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Interleukin-27 (IL-27) Mediates Susceptibility to Visceral Leishmaniasis by Suppressing the IL-17–Neutrophil Response

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The relationship established between *Leishmania infantum* and the vertebrate host can lead to a self-healing infection or to the manifestation of visceral leishmaniasis, a chronic systemic infection associated with high rates of mortality. We hypothesized that regulatory cytokines, such as interleukin-27 (IL-27), play a role in susceptibility to *L. infantum* infection. IL-27 is a heterodimeric cytokine composed of IL-27p28 and EBi3 subunits which, when combined, bind to IL-27R, leading to STAT-1 and -3 activation, playing a role in the regulation of the immune response. We observed in this work that IL-27 regulates the Th1/Th17 profiles in a mouse model of visceral leishmaniasis (VL) caused by *L. infantum*. We showed here that the pathogen recognition by endosomal Toll-like receptors triggers a type I interferon (IFN) response, which acts through the type I IFN receptor and interferon regulatory factor 1 to induce IL-27 production by macrophages. Furthermore, IL-27 plays a major regulatory role *in vivo*, because *Ebi3*$^{-/-}$ mice can efficiently control parasite replication despite reduced levels of IFN-γ compared to wild-type mice. On the other hand, the absence of *Ebi3* leads to exacerbated IL-17A production in the infected organs as well as in a coculture system, suggesting a direct regulatory action of IL-27 during *L. infantum* infection. As a consequence of exacerbated IL-17A in *Ebi3*$^{-/-}$ mice, a greater neutrophil influx was observed in the target organs, playing a role in parasite control. Thus, this work unveiled the molecular steps of IL-27 production after *L. infantum* infection and demonstrated its regulatory role in the IL-17A–neutrophil axis.

Visceral leishmaniasis (VL), or kala-azar, is a systemic chronic disease with high mortality rates if not treated. It is caused by the parasites *Leishmania infantum* and *L. donovani*, and it is estimated that 300,000 new cases and 20,000 deaths occur annually (http://www.who.int/mediacentre/factsheets/fs375/en/). After dermal inoculation by the sandfly vector, the parasite disseminates to the liver, spleen, bone marrow, and lymph nodes of susceptible hosts, causing symptoms such as hepatosplenomegaly, lymphadenopathy, anemia, constant fever, and immunosuppression (1). Even though it is considered one of the six most important parasitic diseases affecting humans, the immunobiology of VL is not completely understood and novel therapeutic approaches are desired.

The immune response against *Leishmania* spp. is critically mediated by gamma interferon (IFN-γ)-producing Th1 cells, which activate macrophages to produce leishmanicidal compounds, such as nitric oxide (NO) (2). Together with IFN-γ, the inflammatory cytokine interleukin-17A (IL-17A) also mediates protection against *L. infantum* (3, 4). On the other hand, Th2 cytokines are involved in susceptibility to leishmaniasis (2, 5). Moreover, the regulatory cytokines IL-10, IL-21, and IL-27 are produced in the bone marrow of VL patients, suggesting their association with the disease (6).

The cytokine IL-27 is a dimer composed of p28 (IL-30) and EBi3, which together bind to the IL-27R receptor, also a dimer of IL-27Rα and gp130 (7). It was first demonstrated that EBi3 is produced largely by Epstein-Barr virus-infected B cells, but later studies also reported its production by T cells (8), antigen-presenting cells (9), and even keratinocytes (10). EBi3 can form heterodimeric cytokines when combined with IL-12p35, forming IL-35, or with IL-27p28, forming IL-27. The latter can regulate a variety of T cell-mediated immune responses, such as inhibition of Th2 (11) and Th17 development (12), induction of the regulatory molecule Tim3 in Th1 cells (13), and induction of IL-10 secretion by Th1 lymphocytes and CD8^{+} T cells (14, 15).

The role of IL-27 in *Leishmania* infection is dependent on the species and/or experimental setting utilized. In the case of cutaneous leishmaniasis (CL), caused by *L. major* (16), IL-27 signaling is involved in host resistance, since *Il-27ra*$^{-/-}$ mice present increased amounts of parasites. However, during *L. amazonensis* infection, IL-27 administration leads to higher parasite replication, both *in vitro* and *in vivo* (17). In the context of VL caused by *L. donovani*, *Il-27ra*$^{-/-}$ mice present reduced parasite burdens in the spleen and liver (18). Therefore, the role of IL-27 in the regulation of the immune response can lead to higher parasite replication or to the control of infection. It was recently observed that visceral leishmaniasis patients present high levels of IL-27 in serum, which decreases after successful treatment (19). Thus, we hypothesized that IL-27 plays a role in the regulation of immune

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response during *L. infantum* infection, which could exacerbate the disease in a mouse model of visceral leishmaniasis.

We showed here that IL-27 is a regulatory cytokine that drives host susceptibility to *L. infantum* infection. Moreover, we described the mechanisms that control IL-27 production by infected cells, as well as its role in the regulation of the IL-17A–neutrophil axis. The identification of the regulatory mechanisms triggered after *Leishmania* infection can contribute to the development of rational immune interventions, treatment, and prophylaxis for leishmaniasis.

MATERIALS AND METHODS

Mice. C57BL/6, 129SVev, *Ebi3*$^{-/-}$, *Trif*$^{-/-}$, *Irf-1*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, *Myd88*$^{-/-}$, and *Il-17ra*$^{-/-}$ mice were used for the experiments. Animals were maintained in temperature-controlled rooms (22 to 25°C) at the animal facility of the Ribeirão Preto Medical School, University of São Paulo, receiving water and food *ad libitum*. All experimental procedures were approved by the Ethics in Animal Experimentation Committee (CETEA) from Ribeirão Preto Medical School (approval 101/2013).

***L. infantum* infection.** The isolate HU-UFS14 of *L. infantum* (4) was used throughout this work. The parasite was grown in Schneider's medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum (GIBCO BRL) plus 1% (vol/vol) penicillin-streptomycin (Sigma-Aldrich Co, Saint Louis, MO) and 2% (vol/vol) human male urine at 25°C for 5 days. To obtain *L. infantum* lysates, pellets of parasites were submitted to a freeze-thawing process, and protein concentration was determined through a Bradford assay (Sigma-Aldrich Co., St. Louis, MO). To perform infections, mice were anesthetized using 100 mg/kg ketamine–12.5 mg/kg xylazine (intraperitoneally [i.p.]) and were intravenously infected with 100 μl containing 10^7 *L. infantum* promastigotes injected in the retro-orbital plexus. Spleen and liver were collected to determine the parasite titers by quantitative limiting-dilution assay (20, 21). For *in vitro* experiments, cells were infected with *L. infantum* at a multiplicity of infection (MOI) of 5.

Macrophage and DC differentiation and stimulation. Bone marrow-derived macrophages (BMDMs) were obtained as previously described (22, 23), with some modifications. Briefly, isolated femurs and tibias were flushed with phosphate-buffered saline (PBS), and suspensions were grown in RPMI supplemented with 30% L929-cell conditioned RPMI medium with 10% (vol/vol) fetal bovine serum (FBS; GIBCO BRL) and 1% (vol/vol) penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO) to obtain BMDMs after 7 days of culture. Bone marrow-derived dendritic cells (BMDCs) were obtained by culturing bone marrow suspensions in RPMI supplemented with 10% (vol/vol) FBS (GIBCO BRL), 1% (vol/vol) penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MO), and 20 ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ, USA) for 7 days. After differentiation, cells were harvested and infected with *L. infantum*. Where indicated, 1,000 U/ml of recombinant mouse IFN-β or 5 μg/ml of rat anti-mouse IFN-β (PBL Assay Science, NJ, USA) was added to the culture medium. Cultures were kept under 5% CO_2 at 37°C for 24 h, when supernatants were collected for posterior analysis.

Cytokine assay. The concentration of cytokines was determined in cell culture supernatants or in tissue homogenates. To obtain spleen and liver homogenates, samples were collected, weighed, and triturated in 0.5 ml of PBS containing Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Spleen cells were stimulated with 25 μg of *L. infantum* lysate for 48 h, and supernatants were collected to further cytokine analysis. Determination of IL-27p28, IL-10, IL-17A, and IFN-γ was performed using DuoSet kits (R&D Systems, Minneapolis, MN, USA). IFN-β levels were assessed using Legend Max mouse enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA). All procedures were done according to the manufacturer's instructions.

Quantitative PCR (qPCR). mRNA from tissue samples was obtained after extraction with an SV total RNA isolation system kit (Promega, Madison, WI) according to the manufacturer's instructions. SuperScript III reverse transcriptase (Invitrogen) was used to obtain cDNA. SYBR green mix-based real-time quantitative PCR assays were performed using the StepOnePlus real-time PCR system (Applied Biosystems, Singapore, Malaysia). The primers used in reactions are listed in Table S1 in the supplemental material. The mean threshold cycle (C_T) values were used to calculate the expression of the target genes, which were normalized to the housekeeping genes, using the $2^{-\Delta\Delta CT}$ formula.

Flow cytometry. A fragment from the spleen was harvested and triturated to obtain a single-cell suspension, which was then stimulated and stained. For surface analysis, cells were fixed, washed, and stained with fluorochrome-conjugated anti-class II major histocompatibility complex (MHC) (IA/IE), anti-CD11b, and anti-Ly6G (BD Biosciences, San Diego, CA). For cytokine analysis, cells were stimulated with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) in the presence of the protein transport inhibitor GolgiPlug (BD Biosciences, San Diego, CA) for 4 h at 5% CO_2 and 37°C. Cells then were fixed and permeabilized using a Cytofix/Cytoperm (BD Biosciences, San Diego, CA) kit according to the manufacturer's instructions. Further, cells were stained with the fluorochrome-conjugated antibodies anti-CD3, anti-CD4, anti-IL-17A, and anti-IFN-γ, followed by acquisition with a FACSCanto II (BD Biosciences, San Diego, CA). Data were analyzed using FlowJo software (Tree Star, OR, USA).

Cell sorting. BMDCs were obtained from C57BL/6 and *Ebi3*$^{-/-}$ mice as described above and were stained with fluorochrome-conjugated anti-CD11c and MHC-II (BD Biosciences, San Diego, CA). Highly purified CD11c^{hi} MHC-II^{+} cells were obtained after cell sorting using a FACSAria III (BD Biosciences, San Diego, CA). Cells were plated and either left uninfected or infected with *L. infantum* (MOI of 5). Lymph nodes from C57BL/6 mice were collected and triturated, and single-cell suspensions were stained with fluorochrome-conjugated anti-CD4, anti-CD44, and anti-CD62L. Naive CD4^{+} CD62L^{+} CD44^{-} cells were sorted using a FACSAria III (BD Biosciences, San Diego, CA) and were added to cultures containing DCs in the presence of 4 ng/ml of anti-CD3 (clone 145-2C11; BioXCell, NH, USA). After 5 days of culture, supernatants were collected to determine cytokine production.

Immunohistochemistry. Liver fragments fixed in formalin were subjected to routine paraffin embedding. Serial 5-μm cuts were adhered to silane-coated slides. Antigen recovery was performed using incubation in citrate buffer (pH 6.0) for 15 min at 42°C. Endogenous peroxidase was blocked using 3% H_2O_2, and nonspecific reactions were blocked with 1% bovine serum albumin (BSA). The sections were incubated overnight with a monoclonal rat anti-Ly6G antibody (clone 1A8; BioLegend, San Diego, CA, USA), followed by incubation with a biotinylated secondary antibody and avidin-biotin complex (Vector Laboratories, Ontario, Canada). The reaction was detected with diaminobenzidine, and the sections were counterstained with hematoxylin. The stained area corresponding to Ly6G-positive cells was determined in photomicrographs of 20 fields using ImageJ in concert with the immunohistochemistry (IHC) Toolbox plugin.

Neutralization of IL-17 and IFN-β. To neutralize IL-17A during infection, 20 μg of anti-IL-17A (clone 50104; R&D Systems, Minneapolis, MN) or control IgG was administered i.p. on day −1, day 7, and day 14 of infection. To neutralize IFN-β *in vitro*, monoclonal rat anti-mouse IFN-β (clone RMMB-1; PBL Assay Science, NJ) was added to BMDM cultures at a concentration of 5 μg/ml.

Statistics. Each variable from data collected in the experiments was submitted to an analysis of normal distribution and homogeneity of variance. Parametric analyses such as Student *t* test and analysis of variance (ANOVA) were applied to data presented with normal distribution and variance homogeneity, which were shown as means ± standard errors of the means (SEM). The conclusions took into consideration a significance

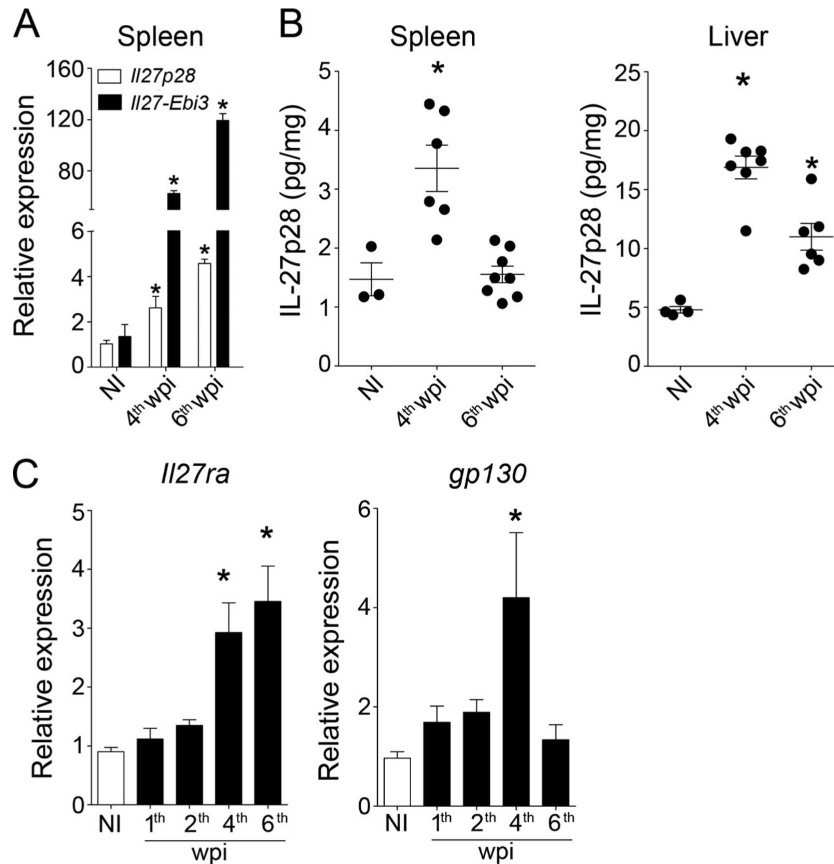

FIG 1 IL-27 and IL-27R are induced in a mouse model of visceral leishmaniasis. C57BL/6 mice were intravenously infected with 10^7 axenic stationary-phase promastigotes of *L. infantum*, and at the 4th and 6th wpi, mRNA and protein from the spleen and liver were analyzed. (A) *Il-27p28* and *Ebi3* mRNA expression in the spleen was analyzed through normalization to the housekeeping genes using the $2^{-\Delta\Delta CT}$ formula. (B) IL-27p28 protein levels in the spleen and liver were evaluated by ELISA. (C) The expression of IL-27R subunits was evaluated in the spleen of infected mice through normalization to the housekeeping genes using the $2^{-\Delta\Delta CT}$ formula. Dots represent individual mice, and each bar represents an independent group of 3 to 5 mice, expressed as mean values (±SEM). Data are representative of those found in 1 to 3 experiments. *, $P < 0.05$ by ANOVA, followed by Bonferroni *post hoc* adjustment on infected versus noninfected (NI) mice.

level of 95% ($P < 0.05$), and distinct statistical groups are represented by an asterisk.

RESULTS

Endosomal TLRs and type I IFN signaling are required for IL-27 production in *L. infantum* infection. We first assessed whether IL-27 is produced during *L. infantum* infection. For this, C57BL/6 mice were intravenously infected with 10^7 axenic stationary-phase promastigotes, and at the 4th and 6th weeks postinfection (wpi), when the hepatosplenomegaly is observed, samples were collected for mRNA and protein analysis. We observed that both *Il-27p28* and *Ebi3* transcripts were upregulated after infection compared to uninfected controls (Fig. 1A). Corroborating these data, high levels of the protein IL-27p28 were observed in the spleen and liver of infected mice (Fig. 1B). To verify if the cytokine could play a biological role, we examined the kinetics of mRNA expression of the IL-27 receptor subunits *Il-27ra* and *gp130*, which were upregulated mainly at week 4 after infection (Fig. 1C), suggesting that *L. infantum* infection induces both IL-27 production and its receptor's expression. As IL-27 is produced *in vivo*, we investigated the mechanisms that lead to its production after *L. infantum* infection. We first observed that both bone marrow-derived macrophages (BMDMs) (Fig. 2A) and dendritic cells (BMDCs) (see Fig. S1 in the supplemental material) were able to produce IL-27 when infected. As macrophages are the major host cell for *Leishmania* parasites and therefore are a key to studying susceptibility/resistance during this infection, we screened for IL-27 production in Toll-like receptor (TLR)-deficient BMDMs after *L. infantum* infection. We observed that the signaling pathways through TRIF and MyD88 are crucial for IL-27 induction, since *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ BMDMs were unable to produce IL-27. Moreover, TLR3 and TLR9 deficiency in infected BMDMs also abrogated IL-27 production, which was not observed in the absence of TLR4 (Fig. 2A).

As it was previously described that type I IFN precedes IL-27 secretion by LPS-stimulated BMDCs (24), we evaluated whether this mechanism also existed in *L. infantum*-infected macrophages. Interestingly, we observed that BMDMs deficient for TLR3 and TLR9, as well as for MyD88 and TRIF adaptor molecules, were not able to produce IFN-β after *L. infantum* infection, indicating their crucial role in this type of cytokine production (Fig. 2B). To understand the role of type I IFN signaling in the production of IL-27, we added IFN-β-neutralizing monoclonal antibodies in BMDM cultures, which reduced the IL-27 levels in supernatants compared to those of controls (Fig. 2C). To further confirm these

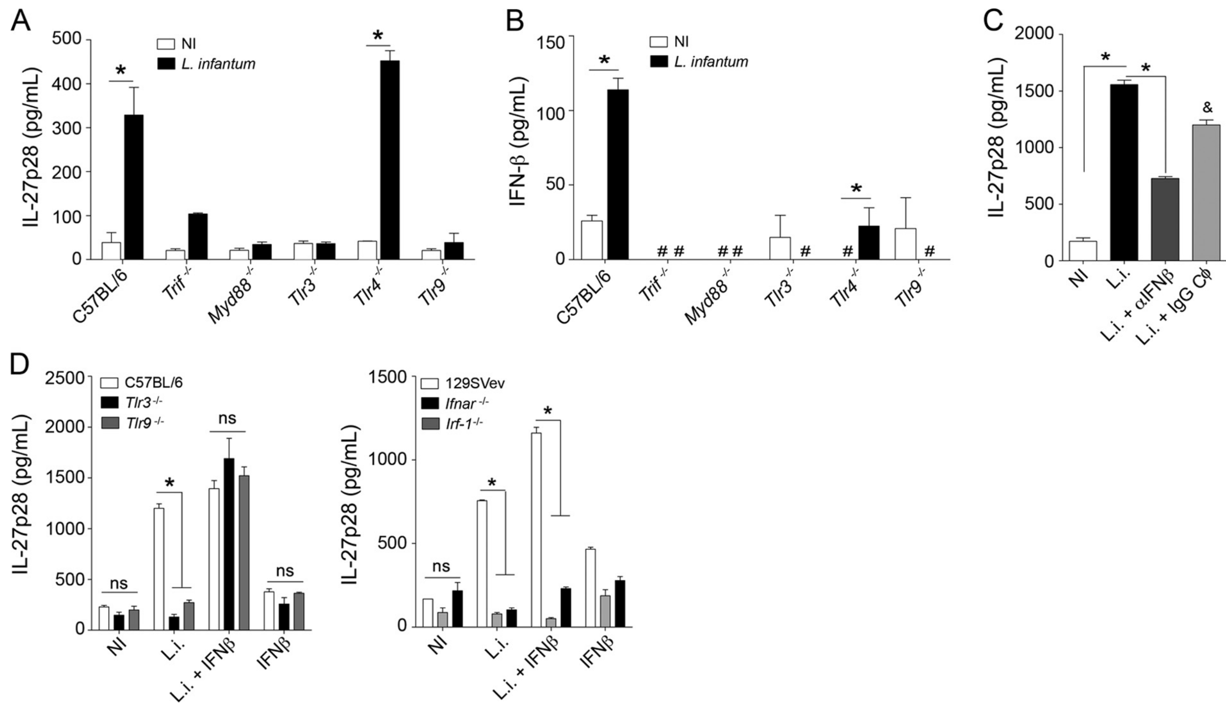

FIG 2 Endosomal TLRs and type I IFN production are critical for IL-27 secretion after *Leishmania infantum* infection. Bone marrow-derived macrophages were generated and infected with *L. infantum* at an MOI of 5, and cytokine levels were evaluated in the supernatants by ELISA. (A) IL-27p28 production by infected BMDMs from C57BL/6, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$ mice. (B) IFN-β production by infected BMDMs from C57BL/6, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, *Trif*$^{-/-}$, and *Myd88*$^{-/-}$ mice. (C) IL-27p28 production by infected BMDMs from C57BL/6 in the presence of monoclonal antibodies against IFN-β (5 μg/ml). (D) IL-27p28 production by infected BMDMs from C57BL/6, *Tlr3*$^{-/-}$, and *Tlr4*$^{-/-}$ mice and from 129SVev, *Irf1*$^{-/-}$, and *Ifnar*$^{-/-}$ mice in the presence of recombinant mouse IFN-β (1000 U/ml). Each bar represents the means ± SEM from triplicate wells. Data are representative of those found in 1 to 5 experiments. *, $P < 0.05$ by unpaired Student *t* test (infected versus noninfected) or ANOVA followed by Bonferroni's *post hoc* adjustment (C57BL/6 versus knockouts). &, $P < 0.05$ for infected versus noninfected groups. Abbreviations: NI, noninfected; *L.i.*, *L. infantum*; ns, nonsignificant; CØ, control. Symbol: #, not detected.

results, we added recombinant IFN-β in cultures of *Tlr3*$^{-/-}$ and *Tlr9*$^{-/-}$ BMDMs infected with *L. infantum*. The addition of IFN-β rescued IL-27 production by infected macrophages, bypassing the need for TLR3 and TLR9 signaling (Fig. 2D). These results were confirmed and extended using *Ifnar*$^{-/-}$ and *Irf-1*$^{-/-}$ BMDMs, which are still defective in IL-27 production even when IFN-β was added to the cultures (Fig. 2D). Together, these results demonstrate that the production of IL-27 induced by *L. infantum* is dependent on TLR3 and TLR9, as well as on the adaptors TRIF and MyD88, which are crucial for IFN-β induction. Moreover, IFN-β triggers type I IFN receptor (IFNAR) and interferon regulatory factor 1 (IRF1) signaling, leading to IL-27 production in response to *L. infantum* infection.

IL-27 confers susceptibility to *L. infantum* infection by inhibiting Th17 cells. To address the role of IL-27 during *L. infantum* infection, we infected *Ebi3*$^{-/-}$ and C57BL/6 wild-type mice with 10^7 promastigotes in stationary growth phase and evaluated the parasite titers in the liver and spleen at the 4th and 6th wpi. We observed that *Ebi3*$^{-/-}$ mice showed significantly reduced parasite numbers during infection in both organs analyzed (Fig. 3A) as well as reduced hepatosplenomegaly, demonstrated by diminished organ weight at the 6th wpi (Fig. 3B). Therefore, these data clearly indicated that IL-27 plays a major role in host susceptibility to *L. infantum* and suggest that IL-27 can modulate the levels of other proinflammatory mediators. Once the Th1 response is classically associated with host protection during *Leishmania* species infection (2), we determined the numbers of CD3$^+$ CD4$^+$ IFN-γ$^+$ cells in the spleen of infected mice. Although more resistant, reduced frequencies of Th1 cells were found in the spleen of *Ebi3*$^{-/-}$ mice than in infected C57BL/6 mice (Fig. 4A and B). Moreover, IFN-γ production in tissue homogenates was also reduced in the absence of IL-27 (Fig. 4C).

To further understand the mechanism that leads to the resistance observed in *Ebi3*$^{-/-}$ mice, we evaluated the levels of IL-10 and IL-17A after infection, since these cytokines play a role in host resistance to *L. infantum* infection (4). To address this question, we infected C57BL/6 and *Ebi3*$^{-/-}$ mice and evaluated the accumulation of cytokines in spleen and liver homogenates at the 4th and 6th wpi by ELISA. The data demonstrated that *Ebi3*$^{-/-}$ mice produce significantly higher levels of IL-17A than C57BL/6 mice in both spleen and liver (Fig. 5A). However, IL-10 levels are slightly decreased in *Ebi3*$^{-/-}$ mice only in the spleen at the 4th wpi (see Fig. S2 in the supplemental material), which could not explain the higher resistance observed in these mice. We also observed higher numbers of IL-17-producing cells in the spleen of *Ebi3*$^{-/-}$ mice at the 4th wpi, as observed through gating in FSC^{lo} SSC^{lo} and IL-17$^+$ cells (Fig. 5B). IL-17A production was mediated by antigen-specific spleen cells, since *ex vivo* restimulation of splenocyte cultures with *L. infantum* lysate for 48 h led to significant IL-17A accumulation in the supernatants (Fig. 5C). We also explored the regulatory role of IL-27 using *in vitro* coculture systems. For this purpose, highly purified CD11c$^+$ MHC-II$^+$ cells were obtained

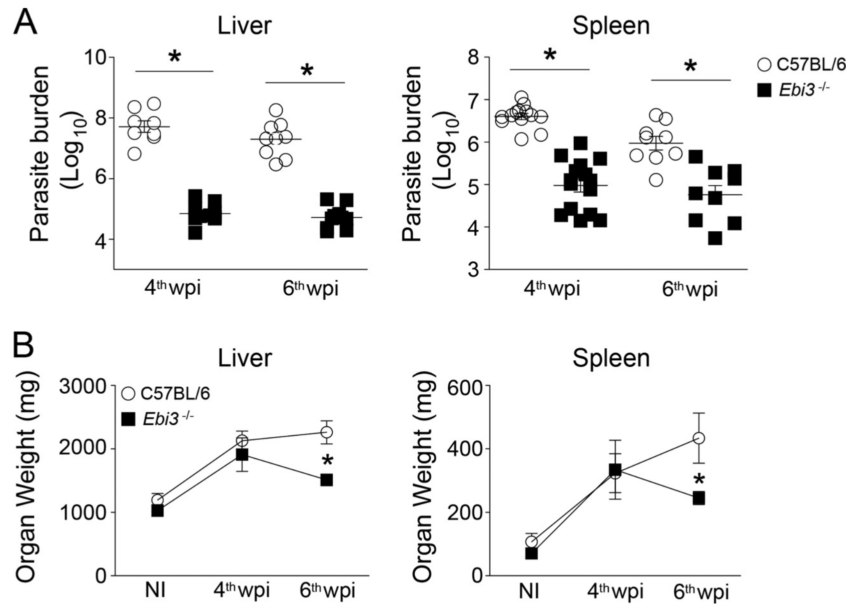

FIG 3 *Ebi3* mediates susceptibility to *L. infantum* infection. C57BL/6 and $Ebi3^{-/-}$ mice were intravenously infected with 10^7 axenic stationary-phase promastigotes of *L. infantum*, and parasite burden was evaluated. (A) At the 4th and 6th wpi, samples from liver and spleen were collected for assessment of parasite titers through limiting-dilution assay in 96-well plates. (B) Liver and spleen were weighed to assess hepatosplenomegaly. Each dot represents a single animal, and the horizontal lines represent the means ± SEM. Each symbol represents an independent group of 3 to 5 mice, expressed as mean values (±SEM). Data are pooled from 2 independent experiments and are representative of those found in 3 to 5 experiments. *, $P < 0.05$ by unpaired Student *t* test (C57BL/6 versus $Ebi3^{-/-}$).

from *in vitro*-differentiated BMDCs, infected with *L. infantum*, and subsequently cocultured with naive T cells in the presence of anti-CD3. After 5 days, the supernatants were collected to determine the levels of IL-17A, which was produced in culture containing $Ebi3^{-/-}$ DCs and T cells but was not observed in cocultures containing wild-type DCs (Fig. 5D). These findings showed that IL-27 suppresses the development of IL-17-producing cells during *L. infantum* infection. Collectively, these data demonstrated the complex role of IL-27 in modulating the inflammatory microenvironment in VL.

Blockade of IL-17A abrogates resistance of $Ebi3^{-/-}$ mice. Once IL-17A was overproduced in the absence of *Ebi3* during *L. infantum* infection, we investigated the migration of neutrophils to the spleen and liver of infected mice. It was observed that $Ebi3^{-/-}$ mice present a peak in neutrophil migration at the 4th wpi both in the spleen and liver (Fig. 6A and B). Moreover, higher

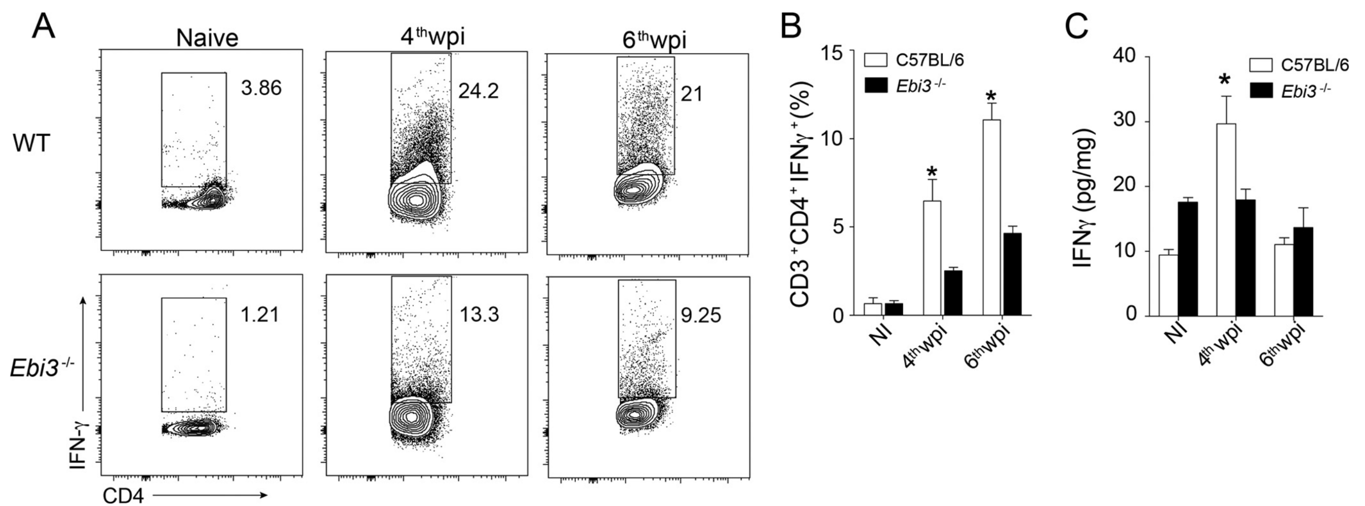

FIG 4 Absence of *Ebi3* compromises Th1 response in *L. infantum*-infected mice. C57BL/6 and $Ebi3^{-/-}$ mice were intravenously infected with 10^7 axenic stationary-phase promastigotes of *L. infantum*, and at the 4th and 6th wpi, development of Th1 response was evaluated in the spleen. (A and B) Cellular suspensions of the spleen were stimulated with PMA (20 ng/ml) plus ionomycin (500 ng/ml) in the presence of GolgiStop for 6 h. The cells were stained for surface expression of CD3 and CD4, as well as intracellular accumulation of IFN-γ, followed by flow cytometry analysis. Populations were gated as FSC^{lo} SSC^{lo} $CD3^+$ $CD4^+$ and displayed as $CD4^+$ IFN-γ^+. Bars are representative of dot plots and are displayed as percentages of $CD3^+$ $CD4^+$ IFN-γ^+ cells. (C) The production of IFN-γ was assessed in the spleen through ELISA. Dots represent individual mice, and each bar represents an independent group of 3 to 5 mice, expressed as mean values (±SEM). Data are representative of those found in 1 to 3 experiments. *, $P < 0.05$ by unpaired Student *t* test (C57BL/6 versus $Ebi3^{-/-}$).

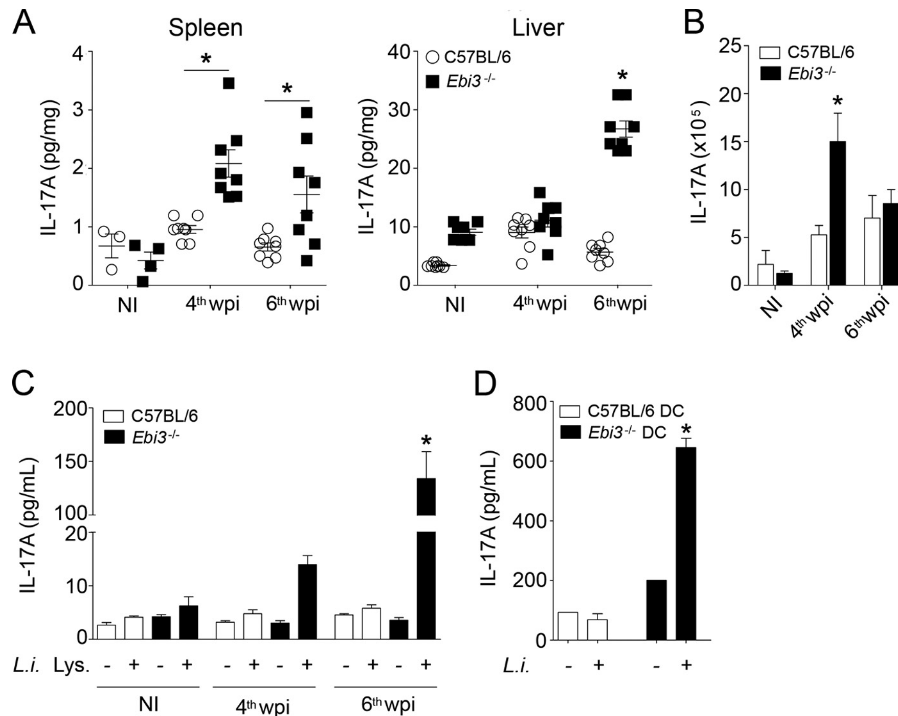

FIG 5 Absence of *Ebi3* leads to exacerbated IL-17 response in *L. infantum*-infected mice. C57BL/6 and *Ebi3*$^{-/-}$ mice were intravenously infected with 10^7 axenic stationary-phase promastigotes of *L. infantum*, and during the 4th and 6th wpi, the production of IL-17 was evaluated in the spleen and liver. (A) The levels of IL-17 were determined in the organs by ELISA. (B) Absolute numbers of IL-17-producing cells as determined by flow cytometry. (C) Spleen cell suspensions were stimulated with *L. infantum* lysate (25 μg/ml), and after 48 h, the levels of IL-17 in the supernatants were evaluated. (D) CD11c$^+$ MHC-II$^+$ BMDCs from C57BL/6 and *Ebi3*$^{-/-}$ mice were infected with *L. infantum* (MOI of 5), and CD4$^+$ CD62L$^+$ CD44$^-$ naive T cells from C57BL/6 mice were added to cultures in the presence of 4 ng/ml of anti-CD3. After 5 days, IL-17 production in the supernatants was evaluated by ELISA. Dots represent individual mice, and each bar represents an independent group of 3 to 5 mice, expressed as mean values (±SEM). Data are representative of those found in 1 to 3 experiments. *, $P < 0.05$ in unpaired Student *t* test (C57BL/6 versus *Ebi3*$^{-/-}$). Abbreviations: NI, noninfected; Lys, lysate.

expression of *Cxcl1*, the main chemoattractant for neutrophils, was observed in the spleen of *Ebi3*$^{-/-}$ mice (Fig. 6C). The IL-17A–neutrophil axis was confirmed through infection of *Il-17ra*$^{-/-}$ mice, which presented a reduction in the frequency of neutrophils in the spleen (Fig. 6D). Altogether, the results demonstrated that high levels of IL-17A are related to an increased recruitment of neutrophils.

To confirm that the resistance observed in *Ebi3*$^{-/-}$ mice during *L. infantum* infection was due to the IL-17A–neutrophil axis, we blockaded this cytokine *in vivo* with monoclonal anti-IL-17A (20 μg; administered at −1, 7, and 14 days of infection) and determined the parasite burden at the 4th wpi in the spleen. The data showed that IL-17A blockade in *Ebi3*$^{-/-}$ mice abrogated the previously observed host resistance (Fig. 7A). Moreover, the neutrophil influx was also mitigated by IL-17A blockade (Fig. 7B). These data confirm that the inhibition of IL-17A by IL-27 during *L. infantum* infection leads to host susceptibility through regulating neutrophil influx.

DISCUSSION

The host immune response against intracellular parasites can determine if the individual will develop a benign, self-healing infection or disease. The production of IL-27 has been observed in the serum of patients with VL, which correlated with the severity of the disease (19). Thus, we hypothesized that IL-27 could mediate susceptibility to VL. In this work, we observed that after *L. infantum* infection, EBi3, a component of IL-27, suppressed the host inflammatory response, leading to a susceptibility profile. Infected *Ebi3*$^{-/-}$ mice presented highly reduced parasite burdens in both spleen and liver compared to C57BL/6 WT mice. Moreover, the target organs of infection were downsized in *Ebi3*$^{-/-}$ mice compared to organs from C57BL/6 mice, demonstrating that control of parasite replication in these mice is accompanied by reduced signs of active disease. Considering that EBi3 also can bind to IL-12p35 and form IL-35 (8), we observed that *Il-12p35* transcripts were very scarce in the organ analyzed (data not shown). In contrast, *Il-27p28* and *Ebi3* were highly expressed at the same time points, ruling out the role of IL-35 in this context. Moreover, both IL-27R subunits are upregulated at critical time points of infection, where the parasite is efficiently controlled in the absence of *Ebi3*.

Previous reports have described divergent roles of IL-27 during leishmaniasis, which differ depending on the species of the parasite employed. For example, *Il-27r*$^{-/-}$ mice are susceptible to infection with a nonhealing strain of *L. major*, presenting exacerbated IL-17 with reduced IL-10 production in the ears and lymph nodes (16). On the other hand, *Il-27r*$^{-/-}$ mice are more resistant to *L. donovani* infection; however, severe liver lesions mediated by CD4$^+$ T cells producing IFN-γ and tumor necrosis factor (TNF) (18) were observed in these mice. In our model of *L. infantum* infection, *Ebi3* deficiency is not involved with immunopathology once reduced signs of hepatosplenomegaly were observed, as

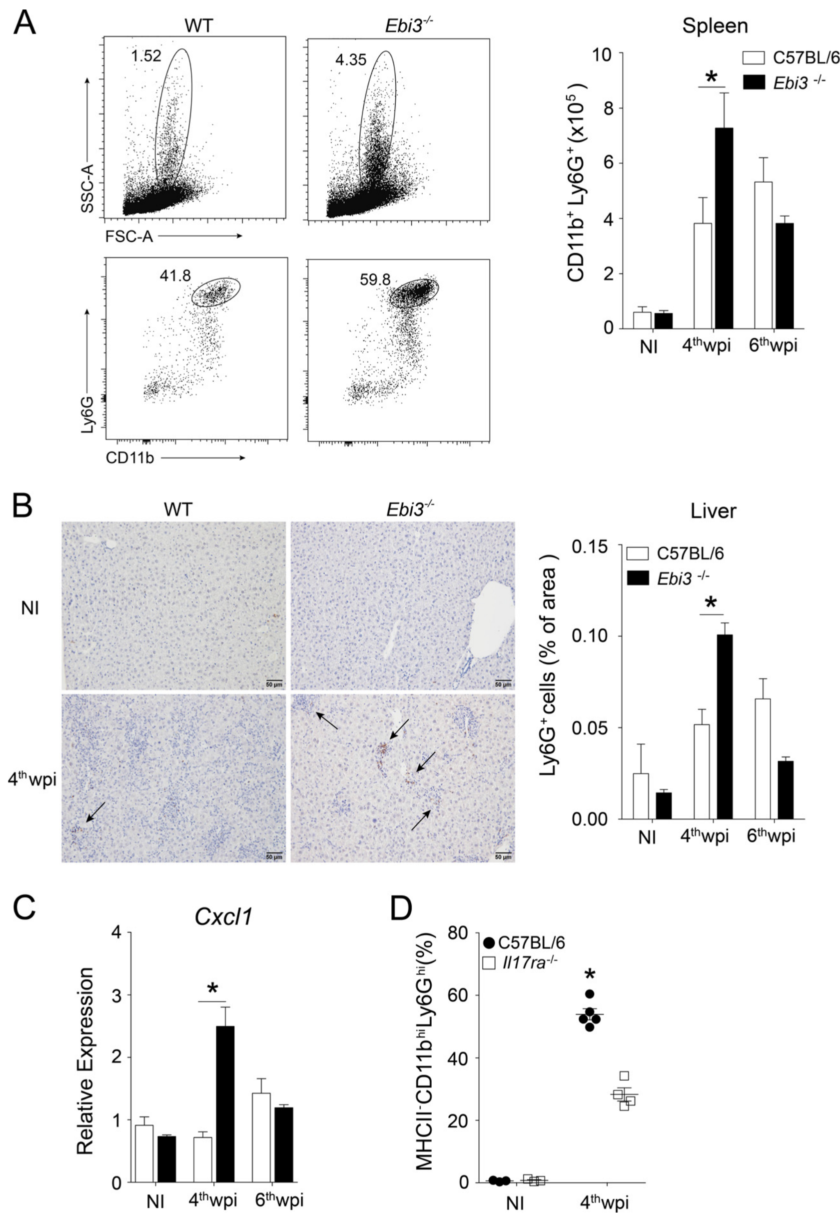

FIG 6 Neutrophils infiltrate the spleen and liver of infected *Ebi3*$^{-/-}$ mice. C57BL/6 and *Ebi3*$^{-/-}$ mice were intravenously infected with 10^7 axenic stationary-phase promastigotes of *L. infantum*, and at the 4th and 6th wpi, neutrophil infiltration was evaluated in the spleen and liver. (A) Representative dot plots and absolute numbers of FSC^{lo} SSC^{hi} CD11b$^+$ Ly6G$^+$ neutrophils in the spleen of C57BL/6 and *Ebi3*$^{-/-}$ mice analyzed through flow cytometry. (B) Representative photomicrographs of Ly6G-stained liver sections are shown at a magnification of ×400, and the scale bar represents 50 μm. Arrows indicate Ly6G$^+$ cell agglomerations, and bars represent the percentages of stained surface as analyzed by ImageJ-IHC Tool Box in photomicrographs of 20 fields per animal. (C) *Cxcl1* mRNA expression in the spleen of infected C57BL/6 and *Ebi3*$^{-/-}$ mice was analyzed through normalization to the housekeeping genes using the $2^{-\Delta\Delta CT}$ formula. (D) C57BL/6 and *Il-17ra*$^{-/-}$ mice were intravenously infected, and at the 4th wpi, neutrophil infiltration in the spleen was evaluated by flow cytometry. Dots represent individual mice, and each bar represents an independent group of 3 to 5 mice, expressed as mean values (±SEM). Data are representative of those found in 1 to 3 experiments. *, $P < 0.05$ by unpaired Student *t* test (C57BL/6 versus *Ebi3*$^{-/-}$ or C57BL/6 versus *Il-17ra*$^{-/-}$).

mentioned above. In general, the regulatory role of IL-27 can be important to prevent tissue damage in organs such as the skin and viscera; however, this action can lead to higher parasite replication.

In this work, we observed that in the absence of IL-27 there is an accumulation of neutrophils in the target organs. Moreover, we observed that the key mediator of neutrophil infiltration in the spleen of infected mice is IL-17, which induces CXCL1 expression, an important neutrophil chemoattractant. Neutrophils can counteract infections by several mechanisms, such as production of cytokines and chemokines that lead to inflammatory cell recruitment as well as release of granules and neutrophil extracellular traps (25). The involvement of neutrophils in host resistance to *Leishmania* species parasites seems to be dependent on the host genetic background as well as the *Leishmania* species considered. During *L. major* infection, neutrophils that ingest the parasites display apoptotic markers on their surfaces, and those regulatory "eat me" signals lead to dendritic cell acquisition of parasites in an

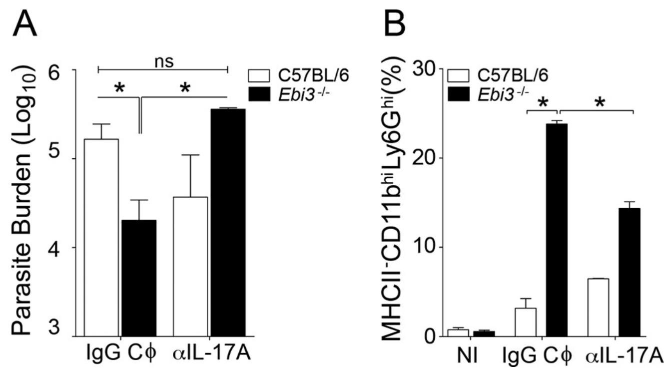

FIG 7 *Ebi3* mediates susceptibility to *L. infantum* infection through negative regulation of IL-17A–neutrophil axis. C57BL/6 and $Ebi3^{-/-}$ received i.p. 20 μg of anti-IL-17A (clone 50104; R&D Systems, Minneapolis, MN) or control IgG on day −1, day 7, and day 14 of infection of mice with *L. infantum* promastigotes. (A) At the 4th wpi, samples from spleen were collected for assessment of parasite titers through limiting-dilution assay in 96-well plates. (B) At the 4th wpi, samples from spleen were collected for assessment of neutrophil infiltration through flow cytometry. Each bar represents the means ± SEM. Data are representative of those found in 1 to 2 experiments. *, $P < 0.05$ by ANOVA followed by Bonferroni's *post hoc* adjustment (C57BL/6 IgG and anti-IL-17 versus *Ebi3* IgG and anti-IL-17). Abbreviations: α, antibody; CØ, control.

anti-inflammatory context, culminating in *L. major* replication (26). However, during infection with *L. amazonensis*, the presence of neutrophils reduces the amount of IL-10 produced in the skin, favoring parasite control by the host (27). In *L. infantum* infection, the depletion of neutrophils by anti-Ly6G monoclonal antibody administration resulted in increased susceptibility to parasite growth (28). In accordance with this, during infection with *L. donovani*, antibody-mediated depletion of neutrophils increases parasite replication in the target organs (29, 30). Collectively, those reports suggest that during visceral leishmaniasis, neutrophils play a protective role.

Regarding the role of IL-17-mediated protection, our group has already observed that this cytokine is involved in host resistance, since $Il\text{-}17ra^{-/-}$ mice are susceptible to *L. infantum* infection. IL-17 can also regulate IL-10 production, and it induces nitric oxide (NO) production by infected macrophages when in combination with IFN-γ (4). In this work, we observed a critical role of IL-17 in the recruitment of neutrophils to infected organs. Curiously, during *L. infantum* infection of C57BL/6 mice, IL-17 levels are reduced in the infected organs. We observed that negative regulation of IL-17 is mediated by IL-27 production, since *Ebi3* absence leads to exacerbated IL-17 secretion. When IL-17 was blocked through antibody administration, the previously resistant $Ebi3^{-/-}$ mice became as susceptible as the C57BL/6 control, demonstrating the ability of IL-27 to directly block IL-17 release. Moreover, neutrophils were also reduced after anti-IL-17 treatment, confirming the role of IL-17 in neutrophil recruitment to infected organs in parallel to induction of NO production by macrophages, as previously shown by our group. The regulatory role of IL-27 on IL-17 response has been demonstrated under autoimmune inflammatory conditions, such as in experimental autoimmune encephalomyelitis (12) as well as in collagen-induced arthritis (31). Here, we suggest that IL-27 plays a major role as an important regulatory cytokine implicated in infectious disease.

Regarding the role of IL-27 in the regulation of the Th1 response, we observed that although $Ebi3^{-/-}$ mice are more resistant to infection, there is a reduction in the overall production of IFN-γ in the infected organs. This result demonstrated that other aspects of the inflammatory response, such as IL-17 and neutrophils, can lead to parasite control even in the absence of a strong Th1 response. It was previously observed that $Ebi3^{-/-}$ mice infected with *L. major* present reduced levels of IFN-γ in the ear (32), in accordance with our results. Thus, IFN-γ is important to control parasite replication but also can lead to tissue damage, as previously observed (18).

As we observed that IL-27 is a mediator involved in host susceptibility, we investigated the molecular components necessary for IL-27 production in macrophages. We observed that macrophages deficient for TLR3 and TLR9 completely fail to produce IL-27, suggesting that nucleic acids from *L. infantum* could activate those receptors to trigger the production of regulatory cytokines. Interestingly, we could not observe a redundant role of these receptors in the induction of IL-27, possibly because the absence of any endosomal TLR abrogates sufficient IFN-β production to trigger further IL-27 secretion. The mechanisms of endosomal TLR activation in the context of parasite replication is an important question to be addressed in the future.

More interestingly, we observed that TLR recognition of *L. infantum* does not induce direct IL-27 production; instead, type I IFN are intermediary cytokines involved in this response. Our results demonstrated that endosomal TLR3 and TLR9 are involved in IFN-β production. Moreover, type I IFN production is upstream of IL-27 production once IFN-β blockade with monoclonal antibodies reduces IL-27 levels. The signaling pathways involved in type I IFN-dependent IL-27 production are dependent on IFNAR and subsequent IRF1 activation to induce IL-27 production after *L. infantum* infection. These results are in accordance with TLR4-dependent production of IL-27, which also relies on the type I IFN intermediary response (33). In the context of TLR activation, classical protective cytokines, such as IL-12, TNF, and IFN-β, tend to be secreted at early time points in order to allow for adaptive response. However, the production of regulatory cytokines occurs at delayed time points in order to regulate the responses triggered by infection. Our results are in accordance with this scenario, once IL-27 is produced after the earlier secretion of type-1 IFN.

Altogether, our results demonstrated that *L. infantum* infection induces IL-27 production in a sequential pathway that involves the engagement of TLR3 and TLR9, production of type I IFN, and activation of IRF1 in macrophages. Secretion of IL-27 is important to drive the Th1 response but also plays a negative regulatory role in the production of IL-17, which impacts the host response by the reduction of neutrophil recruitment to the target organs (see Fig. S3 in the supplemental material). Thus, inhibition or blockade of IL-27 release could be an interesting target for the design of future interventions in the context of visceral leishmaniasis.

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G.F.S.Q., M.S.L.N., and J.S.S. designed the work; G.F.S.Q., M.S.L.N., M.D.-F., L.A.S., and M.H.F.L. designed and performed the experiments;

R.P.A., V.C., and J.S.S. helped with data interpretation; G.F.S.Q., M.S.L.N., and J.S.S. wrote the manuscript.

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