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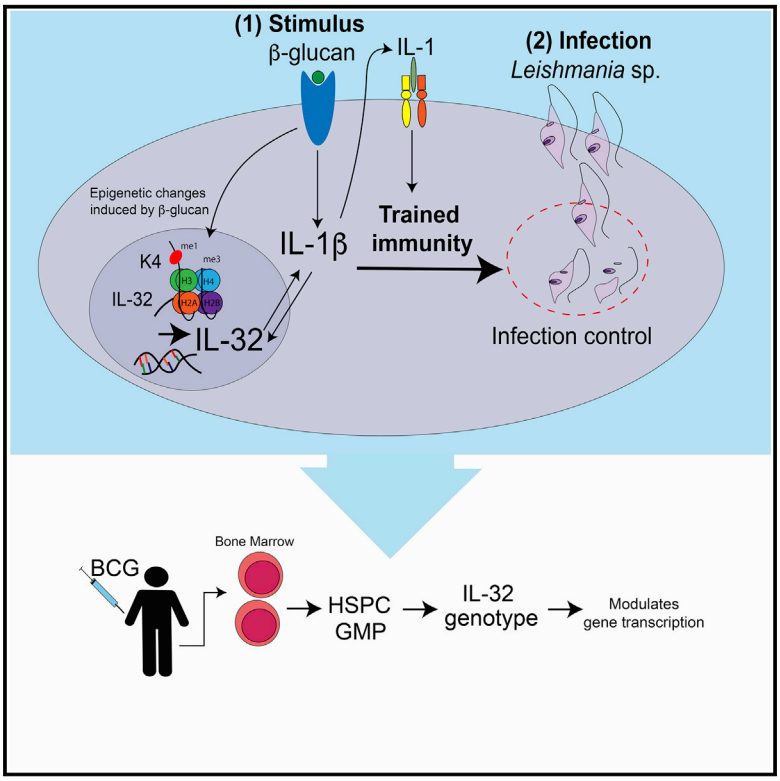
β-Glucan-Induced Trained Immunity Protects against *Leishmania braziliensis* Infection: a Crucial Role for IL-32

Graphical Abstract



Highlights

- Trained immunity induced by β-glucan protects against *L. braziliensis* infections
- β-glucan-induced protection against *Leishmania* is mediated by IL-32 and IL-1
- Bone marrow of IL-32TG mice shows increased responsiveness after β-glucan exposure
- IL-32 modulates gene transcription of HSPCs and GMP in BCG-vaccinated subjects

Authors

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In Brief

dos Santos et al. describe that trained immunity induced by β-glucan confers protection against *L. braziliensis* infections. Infection control is associated with IL-32 and IL-1 induction. Genetic variation in the *IL-32* gene enhances induction of trained immunity leading to proinflammatory gene transcription in bone marrow hematopoietic stem and progenitor cells.

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β-Glucan-Induced Trained Immunity Protects against *Leishmania braziliensis* Infection: a Crucial Role for IL-32

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SUMMARY

American tegumentary leishmaniasis is a vector-borne parasitic disease caused by *Leishmania* protozoans. Innate immune cells undergo long-term functional reprogramming in response to infection or Bacillus Calmette-Guérin (BCG) vaccination via a process called trained immunity, conferring non-specific protection from secondary infections. Here, we demonstrate that monocytes trained with the fungal cell wall component β-glucan confer enhanced protection against infections caused by *Leishmania braziliensis* through the enhanced production of proinflammatory cytokines. Mechanistically, this augmented immunological response is dependent on increased expression of interleukin 32 (IL-32). Studies performed using a humanized IL-32 transgenic mouse highlight the clinical implications of these findings *in vivo*. This study represents a definitive characterization of the role of IL-32γ in the trained phenotype induced by β-glucan or BCG, the results of which improve our understanding of the molecular mechanisms governing trained immunity and *Leishmania* infection control.

INTRODUCTION

Leishmaniases are vector-borne tropical diseases caused by protozoa of the *Leishmania* genus, characterized by a wide clinical spectrum and symptoms ranging from self-healing cutaneous lesions to lethal visceral disease. Leishmaniases are among of the most neglected infectious diseases with endemicity in 88 countries and affecting 12 million people worldwide. The annual incidence is around 2 million cases, including both visceral and cutaneous diseases (Alvar et al., 2012). In the Americas, *L.* (*Viannia*) *braziliensis* is the predominant species causing American tegumentary leishmaniasis (ATL), characterized by localized cutaneous leishmaniasis (LCL), disseminated cutaneous leishmaniasis (DL), and mucosal leishmaniasis (ML) (Goto and Lauletta Lindoso, 2012). In the absence of efficient strategies to prevent infections with *Leishmania* parasites and to control the transmission of the parasites by vector insects, the treatment of leishmaniasis relies on drug therapy. Pentavalent antimony has been the first line of treatment for decades. This drug is expensive for low and middle-income countries and requires several injections that are toxic and painful. Furthermore, there is emergence of parasite resistance to antimonial drugs, and the rate of treatment failure affects more than 47% of the cases. In some endemic areas, where the efficacy of pentavalent antimonial is very low, it is replaced by other more effective drugs such as amphotericin B and miltefosine (Barroso et al., 2007; Khamesipour, 2014; Machado et al., 2010).

Leishmania is an intracellular parasite that can be taken up by phagocytic cells, including neutrophils, monocytes, macrophages, and resident dendritic cells. In macrophages, there is the best evidence for parasite replication and long term-survival (Beattie and Kaye, 2011). Therefore, targeting macrophages to enhance or modulate their antimicrobial function may facilitate the development of novel therapeutic strategies. Among the effector mechanisms used by macrophages to control *Leishmania* replication are reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). Activation of macrophages is

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dependent on interferon gamma (IFNγ) and tumor necrosis factor (TNF-α) (Bottrel et al., 2001; Ibraim et al., 2013; Liese et al., 2008). Additionally, studies have shown that macrophage interleukin (IL)-1β signaling is important for controlling parasite replication and influences the clinical course of Leishmaniasis (Lima-Junior et al., 2013; Novais et al., 2017).

Recently, our group identified interleukin 32 (IL-32), an intracellular cytokine produced in different isoforms by immune and non-immune cells, as a pivotal mediator in controlling infections caused by New World *Leishmania* spp. (Dos Santos et al., 2018). IL-32γ is expressed in lesions of patients infected with *L.* (*V.*) *braziliensis* or *L.* (*L.*) *amazonensis* (Galdino et al., 2014; Gomes et al., 2017). In addition, infection of human macrophages with *Leishmania* induces intracellular IL-32γ expression. Once produced, IL-32γ is associated with the production of TNF-α, IL-8, nitric oxide (NO), and antimicrobial peptides such as cathelicidin and β-defensin 2 (Dos Santos et al., 2017b). Moreover, *Leishmania* infection of human IL-32γ transgenic (IL-32γTG) mice resulted in improved control of lesions caused by *L. braziliensis* in association with an increase of IFNγ, TNF-α, and IL-10 production (Gomes et al., 2017).

Clinical improvement in the treatment of ATL with Bacillus Calmette-Guérin (BCG) vaccination has been reported previously (Convit et al., 2004). In addition, we recently confirmed that BCG vaccination in combination with conventional treatment can increase circulating natural killer (NK) cells and proinflammatory monocytes, contributing to clinical remission of several lesions in a patient with diffuse cutaneous leishmaniasis (DCL) caused by *L*. (*L.*) *amazonensis* (Pereira et al., 2009). Similarly, Obaid et al. (1989) observed that β-glucan (a cell wall component of *Candida albicans*) as adjuvant together with *Leishmania* antigens lead to protection against *L. (L.) donovani* in hamsters.

In monocytes and macrophages, BCG and β-glucan induce non-specific protection to secondary infections by long-term functional reprogramming via a process called trained immunity, which is dependent on metabolic and epigenetic changes, such as lysine methyl modifications (H3 histones monomethylated [H3K4me1] or trimethylated [H3K4me3] at lysine 4) (Arts et al., 2016; Netea et al., 2016; Quintin et al., 2012). We therefore hypothesized that exposure of monocytes to β-glucan may induce non-specific protection against intracellular infection caused by *Leishmania* spp., which could offer novel immunotherapeutic strategies to patients with leishmaniasis. In both human primary cells and a murine model of trained immunity, we assessed the effects of β-glucan or BCG training in monocytes and macrophages using various biochemical and molecular approaches. In addition, we investigated the role of IL-32γ and IL-1 signaling for β-glucan-induced trained immunity.

RESULTS

β-Glucan-Induced Trained Immunity Controls *L. braziliensis* Infection in Primary Human Macrophages

Because trained immunity has been reported to protect against non-specific infections, we investigated whether training induced by β-glucan could control infection caused by *L. braziliensis.* Human primary monocytes incubated with β-glucan for 24 h were washed and incubated in normal culture conditions for an additional 5 days and infected at day 6 with promastigote forms of *L. braziliensis*. Production of TNF-α, IL-6, and IL-10 was evaluated after 2 h, 4 h, and 24 h of infection (Figure 1A). Training of monocytes with β-glucan induced higher TNF-α and IL-6 production after stimulation with LPS, compared to control cells (Figures S1A and S1B). *L. braziliensis* infection did not induce these cytokines in naive macrophages, whereas a significant increase in IL-6 and IL-10 production was observed after 2 h and 4 h of infection in β-glucan-trained macrophages (Figures 1B–1D). In parallel, a greater percentage of macrophages had ingested the parasite after training with β-glucan at these time points (Figure 1E). Remarkably, after 24 h of infection, the percentage of infected macrophages strongly decreased in β-glucan-treated macrophages (Figure 1E). Similar results were observed with regard to the number of parasites per infected cell and infection index (Figures 1F and 1G). Interestingly, the number of parasites per infected cell was significantly decreased as early as 4 h after infection, indicating rapid killing of the parasites in β-glucan-trained cells (Figure 1F). Accordingly, β-glucan-trained macrophages contained 3-fold less live parasites compared to control macrophages (Figure S1C). Macrophages death was monitored by measuring the release of lactate dehydrogenase (LDH). LDH concentrations remained stable in both β-glucan-trained and control macrophages after infection (Figure S1D).

To investigate potential mechanisms underlying these initial observations, we evaluated whether known microbicidal molecules such as ROS, NO, cathelicidin, and β-defesin could explain the better parasite control in β-glucan-trained primary macrophages. No differences in ROS or NO production were observed when comparing trained or non-trained macrophages (Figures S2A and S2B). In contrast, *cathelicidin* and *β-defensin 2* mRNA expression were significantly increased in β-glucan-trained macrophages (Figures S2C and S2D).

β-Glucan-Induced Epigenetic Modifications Results in Upregulation of IL-32 Expression in Human Macrophages

We previously described a role for IL-32-dependent *cathelicidin* and *β-defensin 2* expression in host defense against *Leishmania* spp. (Dos Santos et al., 2017b), thus prompting us to explore if IL-32 was involved in β-glucan induced protection against *L. braziliensis* infection in human macrophages. In accordance with our hypothesis, *IL-32γ* mRNA expression as well as intracellular IL-32 protein levels were significantly increased in β-glucan-trained macrophages (Figures 2A and 2B). Furthermore, when β-glucan-trained cells were infected with *L. braziliensis*, the intracellular IL-32 protein levels were increased compared to control cells (Figure 2C). These results indicated that IL-32 is upregulated on a transcriptional level. Indeed, intracellular IL-32 expression was previously shown to be influenced by genetic variation in the IL-32 promoter region (https://genenetwork.nl/bloodeqtlbrowser; Westra et al., 2013). Using the 200FG cohort (Li et al., 2016) of healthy volunteers, we examined whether the *IL32* promoter single nucleotide polymorphism (SNP) rs4786370 could interfere with β-glucan-induced trained immunity. The presence of CC genotype was associated with greater

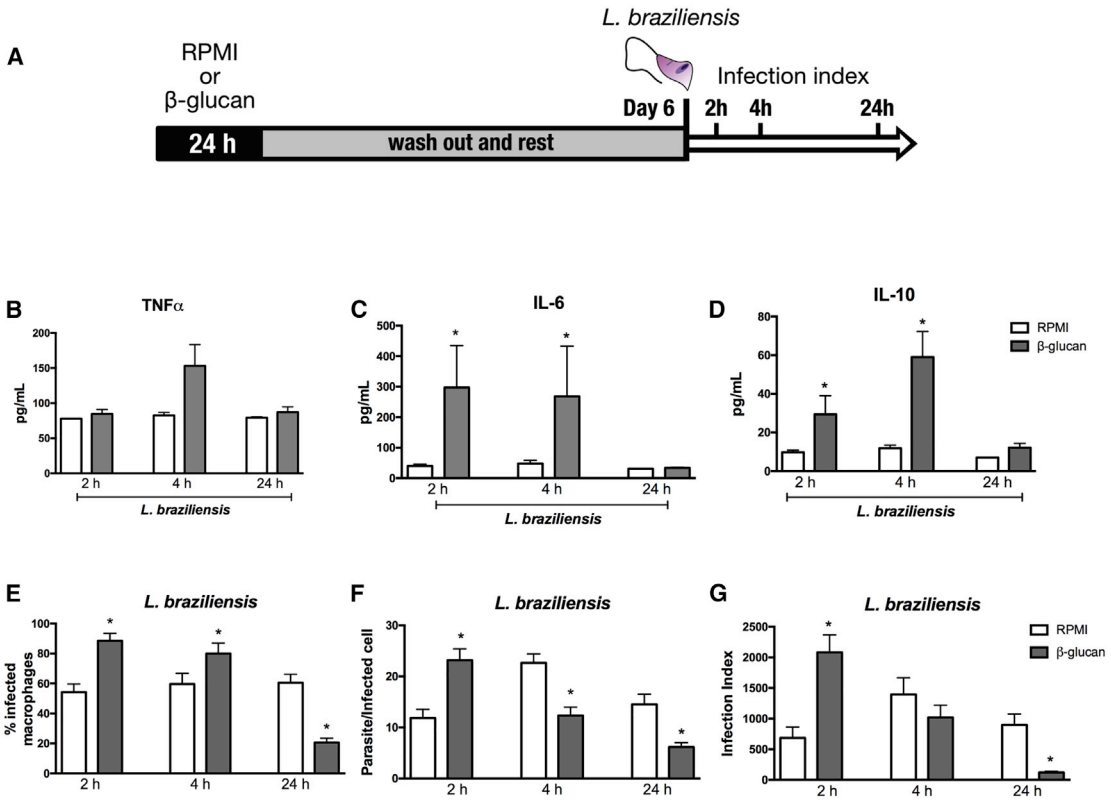

Figure 1. Trained Immunity with β-Glucan Is Important for Controlling *L. braziliensis* Infection in Human Macrophages

Monocytes were trained with RPMI or β-glucan for 24 h.

(A) On day 6 after β-glucan exposure, macrophages were infected with stationary-phase *L. braziliensis* promastigotes for 2 h, washed, and incubated for a total of 24 h infection.

(B–D) Production of TNF-α (B), IL-6 (C), and IL-10 (D) was measured in supernatants by ELISA.

(E) Percentage of infected macrophages after 2 h, 4 h, and 24 h of infection.

(F) Number of parasites per infected cell after 2 h, 4 h, and 24 h of infection.

(G) Infection index after 2 h, 4 h, and 24 h of infection. The data shown are the mean ± SEM. Results are compiled from at least two experiments. n = 6. *p < 0.05 (RPMI versus β-glucan; by Wilcoxon test).

production of TNF-α and IL-6 compared with the CT and TT genotypes (Figures 2D and 2E). Additionally, in individuals carrying the CC genotype, a tendency to higher production of lactate was observed compared with the TT genotype (Figure 2F).

To investigate whether the *IL32* rs4786370 SNP could influence the production of intracellular IL-32 and infection index during the training with β-glucan, we stratified the healthy volunteers based on their genotype. The tendency of higher production of IL-32 was observed in individuals carrying the CC genotype compared with those carrying the TT genotype in β-glucan-trained macrophages after the infection with *L. braziliensis* (Figure 2G). In parallel, the infection index pointed toward a decreased infection rate in the individuals carrying the CC genotype compared with the TT genotype (Figure 2H). Interestingly, a negative correlation between the levels of intracellular IL-32 and the infection index in the individuals carrying the CC genotype was observed (Figure 2I). These results suggested that trained immunity phenotype induced by β-glucan is mediated by IL-32.

We next examined the chromatin landscape surrounding IL-32 expression in cells trained with β-glucan. Levels of H3 histones trimethylated at lysine 4 (H3K4me3), a marker generally associated with transcriptional activation, were unexpectedly depleted from the IL-32 promoter region in trained cells (Figure 2J). Furthermore, transcriptionally repressive lysine 9 trimethylation of H3 histones (H3K9me3) was not significantly altered by β-glucan training (Figure 2K). A recent study identified a distal regulatory element that is crucial for IL-32 transcriptional regulation in $CD4^+$ T cells (Palstra et al., 2018). Activation of such elements can be epigenetically distinguished by enrichment for H3 histones monomethylated at lysine 4 (H3K4m1) (Catarino and Stark, 2018). Paralleling the transcriptional upregulation of IL-32, we observed significant enrichment of H3K4m1 at the distal enhancer region (Figure 2L).

Human Monocyte Training with β-Glucan Is Dependent on IL-32γ Expression and IL-1 Signaling

Trained immunity induced by β-glucan is reported to be dependent on bioactive IL-1β production in mice (Christ et al., 2018; Mitroulis et al., 2018). Moreover, IL-1β induces IL-32 production (Kim et al., 2005). To further investigate whether IL-1β and IL-32

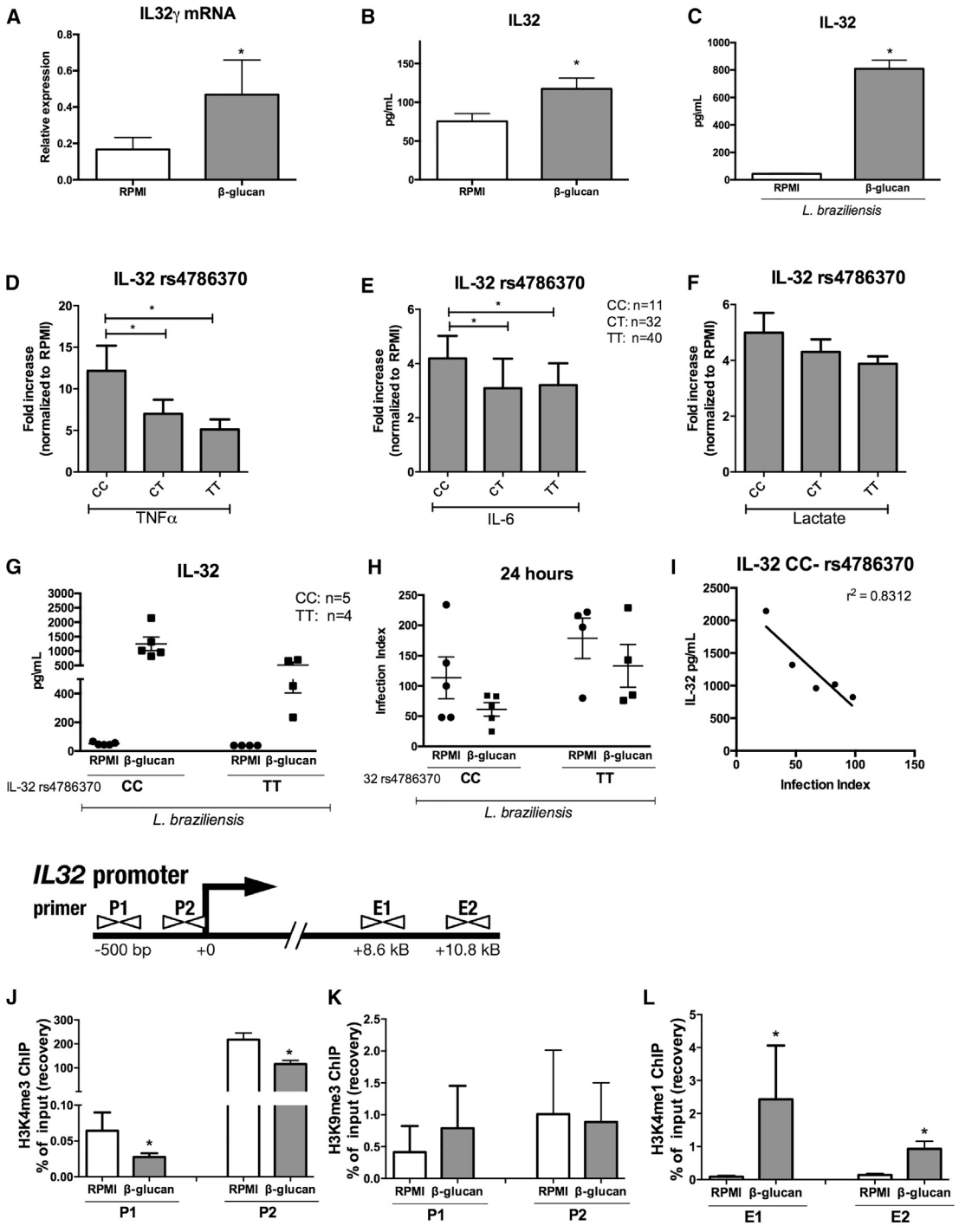

Figure 2. IL-32γ Is Upregulated in β-Glucan-Trained Macrophages and Is Relevant for Training

Monocytes were untrained or trained with β-glucan for 24 h.

(A and B) On day 6 after β-glucan exposure, *IL-32γ* mRNA expression (A) and intracellular IL-32 protein (B) were measured.

(C) After β-glucan exposure, macrophages were infected with stationary-phase *L. braziliensis* promastigotes and intracellular IL-32 protein was measured.

(D–F) Genetic variations in IL-32 were assessed in DNA samples from 83 healthy volunteers from the 200FG cohort. On day 6, after LPS restimulation, TNF-α (D), IL-6 (E), and lactate (F) production in β-glucan-trained macrophages was assessed in the 3 genotypes for *IL-32* rs4786370 SNP. The data shown are fold increased normalized to RPMI. Mean ± SEM; *p < 0.05 (Mann-Whitney U test).

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was associated with the training induced by β-glucan in primary human cells, the production of IL-1β were assessed in the individuals carrying the different genotypes of the *IL32* rs4786370 SNP. The results showed a higher production of both secreted IL-1β and intracellular IL-1β by individuals carrying the CC genotype compared with the TT genotype during the first 24 h of exposure with β-glucan (Figures 3A and 3B). The treatment of primary monocytes with human IL-1β resulted in a significant increase in *IL-32γ* mRNA expression as well as intracellular IL-32 protein levels (Figures 3C and 3D). Further, we examined the effect of genetic variations in *IL-1/IL-1R* genes on β-glucan-induced trained immunity. Cytokine production was significantly decreased when there were polymorphisms located in the promoter region of the IL-1β-encoding gene (*IL-1β*; rs16944) (Figures 3E and 3F) or a missense SNP in the *IL-1α* gene (*IL-1α*, rs17561) (Figures 3G and 3H), respectively. Similarly, an intronic SNP on *IL1RAP* (interleukin 1 receptor accessory protein; rs34590034) gene also influenced the training capacity of monocytes by decreasing the production of TNF-α by individuals carrying the TT genotype compared to AA and AT genotypes (Figure 3I).

The effects of impaired IL-1 signaling could be ascribed to a decrease in IL-32 production. In fact, when monocytes were trained with β-glucan in the presence of interleukin-1 receptor antagonist (IL-1Ra), we observed lower levels of *IL-32γ* mRNA than monocytes trained in the absence of IL-1Ra (Figure 3J). Additionally, when these macrophages were infected with *L. braziliensis,* we observed a significant higher infection index than monocytes trained in the absence of IL-1Ra (Figure 3K). The importance of IL-1β for β-glucan-induced training was confirmed by a reduction in TNF-α and IL-6 production when cells were trained in the presence of IL-1Ra and challenged after 6 days of culture with LPS (Figures S3A and S3B). Thus, we showed that β-glucan-induced trained immunity is regulated by the expression of IL-32 and IL-1 signaling, which is important for the control of *L. braziliensis* infection.

β-Glucan-Induced Trained Immunity in Human IL-32γ Transgenic Mice Improves Resistance to *Leishmania braziliensis*

Because IL-32 is not expressed in rodents to further confirm that IL-32γ is important for β-glucan training and control of *L. braziliensis* infection, we performed experiments with human IL-32γ transgenic mice (IL-32γTG) (Choi et al., 2010). Recently, it was reported that β-glucan-induced trained immunity in C57BL/6 mice induced proliferation of bone marrow progenitors of myeloid lineage, which was associated with increased production of innate immune mediators such as IL-1β, and with adaptations in cell metabolism (Mitroulis et al., 2018). Next, we investigated whether β-glucan also induces changes in bone marrow cells (BM) of IL-32γTG mice. Human IL-32γTG mice received intra-peritoneal β-glucan injections, and after 7 days, bone marrow cells were analyzed. An increased cellularity was observed in the β-glucan-injected group compared to the PBS control animals (Figure 4A). Furthermore, β-glucan-treated mice displayed higher expression of *mki67* and *csf2rb* mRNA, which are genes related to cell-cycle regulation and a myeloid lineage marker, respectively (Figures 4B and 4C). Of note, *rag2* gene expression that is a lymphoid cell marker was downregulated in β-glucan-injected group compared to the PBS control animals (Figure 4D). We next analyzed IL-32 expression after 24 h of *ex vivo* stimulation with LPS or *L. braziliensis* lysates. Levels of *IL-32γ* and *IL-1β* mRNA were significantly higher in BM cells from the β-glucan-injected mice compared with cells from the PBS group after stimulation with either LPS or lysates of *L. braziliensis* (Figures 4E and 4F). Besides cytokines, expression of genes encoding key regulatory enzymes of the glycolytic pathway was evaluated. We observed significantly increased expression of *hif-1a*, *pfkp3*, and *hk3* mRNA expression in BM cells from β-glucan-injected mice in comparison with cells from PBS-injected IL-32γTG mice (Figures 4G–4I).

These results indicated that IL-32γTG mice can be trained by β-glucan, leading to metabolic alterations and an improved immune response to *L. braziliensis* antigens. Thus, we further explored whether β-glucan could induce protection against *L. braziliensis* infection *in vivo* in IL-32γTG mice. To this end, either wild-type (WT) C57BL/6 or IL-32γTG mice were injected with β-glucan or PBS and infected 7 days later with stationary-phase promastigotes of *L. braziliensis* (Figure 5A). β-glucan-trained IL-32γTG mice showed a significant increase in lesion size after 3 weeks of infection compared with the PBS control group. Remarkably, after week 5 of infection, a significant decrease in lesion size was observed in β-glucan-trained mice. Nevertheless, no significant differences in lesion size were observed in β-glucan-trained WT mice compared with the PBS control group (Figure 5B). At the later stages of the infection, a significant decrease in parasite load was observed in β-glucan-trained IL-32γTG mice compared with the PBS group. Consistent with the lesion size, no differences in parasite load were observed in β-glucan-trained WT mice (8 weeks, Figure 5C). Furthermore, no significant differences in TNF-α and IL-32 levels were observed at this time point (Figures 5D and 5E).

The increased size of the lesion on the third week of infection observed in the β-glucan-trained mice could be ascribed to a marked increase in the inflammatory infiltrate (Figures 6B–6D). After 8 weeks of infection, β-glucan-trained IL-32γTG mice showed a significant reduction of inflammation when compared with the PBS group (Figures 6E–6G). Contrary to the IL-32γTG mice, when β-glucan-trained WT mice were compared with the PBS group, no significant difference in the inflammatory infiltrate was observed (Figures 6H–6J).

(G and H) After training with β-glucan, macrophages from individuals carrying the CC and TT genotypes of *IL32* rs4786370 SNP were infected with stationary-phase *L. braziliensis* promastigotes. Intracellular IL-32 protein (G) was measured and the infection index (H) was determined. The data shown are the mean ± SEM; n = 5 CC and n = 4 TT.

(I) Correlation of IL-32 with infection index in individuals carrying the CC genotype of *IL32* rs4786370 SNP.

(J–L) Chromatin was fixated on day 6 and chromatin immunoprecipitation (ChIP)-qPCR was performed. H3K4me3 (J) and H3K9me3 (K) were determined at the promoter of *IL-32* gene (P1 and P2). H3K4me1 (L) was determined at the distal enhancer region of *IL-32* gene (E1 and E2). The data shown are the mean ± SEM. Results are compiled from at least two experiments. n = 6. *p < 0.05 (RPMI versus β-glucan; by Wilcoxon test).

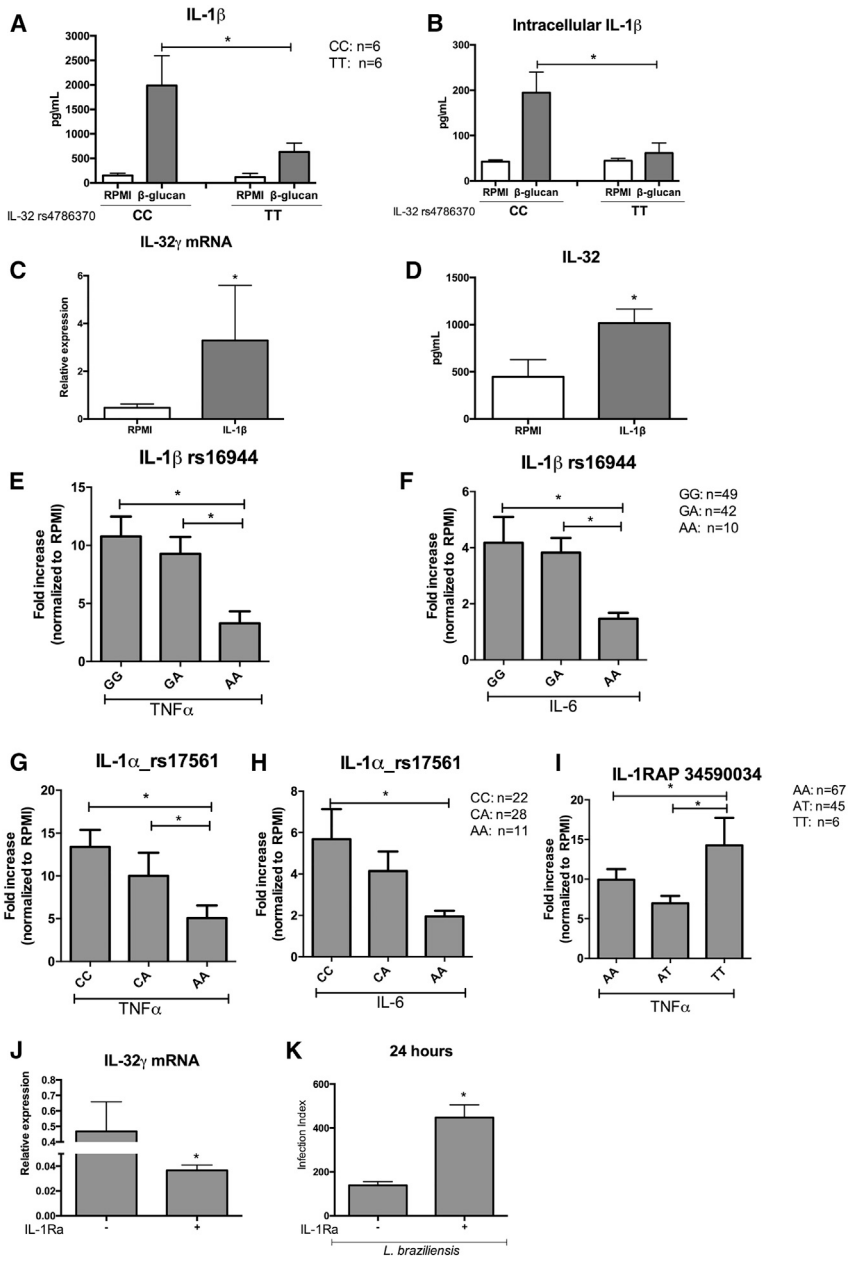

Figure 3. IL-1β and IL-32γ Expression Mediate β-Glucan Training

(A and B) Monocytes from individuals carrying the CC and TT genotypes of *IL32* rs4786370 SNP were trained with RPMI or β-glucan for 24 h. Secreted (A) and intracellular (B) IL-1β protein were measured.

(C and D) IL-32γ mRNA expression (C) and intracellular IL-32 protein (D) were measured in monocytes after 24 h stimulation with rhIL-1β. Monocytes were trained with RPMI or β-glucan for 24 h.

(E–I) Genetic variations in IL-1β (rs16944) (E and F), IL-1α (rs17561) (G and H), and IL-1RAP (rs34590034) (I) were assessed in DNA samples from healthy volunteers from the 200FG cohort. Following LPS restimulation on day 6, TNF-α and IL-6 production in β-glucan-trained macrophages were assessed. The data shown are fold increased normalized to RPMI. Mean ± SEM; *p < 0.05 (Mann-Whitney U test). Monocytes were trained with β-glucan ± rhIL-1Ra for 24 h. On day 6 after β-glucan exposure, macrophages were infected with stationary-phase *L. braziliensis* promastigotes.

(J) IL-32γ mRNA expression.

(K) Infection index after 24 h of infection. The data shown are the mean ± SEM. Results are compiled from at least two experiments. n = 6. *p < 0.05 (RPMI versus β-glucan; by Wilcoxon test).

Because IL-32 and IL-1β seem to play a pivotal role in the control of the infection caused by *L. braziliensis* in β-glucan-trained macrophages, we evaluated whether these two cytokines as well as macrophages-like cells were present in the lesion of IL-32γTG mice. The predominant cells in the lesions were macrophage-like (F4/80^{+}) cells in both β-glucan-trained or PBS-treated IL-32γTG mice after 3 weeks and 8 weeks of infection. Furthermore, we observed that IL-32 protein was mainly expressed in β-glucan-trained mice. Of note, the high expression of IL-1β at week 3 after infection in β-glucan-trained mice compared with the PBS group provide an extra evidence supporting the concept that IL-32 and IL-1β are closely associated with the β-glucan-induced trained immunity (Figure S4).

Intracellular IL-32 Expression Determines the Gene Transcription Profile in Bone Marrow-Derived HSPC and GMP Cells 90 Days after BCG Vaccination of Human Volunteers

BCG vaccination has been shown to induce effects similar to that of β-glucan at the level of the hematopoietic stem cells within the bone marrow resulting in an enhanced training ability (Kaufmann et al., 2018; Mitroulis et al., 2018). Because β-glucan is not yet ready to be used as a therapeutic option to treat human diseases (Leentjens et al., 2014), to gain further insight into the mechanism by which *IL32* rs4786370 SNP enhances the induction of trained innate immunity, we evaluate the gene expression in bone marrow hematopoietic stem and progenitor cells (HSPC, consisting of hematopoietic stem cells and multi-potent progenitors) and granulocyte macrophage progenitors (GMP) of healthy volunteers before BCG vaccination on day 0 (D0) and on day 90 (D90) post BCG vaccination (Figure 7A). We focused our analyses on genes differently expressed specifically between rs4786370 CT_TT and CC BM cells. At day 0, prior to BCG vaccination, *IL32* expression was similar in the individuals carrying the *IL32* rs4786370 CT_TT and CC genotypes in both GMP and HSPC BM cells (Figure 7B). We next analyzed *IL32* expression in GMP cells after BCG vaccination. Of note, mean *IL32* expression in GMP cells was already modulated at day 0 after BCG exposure in individuals carrying the *IL32*

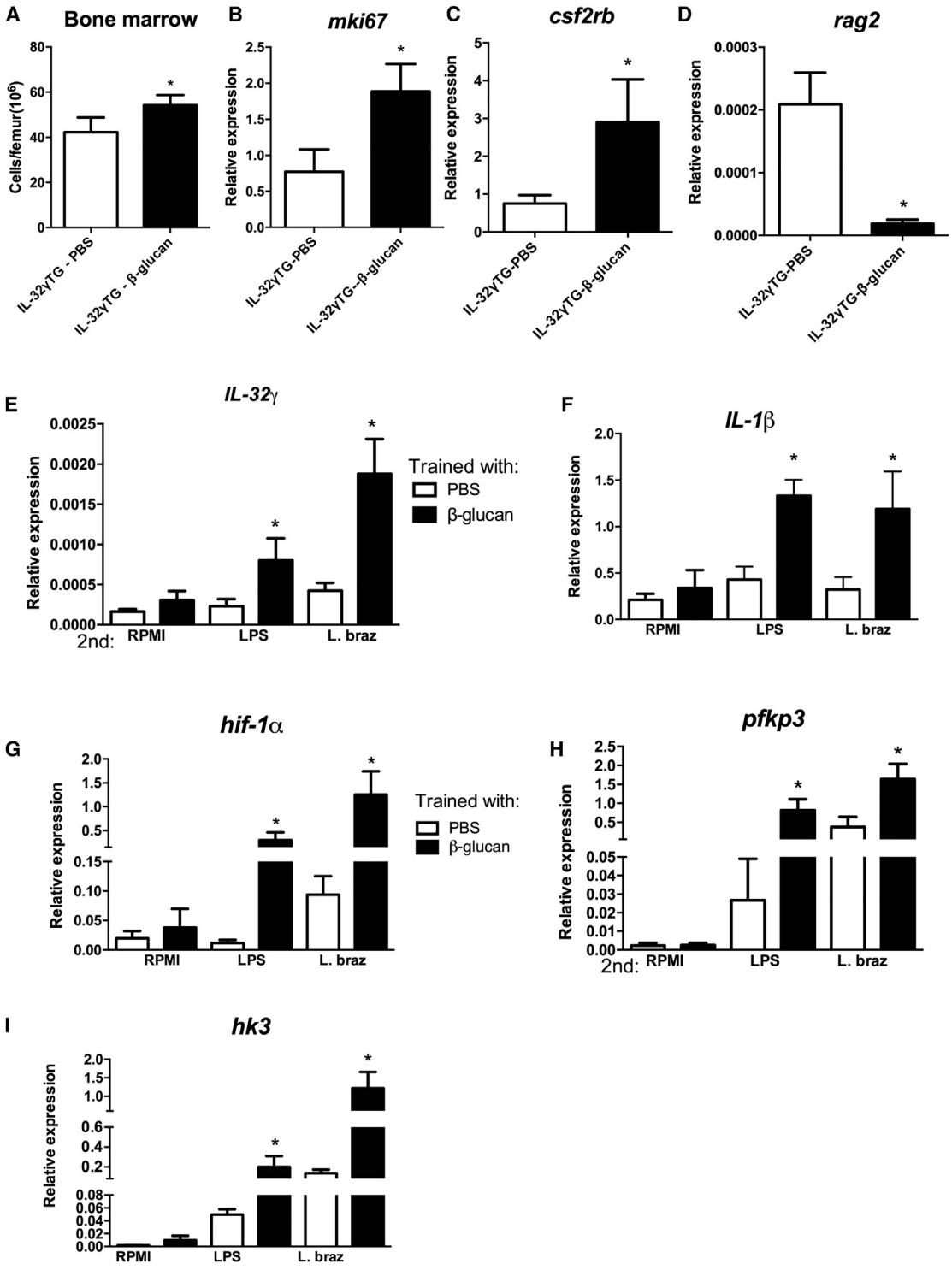

Figure 4. β-Glucan-Induced Trained Immunity Acts in the Bone Marrow of IL-32γ Transgenic Mice

Humanized IL-32γ transgenic (IL-32γTG) mice were injected with β-glucan or PBS, and bone marrow analyses were performed after 7 days. (A–D) On day 7, the number of cells in the bone marrow (femur) (A) and *mki67* (B), *csfr2b* (C), and *rag2* (D) mRNA expression were measured. (E–I) *IL-32γ* (E), *IL-1β* (F), *hif-1α* (G), *pfkp3* (H), and *hk3* (I) mRNA expression after *ex vivo* 24 h stimulation with LPS or lysates of *L. braziliensis* (L. braz). The data shown are mean ± SEM of representative group of experiments performed with 5 mice per group. *$p < 0.05$ (PBS versus β-glucan; by Student's t test).

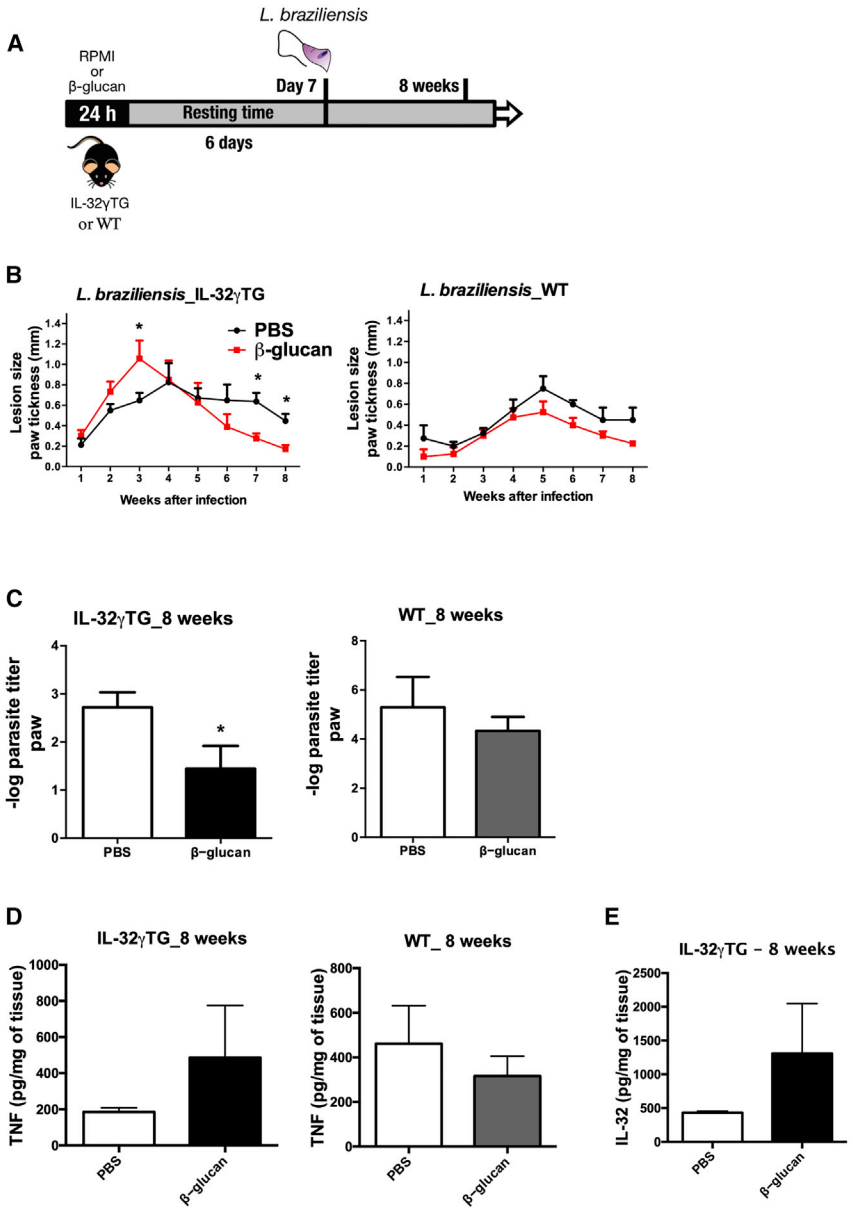

Figure 5. β-Glucan Training in IL-32γTG Mice Improves the Control of Infection Caused by *L. braziliensis*

(A) WT and IL-32γTG mice were injected with β-glucan or PBS and subcutaneously infected in footpad with stationary-phase *L. braziliensis* promastigotes after 7 days.

(B) Lesion size (in millimeters; for each mouse, the size of the infected footpad was subtracted by size of the uninfected control footpad) was monitored weekly.

(C) Parasite load of tissue measured after 8 weeks of infection.

(D and E) TNF-α (D) and IL-32 (E) production per mg of tissue measured in the footpad macerated after 8 weeks of infection. The data shown are mean ± SEM of representative group of experiments performed with 6 mice per group. *$p < 0.05$ (PBS versus β-glucan; by Student's t test).

rs4786370 CC genotype and remained unaltered after 3 months of vaccination (Figure 7B). However, after BCG vaccination, *IL32* expression in HSPC cells was modulated within the different time points. Interestingly, 3 months (M3) after exposure to BCG, *IL32* expression was higher in the individuals carrying the *IL32* rs4786370 CC genotype when compared with individuals carrying *IL32* rs4786370 CT_TT (Figure 7B).

We next investigated genes that were differentially expressed in GMP and HSPC BM cells of individuals carrying the *IL32* rs4786370 genotypes post BCG vaccination. When we looked at the genes expressed in the GMP BM cells, we found some genes that were specifically upregulated in the *IL32* rs4786370 CC individuals 3 months (D90) post vaccination. Among these were genes related to cell metabolism such as oxidative stress-associated Src activator (*FAM120A*), which is a critical component of the oxidative stress-induced survival signaling (Tanaka et al., 2009), C2 calcium-dependent domain containing 4 (*C2CD5*), which encodes one of the proteins required for insulin-stimulated glucose transport, and glucose transporter SLC2A4/GLUT4 translocation to the plasma membraned of cells (Yu et al., 2013), as well as long chain fatty acid transport protein 1 (*SLC27A1*), which plays a pivotal role mediating the ATP-dependent import of long-chain fatty acids into the cell by mediating their translocation at the plasma membrane (Ochiai et al., 2019) (Figure 7C). In addition, the results also showed a clear pattern of genes that were only expressed in HSPC BM cells of *IL32* rs4786370 CC individuals at D90 post vaccination (Figure 7D). These analyses revealed upregulation of genes associated with inflammatory immune response, DNA-binding transcription factor activity such as nuclear factor κB (NF-κB) subunit p65 (*RELA*) (Kunkl et al., 2019), bromodomain-containing 7 (*BRD7*), and hepatocyte nuclear factor 1-beta (*HNF1β*) (Wu et al., 2017; Park et al., 2014), a gene associated with the activation of inflammasome machinery such as NLR family pyrin domain containing 1 (*NLRP1*) (Finger et al., 2012), as well as transmembrane signaling receptor activity such as tumor necrosis factor receptor superfamily member 8 (*TNFRSF8*) (Luo et al., 2018) (Figure 7D).

These data strengthen the finding that IL-32 is a crucial component in the regulation of events occurring at the level of the hematopoietic stem progenitor cells, which confer the development of the trained immunity phenotype leading to protection against *L. braziliensis* infections.

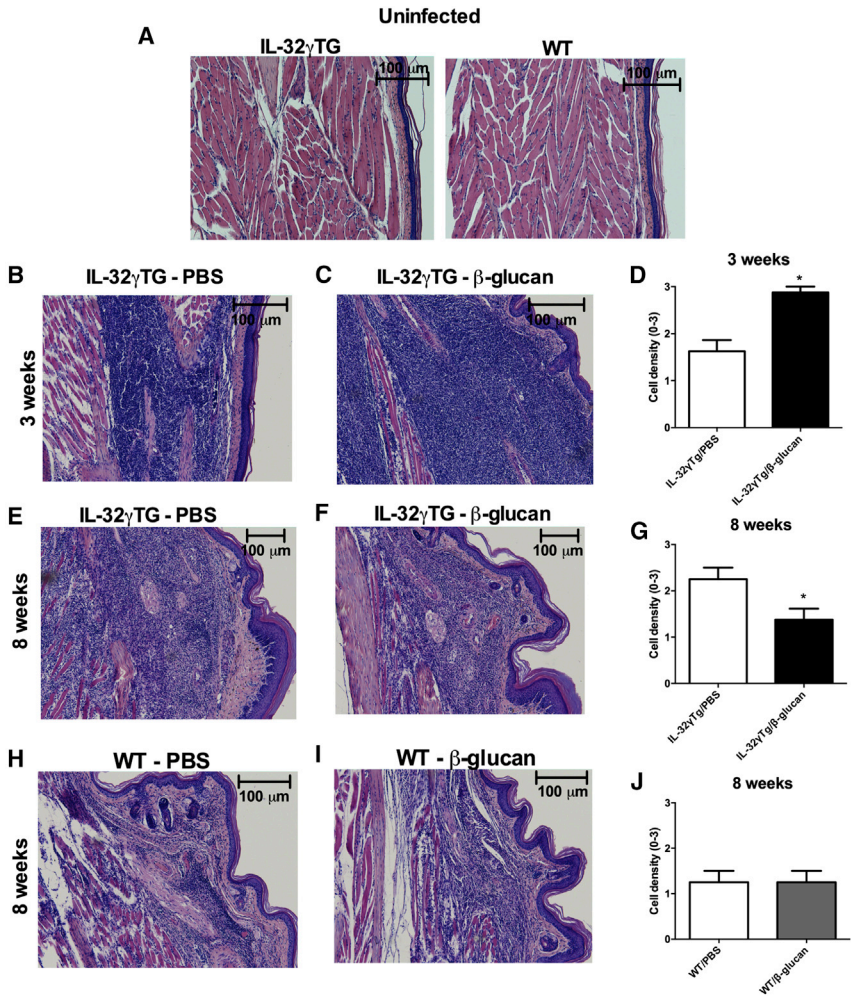

Figure 6. Histopathological Analyses of Paw's Lesions of IL-32γTG Mice Previously Treated with β-Glucan and Infected with *L. braziliensis*

WT and IL-32γTG mice were injected with β-glucan or PBS and subcutaneously infected in footpad with stationary-phase *L. braziliensis* promastigotes after 7 days.

(A–C) Representative photomicrographs of histopathological profile of footpad lesions: (A) uninfected paw of IL-32γTG and WT mice, (B) PBS group after 3 weeks of infection, and (C) β-glucan group after 3 weeks of infection.

(D) Inflammation scores after 3 weeks of infection.

(E–J) PBS group after 8 weeks of infection (E and H). β-glucan group after 8 weeks of infection (F and I). Inflammation scores after 8 weeks of infection (G and J). Scores were based on cell density as 0: no inflammation; 1: mild; 2: moderate; 3: intense; scale bar is 100 μm. The data shown are mean ± SEM of representative group of experiments performed with 4 mice per group. *p < 0.05 (PBS versus β-glucan; by Student's t test).

DISCUSSION

Several recent studies describe the ability of human innate immune cells to build a *de facto* immunological memory against pathogens and microbial products (Kleinnijenhuis et al., 2012; Netea et al., 2016). The fungal cell wall component β-glucan is a prototypical inducer of trained immunity *in vitro* and *in vivo*, stimulating a long-term pro-inflammatory macrophage phenotype capable of mounting an augmented response to infection. Importantly, the trained immunity response is heightened non-specifically, triggered by infections unrelated to the initial training stimulus (Ifrim et al., 2014). In this series of experiments, we demonstrated that induction of trained immunity by β-glucan modulates IL-32-mediated control of *Leishmania* infection via IL-1 signaling and epigenetic changes. These findings improve our understanding of the mechanism of trained immunity induced by β-glucan and simultaneously point toward different avenues for pharmacotherapy for improved treatment of leishmaniasis.

Specifically, we have shown that induction of trained immunity by β-glucan increases the efficiency of phagocytosis and killing of *L. braziliensis*, in parallel with increased cytokine production, specifically IL-6 and IL-10. It has previously been demonstrated that dectin-1 and the complement receptor 3 (CR3) receptors are required to induce training of monocytes with β-glucan (Quintin et al., 2012). These findings could at least partly explain the increased capacity for phagocytosis by β-glucan-trained cells, because both dectin-1 and CR3 are reportedly involved in *Leishmania* uptake by macrophages (Martínez-López et al., 2018). Considering macrophage microbicidal activity, we assessed induction of ROS and NO production (Novais et al., 2009). However, β-glucan training did not alter any of these mediators. These results are in line with previous findings (Bekkering et al., 2016) that reported no differences in ROS and NO induction in β-glucan-trained cells compared with naive cells. On the other hand, β-glucan-trained cells produced higher levels of antimicrobial peptides such as *cathelicidin* and β-*defensin 2*, which have been previously associated with improved killing of *Leishmania* spp. in human macrophages (Dos Santos et al., 2017b; Kulkarni et al., 2011).

Because IL-32 has been described as a crucial mediator to control *Leishmania* infections through transcriptional induction of antimicrobial peptides, we explored the role of IL-32 in β-glucan-induced trained immunity. Significant induction of *IL-32* expression suggested that β-glucan training influenced gene regulatory mechanisms. Indeed, histone methylation changes, namely H3K4me3 enrichment, are induced by β-glucan at the promoters of numerous pro-inflammatory genes such as *TNF-α* and *IL-6* (Quintin et al., 2012). In contrast, we observed that the *IL-32* gene promotor region became depleted of H3K4me3 upon training with β-glucan. Importantly, we identified a training-specific H3K4me1 enhancer signature located downstream of the *IL-32* gene transcription start site. Enhancers

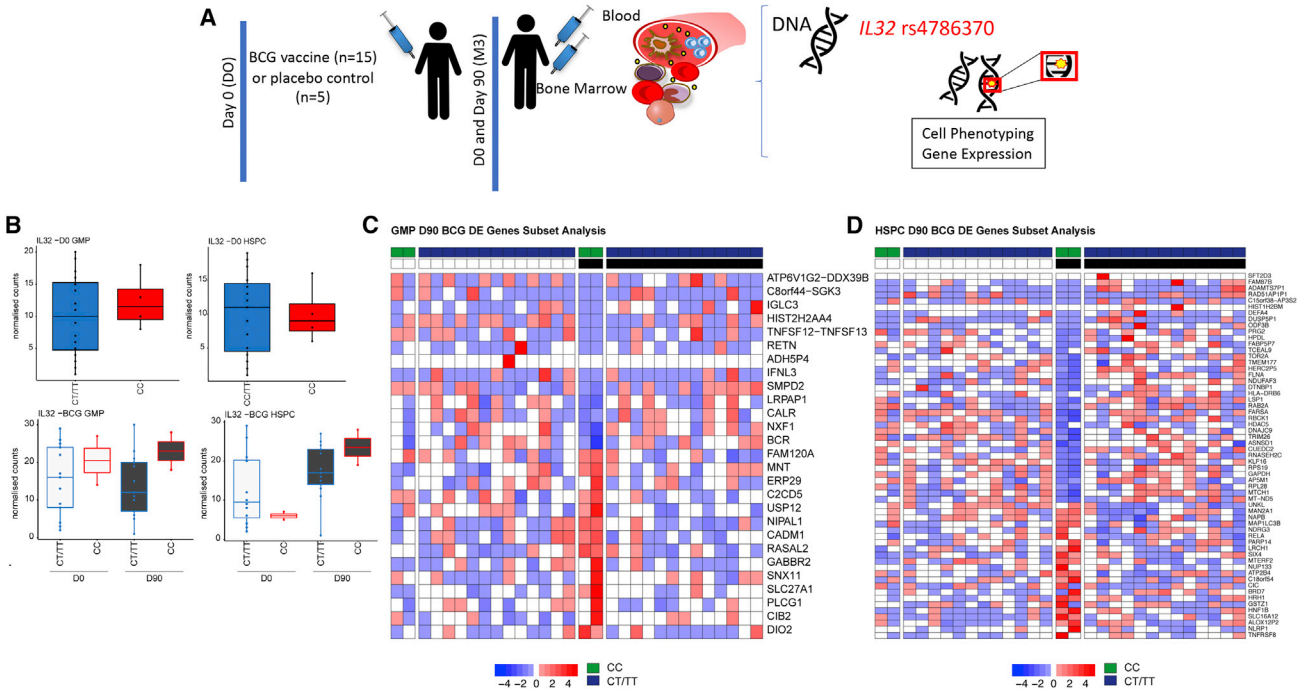

Figure 7. Human Bone Marrow Analyses in BCG-Vaccinated Individuals Stratified Based on *IL32* rs4786370 Genotypes

(A) Study design including BCG-vaccinated individuals (n = 15; *IL32* rs4786370 CC genotype = 2) and placebo-treated controls (n = 5; *IL32* rs4786370 CC genotype = 2). Bone marrow aspirations were analyzed before day 0 (D0) and day 90 (D90) after vaccination.

(B) *IL32* expression in granulocyte macrophages progenitors (GMPs) and in hematopoietic stem and progenitor cells (HSPCs) from individuals stratified for the different *IL32* rs4786370 genotypes (CT/TT and CC) at D0 and D90 after BCG-vaccination.

(C and D) Heatmap representation of differentially expressed genes in *IL32* rs4786370 CT/TT versus CC GMPs (C) and HSPCs (D) (BCG, D0 versus D90). Samples are separated in columns based on time point and *IL32* rs4786370 genotypes. Names of genes associated with the different genotypes are shown.

are distal *cis*-regulatory elements essential to controlling gene expression programs (Schaffner, 2015) and are thought to play crucial roles in the establishment of distinct macrophage phenotypes (Denisenko et al., 2017). Recently, it has been demonstrated that this particular enhancer element modulates the expression of the different isoforms of IL-32 to regulate inflammatory states of immune cells during HIV-1 infection (Palstra et al., 2018). Thus, from our limited profiling of the chromatin landscape surrounding *IL-32* transcriptional regulation, it might be tempting to speculate that activating histone modifications at distal regulatory elements may be more important for *IL-32* expression than those at proximal elements.

However, the importance of the *IL-32* promoter cannot be overlooked, as shown by the fact that the expression of *IL-32* is also altered by promoter SNPs (Westra et al., 2013). The CC genotype of IL-32 rs4786370 SNP was previously associated with enhanced IL-32 protein production. We therefore examined the effect of this particular SNP in β-glucan-induced trained immunity. In accordance with previous reports (Plantinga et al., 2013), we found that TNF-α, IL-6, and lactate production were higher in individuals carrying the CC genotype compared with those carrying the TT genotype in β-glucan-trained cells. Moreover, by assessing the influence of the *IL32* promoter SNP on the infection index, we demonstrated that besides its important role for cytokine production in LPS restimulated macrophages, IL-32 levels were negatively associated with infection index rates in individuals carrying the CC genotype. What is the direct role of IL-32 in the induction of trained innate immunity is not known at this moment. However, because IL-32 is mainly expressed intracellularly in β-glucan-trained macrophages, it might be that IL-32 is acting like other intracellular cytokines such as IL-1α, IL-33, and IL-37 (Dinarello, 2011; Moorlag et al., 2018). In addition, a recent study has pointed that IL-32 also can function as a transcription factor, because it appears to bind to DNA impairing the transcription and viral replication during infections (Kim et al., 2018). Further work is required in order to understand the mechanistic significance of IL-32 in β-glucan-trained macrophages, including epigenetic mechanisms not explored in the current study such as DNA methylation (Meyer et al., 2015), as well as transcription factor binding.

It has previously been shown that IL-32 is able to induce several proinflammatory cytokines, such as IL-1β, IL-8, and TNF-α. Furthermore, IL-1 and TNF-α can induce IL-32 (Heinhuis et al., 2011; Kim et al., 2005; Netea et al., 2005). Recent reports pointed toward a role for IL-1 in the induction of β-glucan-induced trained immunity *in vivo* (Mitroulis et al., 2018). In addition, Arts et al. (2018) demonstrated that IL-1β itself can induce trained immunity. In the present study, we were able demonstrate that IL-32 and IL-1 regulate the expression of each other in the context of trained innate immunity. Here, we showed that the *IL32* rs4786370 is associated with IL-1β production during the early stages of training with β-glucan. Additionally, IL-1β

protein was higher at the lesion of β-glucan-treated IL-32γTG mice after 3 weeks of infection. On the other hand, IL-1β itself also upregulates the production of IL-32. Moreover, we confirmed the role of IL-1 in the induction of trained immunity by using functional genomic analysis and IL-1 receptor blockade. Inhibition of IL-1 signaling downregulated *IL-32* expression, which in turn decreased the killing of *Leishmania* parasites. Of note, we cannot exclude that the presence of IL-1β as a detrimental consequence, resulting in of chronic inflammation. IL-1β has been previously associated with $CD8^+$ cell-mediated toxicity in lesions of patients with cutaneous leishmaniasis (Novais et al., 2017). Furthermore, it cannot be discounted that the IL-1-dependent TNF-α and IL-6 induction after β-glucan training might also play a role in the induction of IL-32, therefore contributing to the killing of *Leishmania* parasites. As demonstrated previously (Heinhuis et al., 2011), IL-32 and TNF-α are part of an inflammatory loop. Moreover, TNF-α has also been associated with IL-32 in ML caused by *L. braziliensis* (Galdino et al., 2014).

As the results indicated that IL-32 is important for β-glucan-induced trained immunity in human macrophages *in vitro*, we evaluated the effect of β-glucan-induced trained immunity in human IL-32γTG mice infected with *L. braziliensis.* In these mice, β-glucan training induced proliferation of bone marrow cells. In addition, in agreement with the results previously shown by Mitroulis et al. (2018) using WT mice, we suggested that myeloid cells might be playing the major role in control the infection caused by *L. braziliensis* in IL32γTG mice. Furthermore, an enhanced IL-32 and IL-1β expression, as well as upregulation of genes involved in glycolysis, upon exposure to LPS or lysates of *L. braziliensis* was reported (Figure 4). Once again, these findings are in line with those from a previous study, in which changes in progenitor cells of WT mice such as increased production of IL-1β and enhanced glycolysis accompany the induction of trained innate immunity (Cheng et al., 2014; Mitroulis et al., 2018). Moreover, it was also shown that BCG influences hematopoietic stem cells to generate epigenetically modified macrophages that provide significantly better protection against *M. tuberculosis* infection (Kaufmann et al., 2018). Of great interest, as a proof of concept, we were able to demonstrate that after BCG vaccination of healthy individuals, IL-32 has effects at the level of BM progenitor cells by modulating signature genes associated with inflammation, DNA binding transcript factors, and metabolism, including pathways known to be important for the long-term induction of trained immunity (Mulder et al., 2019). Netea et al. (2008) previously described that IL-32 contributes to differentiating monocytes into macrophage-like cells, resembling the phenotypical changes seen after β-glucan exposure. Additionally, IL-32 modulates the expression of ABCA1 and ABCG1 resulting in changes in lipid concentrations in primary human hepatocytes (Damen et al., 2018). Although we have not evaluated deviations in lipid metabolism, it is likely that IL-32 plays a role in metabolic changes necessary for cell adaptation during the induction phase of trained immunity with β-glucan.

Successful treatment of leishmaniasis is limited in the most parts of the world due to the toxicity and costs of effective medication. Moreover, a functional vaccine against the *Leishmania* spp. infections in human is still under development. However, a safe and effective leishmaniasis vaccine might be based on whole irradiated live parasites or *Leishmania* fractions, such as *Leishmania*-derived recombinant poly-protein (LEISH-F), as well as DNA vaccines (Rezvan and Moafi, 2015). It would be of considerable interest to combine vaccine components that target the enhancement of Th1 responses against *Leishmania* with strategies such as induction of trained immunity, especially to improve memory development for long-term protection. In therapy, especially in the cases where an acquired immune response is not present or is not effective such as in DCL, induction of trained immunity may be crucial to contain the parasite proliferation and dissemination. As a proof of concept, treatment of patients with DCL with BCG provides control of parasites and long periods without relapses, improving the quality of the patient life (Convit et al., 2004; Pereira et al., 2009). Thus, in addition to BCG training, β-glucan training represents another approach to control leishmaniasis in these patients. Moreover, β-glucan treatment may be an alternative when BCG, after several injections, could no longer be used due to the induction of strong acquired immune response in the local site of injection (Pereira et al., 2009).

In conclusion, we have shown that β-glucan improves host defense against *L. braziliensis* via the induction of trained immunity, highlighting IL-1 signaling and IL-32γ as important mediators for the eradication of *Leishmania* parasites. This study represents a definitive characterization of the role of IL-32γ in the trained phenotype induced by β-glucan. Our results begin to unravel the molecular mechanisms governing trained immunity and *Leishmania* infection control. Finally, the demonstration that β-glucan is able to improve the control of *Leishmania* infection using several mechanisms opens the gates for treatment strategies using trained immunity.

STAR★METHODS

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Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.08.004.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.A.B.J., F.R.-D, M.G.N., and A.S.; Methodology, J.C.d.S., A.M.B.d.F., M.V.T.S., M.S.M.A.D., S.J.C.F.M.M., R.S.G., M.D.O., M.M.H., S.T.K., B.C., and L.C.J.B.; Investigation, J.C.d.S., A.M.B.d..F., M.V.T.S., M.S.M.A.D., S.J.C.F.M.M., R.S.G., M.O., M.M.H., S.T.K., B.C., and L.C.J.d.B.; Writing – Original Draft, J.C.d.S., A.M.B.d.F., M.V.T.S., M.S.M.A.D., S.J.C.F.M.M., R.S.G., and M.O.; Review & Editing, S.T.K., L.A.B.J., F.R.-D., A.S., B.C., and L.C.J.d.B.; Supervision, L.A.B.J. and F.R.-D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-H3K4me3	Diagenode	Cat#pab-003-050, C15410003-50; RRID: AB_2616052
Rabbit polyclonal anti-H3K4me1	Diagenode	Cat#pAb-194-050, C15410194; RRID: AB_2637078
Rabbit polyclonal anti-H3K9me3	Diagenode	Cat#pab-193-050, C15410193; RRID: AB_2616044
F4/80 antibody	AbD Serotec	Cat#MCA497GA; RRID: AB_323806
goat primary polyclonal IL-32 antibody	R&D Systems	Cat#AF3040; RRID: AB_2124022
rabbit primary polyclonal IL-1β antibody	ThermoFisher	Cat#P420B; RRID: AB_223478
biotinylated rabbit anti-rat antibody	Vector Laboratories	Cat#BA-4000; RRID: AB_2336206
biotinylated horse anti-goat antibody	Vector Laboratories	Cat#BA-9500; RRID: AB_2336123
biotinylated goat anti-rabbit antibody	Vector Laboratories	Cat#BA-1000; RRID: AB_2313606
APC anti-human CD10	BioLegend	Cat#312210; RRID:AB_314921
APC/Cyanine7 anti-human CD10	BioLegend	Cat#312212; RRID:AB_2146550
PE-CF594 anti-human CD110	BD Biosciences	Cat#562416; RRID:AB_11154044
BV785 anti-human CD123	BioLegend	Cat#306032; RRID:AB_2566448
PE anti-human CD135	BD Biosciences	Cat#558996;RRID:AB_397175
BV421 anti-human CD14	BioLegend	Cat#325628;RRID:AB_2563296
APC/Fire750 anti-human CD15	BioLegend	Cat#323042; RRID:AB_2572103
BV510 anti-human CD16	BioLegend	Cat#302048; RRID:AB_2562085
PE/Cyanine7 anti-human CD16	BioLegend	Cat#302016; RRID:AB_314216
APC/Cyanine7 anti-human CD19	BioLegend	Cat#363010; RRID:AB_2564193
APC/Cyanine7 anti-human CD20	BioLegend	Cat#302314; RRID:AB_314262
APC/Cyanine7 anti-human CD3	BioLegend	Cat#317342; RRID:AB_2563410
AF700 anti-human CD34	BioLegend	Cat#343526; RRID:AB_2561495
BV510 anti-human CD38	BioLegend	Cat#356612; RRID:AB_2563875
PerCP anti-human CD45	BioLegend	Cat#304026; RRID:AB_893337
FITC anti-human CD45RA	BioLegend	Cat#304106; RRID:AB_314410
APC-eFluor anti-human CD7	Thermo Fisher Scientific	Cat#47-0079-42; RRID:AB_2573942
BV711 anti-human CD90	BD Biosciences	Cat#740786; RRID:AB_2740449
BV570 anti-human HLA-DR	BioLegend	Cat#307637; RRID:AB_10895753
Chemicals, Peptides, and Recombinant Proteins		
β−glucan (β−1,3-(D)-glucan	Professor David Williams, College of Medicine, Johnson City, USA	N/A
β-D-glucan from barley	Sigma-Aldrich	Cat#9041-22-9
Lipopolysaccharide	Sigma-Aldrich	Cat#L2880From *E. coli* serotype 055:B5,
Recombinant human Interleukin-1Ra	Sobi	Kineret® Anakinra
Recombinant human Interleukin-1β	R&D systems	Cat#201-LB-005
Grace's insect medium	GIBCO, Life Technologies	Cat#11595-030
Heat-inactivated fetal bovine serum (FBS)	GIBCO, Life Technologies	Cat#10500064
Protease inhibitor cocktail	Sigma-Aldrich	Cat#P8465
Giemsa	Merck Millipore	Cat#1.09204.0100
Percoll	Sigma-Aldrich	Cat#P1644
Ficoll-Paque	GE Healthcare	Cat#17-1440-03
Roswell Park Memorial Institute medium (RPMI)	Invitrogen	Cat#22406031
iScript reverse transcriptase	Bio-Rad	Cat#1708840

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TRIzol reagent	Life Technologies	Cat#15596018
SYBR Green	Applied Biosciences	Cat#4368708
16% Formaldehyde	Fisher Scientific	Cat#28908, 11835835
Zymosan	Sigma-Aldrich	Cat#Z4250
Griess reagent	Sigma-Aldrich	Cat#G4410
DAB (3′3 diaminobenzidine)	Sigma-Aldrich	Cat#D12384
DRAQ7	BioLegend	Cat#424001
Critical Commercial Assays		
Pierce BCA protein assay kit	ThermoFisher Scientific	Cat#23225
Human TNFα ELISA	R&D systems	Cat#DY210
Human IL-6 ELISA	R&D systems	Cat#DY206
Human IL-10 ELISA	R&D systems	Cat#DY217B
Human IL-32 ELISA	R&D systems	Cat#DY3040
Human IL-1β ELISA	R&D systems	Cat#DY201
Lactate Fluorometric Assay kit	Biovision	Cat#K607
MinElute PCR purification Kit	QIAGEN	Cat#28006
iScript cDNA Synthesis Kit	Bio-Rad	Cat#1708891
Cytotox 96 assay	Promega	Cat#G1780
DNeasy Blood & Tissue Kit	QIAGEN	Cat#69504
TaqMan SNP assay IL32 rs4786370	Applied Biosystems	Cat#4351379
miRNeasy Micro Kit	QIAGEN	Cat#217084
QIAseq FX Single Cell RNA Library Kit	QIAGEN	Cat#180733
KAPA Library Quantification Kits	Kapa Biosystems	Cat#07-KK4852-01
Deposited Data		
Raw and analyzed data	This paper	GEO: GSE124220
Raw and analyzed data	This paper	GEO: GSE132155
Blood eQTL browser	N/A	https://genenetwork.nl/bloodeqtlbrowser/
Experimental Models: Organisms/Strains		
200FG cohort (Human Functional Genomics project)	N/A	http://www.humanfunctionalgenomics.org
Transgenic mice for human IL-32 (IL-32TGγ)	Choi et al., 2010	N/A
Leishmania Viannia braziliensis	Oliveira et al., 2010	MHOM/BR/2003/IMG
BCG vaccine (Bulgaria strain)	Intervax	N/A
Oligonucleotides		
See Table S1	This paper	N/A
Software and Algorithms		
GraphPad Prism	Graphpad Software	https://www.graphpad.com
VisionTEK	Sakura	https://www.visiontek.com
FlowJo	Tree Star	RRID:SCR_008520
R statistical programming	N/A	RRID:SCR_001905
DESeq2	N/A	RRID:SCR_015687
Bioconductor	N/A	RRID:SCR_006442
clusterProfiler	N/A	RRID:SCR_016884
pheatmap	N/A	RRID:SCR_016418
ggplot2	N/A	RRID:SCR_014601
fGSEA	Bioconductor	https://bioconductor.org/packages/fgsea
tximport	N/A	RRID:SCR_016752
flowCore	N/A	RRID:SCR_002205

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Leo Joosten (leo.joosten@radboudumc.nl). This study did not generate unique reagents. There are no restrictions to the availability of reagents used in the current study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Experiments were conducted according to the principles expressed in the Declaration of Helsinki. All blood donors (Sanquin Blood Bank, Nijmegen, the Netherlands) gave written informed consent before donating the blood. The volunteers (n = 120) from the 200FG cohort (http://www.humanfunctionalgenomics.org) were between 23 and 73 years old and consisted of 77% males and 23% females. A randomized controlled trial (Trial NL6003 [NTR6501] https://www.trialregister.nl/trial/6003) was conducted at the Radboud University Medical Center (Nijmegen, the Netherlands) from January 2017 to July 2017. Prior to inclusion, volunteers were medically screened and provided written informed consent. The trial was approved by the Arnhem-Nijmegen Ethical Committee (approval number NL55825.091.15). The healthy volunteers (n = 20) were between 18 and 50 years old and consisted of 65% males and 35% females.

Animals

Transgenic mice for human IL-32 were generated (Choi et al., 2010) and kindly donated by Professor Charles Dinarello (University of Colorado, Denver, USA). Wild-type C57BL6J and IL-32γTG mice (females) between 8-10 weeks old were used in the experiments. Mice were maintained in animal facility of Instituto de Patologia Tropical e Saúde Pública of Federal University of Goiás, Goiás, Brazil. The study was approved by Ethics Committee of Radboud University Nijmegen, the Netherlands (approval number 42561.091.12) and also by Ethics Committee for animal research of Universidade Federal de Goiás (CEUA/PRPI/UFG, protocol 042/16). All animal experiments were in accordance with the guidelines of legislation of ethics of the Brazilian Society of Science in Laboratory Animals (SBCAL) and National Council of Control of Animal Experimentation (CONCEA).

Leishmania braziliensis parasites

L. braziliensis (MHOM/BR/2003/IMG) strain was obtained as previously described (Oliveira et al., 2010). Promastigote forms 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), 100 U/mL of penicillin/streptomycin (Sigma-Aldrich) at 26°C. Stationary-phase parasites were obtained on 6th day of growth and washed three times with phosphate-buffered saline (PBS; 1000xg, 10 min 10°C). After, they were suspended in PBS and quantified by using hemocytometer after fixation with PBS/0.4% formaldehyde. Live parasites were used in macrophage infection experiments. In addition, parasite lysates were obtained by 5 freeze-thaw cycles of promastigotes in the presence of protease inhibitors (Protease inhibitor cocktail, Sigma-Aldrich), in liquid nitrogen followed by water bath at 37°C. Protein quantification was performed by using Pierce BCA protein assay kit (ThermoFisher Scientific, USA).

METHOD DETAILS

Peripheral Blood Mononuclear Cells (PBMC) and monocyte isolation

PBMC isolation was performed by dilution of blood in pyrogen-free PBS and differential density centrifugation over Ficoll-Paque (GE healthcare, UK) as previously described (Dos Santos et al., 2017a). Percoll isolation of monocytes was performed as previously described (Repnik et al., 2003). 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 minutes 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 (Roswell Park Memorial Institute medium; Invitrogen, USA) supplemented with 50 μg/mL gentamicin, 2 mM glutamax (GIBCO, Life Technologies, USA), and 1 mM pyruvate (GIBCO) and quantified. Cells (10 × 10^6 cells/10 mL) were allowed to adhere on Petri dishes plates (Corning, NY, 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.

Monocyte training, treatments and macrophage infection

Trained immunity was induced in adherent monocytes as described previously (Bekkering et al., 2016). Cells were incubated either with culture medium containing 10% pooled human serum, referred as complete medium, as a negative control or 5 μg/mL of β-glucan (β-1,3-(D)-glucan was kindly provided by Professor David Williams, College of Medicine, Johnson City, USA). After 24 h (37°C), cells were washed once with 10 mL of warm PBS and incubated for 5 days with one change of complete medium. On day 6, cells were harvested and counted. 1 × 10^6 cells were stored in 200 μL of TRIzol (Invitrogen) at −80°C until RNA extraction. 1 × 10^6 cells were stored in 100 μL of 0.5% Triton X-100 (Sigma-Aldrich) for intracellular IL-32 measurement. 5 × 10^5 cells in a final volume of 500 μL of complete medium were added into 24-well plates on 12 mm coverslips (Corning, NY, USA), adhered for 1 h at

37°C and infected with stationary-phase promastigotes of *L. braziliensis* (2.5 × 10^6/well; MOI 5:1). Percentage of infected macrophages, number of parasites per infected cells and infection index were measured after 2 h, 4 h and 24 h of infection. 1 × 10^5 cells in a final volume of 100 μL of culture medium were added into flat-bottom 96-well plates (Corning, NY, USA), adhered for 1 h at 37°C and restimulated with 10 ng/mL of lipopolysaccharide (LPS) from *E. coli* serotype O55:B5, (Sigma-Aldrich), further purified as described (Battisti and Minnick, 2008) and stationary-phase *L. braziliensis* promastigotes (5 × 10^5/well; MOI 5:1). Supernatants were collected after 2 h, 4 h and 24 h and stored at −20°C. In some experiments cells were pre-incubated (before β-glucan) for 1 h with 10 μg/mL of recombinant interleukin-1 receptor antagonist (IL-1Ra; Synergen, USA) to evaluate the IL-1 effects during the priming with β-glucan. After 24 h stimuli and inhibitors were washed away and the assay continued as described above. Additionally, 1 × 10^6 monocytes were stimulated with 10 ng/mL of rhIL-1β (R&D systems) for 24 h at 37°C. After 24 h cells were stored in 200 μL of TRIzol until RNA extraction.

Evaluation of Macrophage infection

Infection index were performed as previously described (Dos Santos et al., 2017b). Coverslips were collected, cells were fixed and stained with Giemsa (Merck Millipore) and analyzed under a light microscope (1000x) to determine the infection index. Three hundred cells were analyzed and the percentage of infected cells and the mean number of intracellular parasites per infected cell (at least 50) were determined. Infection index = percentage of infected cells × mean number of parasites per infected cell.

Reactive oxygen species and nitric oxide measurements

For measurement of ROS production, a luminol-enhanced luminescence assay was used. After detachment and counting of trained monocytes, a total of 1 × 10^5 cells were added per well of a 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* (5 × 10^5/well; MOI: 5:1). Luminol (145 μg/mL) was added and chemiluminescence was measured for 1 h. For NO, 1 × 10^6 cells were trained and nitrite was determined in supernatants on day 6 by using Griess reagent (Sigma-Aldrich). Prior to the assay, the human serum was precipitated from the supernatants using perchloric acid (PCA 13.5% - Fluka) and neutralized with 4 N NaOH.

Chromatin immunoprecipitation (ChIP)

Monocytes were isolated and trained with β-glucan as described above. On day 6, cells were harvested and fixed in 1% methanol-free formaldehyde. Fixed cell preparations were sonicated using Diagenode Bioruptor Pico sonicator using 5 cycles of 30 s on/30 s off and CHIP was performed using antibodies against H3K4me3, H3K9me3, H3K4me1 (Diagenode, Seraing, Belgium). A MinElute PCR purification Kit (QIAGEN) was used for DNA isolation. Afterward, qPCR analysis was performed using SYBR Green method and samples were analyzed by a comparative Ct method. Primers are listed in Table S1.

Genetic Analysis in the 200FG cohort

Genetic variation was assessed in the genes of IL-32 and IL-1β in healthy individuals of Western European descent from the 200FG cohort (Li et al., 2016). Monocytes were isolated and trained with β-glucan as described above. After 24 h, supernatants were collected and IL-6 and TNFα production was measured by ELISA. In some experiments, the individuals were selected according with their genotypes for the *IL32* rs4786370 SNP. PBMCs isolation and training protocol were performed as described above. After 24 hours of training, IL-1b production was measured by ELISA. On day 6, trained macrophages were infected with *L. braziliensis* promastigotes (5 × 10^5/well; MOI 5:1) and the infection index was measured as described above.

In vivo β- glucan training experiments

Wild-type C57BL6 and IL-32γTG mice were injected intraperitoneally (i.p.) with 1 mg (Cheng et al., 2014) of β-D-glucan from barley (Sigma- Aldrich) in 200 μL of PBS; as control, i.p. injection of PBS alone was performed; After 7 days, animals were infected with stationary-phase of *L. braziliensis* promastigotes (1 × 10^5/50 μL of PBS per mice) subcutaneously into the left posterior footpad. Lesion size (mm) was measured weekly using a digital caliper and expressed as the difference between the thickness of the infected and uninfected footpad. After 3 weeks and 8 weeks of infection, mice were euthanized and their footpads were removed for analyses. Infected paw was weighed and macerated in PBS. Parasite load was then analyzed by the limiting dilution assay. The results were expressed as the negative logarithm of the parasite titer per footpad (de Souza-Neto et al., 2004). To measure cytokines concentrations in the lesion, tissue samples were prepared as previously described (Gomes et al., 2018).

Ex vivo bone marrow stimulation after *in vivo* β- glucan training

Mice were injected with β-glucan as described above. On day 7, bone marrow from both femurs and tibiae was harvested in culture RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Cells were counted and the concentration was adjusted for 5 × 10^6 cells/mL. 1 × 10^6 cells were stored in 200 μL of TRIzol at −80°C for RNA extraction. Subsequently, 5 × 10^5 cells in 100 μL were seeded in flat-bottom 96-well plates, stimulated with 100 μL of LPS (10 ng/mL) or *L. braziliensis* lysates (50 μg/mL) and incubated for 24 h, at 37°C. Supernatants were collected for cytokine measurements and cell monolayers were stored in 200 μL of TRIzol at −80°C for RNA extraction.

Cytokine and lactate measurements

Cytokine production was determined in supernatants using commercial ELISA kits (R&D Systems) for human TNFα, IL-6, IL-10, IL-32 and IL-1β. For mouse, bone marrow culture supernatants, and supernatants from footpad homogenates were used to measure TNFα and IL-32 (kits from R&D Systems), according the instructions of the manufacturer. Lactate concentration was measured in supernatants of human macrophages using Lactate Fluorometric Assay kit (Biovision, CA, USA).

RNA isolation and qPCR

RNA was isolated using Trizol (Chomczynski and Sacchi, 1987). RNA was precipitated with isopropanol and washed with 75% ethanol followed by reconstitution in RNase-free water. Subsequently, RNA was reversely transcribed into cDNA by using iScript (Bio-Rad, Hercules, CA, USA). Diluted cDNA was used for qPCR that was done by using the StepOnePlus sequence detection systems (Applied Biosystems, Foster City, CA, USA) with SYBR Green Mastermix (Applied Biosystems). Genes evaluated in humans were: *IL-32*, *cathelicidin*, *β-defensin 2,* and in mice were: *mki67, csf2rb, rag2, IL-1β, hif-1α, pfkp3, hk3*. The mRNA analysis was done with the 2^-dCt x 1000 method and normalized against the housekeeping gene β2M for humans and 18 s for mouse. Primer sequences are listed in Table S1.

Histopathological analysis

Paraformaldehyde fixed-footpad lesions of *L. braziliensis*-infected mice (3 or 8 weeks of infection) were embedded in paraffin to be processed for histological analysis. Sections of 5 μm in thickness were stained with Hematoxylin and Eosin (H&E). Histopathological changes between PBS and β-glucan group mouse lesions were analyzed using a digital microscope VisionTek (Sakura, Japan), determining the cell density in the inflammatory infiltrate. One hundred microscope fields per section were analyzed. Semiquantitative score was performed according with the number of cells per field and classified in 0: none; 1: mild; 2: moderate; 3: intense.

Immunohistochemical (IHC) analysis for F4/80

Paraffin-embedded sections of footpad lesions were stained by the DAB (3′3 diaminobenzidine) method using: rat primary monoclonal anti-mouse F4/80 antibody (AbD Serotec, UK) followed by secondary biotinylated rabbit anti-rat antibody (Vector Laboratories, USA); goat primary polyclonal antibody anti-human IL-32 (R&D Systems, MN, USA) followed by secondary biotinylated horse anti-goat antibody (Vector Laboratories, USA); rabbit primary polyclonal antibody anti-mouse IL-1β (ThermoFisher Scientific, USA) followed by secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, USA). Tissues were counterstained with Meyer's hematoxylin. Macrophages were visualized with 3,3-diaminobenzidene precipitation (DAB, Vector Laboratory). All sections were analyzed using VisionTek (Sakura, Japan) Live Digital microscope.

Human Bacille Calmette-Guérin vaccination

Twenty healthy BCG-naive volunteers were included and randomly assigned to two groups: 15 subjects (10 males and 5 females) received standard dose (0.1 mL of the reconstituted vaccine) of intradermal BCG vaccination (BCG Bulgaria, Intervax), and 5 (3 males and 2 females) received 0.1 mL of vaccine diluent as a placebo control. At baseline (D0) and after three months (D90), bone marrow was extracted by aspiration from the iliac crest by an experienced physician assistant after local anesthetics with lidocaine. 30 mL of bone marrow was aspirated in total and collected into two 20 mL syringes, prefilled with 5 mL (150 IE/ml) sodium heparin per syringe. EDTA blood was collected and stored at −80°C until further processing for *IL32* genotyping.

Isolation of genomic DNA and genetic assessment of IL32 rs4786370 SNP

DNA was isolated from EDTA venous blood using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, the Netherlands), according to the manufacture's protocol. Genotyping of individuals was performed by TaqMan® SNP assay (*IL32* rs4786370, Applied Biosystems), according to the manufacture's protocol on the StepOnePlus qPCR system (Applied Biosystems). Quality control was performed by the incorporation of positive and negative controls and duplication of random samples.

Flow cytometric analysis and sorting

Bone marrow aspirates were collected and enriched for the mononuclear cell fraction (BM MNC) on D0 and D90. Cells were washed in FACS-buffer (0.5% BSA, 2 mM EDTA, PBS), resuspended in blocking buffer (1% rat serum (R9759-10ML; Sigma-Aldrich, USA), 1% mouse serum (M5905-5ML; Sigma-Aldrich, USA), 5% human serum (H4522-100ML; Sigma-Aldrich, USA) in FACS-buffer) and incubated with the respective antibody cocktails for two hours on ice in the dark (Table S2). Cells were washed, resuspended in FACS-buffer and incubated with the live/dead marker DRAQ7 (1:1000, BioLegend, USA) for 5 min at room temperature. Samples were acquired using BD FACS ARIAIII (BD Biosciences) and the FACS Diva software (BD Biosciences, USA). For RNA isolation from hematopoietic stem and progenitor cells HSPCs (identified as alive $Lin^-CD34^+CD38^-CD45RA^-CD90^{+/-}$) and GMPs (identified as alive $Lin^-CD34^+CD38^+CD45RA^+CD10^-$), cells were first sorted into cooled 1.5 mL reaction tubes containing FACS-buffer. Cells were pelleted at 2200 rpm for 7 min at 4°C and vigorously resuspended in 500 μl Qiazol. Tubes were stored at −80°C until further processing.

Generation and sequencing of cDNA libraries for transcriptome analysis

Total RNA was isolated using the miRNeasy Micro Kit (QIAGEN GmbH, Germany) according to the manufacturer's guidelines. RNA concentration and quality were assed using the Tapestation 2200 system and high sensitivity reagents (Agilent Technologies, USA). Sequencing libraries of FACS-enriched populations were prepared following the QIAseq FX Single Cell RNA Library Kit protocol (QIAGEN GmbH, Germany). For QIAseq FX Single Cell RNA Libraries, concentration of cDNA was assessed by KAPA Library Quantification Kits (Kapa Biosystems, USA) before sequencing single read 75bp on a HiSeq1500 instrument using TruSeq SBS v3-HS chemistry.

RNA-sequencing pre-processing and data analysis

For pre-processing, data were demultiplexed using *bcl2fastq2* (v.2.20). Quality controls were performed by *FastQC*, reads aligned to human reference transcriptome hg38 from UCSC and transcript abundance quantified using *kallisto* (v0.440) with default parameters. Further analysis was based on at least 5 million sequenced reads. Samples were imported with *tximport* (v1.8.0) and transcripts were filtered to remove those not corresponding to HGNC gene symbols, read sum lower than 10 across all samples or matching antisense, LINC or pseudogenes. One sample (derived from donor 9 HSPC, D0) was identified as outlier and omitted due to prominent de-clustering of the sample in the PCA based on normalized reads of the top 5000 most variable transcripts. Core analysis of the cleaned RNA-sequencing dataset is based on the *DESeq2* package (v1.20.0) in R and potential confounding effects are taken into account by incorporating gender and batch of library preparation into the *DESeq2* object design matrix. The individuals were stratified based on their *IL32* rs4786370 genotypes and genes were called differentially expressed if reaching an adjusted *p* value (padj) < 0.05 after multi-testing correction after Benjamini-Hochberg. Expression values were adjusted using variance stabilizing transformation (vst) and heatmaps were visualized with *pheatmap* (v1.0.10).

QUANTIFICATION AND STATISTICAL ANALYSIS

Differences were analyzed using Student's t test, Wilcoxon signed-rank test and Mann-Whitney *U* test, where applicable. For cytokine production before and after β-glucan training, the data are shown as fold increases or raw cytokine compared to the RPMI control. The statistics analyses were performed on these ratios. Analyses were performed using Prism software version 6.0 (GraphPad, San Diego, CA, USA). Significance was established as $p < 0.05$.

DATA AND CODE AVAILABITY

RNA sequencing data are available under GEO Accession number GEO: GSE124220 and GEO: GSE132155 for HSPC and GMP, respectively; Scripts used to analyze these data will be shared upon request.

Additional Resources

A randomized controlled trial (Trial NL6003 [NTR6501] https://www.trialregister.nl/trial/6003) was conducted at the Radboud University Medical Center (Nijmegen, the Netherlands) from January 2017 to July 2017.