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The role of IL-32 in Bacillus Calmette-Guérin (BCG)-induced trained immunity in infections caused by different *Leishmania* spp.

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ABSTRACT

Background: Cells of the innate immune system undergo long-term functional reprogramming in response to Bacillus Calmette-Guérin (BCG) exposure via a process called trained immunity, conferring nonspecific protection to unrelated infections. Here, we investigate whether BCG-induced trained immunity is able to protect against infections caused by different *Leishmania* spp., protozoa that cause cutaneous and mucosal or visceral lesions.

Methods: We used training models of human monocytes with BCG and subsequent infection by *L. braziliensis*, *L. amazonensis* and *L. infantum*, and the vaccination of wild-type and transgenic mice for IL-32γ before *in vivo* challenge with parasites.

Results: We demonstrated that monocytes trained with BCG presented enhanced ability to kill *L. braziliensis*, *L. amazonensis* and *L. infantum* through increased production of reactive oxygen species. Interleukin (IL)-32 appears to play an essential role in the development of trained immunity. Indeed, BCG exposure induced IL-32 production in human primary monocytes, both mRNA and protein. We have used a human IL-32γ transgenic mouse model (IL-32γTg) to study the effect of BCG vaccination in different *Leishmania* infection models. BCG vaccination decreased lesion size and parasite load in infections caused by *L. braziliensis* and reduced the spread of *L. amazonensis* to other organs in both infected wild-type (WT) and IL-32γTg mice. In addition, BCG reduced the parasite load in the spleen, liver and bone marrow of both WT and IL-32γTg mice infected with *L. infantum*. BCG vaccination increased inflammatory infiltrate in infected tissues caused by different *Leishmania* spp. In all infections, the presence of IL-32γ was not mandatory, but it increased the protective and inflammatory effects of BCG-induced training.

Conclusions: BCG's ability to train innate immune cells, providing protection against leishmaniasis, as well as the participation of IL-32γ in this process, pave the way for new treatment strategies for this neglected infectious disease.

1. Background

Leishmaniasis are infectious diseases caused by *Leishmania* protozoa, which are transmitted through the bite of an insect vector of the subfamily *Phlebotominae* [1,2]. Tegumentary leishmaniasis affects the skin and the mucosa of nose, mouth and pharynx whereas visceral leishmaniasis (VL) affects internal organs such as spleen, liver and bone marrow. The World Health Organization (WHO) committee has reported that the current global incidence estimate of cutaneous leishmaniasis is 600,000 to 1 million cases per year. In addition, there are 50,

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00 to 90,000 new cases of VL every year [3]. In the Americas, the most important species of *Leishmania* causing American tegumentary leishmaniasis (ATL) are *L. (Viannia) braziliensis*, associated with cutaneous (CL) or mucosal leishmaniasis (ML), and *L. (L.) amazonensis* and *L. (L.) mexicana*, mainly associated with the development of localized (LCL) or diffuse cutaneous (DCL) diseases. *L. braziliensis* is the most prevalent species in Brazil and about 1–10% of patients with LCL caused by this species develop ML [4]. VL is caused by *L. (L.) infantum* in the Americas [1]. In addition to the parasite species, the host's immune response profile is responsible for the clinical forms and/or severity of leishmaniasis. In fact, the pathogen-host interaction induces different immune mechanisms that can contribute to the parasite's survival and persistence or to its control [5–7].

The commonly used drugs to treat leishmaniasis are pentavalent antimonials and amphotericin B. In general, these therapies are successful but present several side effects. Moreover, there are cases that are difficult to treat, especially in VL, ML and DCL [6,8,9]. Thus, immunotherapy usually combined with chemotherapy to treat leishmaniasis has been proposed for a long time. The Bacillus Calmette-Guérin (BCG) vaccine has been used not only as a strategy to protect against tuberculosis, but also as immunotherapy and adjuvant in vaccines [10–13]. Since 1970′s, several studies with leishmaniasis and BCG have been performed with mice or hamsters [14–18] as well as with patients and healthy individuals using both prophylactic and therapeutic approaches [19–23]. Most of the studies showed that BCG can contribute to parasite control, especially alleviating DCL. The mechanisms proposed involve acquired immunity-cross protection and innate immune activation [9,24–26]. In light of the new concept of trained immunity [27], it has been shown that BCG can train monocytes/macrophages by promoting metabolic and epigenetic alterations that contribute to increased cytokine production and innate receptor expression in secondary or unrelated infections [28–31]. Monocytes/macrophages are host cells for *Leishmania* spp. and they are crucial cells in defense against the parasites [32–34]. Thus, trained immunity could at least partially explain the positive effects observed in leishmaniasis treated with BCG.

BCG can induce the production of interleukin 32 (IL-32) in human monocytes [35]. This cytokine is expressed in cutaneous and mucosal lesions of patients infected with *L. braziliensis* or *L. amazonensis* [36,37]. IL-32γ produced during *Leishmania* spp. infections positively regulates the production of cytokines and microbicidal molecules, including NO and antimicrobial peptides [38]. In addition, human IL-32γ transgenic mice (IL-32γTg) present improved control of *L. braziliensis* infections and containment of *L. amazonensis* parasites at the site of infection which prevents parasite spread to other organs [37]. Moreover, *L. infantum* induces IL-32γ in human cells and VL is ameliorated in the IL-32γTg mouse model, which is associated with a mixed Th1/Th17 immune response and increased NO production [39]. Since rodents do not express IL-32 [40], the IL-32γTg mouse model is an important tool to elucidate the mechanisms of IL-32 actions in leishmaniasis.

Recently, we have demonstrated that IL-32 is crucial for β-glucan-induced trained immunity to protect against *L. braziliensis*. In addition, IL-32 expression determines the gene transcription profile in bone marrow hematopoietic stem and progenitor cells as well as in granulocyte macrophage progenitors after human BCG vaccination, thus suggesting the participation of IL-32 in the generation of BCG-induced trained immunity [41]. Overall, these studies suggest that BCG can ameliorate leishmaniasis through trained immunity dependent on IL-32. Thus, and to deepen the knowledge about trained immunity in leishmaniasis, we used a protocol for training human monocytes with BCG [42] and evaluated the capacity of trained macrophages to control the infection caused by *L. amazonensis*, *L. braziliensis* or *L. infantum* in this study. Further, clinical outcome of infections with these *Leishmania* spp. in BCG-trained mice were evaluated in the IL-32γTg mouse model. Since there is no vaccine for leishmaniasis and current treatments are usually toxic, the innate training of monocyte/macrophages can open a new perspective for the control of leishmaniasis.

2. Methods

2.1. Ethical considerations

The study with murine model was approved by Ethics Committee for animal research of Universidade Federal de Goiás (CEUA/PRPI/UFG, protocol 042/16). The human study was approved by Ethics Committee of Radboud University Nijmegen, The Netherlands (approval number 42561.091.12).

2.2. Leishmania parasites

L. braziliensis (MHOM/BR/2003/IMG), *L. amazonensis* (IFLA/BR/67/PH8) and *L. infantum* (MHOM/BR/74/PP/75) were cultured in Grace's insect medium (Gibco, Life Technologies, USA) supplemented with 20% of heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, USA) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich) at 26 °C. Stationary-phase promastigotes were obtained on the 6th day of growth, washed three times with phosphate-buffered saline (PBS; 1000×*g*, 10 min, 10 °C), resuspended in PBS and quantified by using hemocytometer after fixation with PBS/0.4% formaldehyde.

2.3. Peripheral blood mononuclear cells (PBMCs) and monocyte isolation

PBMCs from healthy donors were isolated by differential density centrifugation over Ficoll-Paque (GE healthcare, UK) and monocyte isolation was obtained by Percoll (Sigma-Aldrich, USA), as described previously [43,44]. No blood donors were vaccinated with BCG. Briefly, 150–200 × 10^6 PBMCs were layered on a hyper-osmotic Percoll solution (48.5% Percoll, 41.5% sterile water, 0.16 M NaCl) and centrifuged for 15 min at 580×*g* (4 °C). The interphase layer was collected and cells were washed with cold PBS. Cells were resuspended in RPMI 1640 culture medium (Invitrogen, USA) supplemented with 50 μg/mL gentamicin, 2 mM glutamine (Gibco, Life Technologies, USA), and 1 mM pyruvate (Gibco). Cells (10 × 10^6 cells/10 mL) were allowed to adhere on petri dishes (Corning, USA) for 1 h at 37 °C. Non-adherent cells were washed out with warm PBS and monocytes were recovered from the plates by adding 6 mL of versene (Gibco, Life Technologies, USA) for 30 min at 37 °C.

2.4. Monocyte training, treatments and macrophage infection

Trained immunity was induced in adherent monocytes as described previously [42]. Monocytes (1 × 10^6/mL; 10 mL) were cultured in RPMI 1640 medium containing 10% pooled human serum, referred to as complete medium, as negative control (non-trained cells) or in 5 μg/mL of Bacillus Calmette–Guérin (BCG, 1 × 10^4 CFU/mL) for trained cells. Lyophilized-BCG strain Moreau, 2 × 10^6 CFU/mg was acquired from Ataulpho de Paiva Foundation and checked for viability and CFU determination by National Institute of Quality Control in Healthy INCQS-FIOCRUZ, Rio de Janeiro, Brazil. Bacteria were reconstituted in culture medium and immediately used for training. After 24 h (37 °C), cells were washed once with 10 mL of warm PBS and incubated for five days with one change of complete medium. On day six, cells were harvested and quantified, with viability >90% as observed by trypan blue (0.1% in PBS) exclusion.

Trained and non-trained monocytes/macrophages (2 × 10^5 cells/500 μL of complete medium) were added into 24-well plates on 12 mm coverslips (Corning, NY, USA), adhered for 30 min at 37 °C, and then infected with stationary-phase promastigotes of *L. braziliensis*, *L. amazonensis* and *L. infantum* (1 × 10^6/well; MOI 5:1). Wells were washed after 2 h with warm PBS to eliminate non-internalized parasites, the medium was refreshed and cells were incubated for 4 h, 24 h and 48 h. Cells on coverslips were fixed at 2 h, 4 h and 48 h after infection, stained with Giemsa (Merck Millipore) and analyzed under a light microscope (1000x) to determine the infection index. Cells (300 per

coverslip) were analyzed and the percentage of infected cells as well as the mean number of intracellular parasites per infected cell were determined. The infection index was calculated by multiplying the percentage of infected cells with the mean number of parasites per infected cell.

2.5. Cytokine evaluation

For cytokine evaluation, 2×10^5 cells/well trained and non-trained cells in a final volume of 100 μL of culture medium were added to flat-bottom 96-well plates (Corning), adhered for 1 h at 37 °C and restimulated with 10 ng/mL of lipopolysaccharide (LPS) from *E. coli* serotype O55:B5 (Sigma-Aldrich), purified as described previously [45]. Supernatants were collected after 24 h of incubation and stored at −20 °C. The monolayer of cells was collected in 100 μL of 0.5% TritonX-100 for intracellular IL-32 measurement. Cytokine production was determined in supernatants using commercial ELISA kits (R&D Systems) for human TNFα, and IL-32 under manufacturer conditions. Results are shown in pg/mL; limit of detection 32.25 pg/mL for both cytokines.

2.6. IL-32γ mRNA expression by qPCR

RNA was isolated using TRIzol [46]. RNA was precipitated with isopropanol and washed with 75% ethanol followed by reconstitution in RNase-free water. Subsequently, mRNA was transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Diluted cDNA was used for qPCR which was performed using the StepOnePlus sequence detection systems (Applied Biosystems, Foster City, CA, USA) with SYBR Green Mastermix (Applied Biosystems). Primer sequences for human IL-32γ (FW primer: 5′-AGGCCCGAATGG-TAATGCT-3'; RV primer: 5′-CCACAGTGTCCTCAGTGTCACA-3′) and GAPDH (FW primer: 5′-AGG-GGA-GAT-TCA-GTG-TGG-TG-3'; RV primer: 5′-CGA-CCA-CTT-TGT-CAA-GCT-CA-3′) were used (Biolegio, Nijmegen, The Netherlands). The mRNA was analyzed using the $2^{\wedge -dCt}$ x 1000 method and normalized against the housekeeping gene GAPDH for humans.

2.7. Reactive oxygen species detection and nitric oxide measurements

For measurement of ROS production, a luminol-enhanced luminescence assay was used. Monocytes were trained as described above and 1×10^5 cells were added per well (white-96 well assay plate; Corning) in a volume of 200 μL. Cells were either stimulated with zymosan from *Saccharomyces cerevisiae* (1 mg/mL; Sigma-Aldrich) or with stationary-phase promastigotes of *L. braziliensis*, *L. amazonensis* and *L. infantum* (5×10^5/well; MOI 5:1). Luminol (145 μg/mL) was added and chemiluminescence was measured for 1 h. To measure nitric oxide (NO), 2×10^5 cells/well (100 μL) were trained and nitrite was determined in supernatants after 48 h of infection with stationary-phase promastigotes of *L. braziliensis*, *L. amazonensis* and *L. infantum* (1×10^6/well; MOI 5:1) using Griess reagent (Sigma-Aldrich). Prior to the assay, serum was precipitated from supernatants using perchloric acid (PCA 13.5% - Fluka) and neutralized with 4 N NaOH.

2.8. Mice

Transgenic mice for human IL-32γ (IL-32γTg) [40] were donated by Charles Dinarello (University of Colorado Denver, USA) and maintained at the animal facility of the Federal University of Goiás/IPTSP, Brazil. 6- to 8-week-old C57BL/6 wild-type (WT) and IL-32γTg mice were used in the experiments.

Evaluation of BCG training in interleukin-32γ transgenic and wild type mice: challenge with *L. braziliensis*, *L. amazonensis* or *L. infantum*.

Wild-type and IL-32γTg mice were injected intravenously (i.v.) with BCG ($1.5–2.0 \times 10^6$ CFU in 200 μL of PBS, 750 μg/animal) or PBS (200 μL) [28]. In these experiments, BCG-Intervax, strain Sofia (BB-NCIPD Ltd Sofia, Bulgária) was used. After seven days, mice were infected with 1×10^6 *L. braziliensis* or *L. amazonensis* promastigotes (subcutaneous, left paw) or with 1×10^7 *L. infantum* promastigotes (intraperitoneal). To parasites causing CL, lesion size was measured weekly using a digital caliper and the lesion size was expressed in millimeters (mm) as the difference between the thickness of the infected and uninfected footpad (for 12 weeks in *L. braziliensis* infection or 8 weeks in *L. amazonensis* infection).

Parasite load was analyzed by the limiting dilution assay [5], in the infected footpad (in mice infected with *L. braziliensis* or *L. amazonensis*), spleen and liver (in mice infected with *L. amazonensis*). In mice infected with *L. infantum*, the bodyweight was measured weekly for 4 weeks of infection, and spleen and liver weight were measured 4 weeks post infection. To obtain bone marrow cells, femur and tibia were washed with RPMI complete medium supplemented with fetal calf serum (FCS) 10%. Subsequently, cells were washed twice with PBS and resuspended in 500 μL of supplemented Grace's medium. The parasite load was analyzed in spleen, liver and bone marrow cells by limiting dilution assay. Results are showen as negative - log10.

Additional experiment was performed with bone marrow monocyte-derived macrophages (MDM) to evaluate their capacity to kill *L. infantum*. Bone marrow cells were obtained as described above. The cells (1×10^6 cells/mL) were incubated with RPMI complete medium with FCS 10% for six days to macrophage maturation. Bone marrow MDM (10^6 cells/mL) were infected with *L. infantum* stationary-phase promastigote forms (MOI 5:1) for 4 h, washed with warm medium to eliminate non-internalized parasites, and incubated for 48 h. Then, RPMI medium was replaced by Grace's insect medium. Live L. *infantum* promastigotes were quantified by using hemocytometer after fixation with PBS/0.4% formaldehyde.

2.9. Histopathological analysis in murine infection

Tissue samples from infected footpads (*L. braziliensis*- or *L. amazonensis*-infected mice), spleen and liver (*L. infantum*-infected mice) were collected, cleaved and fixed in 4% paraformaldehyde for 48 h embedded in paraffin, and slices of 5 μm were obtained for histopathological analysis. The Hematoxylin-Eosin (H.E.) staining procedure was performed. Tissue sections were evaluated in Vision Tek digital microscope (Sakura, Japan), magnification 1000×, determining the cell density in the inflammatory infiltrate of lesions caused by *L. braziliensis* or *L. amazonensis* and in spleen and liver from mice infected with *L. infantum*. Number of granulomas and total number of cells in 10 consecutive fields were quantified by using Image J software 1.46r version and TMARKER software 2.162 version, respectively, according to Schuffler et al. [47].

2.10. Statistical analysis

Normality was assessed by D'Agostino & Pearson test and Shapiro-Wilk test. All samples had a normal distribution. The data are presented as mean and standard error of the mean (SEM) and were analyzed by Student's *t*-test and ANOVA with Bonferroni post hoc test. Analyses were performed using Prism software version 6.0 (GraphPad, San Diego, CA, USA). Significance was established as $p < 0.05$.

3. Results

BCG-induced trained immunity contributes to the control of *Leishmania* spp. infection in primary human macrophage.

The effects of BCG training on the ability of human macrophages to uptake and kill *Leishmania* spp. were evaluated (Fig. 1A). The efficacy of BCG for the induction of training was confirmed in separate experiments showed in Fig. S1. *L. braziliensis* infected approximately 80% of macrophages after 2 h and the infection index was significantly reduced after

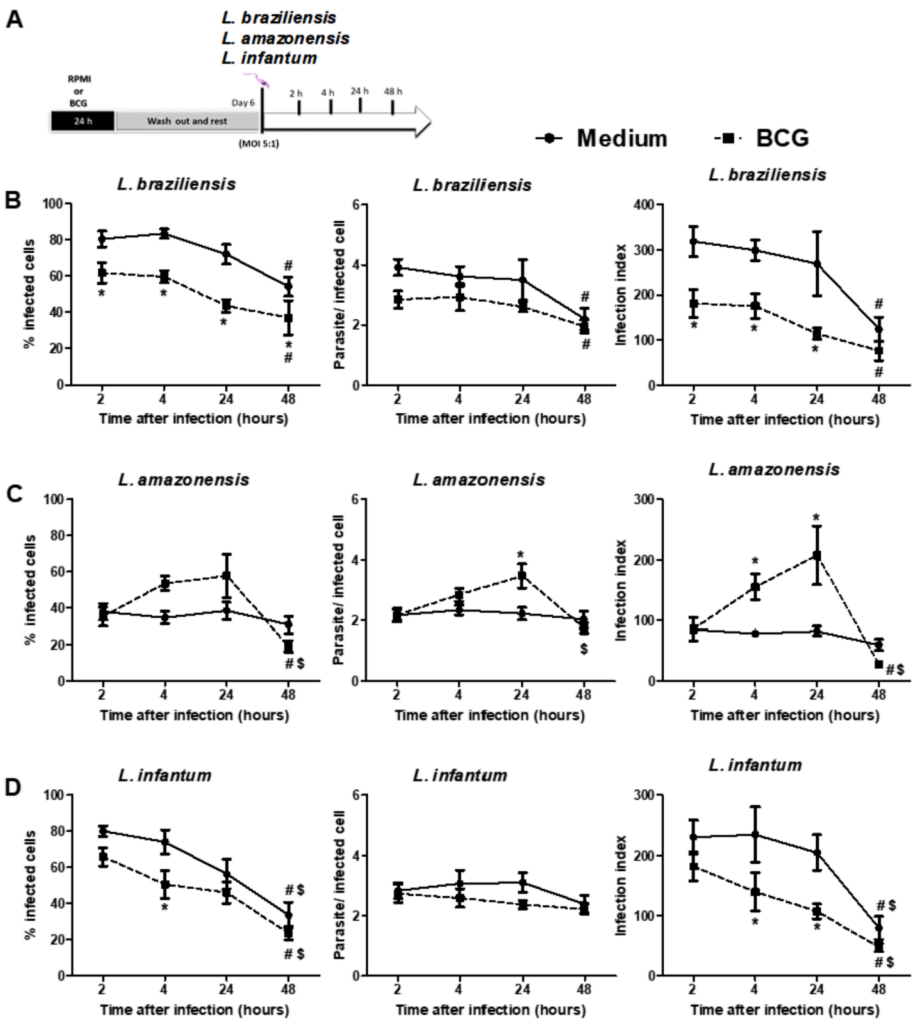

Fig. 1. Trained immunity induced by BCG contributed for controlling *Leishmania* spp. infection in primary human macrophages. (A) Monocytes were incubated with RPMI (medium) or BCG (5 μg/mL; 1 × 10^4 CFU/mL; Moreau strain) for 24 h, washed and rested for 5 days. On day 6 after BCG exposure, macrophages were infected with stationary-phase *L. braziliensis*, *L. amazonensis* or *L. infantum* promastigotes (MOI 5:1) for 2 h, washed, and incubated for 4 h, 24 h, and 48 h. Percentage of infected macrophages, number of parasites per infected cell and infection index (parasites per 100 macrophages) were evaluated. B) *L. braziliensis*, (C) *L. amazonensis*, and (D) *L. infantum*. The data shown are the mean ± SEM. Results are from two independent experiments, n = 6. *$p < 0.05$ (RPMI medium *vs.* BCG), #$p < 0.05$ (2 h and 4 h *vs.* 48 h in the same group), $$p < 0.05$ (2 h or 4 h, 24 h *vs.* 48 h in the same group), all by two-way ANOVA/Bonferroni post hoc test.

48 h, indicating killing of the parasites ($p < 0.05$, 2 h *vs.* 48 h, Fig. 1B). Training with BCG significantly decreased the percentage of macrophages infected with *L. braziliensis* (from 2 h to 48 h) and the infection index (2 h, 4 h, and 24 h, $p < 0.05$, BCG *vs.* medium, Fig. 1B). Average number of parasites per infected cell was similar comparing trained and non-trained cells but was reduced from 2 h to 48 h in both groups (Fig. 1B). Non-trained macrophages were permissive to *L. amazonensis* infection during 48 h of culture (Fig. 1C). Training with BCG increased the average number of parasites (24 h) and infection index (4 h and 24 h) in macrophages infected with *L. amazonensis* in comparison with non-trained cells ($p < 0.05$, BCG *vs.* medium; Fig. 1C). However, the trained cells were able to kill the parasites after 48 h ($p < 0.05$, BCG 4 h and 24 h *vs.* 48 h, Fig. 1C). For L. *infantum* infection, non-trained macrophages controlled the infection seen in a reduced parasite load after 48 h of culture ($p < 0.05$; Medium 48 h *vs.* 2 h, Fig. 1D). BCG-trained macrophages presented lower percentage of infected cells after 4 h and infection index after 4 h and 24 h than non-trained cells ($p < 0.05$; BCG *vs.* medium; Fig. 1D). In both groups the infection index was similarly reduced after 48 h in comparison with 2 h ($p < 0.05$, BCG or medium 48 h *vs.* 2 h, 4 h, 24 h, Fig. 1D).

To better determine the parasite load in the macrophages, the area under the infection curve was evaluated. Training with BCG reduced the area under the curve of the three parameters evaluated (% infected cells, number of parasites/infected cell and infection index) compared with non-trained cells during infection with *L. braziliensis* (Fig. S2A) and *L. infantum* (Fig. S2C). Conversely, the area under the *L. amazonensis* infection curve was increased in macrophages trained with BCG (Fig. S2B). Overall, the data showed that training with BCG reduced the parasite load in *L. braziliensis*- and *L. infantum*-infected macrophages whereas it increased the parasite load in *L. amazonensis*. However, in the end, the infection with this parasite was also controlled (Fig. 1 and Fig. S2).

Human monocytes trained with BCG showed enhanced ROS production capacity after infection with *Leishmania* spp.

To investigate the microbicidal mechanisms used by BCG-trained human monocyte-derived macrophages to control *Leishmania* infection, reactive oxygen species (ROS) and NO production were evaluated. *Leishmania* spp. induced low levels of ROS in non-trained macrophages. However, significant increase of ROS was detected in cultures of BCG-trained macrophages after infection with all three *Leishmania* spp. Notably L. *braziliensis* infection induced higher production of ROS in BCG-trained macrophages in comparison with *L. amazonensis* or *L. infantum* ($p < 0.05$; Fig. 2A). No significant production of NO was detected in any culture conditions (Fig. 2B).

3.1. BCG exposure induces IL-32 in human primary monocytes

As it was previously shown that BCG induces IL-32 production [35] and that IL-32 is involved in β-glucan training [41], the production of IL-32 was investigated during the first step of training with BCG. Intracellular IL-32 was measured after incubation of human primary monocytes with BCG for 24 h. In accordance with previous study, IL-32 production and IL-32γ mRNA expression were significantly increased in BCG-stimulated monocytes compared with controls (Fig. 3).

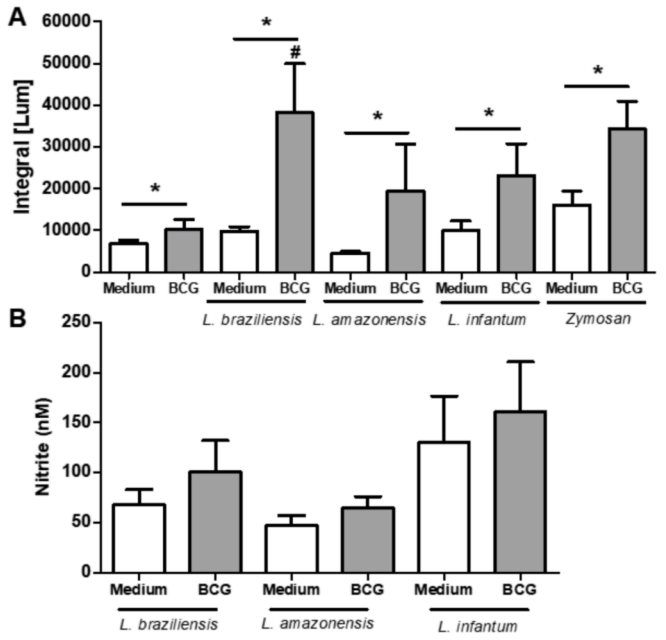

Fig. 2. Increased ROS production in BCG-trained human macrophages after infection with different *Leishmania* spp. Monocytes were incubated with medium or BCG (5 μg/mL; 1×10^4 CFU/mL; Moreau strain) for 24 h, washed and rested for 5 days. On day 6 after exposure to BCG, macrophages were infected with stationary phase-promastigotes of *L. braziliensis*, *L. amazonensis* or *L. infantum* (MOI 5:1) for 1 h. As positive control, ROS production was measured after Zymosan stimulation (1 mg/mL). (A) ROS was detected using luminol-enhanced luminescence assay. (B) To evaluate NO production, cells were incubated for 48 h and nitrite was determined in supernatants of the cultures using Griess reaction. Results are presented in nM of nitrite. The data shown are the mean ± SEM (n = 6 individuals from 2 independent experiments). *p < 0.05 (medium *vs.* BCG. #p < 0.05 (*L. braziliensis vs*. other species). All by two-way ANOVA/Bonferroni post hoc test.

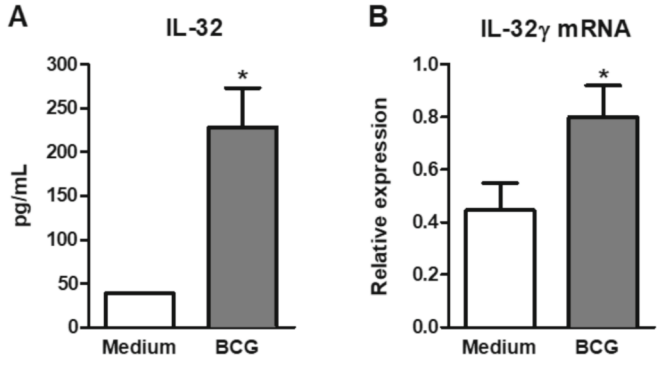

Fig. 3. IL-32 is induced by BCG during the first 24 h of training protocol of human monocytes. Monocytes were incubated with medium or BCG (5 μg/mL; 1×10^4 CFU/mL; Moreau strain) for 24 h and (A) intracellular IL-32 production was measured by ELISA. (B) mRNA expression of IL32γ isoform was determined by quantitative real-time PCR. The data shown are the mean ± SEM (n = 6 individuals from 2 independent experiments). *p < 0.05 (Medium *vs.* BCG; Student's *t*-test).

3.2. BCG administration improves the control of Leishmania infection in mice

The confirmation that IL-32 was induced by BCG in human monocytes and the positive effects of BCG-induced training on macrophage control of *Leishmania* spp. *in vitro* prompted us to evaluate the participation of IL-32 in the clinical outcome of leishmaniasis upon induction of trained immunity with BCG *in vivo*. Human IL-32γTg and WT mice received i. v. BCG injection and were infected with *Leishmania* spp. after seven days. BCG increased the lesion size in WT mice at week 7 post infection with *L. braziliensis* ($p < 0.05$), but the resolution's course of the lesion was similar between PBS-injected and BCG-trained WT mice (Fig. 4A). The course of infection in IL-32γTg mice was similar between the non-infected and BCG-injected group (Fig. 4B). When comparing WT vs. IL-32γTg mice it was observed that the infection of IL-32γTg mice with BCG caused a stronger decrease in lesion size than in BCG-trained WT mice at weeks 7 and 8 post infection ($p < 0.05$, WT BCG *vs.* IL-32γTg mice BCG; Fig. 4C). The parasite load was reduced in the lesions of both BCG-trained WT and IL-32γTg mice compared with non-trained mice after 12 weeks of infection. The presence of IL-32 contributed to the reduction of tissue parasitism (Fig. 4D) and to increase the number of inflammatory cells in the lesions (Fig. 4E and F).

During L. *amazonensis* infection, BCG did not alter the course of the lesion development in WT mice or IL-32γTg mice (Fig. 5A and B). Nevertheless, the lesion size of the BCG-trained IL-32γTg mice was significantly larger than BCG-trained WT mice at the end points of the infection ($p < 0.05$; weeks 7 and 8; Fig. 5C). Despite this, BCG did not significantly affect the parasite load in lesions of the footpad in both IL-32γTg and WT groups in comparison with PBS control groups (week 8; Fig. 5D). The increased size on week 8 of infection observed in BCG-injected IL-32γTg compared with BCG-injected WT mice appears to be due to an increased number of cells in the lesions (Fig. 5G and H), evaluated in histopathological preparations. We have previously shown that *L. amazonensis* has the ability to disseminate from the cutaneous lesion to other organs [37]. Therefore, the parasite burden in both spleen and liver of the *L. amazonensis*-infected mice was evaluated. The BCG administration resulted in fewer parasites in the spleen and liver of both WT and IL-32γTg mice compared with untreated mice (Fig. 5E and F). Importantly, parasites were not found in the liver of BCG-trained IL-32γTg mice (Fig. 5F). Thus, BCG-induced training prevents *L. amazonensis* dissemination, which seems to be more effective in the presence of human IL-32γ.

Further, the effects of BCG in experimental VL caused by *L. infantum* were evaluated in the absence or presence of IL-32γ. The total body weight of mice was evaluated weekly, and no significant difference between WT and IL-32γTg mice both exposed and not exposed to BCG was observed (Fig. S3). At the end of four weeks after challenge with *L. infantum*, there was a significant increase in the relative spleen and liver weight after BCG injection in both WT and IL-32γTg mice compared with the control group (Fig. 6A and B). This indicates that BCG increased hepatosplenomegaly in higher levels than *L. infantum* alone (PBS group). BCG administration reduced the amount of *L. infantum* in the spleen, liver, and bone marrow of both WT and IL-32γTg mice compared with untreated mice (Fig. 6C–E). The synergism between IL-32γ and BCG was observed in the control of parasites in bone marrow after 4 weeks of infection ($p < 0.05$, BCG WT *vs.* BCG IL-32γTg; Fig. 6E). In accordance, bone marrow MDM from IL-32γTg (with PBS or BCG) and BCG-trained WT mice controlled parasitism better than PBS-injected WT cells ($p < 0.05$, Fig. 6F).

The challenge with *L. infantum* after BCG training induced an increase in the number of mononuclear cells in both WT and IL-32γTg spleens compared with controls (Fig. 7A and B). The number of erythrocytes in the spleens of IL-32γTg mice were reduced, especially in animals that were injected with BCG (Fig. 7C), suggesting that BCG exposure followed by *L. infantum* infection reduces spleen congestion in the presence of IL-32γ. BCG induced an increase in granuloma areas in both WT and IL-32γTg mice after challenge with *L. infantum* (Fig. S4).

4. Discussion

The number of cases of leishmaniasis has increased around the world in recent years related to unsuccessful treatment and/or ineffective immune response. In the present study, the positive impact of BCG-

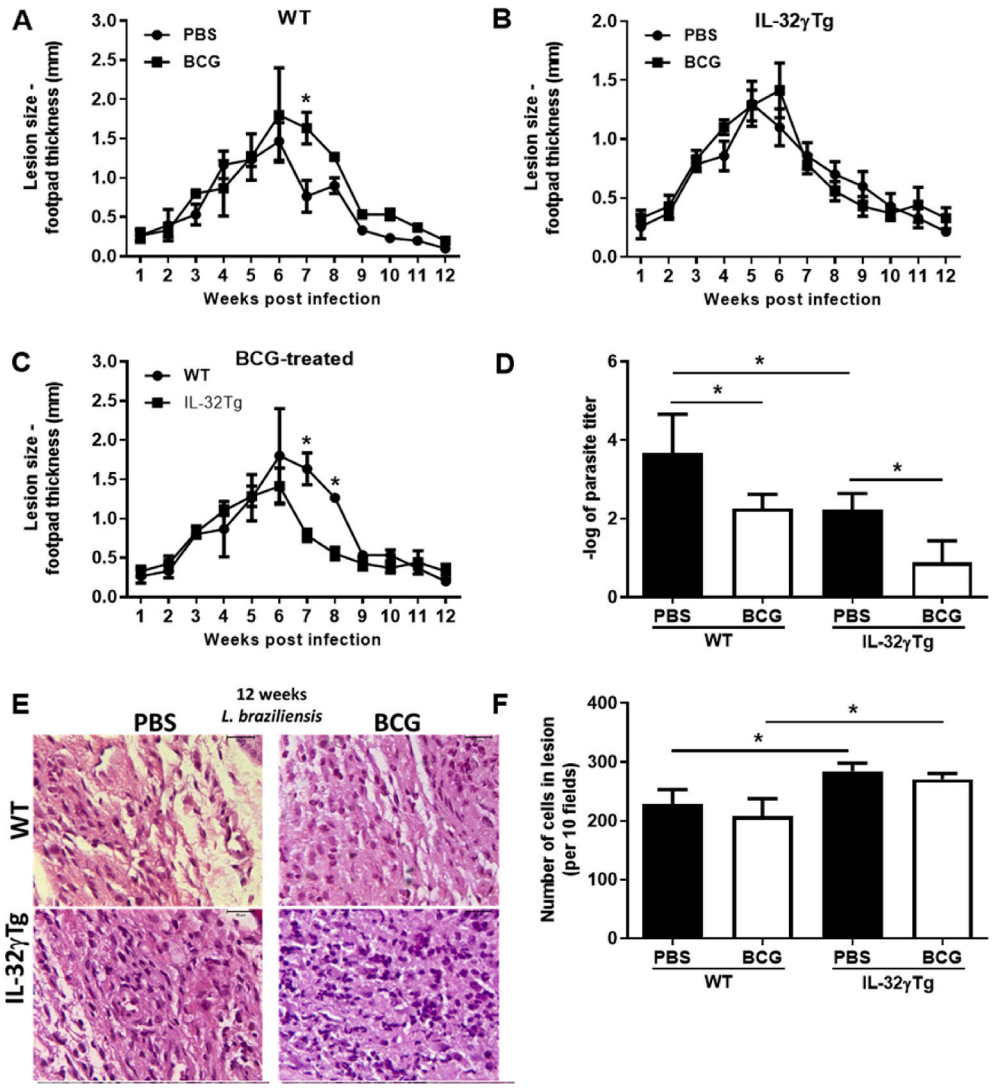

Fig. 4. IL-32γ favors the control of *L. braziliensis* in BCG trained mice. The wild type (WT) and transgenic mice for IL-32 (IL-32γTg) were treated with PBS or BCG (750 μg, intravenously; 1.5–2.0 × 10^6 CFU; BCG-Intervax/Sofia strain). After 7 days, they were infected with 1 × 10^6 stationary phase-promastigotes of *L. braziliensis* in the footpad of the posterior left paw. (A, B e C) Lesion size (mm) in WT and IL-32γTg mice was measured weekly during 12 weeks and represented by the difference between infected-footpad lesion and non-infected footpad. (D) Parasite load was determined by limiting dilution technique 12 weeks post infection. (E) Histopathological micrographs (1000× magnification) of lesions in the footpad were obtained from BCG-injected (at right) or non-injected (at left) WT and IL-32γTg mice after 12 weeks of infection with *L. braziliensis*. (F) Number of inflammatory cells counted in footpad lesions (10 fields evaluated). The results represent mean ± SEM of 4 WT and 7 IL-32γTg mice. In A - C, *p < 0.05, by two-way ANOVA/Bonferroni post hoc test (PBS *vs.* BCG, in each time). In D - F, *p < 0.05, by one-way ANOVA/Bonferroni post hoc test.

induced trained immunity on the control of infections caused by different *Leishmania* spp. was shown. Previously, we have demonstrated that non-stimulated primary human macrophages present low capacity to eliminate *Leishmania* spp. *in vitro* [32,34,48]. Here, it was observed that BCG-trained monocyte-derived human macrophages are more capable of killing *L. braziliensis* and *L. infantum* than non-trained cells. The BCG effects were differentially observed depending on the *Leishmania* spp. For L. *braziliensis*, BCG decreased the uptake of parasites (2 h) and infection index during the whole incubation time (4 h–48 h). For L. *amazonensis*, BCG increased the infection index during the first 24 h, but then trained macrophages controlled the parasites similarly to non-stimulated cells (48 h). Concerning L. *infantum*, the parasites were controlled early upon infection in BCG-trained macrophages compared to non-trained cells. Considering that the infection outcomes are dependent on the *Leishmania* spp. [1,49], the obtained results are not surprising. Although *Leishmania* spp. have macrophage uptake similar characteristics [50,51], there are variations in some batches of parasites due to culture conditions that can explain why in the sets of the present study *L. amazonensis* was less phagocytosed than the other species. Considering the BCG-promoting effects in the infection caused by *L. amazonensis* (during 24 h of culture), this could be attributed to the high capacity of this species to subvert the host's immune response, including ROS- and NO-dependent mechanisms [52–56]. Despite that at the end of the time evaluation (48 h) BCG contributed to the control of these parasites.

When monocyte-derived macrophages were infected with *L. braziliensis* after β-glucan-training using the same protocol as in the present study, macrophages presented increased capacity of parasite uptake [41], as opposing to BCG training. BCG also reduced uptake of *L. infantum* parasites, but not of *L. amazonensis*. It has been demonstrated that BCG increases expression of CD11b and TLR4, which are receptors involved in *Leishmania* uptake [28,57–59]. Our results suggest differences in modulation of the phagocytosis receptors for *Leishmania* spp. depending on whether dectin-1 or NOD2 receptors are activated by β-glucan or BCG, respectively. This hypothesis deserves further investigation in leishmaniasis.

In this study, for all three *Leishmania* spp. the BCG-induced control of parasites was mediated by the increased ROS production in trained macrophages. In fact, human monocytes and macrophages produced ROS after infection with *L. braziliensis* or *L. amazonensis* and *Leishmania*-induced production of ROS is crucial for the killing of the parasite by human cells [32,48,60,61]. Indeed, the inhibition of ROS production in human macrophages increases the progression of *Leishmania* infection rates [32,34,48]. Furthermore, it is known that trained immunity with BCG induces high production of ROS in human macrophages [42]. Thus, BCG can synergize with *Leishmania* to increase ROS production leading to a better control of the infection in human macrophages.

In the present report, BCG induced IL-32γ expression and

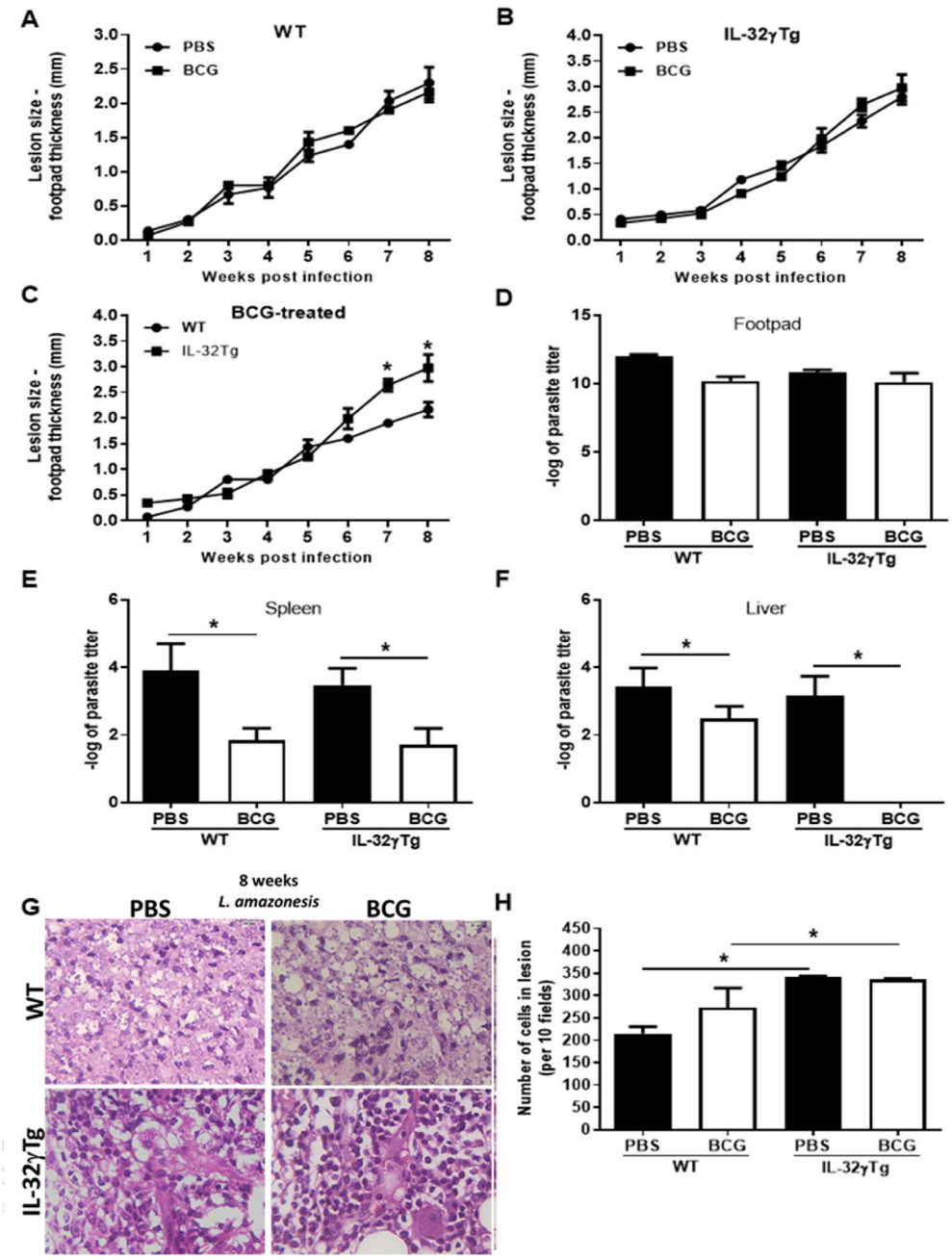

Fig. 5. BCG administration increases inflammation and reduces parasite dissemination during *L. amazonensis* infection in mice, which is potentiated by IL-32γ in the liver. The wild type (WT) and transgenic mice for IL-32 (IL-32γTg) were injected with PBS or BCG (750 μg, intravenously; 1.5–2.0 × 10^6 CFU; BCG-Intervax/Sofia strain). After 7 days, they were infected with 1 × 10^6 stationary phase-promastigotes of *L. amazonensis* in the footpad of the posterior left paw. (A–C) Lesion size (mm) of WT and IL-32γTg mice was measured weekly during 8 weeks and represented by the difference between infected-footpad lesion and non-infected footpad. (D–F) Parasite load was determined by limiting dilution 8 weeks post infection in the footpad, spleen and liver. (G) Histopathological micrographs (1000× magnification) of lesions in the footpad from BCG-injected (at right) or non-injected (at left) WT and IL-32γTg mice were obtained after 8 weeks of infection with *L. amazonensis*. (H) Number of inflammatory cells in footpad lesions was evaluated per 10 fields. The results are represented as mean ± SEM of 4 WT and 7 IL-32γTg mice. In A - C, *p < 0.05, by two-way ANOVA/Bonferroni post hoc test (PBS *vs.* BCG, in each time). In D - F, *p < 0.05, by one-way ANOVA/Bonferroni post hoc test.

intracellular IL-32 in monocytes during the first 24 h of the training, corroborating the results of Netea et al. [35]. In addition, IL-32 appears to synergize with NOD2, the receptor that mediates BCG-trained immunity [28,62]. These results prompted us to investigate the participation of IL-32 in BCG-trained immunity on leishmaniasis outcomes in murine infections. In IL-32γTg mice, the presence of this cytokine improves the host response against all three *Leishmania* spp. evaluated in this study [37,39]. In addition, in this study it was shown that IL-32γ increases the effects of BCG on the control of *Leishmania* spp. infection. In the L. *braziliensis* model, the decrease of lesion size started earlier in BCG-trained IL-32γTg mice than in BCG-trained WT mice. At 12 weeks of infection, although the number of parasites was fewer in BCG-trained IL-32γTg mice than in BCG-trained WT mice, statistical significance was not achieved. As IL-32γ contributes to decrease parasites but also to increase the number of cells in the lesions, differences in the size of lesion can barely be detected. In addition, the C57BL/6 mouse presents acquired immune resistance to *L. braziliensis* [63,64], which can difficult the detection of the immune stimulation effects in this model. Despite this, we could observe that IL-32γ and BCG can work together to improve the control of this parasite.

In the present study, BCG was not able to contribute for controlling the cutaneous lesion caused by *L. amazonensis* infection. The C57BL/6 mouse strain is susceptible to *L. amazonensis* developing ulcerated lesions that can cause paw amputation and also visceralization [63,65,66]. *L. amazonensis* is reported to visceralize also in humans [67]. In this study, BCG prevented the spread of the parasites to spleen and liver. IL-32γ presence improved the effects of BCG in controlling parasitism in liver, an organ that presents high levels of IL-32γ in the transgenic mouse used here [40]. Thus, despite of *L. amazonensis* can escape from immune responses activated by BCG and IL-32γ, resulting in increased lesions at the site of infection, these treatments can alleviate the disease by controlling the dissemination of the parasite.

BCG injection seven days before challenge with *L. infantum* reduced parasitism in the spleen, liver and bone marrow. In this model of

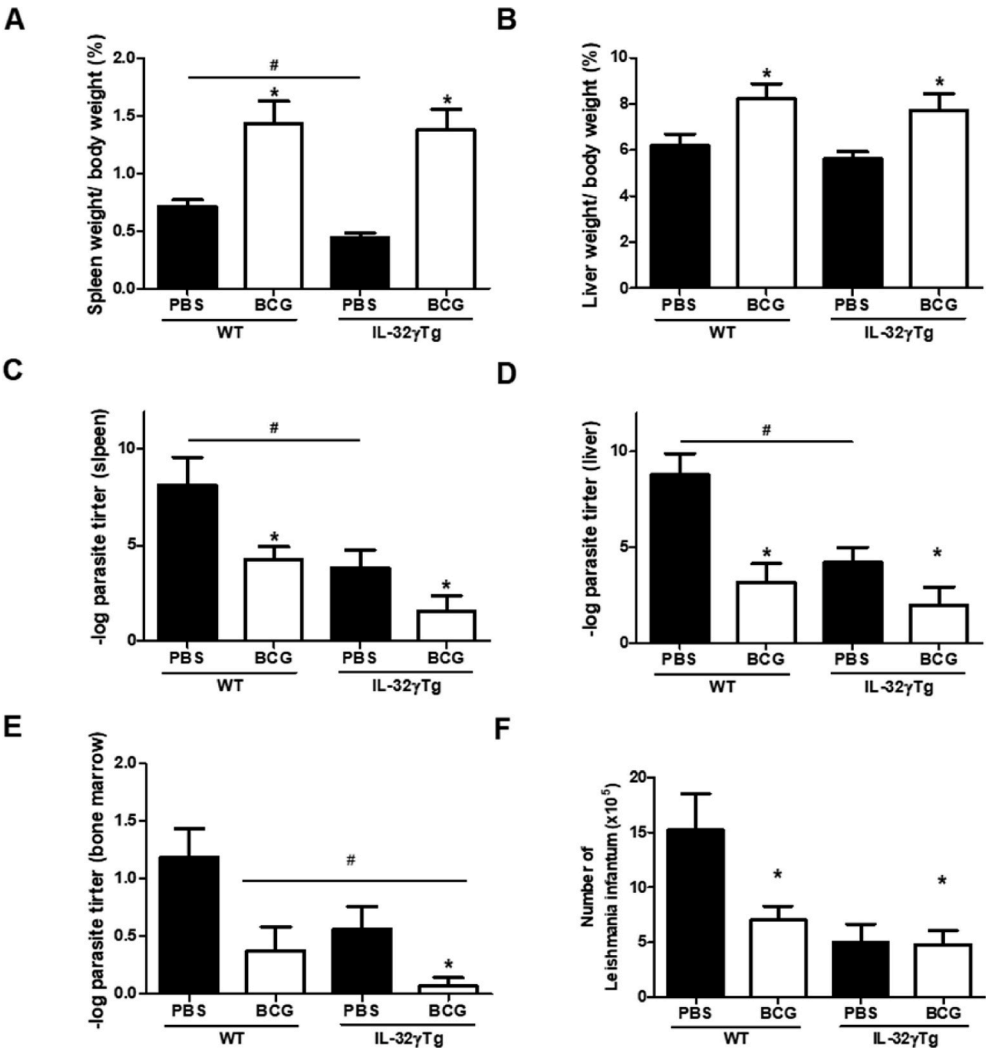

Fig. 6. Training with BCG increases the control of *Leishmania infantum* infection mainly at bone marrow level in IL-32γ transgenic mouse. The wild type (WT) and IL-32γTg mice were injected (i.v.) with PBS or BCG (750 μg; 1.5–2.0 × 10^6 CFU; BCG-Intervax/Sofia strain) and after 7 days they were infected with 1 × 10^7 stationary-phase promastigote forms of *L. infantum* (i.p.). (A) Spleen weight/animal weight ratio obtained after 4 weeks post infection. (B) Liver weight/animal weight ratio obtained after 4 weeks post infection. Parasite load was determined by limiting dilution technique 4 weeks post infection in (C) spleen, (D) liver and (E) bone marrow. (F) Seven days after *in vivo* BCG administration, bone marrow MDM were infected with stationary-phase promastigote forms (MOI 5:1) for 48 h. Then, RPMI medium was replaced by Grace's insect medium and live *L. infantum* were quantified after 2 days. The results are represented as mean ± SEM of 8 WT and 8 IL-32γTg mice in two independent experiments. *$p < 0.05$, by one-way ANOVA/Bonferroni post hoc test (PBS *vs.* BCG). $^{\#}p < 0.05$, by one-way ANOVA/Bonferroni post hoc test, as indicated.

infection, presence of IL-32γ improved the effects of BCG in controlling parasitism especially in bone marrow. The effects of IL-32γ in *L. infantum* mouse infection were clearly demonstrated before [39] and it was shown in the present study that IL-32γ and BCG can synergize to control parasites mainly in bone marrow. In fact, bone marrow MDM trained with BCG *in vivo* in both WT and IL-32γTg mice were able to kill *L. infantum* in higher levels compared with WT non-trained cells. Dos Santos et al. [41] demonstrated that after BCG vaccination of healthy individuals, IL-32 has effects on bone marrow progenitor cells, modulating genes associated with inflammation, DNA-binding transcription factors and metabolism, including pathways known to be involved in trained immunity. Thus suggesting that BCG or IL-32γ can improve the macrophage capacity to kill parasites. Moreover, although BCG injection has induced splenomegaly, similarly to IL-32γ it decreased the number of erythrocytes in the spleens in animals infected with *L. infantum*. Thus, suggesting that both BCG and IL-32γ can reduce the spleen congestion. Taken together, our results suggest that the participation of IL-32γ in the induction of trained immunity could contribute to the protective role of BCG training in VL. That BCG induces epigenetic alterations is already known [28,68], but whether transcriptional alterations caused by IL-32 after BCG vaccination of healthy individuals or treatment of IL-32γTg mice with BCG involves epigenetic alterations needs further investigation. Mechanistically, IL-32 can synergize with NOD2 receptor [62], which is pivotal for BCG signaling [28] to increase the training. In addition, as IL-32 activates vitamin D pathway in monocytes/macrophages [69], which leads to epigenetic modifications [70], IL-32-induced vitamin D activation can be involved in trained immunity.

Macrophages have an essential role in lesion establishment, chronic inflammation and tissue remodeling [71,72]. It is important to note that although BCG has an effect in reducing tissue parasitism in the present study, the activation of macrophages and other cells can increase tissue damage during leishmaniasis. In fact, IL-32γ is associated with TNFα expression in mucosal leishmaniasis [73], the clinical form with strong Th1 immune response mediating tissue damage [74,75]. Thus, data suggest that the combination of IL-32γ and BCG can increase inflammatory response leading to better acquired immune response against the parasites but also to immunopathogenesis.

The data from the present study indicate that BCG training of human monocytes can generate macrophages with the capacity to kill rapidly *L. braziliensis* and *L. infantum* but also to control *L. amazonensis* infection. Moreover, BCG can ameliorate leishmaniasis in mouse infection, especially when IL-32γ is present. Thus, our results reinforce the effects of BCG as a powerful adjuvant in the immunotherapy of CL [9,19,23] as well as ML [20] caused by New World *Leishmania* spp. In general, in these studies the causal agent of the infections was *L. braziliensis*, but BCG has also been used as immunotherapy combined with chemotherapy in infections caused by *L. amazonensis* or *L. mexicana*, alleviating DCL [9,76,77]. There is no effective treatment and acquired immune response development in DCL, thus BCG-induced trained immunity can be an alternative immunotherapy to help the elimination of the parasites [9,76,77].

The present evaluation of trained immunity in human monocytes/

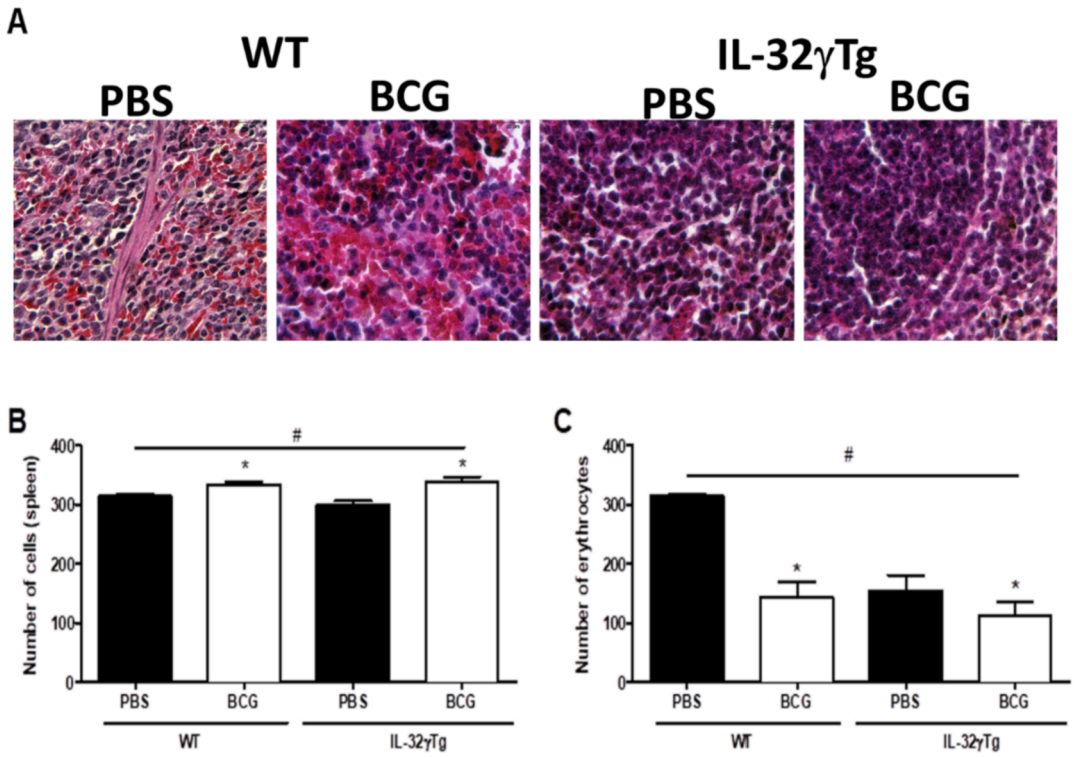

Fig. 7. Histological analysis of spleen from *L. infantum*-infected mice trained with BCG. The wild type (WT) and IL-32γ transgenic mice (IL-32γTg) were treated (i.v.) with PBS or BCG (750 μg; 1.5–2.0 × 10^6 CFU; BCG-Intervax/Sofia strain) and were infected (i.p.) with 1 × 10^7 *L. infantum* stationary-phase promastigote forms after 7 days. (A) Histopathological micrography of spleen after 4 weeks of infection. (B) Total number of nucleated cells (by field) in the spleen after 4 weeks of infection. (C) Number of erythrocytes (by field) in the spleen after 4 weeks of infection. The results represent mean ± SEM of 8 WT and 8 IL-32γTg mice in two independent experiments. *$p < 0.05$, by one-way ANOVA/Bonferroni post hoc test (PBS *vs*. BCG). $^{\#}p < 0.05$, by one-way ANOVA/Bonferroni post hoc test, as indicated.

macrophages was performed with non-BCG vaccinated Dutch volunteers, in The Netherlands, a country that is not an endemic area of tuberculosis or leishmaniasis. Thus, the probability of previous exposure to mycobacterial or leishmanial antigens is very low. However, considering endemic areas of tuberculosis and leishmaniasis, and BCG vaccination, as in Latin America, previous exposure to these antigens must be considered. Studies with *Leishmania* vaccine formulation and BCG for immunoprophylaxis or immunotherapy have shown that previous exposure to BCG does not exacerbate leishmaniasis [20,21,78,79]. BCG together chemotherapy used to treat tegumentary leishmaniasis helps the clinical cure and improve the time of lesion healing, which decreases the use of very toxic drugs and prevent mutilation due to tissue destruction [19,23,77,79]. The use of BCG lead to slight or moderate adverse effects in the local of application (intradermal route; small ulcers), but in some cases it is necessary to suspend it after two doses due to severe local inflammation [22]. Thus, BCG used in vaccine formulations and immunotherapies to leishmaniasis can improve the immune responses against the parasites and contribute to the control of the disease. Mechanistically, one of the beneficial effects of BCG is due to the trained immunity.

Both strains of BCG used in this study induced similar effects in experimental leishmaniasis and *in vitro* cytokine production (Fig. S5). Although, to our knowledge, no comparative data between the Bulgarian (Sofia strain) and Moreau (Brazilian strain) BCG vaccines are available and it is known that the immune responses raised by different BCG vaccines can depend on the strain used [72,73,80,81], both are capable of inducing an inflammatory response and protection against tuberculosis [74,76,82–84].

Here, BCG decreased *L. infantum* infection both *in vitro* as well as *in vivo* indicating the BCG can reduce the severity of the disease. Despite BCG's proven safety and the lack of adverse effects of the conventional treatments against CL, BCG has not been used as therapy against VL. However, increased levels of BCG vaccination reduced the incidence of VL in children from Brazilian Northern region [85] and BCG has been used in vaccine development as an adjuvant to increase the protection against VL in murine or dog models [86–89].

Since BCG is a live attenuated vaccine, when monocyte cultures or mice are exposed to the bacteria suspension for immune training (one week) and further they are infected with *Leishmania* spp., the effects of a co-infection deserve consideration. In mice, training with an i. v. injection of live BCG lead to detection of bacteria up to seven months later [90]. To circumvent the co-infection effects, cells and mice can be treated with antimycobacterial drugs after time of training, before *Leishmania* spp. challenge. Despite this, it is important to highlight that MDP (a NOD2-specific ligand present in BCG) mimics the effects of BCG in trained monocytes/macrophages [28]. We could use MDP instead of BCG for training to avoid misinterpretation of the data due to co-infection. However, BCG has been used around the world as prophylactic and therapeutic live attenuated vaccine, thus we did the experiments with BCG at the light of training effects (trained immunity protocols). In fact, MDP and glycolipopeptides structurally related to it have been used for prophylaxis in leishmaniasis since 1991, using protocols similar to those of trained immunity, showing protective effects [91,92]. In addition, MDP has been used as adjuvant in vaccine against canine leishmaniasis [93]. As live and proliferating BCG can lead to innate activation by other mechanisms besides trained immunity [94], our results shown that BCG-innate activation plays an important role in the control of *Leishmania* spp. infection and indicate that this complex system should be better scrutinized in further studies. Another concern in co-infection is whether *Leishmania* infection could interfere with the BCG load. Although we did not check this point, independent of the effects of *Leishmania* infection in BCG load, during the period of evaluation, the mice presented an increased capacity to control the parasites. In fact, one concern was the co-infection with BCG plus *L. infantum*

because this *Leishmania* species causes immunossupression, affects bone marrow, causes splenomegaly and hepatomegaly, being fatal. However, despite BCG had caused an increase of the weights of spleen and liver, the best results in terms of parasite control were obtained with this *Leishmania* species in the VL model. Thus, we believe that despite co-infection trained immunity mechanisms induced by BCG must contribute prophylactically to improve the resistance to leishmaniasis.

In addition to immunotherapeutic interventions, BCG has been used in immunoprophylaxis approaches to protect against leishmaniasis [22,95,96]. Further larger studies in animals and, especially, in humans vaccinated or not with BCG are needed to determine the benefits of BCG vaccination in the prevention and/or treatment of leishmaniasis.

5. Conclusions

Concerning the mechanisms of BCG's effects against *Leishmania* spp. infections, the stimulation of innate cells and induction of cross-reactive T cells have been discussed in previous studies. The present data showed that additionally trained immunity induced by BCG improves the immune response towards *Leishmania* spp., which was confirmed *in vitro* and *in vivo*. Overall, the results suggest that BCG training favors the control of infection with different *Leishmania* spp. causing CL or VL, especially in the presence of IL-32γ. This study strengthens the concept of IL-32γ as modulator of trained immunity induced by BCG. Thus, BCG and IL-32 could be targets for therapeutic alternatives in leishmaniasis.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MVTS and RSG: *in vitro* and *in vivo* experiments, manuscript preparation and animal care; LABJ, FRD, RSG and MGN: conceived and designed the study, and manuscript preparation; JCS: *in vitro* experiments and revision of the *in vitro* methods of the manuscript; AMBF, GGM: *in vivo* experiments and animal care; LUT: *in vitro* experiments; JXP, SAP: Histological analysis; FRD, LABJ and MGN: Obtaining of funding for the study. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Abbreviations

ATL	American tegumentary leishmaniasis
BCG	Bacillus Calmette-Guérin
CL	cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
IL-32	Interleukin 32, IL- 32γTg Transgenic mouse for human IL-32γ gene
ML	mucosal leishmaniasis
MDM	monocyte-derived macrophages
ROS	reactive oxygen species
WT	Wild type mouse
VL	visceral leishmaniasis

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2021.105088.

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Ethics approval and consent to participate

The study was approved by Ethics Committee of Radboud University Nijmegen, the Netherlands (approval number 42561.091.12) and by Ethics Committee for animal research of Universidade Federal de Goiás (CEUA/PRPI/UFG, protocol 042/16).

Consent for publication

Not applicable.

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