml) was again added.

**NKT cell expansion**

NKT cells were expanded from total PBMCs by treatment with 50 IU/ml rhIL-2 in the presence of 100 ng/ml α-GalCer (KRN7000) or PBS-57, an α-GalCer surrogate (P. B. Savage, submitted for publication) for 2 wk.
Identical results were always obtained with α-GalCer or PBS-57. Abs anti-\textit{Vv}24 and anti-\textit{Vv}11 (Beckman Coulter) were used to isolate iNKT cells by Miltenyi MicroBeads systems, and also to identify them by FACS analysis.

**Abs and flow cytometry analysis**

Primary unconjugated Abs used in this study were: anti-human CD1d clone CD1d42 (BD Pharmingen); anti-human CD1a clone OKT6 culture supernatant (from American Type Culture Collection); anti-human HLA-E clone 3D12 (gift from Dr. J. Geraghty, Seattle, WA); anti-human CD94 clone HP3D9, affinity purified (gift from Dr. M. López-Bolet, Barcelona, Spain); PE- and fluorescein-coupled polyclonal goat anti-mouse IgG (H+L; Caltag Laboratories) were used as secondary Abs in FACS analysis. The following conjugated Abs were used: anti-human CD3 Leu4-PE (BD Biosciences), anti-human CD56-PE (Miltenyi Biotec), anti-human CD86-PE (BD Pharmingen), and anti-human HLA-DR-PE (BD Pharmingen). NKp30-human Ig fusion protein (gift from Dr. O. Handelboim, Jerusalem, Israel) was used to test the expression of NKp30 ligand on target cells. Other Abs used were: FITC-conjugated F(ab')2 goat anti-human total Ig (Kallestad). Anti-human HLA-I clone W6/32 was labeled with FITC following standard protocols. Flow cytometry was performed following standard protocols on an Epics Elite Cytometer (Beckman Coulter).

\[ F(ab')_2 \] production

F(ab')2 of CD1d42, 3D12, and MB40.3 (used as negative control) were generated using an Immunopur F(ab')2 Preparation kit (Pierce), following the manufacturer’s instructions.

DCs or CD56+CD3+ effector cells were incubated with 4 μg/ml specific anti-CD1d F(ab')2, anti-HLA-E F(ab')2, anti-HLA-I F(ab')2, or anti-CD94, or F(ab')2 control, for 30 min at room temperature, washed twice, and used in the cytotoxicity assays.

**Cytotoxicity assay**

Cytotoxicity was measured using the nonradioactive Cytotoxicity Detection kit LDH (Roche), following the manufacturer’s instructions. Briefly, cells were cocultured in a 96 V-well plates for 4 h at different E:T ratios, and the percentage of specific lysis was determined from the amount of lactate dehydrogenase activity detected in culture supernatants. All cytotoxic assays were performed in triplicate. The percent of specific lysis was calculated as follows: 100 \times \text{[experimental value - low control]} / \text{[high control - low control]}. Purified CD56+CD3+ and iNKT were used as effector cells and untreated or treated DCs as target cells.

In some experiments, iDCs were pretreated with 100 ng/ml PBS-57 or α-GalCer in DMSO at 37°C overnight. In other cases, infected, uninfected, and LPG-treated iDCs were incubated for 15 min with 1 μg/ml Isolcetin B4 (Fluorescein bandeiraea (Griffonia) Simplicifolia Lectin I (Isolcetin B4); Vector Laboratories) in PBS 0.1 mM CaCl2 to block the Gal α,1,3 Gal epitope.

**Analysis of cytokine-producing iNKTs**

iNKT cells were cocultured for 8 h with uninfected, amastigote-, or promastigote-infected iDCs or LPG-treated iDCs. The percentage of IL-4 and IFN- γ-secreting iNKT cells was determined using the Secretion Assay Detection kit (Miltenyi Biotec), following the manufacturer’s instructions.

**Statistical analysis**

We analyzed samples with the Kolmogorov-Smirnoff and χ2 tests for normality distribution and Bartlett and Cochran’s test to assure homocedasticity. We used one-way ANOVA to test for differences between experiments. The figures represent the mean ± SEM. Error bars ± SD obtained in triplicates.

**Results**

**Human monocyte-derived iDCs infected by \textit{L. infantum} are resistant to NK-mediated cytosis**

To investigate whether iDCs infected with \textit{L. infantum} were killed by NK cells, we first confirmed that \textit{L. infantum} promastigotes and amastigotes were able to infect human iDCs “in vitro”. Our results indicate that both forms of the parasite efficiently infected iDCs as shown in Fig. 1a. We have previously shown that \textit{L. infantum} amastigotes are unable to induce maturation of monocyte-derived DCs (25). For this reason, we wanted to analyze whether promastigotes behave in the same way. Our results, shown in Fig. 1b, indicate that neither amastigotes nor promastigotes are able to induce maturation markers in iDCs, as measured by FACS analysis.

Interestingly, we observed that CD1d was up-regulated in \textit{L. infantum}-infected iDCs.

It is well-established that autologous NK cells efficiently kill iDCs, whereas mDCs are resistant to NK-mediated killing (3). To investigate the capability of NK cells to kill iDCs infected with \textit{L. infantum}, we performed “in vitro” cytotoxic assays using as effector, autologous purified NK cells CD56+CD3+ preactivated with IL-2. The “in vitro” killing assays showed (Fig. 2a) that \textit{Leishmania} infection renders iDCs resistant to the killing at levels similar to that observed with mature, LPS-treated mDCs. No differences were observed between experiments using promastigotes or amastigotes.

In our experiments, the levels of classical HLA class I on the surface of infected and noninfected iDCs were similar (Fig. 1b). In contrast, much higher levels were observed in LPS-treated mDCs, as expected (see Fig. 1b). Thus, differences in the levels of classical HLA class I on the cell surface of infected iDCs were not likely to explain our results. In contrast, we (26) and others (27,...
28) have previously shown that CD1d expression on the surface of MHC class I-deficient cell lines can protect them from NK-mediated cytolysis. Taking into account that the levels of CD1d were significantly higher on the cell surface of infected iDCs, we performed cytotoxic assays blocking CD1d with specific mAbs. As a control, we also performed blocking experiments using anti-HLA class I mAbs. The results (Fig. 2b) of these blocking experiments showed only a partial effect of the mAbs. A partial blocking effect is also observed using W6/32 that recognizes classical HLA class I molecules (Fig. 2c). The effect of both anti-CD1d and anti-HLA class I are of the same magnitude as the ones observed in the uninfected iDCs. Thus, the effect of blocking HLA class I molecules and CD1d molecules are comparable and this strongly suggests that the protective effect observed against the NK-mediated killing in the infected iDCs is not due to the CD1d up-regulation.

The NK killing of iDCs cells is mediated by signals delivered by the activating receptor NKp30 (5). A possible explanation for our data was that *Leishmania* infection would down-regulate the ligand for NKp30. To investigate this possibility, we measured the amount of NKp30 ligand on the target cells using an NKp30-Fc fusion protein in flow cytometry experiments. No differences in staining between infected and uninfected DC were observed (data not shown), indicating that variations in the levels of the NKp30 ligand levels on the infected iDCs were unlikely to explain our results.

*HLA-E is up-regulated on the cells surface of *L. infantum*-infected iDCs and protects them from NK-mediated cytolysis*

It has been reported that the killing of iDCs is confined to the NK subset that expresses CD94/NKG2A, which become inhibited by the up-regulation of HLA-E that occurs during DC maturation (29). Thus, we decided to measure the levels of HLA-E in the *Leishmania*-infected DCs. Our data (see Fig. 3a) showed that levels of HLA-E were significantly increased in the infected iDCs compared with noninfected control cells. No differences on HLA-E levels were observed between experiments using promastigotes or amastigotes. In contrast, the level of HLA-E on the infected cells was not as high as that observed in LPS-treated mature

**FIGURE 3.** iDCs HLA-E expression and NK-mediated cytolytic activity. *a*, HLA-E expression on uninfected and *L. infantum*-infected iDCs. HLA-E expression was measured by flow cytometry. LPS-matured DCs were used as a positive control. **Left panel,** Histograms from a representative experiment are shown; **right panel,** columns represent the mean percentage of HLA-E-positive cells ± SD obtained from five independent experiments with five different individuals. Significant differences were observed between parasite-infected iDCs and mDCs compared with noninfected iDCs. *L. infantum* amastigote (⁎⁎, p = 0.001); *L. infantum* promastigote (⁎, p = 0.03) and mDCs (⁎⁎⁎, p = 0.0001). *b*, Effect of anti-HLA-E F(ab′)2 (3D12) mAb treatment of target cells on NK cytotoxicity test. Nonparasite-infected iDCs (Uninf), amastigote-infected iDCs (Amas), promastigote-infected iDCs and LPS-matured mDCs were used as target cells in 4-h standard cytotoxicity assays where CD56+CD3+ were used as effector cells. The results are represented as mean percentage of lysis ± SE. The inhibition observed in control-F(ab′)2-treated cells (⁎) was reversed in 3D12-F(ab′)2-treated infected iDCs or mDCs (⁎⁎). ANOVA test shows significant differences with 3D12-F(ab′)2 treatment (n = 3); amastigote-infected iDCs E:T ratio 10:1 (⁎, p = 0.045) and 20:1 (⁎, p = 0.023); promastigote-infected iDCs E:T ratio 20:1 (⁎, p = 0.028); and mDCs E:T ratio 10:1 (⁎, p = 0.03) and 20:1 (⁎⁎, p = 0.011). *c*, Effect of anti-CD94 (HP3D9) mAb treatment of CD56+CD3+ effector cells on NK cytotoxicity test. NK cytotoxicity was tested against noninfected or infected iDCs and mDCs. CD56+CD3+ pretreated with 4 μg of HP3D9 and a control mAb were used in 4-h standard cytotoxicity assays. A reversion of the initial inhibition was observed. Mean percentage of lysis ± SEM is represented (n = 3 different individuals). ANOVA test was used to analyze the differences between the treatments at E:T ratio 10:1: with amastigote iDCs (⁎, p = 0.049); promastigote iDCs (⁎, p = 0.012) and mDCs (⁎⁎, p = 0.007).
DCs. For this reason, we wanted to investigate whether these levels of HLA-E were enough to explain the resistance to the NK-mediated killing of infected iDCs. We performed “in vitro” cytotoxicity assays using mAbs to block HLA-E and classical HLA class I molecules to study the contribution of these molecules to the protection against NK killing in the infected target cells. As shown in Fig. 3b, our results indicate that the inhibitory effect in the NK killing assays is blocked by a mAb specific for HLA-E, and the effect was of a magnitude similar to the one observed with LPS-treated mDCs. These results strongly suggest that the higher levels of HLA-E displayed by infected iDCs could explain the resistance to NK killing. To analyze the contribution of the inhibitory receptor CD94/NKG2A, “in vitro” cytotoxicity assays were performed using anti-CD94 mAb. The data obtained (Fig. 3c) show that most of the inhibitory effect observed in the Leishmania-infected iDCs can be reverted in the presence of anti-CD94 mAbs. Thus, our results indicate that NK-mediated cytolysis is inhibited through the CD94/NKG2A receptors which bind the up-regulated HLA-E molecules on the infected iDCs.

**iDCs infected with *L. infantum* up-regulate the expression of CD1d on the cell surface**

It has been recently reported that infection by *L. donovani* inhibits CD1a, b, and c expression in human DCs (30). Our data indicate that infection by *L. infantum* does not alter the expression of CD1a (Fig. 1b). In contrast, CD1d was up-regulated as measured by FACS analysis. As shown in Fig. 4a, the expression of CD1d is significantly higher in *L. infantum*-infected iDCs when compared with uninfected cells. The expression of CD1d starts to increase at 24 h after infection, and the maximum expression is observed at the end of our infection process (48 h) (Fig. 4b). An increase in CD1d expression was not observed when latent beads or *S. cerevisiae* cells were used in our phagocytosis experiments (data not shown).

**L. infantum-infected iDCs are lysed by iNKT cells**

Taking into account the up-regulation of CD1d expression in *L. infantum*-infected iDCs, we decided to study the possible recognition of these cells by iNKT Vα24^+Vβ11^+ lymphocytes. We performed “in vitro” cytotoxicity killing assays using as effector cells α-GalCer expanded and purified autologous iNKT cells. As shown in Fig. 5a, *L. infantum*-infected iDCs are efficiently killed by iNKT cells. NKT cells can respond to CD1d-presenting cells upon recognition of endogenous iGb3. The recognition of endogenous iGb3 is specifically blocked by the lectin IB4 (31, 15). The data obtained in cytotoxicity experiments including this lectin showed (see Fig. 5b) that IB4 was able to completely block the recognition of amastigote-infected DCs by iNKTs whereas recognition of promastigote-infected cells was partially blocked. As expected, recognition mediated by α-GalCer was not blocked by IB4.

**iNKT cells produce IFN-γ in response to *L. infantum*-infected iDCs**

We also measured the production of cytokines by iNKT cells. Our data (Fig. 5c) showed that IFN-γ-secreting iNKT cells were clearly increased in response to *L. infantum*-infected iDCs compared with uninfected cells; however, the number of IL-4-producing cells was very low (Fig. 5d). In addition, we observed that the production of IFN-γ was completely abrogated in the presence of IB4, as shown in Fig. 5c. Data from the killing assays and cytokine production indicate that the Ag recognized by iNKT cells is iGb3 or a related compound.

**LPG induces up-regulation of CD1d on iDCs**

It has been recently described in mice that LPG from *L. donovani*, the major cell surface component of the promastigote form, binds to murine CD1d and activates a subset of liver NKT cells (20). LPG is a glycolipid that shows a high degree of structural similarity to α-GalCer and iGb3. We decided to incubate LPG with iDCs and we observed that LPG alone was able to induce CD1d expression on the iDC and the effect was of a magnitude similar to that observed in the Leishmania-infected DCs, as shown in Fig. 6a. Next, we performed experiments to analyze the recognition of LPG-treated iDCs by iNKT cells. Our results showed (Fig. 6, b and c) that iNKT efficiently recognized LPG-treated iDC cells, which respond by killing the target cells as well as producing IFN-γ. Incubation of target cells with IB4 completely abrogated the iNKT recognition measured by cytotoxicity and also by IFN-γ, suggesting that iGb3 or a related compound is recognized by the iNKT cells.

**Discussion**

The ability of autologous NK cells to kill monocyte-derived iDCs “in vitro” is well-established, whereas after maturation, DCs became resistant to the killing. However, the physiological significance of this phenomenon is unknown. In the present work, we have shown that, whereas the infection of iDCs with *L. infantum* does not induce maturation of these cells, they became resistant to NK killing. Infected iDCs up-regulate the expression of CD1d on the cell surface. Expression of CD1a, b, and c molecules (32) as well as promastigote-infected iDCs (26, 27) can protect MHC class I-negative target cells from NK-mediated cytolysis. In this case, the expression of CD1d does not confer a significant degree of protection as shown in our blocking experiments using anti-CD1d Abs.

In normal, healthy DCs, HLA-E levels increase significantly on the cell surface upon maturation, for example, by treatment with LPS. This also correlates with an important increase in the levels of classical HLA class I molecules, which can provide leader peptides for binding to HLA-E. In contrast with this, we found that *L. infantum*-infected iDCs up-regulate HLA-E expression without increasing the total amount of classical HLA class I molecules on the cell surface. The expression of HLA-E that we observed in *L.
**FIGURE 5.** iNKT cells efficiently recognize *L. infantum*-infected iDCs. *a,* Cytotoxicity test of autologous iNKT cells against iDCs. Cytolytic activity of iNKT was measured by a 4-h standard cytotoxicity assay. Parasite-infected, uninfected, and 100 ng/ml α-GalCer or PBS-57 pretreatment iDCs were used as target cells. The percentage of lysis was higher with infected iDCs compared with noninfected. iDCs pretreated with α-GalCer or PBS-57 were used as control of maximum lysis. The mean percentage of lysis ± SE of three independent experiments are represented (n = 3 different individuals). ANOVA test showed significant differences when comparing the lysis of iDCs with promastigote-infected iDCs (E:T ratio 15:1 (p = 0.02)) and iDCs pretreated with α-GalCer or PBS-57 (E:T ratio 7.5:1 (p = 0.003) and 15:1 (p = 0.013)). *b,* IB4 effect. Target cells were incubated before the cytolytic assay with 1 μg of IB4 in PBS 0.1 mM CaCl₂. The percentage of lysis was reduced after IB4 incubation of infected target cells. The results are represented as the mean percentage of lysis (n = 3 different individuals) at an E:T ratio of 10:1. Significant differences were observed when comparing the lysis between IB4-treated and nontreated amastigote-infected iDCs (+, p = 0.029). This inhibition was not observed in the case of α-GalCer or PBS-57 pretreated iDCs with IB4. *c,* IFN-γ-secreting iNKT cells. iNKTs were cocultured with uninfected, infected and α-GalCer or PBS-57 pretreated iDCs, for 8 h before the FACS analysis. Percentage of IFN-γ-secreting iNKT cells was higher with infected and α-GalCer-treated iDCs and diminished after IB4 incubation. A representative experiment of four is shown. *d,* Percentage of IL-4-secreting iNKT cells. iNKTs were cocultured with infected, uninfected, or α-GalCer pretreated iDCs similar at the previous analysis. Similar IL-4-secreting iNKTs were observed in all cocultured assays. Cytokine secreting iNKT cells in culture were used as negative control in both analyses. A representative experiment of three is shown.

*iNKT* cells efficiently recognize *L. infantum*-infected iDCs. Mature iDCs are the most potent activators of T cells; however, *L. infantum* parasites are unable to induce the maturation and migration capacity of iDCs, impairing in this way the activation of naive T cells. In this context, a possible role for NK cells would be to eliminate iDCs containing intracellular pathogens that are unable to induce the maturation of the host cell. An escape mechanism of *L. infantum* would be to up-regulate HLA-E to avoid the NK killing, a mechanism used by a number of viruses. These results are in agreement with different studies pointing to a modest role of NK cells in immune protection against *Leishmania* parasites.

A recent study (30) showed that *L. donovani* infection of human DCs produces the inhibition of CD1 group I molecules (CD1a, b, and c), which was interpreted as a mechanism to avoid recognition by CD1-restricted T cells. We did not observe down-regulation of CD1a molecules in *L. infantum*-infected iDCs. This is most likely due to differences between the biology of *L. infantum* and *L. donovani* species (34). In contrast, we did observe an up-regulation in CD1d expression on the *L. infantum*-infected iDCs. Human monocytes express CD1d on the cell surface and this expression is down-regulated during the differentiation from monocytes to iDCs. Interestingly, we also found that iNKT cells can very efficiently recognize infected iDCs, producing cytotoxicity and IFN-γ. CD1d-restricted NKT cells can be activated by two mechanisms. NKT cells can recognize an endogenous lysosomal GSL, iGb3, in a self-limited autoreactive way. After infection, this recognition is amplified by the IL-12 secreted by the DCs in response to LPS via TLRs. In contrast, it has been recently reported that NKT cells are able to recognize in an IL-12-independent way, foreign glycosylceramides with structural similarities to α-GalCer and iGb3, from
CD1d expression by iDCs. LPG (1 µg/ml) added to iDCs and was able to induce the expression of CD1d on iDCs to levels similar to those obtained with Leishmania parasites. The histogram from a representative experiment is shown. Isotype control Ab fluorescence is indicated by a dotted vertical line. Five independent experiments with five different individuals were performed. The percentage of CD1d-positive iDCs after culture with LPG was 52, 41 ± 21, 42 (mean ± SD from five independent experiments). b, Cytotoxicity test of autologous iNKT cells against iDCs. LPG-treated iDCs were used as target cells in a 4-h standard cytolytic assay against autologous iNKT cell. The percentage of lysis was higher in the case of target cells cultured with LPG compared with iDCs alone or with IB4-treated iDCs cultured with LPG. The mean percentage of lysis ± SE of three independent experiments with three different individuals is represented. ANOVA test showed significant differences between iDCs alone and cells incubated with LPG (E:T ratio 7.5:1 (p = 0.034) and 15:1 (p = 0.03)). The differences were also observed in comparisons of LPG-cultured iDCs pretreated or nonpretreated with IB4 (E:T ratio 7.5:1 (p = 0.0019) and 15:1 (p = 0.013)). c, Cytokine-secreting iNKT cells. iNKTs were cocultured with LPG-treated or untreated iDCs and the percentage of cytokine-producing iNKT cells was analyzed by FACS. Cytokine-secreting iNKT cells alone in culture were used as a negative control. The results showed an increase in the IFN-γ (but not IL-4) secreting cells against LPG-treated iDCs compared with that observed with the nontreated iDCs. The incubation with IB4 clearly diminishes this IFN-γ production.

Gram-negative, LPS-negative bacteria such as Ehrlichia muris and Sphingomonas capsulate (15). The lectin IB4 specifically binds the terminal Galα1–3Gal bond of iGb3, and it has been shown that this lectin can block the recognition of iGb3 presented by CD1d to NKT cells. Our data show that recognition of iDCs by iNKT cells is almost completely blocked by the lectin IB4, suggesting that iNKT cells recognize iGb3. One interpretation of this is that infection by L. infantum increases the production of endogenous iGb3, for example, up-regulating the β-hexosaminidase responsible for producing iGb3 from the precursor iGb4 in the lysosoma. We have obtained similar results when treating iDCs with purified LPG from L. donovani. This suggests that LPG is able to activate the same mechanisms in the iDCs as live Leishmania did. It is also possible that a compound with a terminal Galα1–3Gal could be generated inside the cell from precursors such as LPG, glycoconjugates, phospholipids, or other glycolipids from Leishmania (35).

In our IB4-blocking experiments, the lectin almost completely inhibited the IFN-γ production by NKT cells against both amastigote-infected and promastigote-infected iDCs. In contrast, IB4 almost completely blocked the cytotoxicity against the amastigote-infected iDCs, whereas the killing against the promastigote-infected iDCs was only partially inhibited. A possible explanation could be that promastigote-infected iDCs are more susceptible to NKT cell-mediated cytolyis than amastigote-infected iDCs. The main component of the promastigote cell surface is LPG which is almost absent from the amastigote. However, LPG is unlikely to be involved in this phenomenon because data obtained when treating the iDCs with LPG showed an almost complete IB4 blocking in both cytotoxicity as well as IFN-γ production by the NKT cells.

It has been recently reported that LPG from L. donovani binds to CD1d and activates a subset of liver NKT cells in a mouse model (20). In our experiments, one possibility was that LPG could bind to CD1d and be recognized by iNKT cells. We have found that cytotoxicity and IFN-γ production in response to iDCs treated with LPG is completely blocked by IB4. Taking into account that LPG from L. donovani lacks a terminal Galα1–3Gal (35), it is reasonable to think that LPG is not likely to be recognized directly by iNKT cells. It could act by inducing the synthesis of endogenous iGb3 and/or a related LPG-processed compound. A similar mechanism would also act in the amastigote form, which lacks LPG.

In summary, our data provide evidence that NK cells are not activated by iDCs infected with L. infantum. This can be a mechanism of immune evasion against the NK recognition. In contrast, iNKT cells readily recognize these cells producing IFN-γ and a powerful cytotoxic response. This cytotoxic response as well as the IFN-γ could be very important because it can be produced rapidly in early phases of infection, facilitating the development of a Th1 response against Leishmania.

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References


